Immune dysfunction in oral cancer patients: role of tumor microenvironment

By
Asif Amin Dar
(LIFE09201004005)

Tata Memorial Centre
Mumbai

A thesis submitted to the
Board of Studies in Life Sciences
In partial fulfillment of requirements
for the Degree of

DOCTOR OF PHILOSOPHY
of
HOMI BHABHA NATIONAL INSTITUTE

November, 2016

Homi Bhabha National Institute

Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Mr. Asif Amin Dar entitled "**Immune dysfunction in oral cancer patients: role of tumor microenvironment**" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

S. N. Dalal — 4/11/16

Chairman – Dr. Sorab N. Dalal — Date:

S. Chiplunkar — 04/11/16

Guide/Convener – Dr. Shubhada V. Chiplunkar — Date:

Mahimkar — 4/11/2016

Member 1 – Dr. Manoj Mahimkar — Date:

Varma — 04/11/2016

Member 2 – Dr. Ashok Varma — Date:

Gaurisankar Sa. — 04/11/16

External Examiner – Dr. Gaurisankar Sa — Date:

Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to HBNI.

I hereby certify that I have read this thesis prepared under my direction and recommend that it may be accepted as fulfilling the thesis requirement.

Date: 04/11/2016

Place: Navi Mumbai

S. Chiplunkar

Dr. Shubhada V. Chiplunkar

Guide

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at Homi Bhabha National Institute (HBNI) and is deposited in the library to be made available to borrowers under rules of the HBNI.

Brief quotations, from this dissertation, are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the Competent Authority of HBNI when in his or her judgment, the proposed use of the material is in the interests of scholarship. In all the other instances, however, permission must be obtained from the author.

Navi Mumbai
Date: 07/11/2016

Asif Amin Dar

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree/diploma at this or any other Institution or University.

Navi Mumbai
Date: 07/11/2016

Asif Amin Dar

LIST OF PUBLICATIONS ARISING FROM THE THESIS

Journal

1. **Asif A Dar**, Trupti N Pradhan, Dakshayni P Kulkarni, Sagar Shah, Kanury V Rao, Devendra A Chaukar, Anil K D'Cruz, Shubhada V. Chiplunkar "Extracellular 2'5' Oligoadenylate synthetase 2 mediates TCR CD3-ζ chain downregulation via caspase 3 activation in oral cancer" *Immunology* 2015 Nov 23; **doi: 10.1111/imm.12560**
2. Rushikesh S Patil, Sajad A Bhat, **Asif A Dar**, Shubhada V Chiplunkar "The Jekyll and Hyde story of IL17 producing γδT (Tγδ17) cells" *Frontiers in Immunology* 2015 Jan*;* 6:37. **doi: 10.3389/fimmu.2015.00037**
3. **Asif A Dar**, Rushikesh S Patil, Shubhada V Chiplunkar "Insights into the relationship between toll like receptors and gamma delta T cell responses" *Frontiers in Immunology* 2014 Jul; 5:366. **doi: 10.3389/fimmu.2014.00366**
4. Dimpu Gogoi, **Asif A Dar**, Shubhada V Chiplunkar "Involvement of Notch in activation and effector functions of gamma delta T cells" *Journal of Immunology* 2014 Mar; 192(5):2054-62. **doi: 10.4049/jimmunol.1300369**

Conferences (Abstracts published)

1. **Asif A Dar, Trupti Pradhan, Anil D'Cruz, Devendra Chaukar, Shubhada Chiplunkar** S31 "Mechanism of immune dysfunction mediated by HLA-DR$^{-/low}$ CD33^{+} CD11b^{+} myeloid cells in oral cancer patients" *Journal of Carcinogenesis*. 2015; 14 (Suppl 1): S21–S38. Published online 2015 Feb 10
2. **Asif A Dar**, Anil D'Cruz, Devendra Chaukar, Shubhada Chiplunkar "Targeting Myeloid-derived suppressor cells to rejuvenate the antitumor immunity in oral cancer" *European Journal of Cancer*. Feb 2016; http://dx.doi.org/10.1016/S0959-8049(16)31953-0

Presentation at conferences

1. Presented a Poster titled "Contribution of myeloid derived suppressor cells to immune suppression in oral tumor microenvironment" at **"13th FIMSA Advanced Immunology Course"** organized by PGIMER, Chandigarh , India (March 2016)
2. Presented a Poster titled "Targeting Myeloid-derived suppressor cells (MDSCs) to rejuvenate the antitumor immunity in oral cancer" at International conference on **"New Ideas in cancer challenging the dogmas"** organized by Tata Memorial Centre, India (February 2016)
3. Oral presentation titled "Crosstalk of myeloid derived suppressor cells and Regulatory T cells in oral cancer" at 42nd Annual Convention of Indian Immunology Society (IIS), **"Immunology in 21st century and beyond",** Patna (October 2015)
4. Oral presentation/poster titled "Myeloid derived suppressor cells are key players of immune suppression in oral cancer patients" at 34th Annual Convention of Indian Association for Cancer Research (IACR), **"Cancer Research: from Bench to the Bedside",** Jaipur (February 2015)
5. Presented a Poster titled "Mechanism of immune dysfunction mediated by HLA-DR$^{-/low}$ CD33^{+}CD11b^{+} myeloid cells in oral cancer patients" at International conference on "**Molecular pathways to therapeutics: Paradigms and challenges in Oncology"** organized by Carcinogenesis foundation, USA (February 2015)
6. Presented a Poster titled "Immune dysfunction in patients with oral cancer: role of myeloid derived suppressor cells and regulatory T cells" at **Keystone Symposia "Inflammation, Infection and Cancer"**, held at Fairmont Chateau,

Whistler, Canada (March 2014)

7. Presented a Poster titled "Immunosuppressive role of myeloid derived suppressor cells and regulatory T cells in patients with oral cancer" at 'IX-DAE BRNS Life Sciences Symposium LSS-2013' on **"Current Advances in Immunobiology and Cancer"**, held at BARC, Mumbai (November 2013)
8. Presented a poster titled "Immune dysfunction in oral cancer patients: Role of tumor microenvironment" at 32nd Annual Convention of Indian Association for Cancer Research (IACR) and "**International symposium: Infection and Cancer"**, held at ACBR, New Delhi (February 2013)

Navi Mumbai
Date: 07/11/2016

Asif Amin Dar

Dedicated To

I want to dedicate this dissertation to the entire Dar family (All and Sundry) for being an integral part of my life during every phase. In particular to my mother Reshma Begum, for her perpetual sacrifices for the benefit of her children; you are the greatest mother in the world. To my father Mohammad Amin Dar for his unconditional support and advise during the thick and thin of life. To my sister Dr. Rehana Amin, for her unconditional love and for always being my best friend. To my grandparents who have showered immense amount of love, care and taught me moral values.

And lastly, to all the blood donors (OSCC patients and Healthy Volunteers) who have willingly donated their precious blood samples to the cause of this study.

Acknowledgements

After an exhaustive tenure of almost six years, today is the day when I am writing this note of thanks to give the finishing touch on my thesis. It has been a period of intense learning for me, not only in the scientific arena, but also on a personal level. Writing this thesis has had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this period.

Foremost, I would like to express my sincere gratitude to my mentor Professor Shubhada V. Chiplunkar. Her continuous support, patience, encouragement, enthusiasm and immense knowledge were key motivations during my PhD. Her objective based and principled approach towards research has influentially shaped my interest in my work. I am truly thankful for her steadfast integrity and selfless dedication to both my personal and academic development. I could not have imagined a better advisor and mentor for my PhD study and I feel incredibly privileged to have her as my mentor.

I have also benefited greatly from others mentors at ACTREC who were my Doctoral Committee members. I am thankful to Dr. Dalal, Dr. Maru, Dr. Mahimkar, Dr Varma, Dr. Robin, and Dr. (late) Kalraiya for their criticisms and valuable suggestions. I am indebted to our clinical collaborators Dr. Anil D Cruz and Dr. D Chaukar, for the help extended by them in the project.

Special thanks to the Dr. Robin and Dr. Varma Lab members especially Dr. Ajit Chande, Dr. Hemant Dhamne and Dr. Sumeer Raina for their unconditional help in the cloning experiments. Distinctive thanks to Dr. Shobhona Sharma, TIFR, Mumbai for allowing us to use the Imagestream flow cytometry. Special thanks to Dr. Kanury Rao ICGEB, Delhi for his help in the LC-MS/MS experiment.

I would like to thank the Council of Scientific & Industrial Research (CSIR), India, and the Department of Biotechnology (DBT), India for providing me with generous financial support throughout my doctorate. I would like to acknowledge the financial support provided by Department of Biotechnology, India to carry out this work. For scholarships and conference funding, I would like to thank Homi Bhabha National Institute, Sam Mistry foundation, Immunology Foundation, India and Department of Science and Technology, J&K.

I appreciate the generosity and cooperation shown by all the healthy volunteers and oral cancer patients for donating their blood and consenting to be part of the study. Special thanks to Madan Ludbe and all the Sisters at ACTREC for their help and support during sample collection.

I want to acknowledge the rich and supportive academic environment provided by ACTREC, especially Chiplunkar Lab members. They have nurtured me (literally), housed me, and created a lively community of people that I count myself lucky to be a part of. I would like to thank Dr. Jyoti Kode, Trupti Pradhan, Shamal Vetale, Meena Patkar, Rekha Gour, Dakaveji, Desaiji, Sawantji, Kalpesh, Ram, Preeta Menon, Maya Dolas, and Sujata Hake. Special thanks to Trupti Pradhan for her unconditional help during my PhD from carrying out experiments to writing thesis. Shamal and Rekha for teaching flow cytometry and Meena Patkar for being a great help to withdraw blood from healthy donors and for placing purchase orders.

I would like to continue thanking people from flow cytometry, Microscopy, Common Instrument facility, Library Photography, IT department, Security and Administration for their timely support. A special thanks to Mr. Shyam Chavan from Photography for his help in the art related material during my PhD tenure.

I would like to thank Chiplunkar Sir for being such a wonderful host for diverse Lab parties at his abode or at outside venues. I would also like to thank the group of Shamal, Meena, Rushikesh, Sachin, Sajad, Dakave, Desai, Ram and Siddhesh for arranging some surprise picnics and parties.

I found people in Chiplunkar Lab group immensely talented and dedicated. They are passionate, knowledgeable, fascinating, engaged, and friendly; in short, they were wonderful colleagues in every sense. In particular, I would like to thank Bhairav, Nirmala, Dimpu, Babita, Swati, Aparna, Rushikesh, Gauri, Sajad, Sachin, Shalini, Neha, Siddhesh, Sagar, Pradeep, Atul, Devanand, Priyanka, Prasad, for creating such a collegial atmosphere.

I want to thank those who took the time to directly engage with earlier drafts of this thesis/manuscripts, I could not have asked for sharper minds than Rushikesh, Shalini, Neha, and Sachin who took their time to read and re-read very early drafts of the thesis/manuscripts and provided incredibly helpful comments. Special thanks to Chinmay, Abhiram and Shweta who worked with me as summer trainees.

After I emerge out of the cocoon of Lab I would be surrounded by some vibrant friends, roommates and colleagues like Burhan, Sajad, Yaseer, Indrajit, Rushikesh, Shyam, Moquitul, Quadir, Shujath, Zahid, and Dimpu. Interactions with them were the highlight of my day and they have benefited me greatly in different ways. They each taught me how to think about problems, how to marshal and evaluate evidence, how to assess the validity and consistency of arguments, and how to tie together seemingly disparate concepts.

Huge thanks go to my batchmates (Abira, Aditi, Crismita, Gauri, Indrajit, Madhura, Nikhat Piyush, Poonam, Prasad, Raja, Rajan, Ram, Richa, Rushikesh, Saikat, Satish, Shyam, and Yasser) for the all the fun time, get-togethers and other good times besides supporting whole-hearted during difficult times. Thanks to Sajad, Indrajit, Shujath for being such amazing roommates.

I have been blessed with a very loving and supportive family. My grandfather has always stressed the importance of education and I know that this respect for education has shaped my values. My grandmother has been a source of constant and unconditional love. I am grateful to the both of them for all that they have taught me about what it means to be a good person. I am also grateful to my mother, father, and my sister for their love and encouragement. My parents have not only been a source of logistical support, they have also taught me skills that have made me a better researcher. Certainly in my case, I have learned so much from my parents and I appreciate all of the sacrifices that they have made for me. From my mother, I learned empathy, the art of working around obstacles, and the importance of just getting it done. From my father I learned to work hard, to stand up for myself, and to pick apart an argument. I am grateful to them both for being wonderful role models for me.

Last but not the least praise is to ALLAH, the Almighty, on whom ultimately we depend for sustenance and guidance. I would like to thank Almighty Allah for giving me opportunity, determination and strength to do my research. His continuous grace and mercy was with me throughout my life and ever more during the tenure of my research.

Asif Amin Dar

CONTENTS

Homi Bhabha National Institute

Ph. D. PROGRAMME

1. Name of the Student:	Asif Amin Dar
2. Name of the Constituent Institution:	Tata Memorial Centre**,** Advanced Centre for Treatment, Research and Education in Cancer
3. Enrolment No. :	LIFE09201004005
4. Title of the Thesis:	Immune dysfunction in oral cancer patients: Role of tumor microenvironment**"**
5. Board of Studies:	Life Sciences

SYNOPSIS

Introduction:

Oral cancer is the sixth leading cause of human cancer and is considered to be responsible for 3% to 10% cancer-related mortality worldwide [1]. In India, due to the high prevalence of tobacco chewing, smoking, and alcohol consumption, 40% of all cancers diagnosed are oral cancers, and amongst these, most of them are in their advanced stages (stage III and IV) [2]. The single most important causative agent of oral cancer is tobacco. It is used in various forms, most popular being chewing, either alone or in combination with areca nut and betel leaves [3]. Oral squamous cell carcinoma is often associated with a lymphocytic infiltrate that is believed to mount an in vivo immune reaction to the tumor cells. The extent of the lymphocytic infiltrate has been correlated with clinical prognosis, suggesting the relevance of the immune response to tumors [4].

Immune effector cells obtained from the peripheral blood of cancer patients, including oral cancer is reported to have a variety of functional abnormalities [5], which may vary in magnitude from patient to patient and may be related to the extent of disease [6]. These include abnormalities in signaling via the T-cell receptor (TCR) [7], poor proliferative responses [8], defects in lytic capacity [9], decreased ability for cytokine production [10] and increased propensity for spontaneous apoptosis [11]. These significant functional defects have been correlated with decreased expression of the CD3-ζ chain, the key T-cell signaling molecule which is also observed in T cells from oral cancer patients [12].

The tumor microenvironment apart from malignant cell also includes non-malignant cells, secreted proteins, and blood vessels that surround and support the growth of the tumor. Interactions between the various components of the tumor microenvironment are significant and can affect how a tumor grows and spreads [13]. Many tumors are characterized by the overproduction of a range of immunosuppressive cells and cytokines. These not only inhibit the host's antitumor immune response but also thwart attempts to augment anticancer immunity through the use of cancer vaccines. The best characterized immunosuppressive cell subsets are $FoxP3^+$ T regulatory cells (Tregs), tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) [14]. MDSCs arise from myeloid progenitor cells that have failed to terminally differentiate into mature granulocytes and macrophages. They suppress $CD4^+$ and $CD8^+$ T cells and have high intracellular arginase-1 levels that deplete arginine, an essential amino acid for T cell activation [15]. MDSC-produced ROS and peroxynitrite inhibit $CD8^+$ T cells by catalysing the nitration of the TCR and thereby preventing T cell-peptide-MHC interactions [16]. MDSCs also indirectly affect T cell activation by inducing T regulatory cells (Tregs), which in turn down-regulate cell-mediated immunity [17]. Naturally occurring $CD4^+$ Tregs are characterized by the expression of the IL-2 receptor α-chain (CD25). These cells naturally arise in the thymus, and after differentiation,

the naive $CD4^+CD25^+CD45RA^+$ T cells are exported to the periphery [18]. Tregs inhibit the proliferative responses of $CD4^+CD25^-$ T cells and the importance of Tregs in the context of the antitumor immune response is highlighted by the demonstration that interfering with the Tregs function promotes rejection of various types of tumors in animal models [19].

Based on these evidences, the focus of the study was to identify factors secreted by oral tumor that affects the key T signaling events in these patients and to address the crosstalk of immunosuppressive cells such as myeloid-derived suppressor cells and regulatory T cells in inhibiting the antitumor immunity and promoting immune evasion of oral tumors.

Aims and Objectives:

1) Analysis of the tumor-derived factors that may be responsible for defective T cell signaling.

2) To investigate the role of myeloid-derived suppressor cells and regulatory T cells in immune evasion of tumors.

3) To study the mechanism involved in immune suppression mediated by myeloid-derived suppressor cells and regulatory T cells.

Methodology

Study Group: The study was approved by the Institutional Ethics Committee (IEC). After written informed consent, peripheral blood (n=85) and surgically resected tumors (n=52) were obtained from newly diagnosed oral cancer (OC) patients (stage (I-IV)) before initiation of treatment. Blood specimens were also obtained from healthy individuals (HIs).

Cell culture: PBMCs isolated by Ficoll-Hypaque gradient were cultured with RPMI 1640 medium supplemented with 10% FCS. Tumor cells were obtained by enzymatic digestion (0.05% collagenase, 0.02% DNase, and 5U/ml hyaluronidase) (Sigma-Aldrich). Oral tumor cells, oral tumor-derived cell lines (AW13516, AW8507 and AW9803, SSC29B and SSC40) and HEK293 cells were cultured in 10% FCS supplemented IMDM and DMEM respectively.

Magnetic activated cell sorting: MDSCs ($HLADR^{-}CD33^{+}$), Naïve $CD4^{+}$ T cells, Tregs and $CD3^{+}$ T cells were purified from PBMCs using MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The separation procedure was conducted according to the manufacturer's instructions. The purity of separated cells was >95% as determined by flow cytometry (BD Biosciences, San Jose, CA).

Flow cytometry: To characterize MDSCs, TAMs, Tregs, and TH17 cells in freshly isolated PBMCs and tumor cells, multicolour flow cytometry was done using the following Abs (BD Bioscience): CD16, CD163, CD68, CD80, CD14, HLADR, CD11b, CD15, CD33, CD34, CD38, CD66b, CD124, CD62L, CD4, CD25, CD127, Foxp3, IL17, IFN-γ, IL-10, IL12, pSTAT3, PDL1, PDL2, and their respective isotype controls. Cells were acquired using FACSaria flow cytometry (Becton Dickinson), and 100,000 cells were collected to obtain reliable data. The expression of CD3-ζ chain on T cells was checked using anti CD3-ζ chain Ab (Santa Cruz) by flow cytometry.

Cloning and cell transfection: The cDNA encoding OAS2 gene was amplified by PCR using forward primer containing Pme1 restriction site and a reverse primer containing Sal1 restriction site. The PCR product was gel purified and by TA cloning inserted into pTZ57R/T vector. The pTZ57R/t vector was digested with EcoR1 and Sal1 to release the OAS2 cDNA which was purified and cloned into EcoR1 and Sal1 digested pEGFP-N2 vector.

Small interfering RNA: PBMCs were transfected with small interfering RNA (siRNA) specific for caspase 3 and fluorescent oligonucleotide control siRNA (Cell signaling Technology, USA) at a concentration of 100 nM using X-tremeGENE HP transfection reagent (Roche Diagnostics, Indianapolis, IN).

Western blotting: Cells were washed and lysed using 1% Nonidet P40 (NP 40) lysis buffer containing 10mM Tris, 50mM NaCl, 5mM EDTA, 1mM PMSF, 10µg aprotinin, 10µg leupeptin and 1% NP 40. Samples were resolved on SDS-PAGE and then transferred to

nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was probed with the primary antibodies. Immunostaining was performed using appropriate secondary antibody and developed with ECL plus Western blot detection system (Amersham Pharmacia).

Real-time and Semi-quantitative PCR: RNA was extracted from different cells using Trizol reagent (Invitrogen Life Technologies, N.Y) in accordance with the company's instructions. RNA obtained from cells was reverse transcribed into cDNA. The cDNA was used to probe different targets in different cell subsets.

Mass Spectroscopy (LC-MS/MS): The cell-free supernatant (1 ml) from surgically excised oral tumors, oral cancer cells lines AW8501, AW13516 and medium alone (control) were lyophilized. The dried samples were dissolved in 0.5 ml of ammonium bicarbonate buffer (0.1 M, pH 8.5) and the samples were concentrated on a centricon (3kDa cut-off membrane). Samples were dried in a lyophilizer overnight and the dried samples were dissolved in dissolution buffer supplied with the i-TRAQ kit (Applied Biosystem). By differential labeling, peptides labeled with isotope 117 (3-fold excess) were selected by subtracting the common peptides found in AW8501 and AW13516 cell lines from cell-free supernatants of oral tumors. Mascot analysis on them was done to identify the peptide and the protein from which the peptide was derived.

Statistical Analysis: Results are expressed as the mean ± standard error of mean (SEM). Statistical analysis was performed using Prism software (Prism Software, Lake Forest, CA) and the p-value was calculated using the student t-test. Two-sided p-values < 0.05 were considered statistically significant. Densitometry analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA)

Results:

Objective1: Analysis of the tumor-derived factors that may be responsible for defective T cell

1A) Extracellular 2′5′ oligoadenylate synthetase 2 mediates TCR CD3-ζ chain down-regulation via caspase-3 activation in oral cancer

The ability of tumor cells to escape obliteration by immune cells is due to the plethora of strategies including the production of immunosuppressive soluble factors which affect T-cell function in the tumor microenvironment. Oral tumors produced immunosuppressive factors and 2′5′oligoadenylate synthetase 2 (OAS2) was identified using the proteomic approach. Our results established a causative link between CD3-ζ chain downregulation and OAS2 stimulation. The surrogate situation was established by overexpressing OAS2 in HEK293 cell line and cell-free supernatant was collected. These supernatants when incubated with T cells resulted in downregulation of CD3-ζ chain, validates that secreted OAS2 is capable of regulating CD3-ζ chain expression. Incubation of T cells with cell-free supernatants of oral tumors or recombinant human OAS2 (rh-OAS2) induced caspase-3 activation which resulted in CD3-ζ chain downregulation. Caspase-3 inhibition/ downregulation using pharmacological inhibitor or siRNA restored downregulated CD3-ζ chain expression in T cells induced by cell-free tumor supernatant or rh-OAS2. Collectively these results show that OAS2 leads to impairment in CD3-ζ chain expression, thus offering an explanation that might be applicable to CD3-ζ chain deficiency observed in cancer and diverse disease conditions.

1B) Defining the role of TNF-α in down-regulation of T cell signaling molecule CD3-ζ chain in oral cancer patients

Chronic exposure of TNF-α has been found to regulate T cell function by promoting T-cell death and mediating immune escape of tumors. There are two receptors for TNF-α: TNF-RI or p55, also classified as CD120a, and TNF-RII, also referred to as p75 or CD120b. The

present study was proposed to directly address which receptor could be responsible for the downregulation of CD3-ζ chain in T cells induced by chronic TNF-α exposure. TNFR-I expression is upregulated compared to the TNFR-II in CD3+ T cells of oral cancer patients while their expression is comparable in healthy individuals. Further serum levels of TNF-alpha are higher in oral cancer patients compared to the healthy individuals. To mimic this, PBMNCs of HIs were incubated with 0.1% PHA to induce TNFR-I levels. Cells were then stimulated with TNF-α and results showed that there is a selective decrease in the CD3-ζ chain expression in T cells. On blocking TNFR-I and TNFR-II, it was observed that TNFR-I blocking abrogates TNF-α induced downregulation of CD3-ζ chain. Stimulation of T cells with TNF-α increased Ca^{2+} flux which was also reflected at altered p52shc dependent CD28 signaling. Elevated levels of reactive oxygen species and caspase 3 activity in T cells on TNF-α stimulation indicates a possible mechanism of TNF-α induced CD3-ζ chain degradation.

1C) Inhibition of Notch signaling alters CD3-ζ chain expression in human $CD3^+$ αβ T-cells by regulating E3 ubiquitin ligases

The Notch signaling pathway is identified as an important regulator of T cell function. Effector functions of T cells driven by T cell receptor (TCR) and antigen non-specific signals (IL2) are known to be regulated by Notch signaling. The point integrating these pathways in human $CD3^+$ αβ T cells is incompletely understood. This study reported how these signals are synchronized by Notch signaling in human αβ T cells. Human αβ T cells have differential expression of Notch receptors, ligands, and target genes which get upregulated on stimulation with α-CD3/CD28 mAb. Inhibition of Notch signaling appeared to regulate activation of T cells and affect proximal T cell signaling by regulating CD3-ζ chain expression. Inhibition of Notch signaling affected protein levels of the CD3-ζ chain by inducing the expression of an E3 ubiquitin ligase, GRAIL. Further, in the absence of Notch

signaling, α-CD3/CD28 mAb induced activation of αβ T cells and IFN-γ production was down-modulated. The absence of Notch signaling prevented αβ T cell proliferative responses despite strong signaling through TCR and IL-2 receptor. This study shows how TCR signaling and Notch signaling cooperate with each other to support proliferation and activation of human αβ T cells.

Objective 2: To investigate the role of myeloid-derived suppressor cells and regulatory T cells in immune evasion of tumors

To unravel the role of MDSCs in immune suppression, MDSCs were characterized in OC patients (n=75) and it was found that OC patients have significantly higher MDSCs than healthy individuals (HI, n=48). The levels of MDSCs correlated with cancer stage. Monocytic MDSCs (Mo-MDSCs) were prevalent in the periphery while granulocytic subset (G-MDSCs) was dominant in the tumor compartment. Mo-MDSCs expressed toll-like receptors (TLR2 and TLR4) that may respond to microbiota and heat shock proteins (HSPs) and enhance the differentiation and suppressive potential of Mo-MDSCs into macrophages. Tumor microenvironment was enriched by CD68 and CD163 positive macrophages having the $IL10^{high}$ phenotype. Increased MDSCs in oral cancer patients was not due to their increased lifespan as they undergo higher turnover compared to their mature counterparts. They expressed higher levels of phopho-p53 and BAD. MDSCs also expressed higher levels of DR5, cleaved caspase 3 and p66shc. Accordingly, the levels of ROS secreted by these were drastically high which might be regulating the emergency myelopoiesis and thereby marshalling the accumulation of MDSCs in peripheral blood. Increased Tregs were observed in OC patients and correlated with stage of the tumor. The levels of Tregs showed no correlation with the subsite of the tumor of the oral cavity. The Foxp3 expression in the Tregs of tumor compartment was higher than that of the peripheral blood suggesting that the tumor microenvironment is highly immunosuppressive. Further, the oral tumors are infiltrated with

the effector T cells (CD4 and CD8) but majorly they are terminally differentiated and are functionally compromised, expressing lower levels of perforin and granzyme. The cytokine profile of oral cancer patients confirmed that the tumor microenvironment is conducive for the generation and accumulation of Tregs, MDSCs and TAMs as higher levels of IL-10, PGE2, VEGF, IL1β, IL-6, TGF-β and TNF-α were present.

Objective 3: To study the mechanism involved in immune suppression mediated by myeloid-derived suppressor cells and regulatory T cells

MDSCs suppressed T lymphocyte proliferation, decreased CD3-ζ chain expression, and IFN-γ production. The effect of Mo-MDSCs on CD3-ζ chain in $CD8^+$ T cells was more prominent compared to that on $CD4^+$ T cells. However, the CD3-ζ chain was decreased to the same extent in the T cells of oral cancer patients. Mo-MDSCs expressed Arginase 1 which has the capability to alter CD3-ζ chain expression and also express higher levels of COX-2 and STAT3 which are known to regulate Arginase 1. The levels of pSTAT3 in Mo-MDSCs were higher in oral cancer patients and IL-6 induced pSTAT3 in MDSCs regulated expression of PDL1, C/EBPα, C/EBPβ and IL-10. The pSTAT3 inhibited C/EBPα expression and may be dependent on Hes1 to regulate C/EBPα expression. The Hes1 was present in the MDSCs and might co-operate with pSTAT3 to alter the steady-state myelopoiesis. C/EBPβ was induced in a pSTAT3 dependent manner which has the proficiency to induce the emergency myelopoiesis which marks the generation of MDSCs. MDSCs present in the peripheral blood of oral cancer patients expressed higher levels of IL-10 and PDL1/PDL2 which might aid in their ability to alter T cell function and also IL-10 secreted would facilitate crosstalk between MDSCs and regulatory T cells (Tregs). Tregs levels also correlated with the IL-10 levels produced by MDSCs. It was therefore hypothesized that these two immunosuppressive cell populations may interact with each other to execute immunosuppression. However, *in-vitro* data showed that MDSC inhibits the TGF-β driven generation of iTregs from naïve $CD4^+$ T

cells. Cytokine profile of MDSCs suggests that they secrete IL1-β, IL-6, and TNF-α apart from IL-10. These cytokines have the ability to alter the differentiation of naïve $CD4^+$ cells to Th17 cells and *in vitro* data showed the same. The IL17 producing cells were higher in oral cancer patients which correlated with the in vitro data. This suggests that inflammation and T cell tolerance co-exist in oral cancer patients which might enhance tumor growth.

Further, the possible mechanism by which Tregs accumulate in the tumor compartment was studied. Tregs from peripheral blood expressed CCR6, CXCR4, CCR4 and CCR7 but Tregs from tumor compartment expressed higher levels of CCR6 and CXCR4. Oral tumor cells also expressed their respective chemokines CCL20 and SDF1α. Chemotaxis assay confirmed that Tregs have the capability to migrate via CCL20-CCR6 and SDF-1α-CXCR4 axis creating an immunosuppressive tumor microenvironment.

Summary and Conclusion

This study has unravelled different regulators which have the competency to dampen the antitumor immune response in oral cancer patients. For the first time, a potentially important function of tumor-derived OAS2 as a paracrine negative regulator of T-cell functions was identified. The mechanism employed by TNF-α, an important negative regulator of T cell function was also addressed. Blocking TNFR-II enhanced the TNF-α induced T cell dysfunction suggesting that use of antagonist ligands aimed at a single receptor, TNFR-II, might on one hand hold the potential for selectively turning off immunosuppressive microenvironment. However, at the same time, it might lead to TNF-α induced TCR signaling defect in antigen encountered T cells via TNFR-I. Therefore, to design the future therapies using TNF inhibitors these aspects should be taken into account.

The Notch signaling was found to be as one of the positive regulators of T cell signaling and inhibiting this signaling was observed to affect the proximal as well as distal signaling molecules. The study provided insight into mechanisms that integrate TCR and cytokine

signals to determine the outcome of T cell response and identified a central role for notch signaling in this process. Our data highlight novel molecular targets for the manipulation of T-cell dependent immunity with important implications for cancer immunotherapy.

MDSCs were elevated in human OC patients and showed that they contribute to immune suppression and their levels correlated with cancer stage. STAT3 played an important role in MDSC activation and accumulation in OC patients. OC patients had elevated Tregs and correlated with IL-10 produced by MDSCs. However, MDSCs do not have the capability to induce the Treg generation during *in vitro* conditions. MDSCs induced TH17 generation and IL17 producing cells were higher in OC patients. This suggests that MDSCs contribute in enhancing the inflammation as well as inducing T cell tolerance in oral cancer patients. Understanding the mechanism of action of immune suppressive cell populations in OC patients is important in designing effective immunotherapeutic protocol for OC patients.

References

1. Tanaka, T. and R. Ishigamori, *Understanding carcinogenesis for fighting oral cancer.* J Oncol, 2011. **2011**: p. 603740.
2. Moore, S.R., et al., *The epidemiology of mouth cancer: a review of global incidence.* Oral Dis, 2000. **6**(2): p. 65-74.
3. Gupta, P.C. and C.S. Ray, *Smokeless tobacco and health in India and South Asia.* Respirology, 2003. **8**(4): p. 419-31.
4. Begley, J. and A. Ribas, *Targeted therapies to improve tumor immunotherapy.* Clin Cancer Res, 2008. **14**(14): p. 4385-91.
5. Reichert, T.E., et al., *Signaling abnormalities, apoptosis, and reduced proliferation of circulating and tumor-infiltrating lymphocytes in patients with oral carcinoma.* Clin Cancer Res, 2002. **8**(10): p. 3137-45.
6. Zea, A.H., et al., *Alterations in T cell receptor and signal transduction molecules in melanoma patients.* Clin Cancer Res, 1995. **1**(11): p. 1327-35.
7. Lai, P., et al., *Alterations in expression and function of signal-transducing proteins in tumor-associated T and natural killer cells in patients with ovarian carcinoma.* Clin Cancer Res, 1996. **2**(1): p. 161-73.
8. Lockhart, D.C., et al., *Loss of T-cell receptor-CD3zeta and T-cell function in tumor-infiltrating lymphocytes but not in tumor-associated lymphocytes in ovarian carcinoma.* Surgery, 2001. **129**(6): p. 749-56.
9. Choi, S.H., et al., *Alteration of signal-transducing molecules in tumor-infiltrating lymphocytes and peripheral blood T lymphocytes from human colorectal carcinoma patients.* Cancer Immunol Immunother, 1998. **45**(6): p. 299-305.

10. Kono, K., et al., *Decreased expression of signal-transducing zeta chain in peripheral T cells and natural killer cells in patients with cervical cancer.* Clin Cancer Res, 1996. **2**(11): p. 1825-8.
11. Kuss, I., et al., *Clinical significance of decreased zeta chain expression in peripheral blood lymphocytes of patients with head and neck cancer.* Clin Cancer Res, 1999. **5**(2): p. 329-34.
12. Kulkarni, D.P., et al., *Mechanisms involved in the down-regulation of TCR zeta chain in tumor versus peripheral blood of oral cancer patients.* Int J Cancer, 2009. **124**(7): p. 1605-13.
13. Ansell, S.M. and R.H. Vonderheide, *Cellular composition of the tumor microenvironment.* Am Soc Clin Oncol Educ Book, 2013.
14. Ostrand-Rosenberg, S., *Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity.* Cancer Immunol Immunother, 2010. **59**(10): p. 1593-600.
15. Bronte, V., et al., *L-arginine metabolism in myeloid cells controls T-lymphocyte functions.* Trends Immunol, 2003. **24**(6): p. 302-6.
16. Nagaraj, S., et al., *Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer.* Nat Med, 2007. **13**(7): p. 828-35.
17. Serafini, P., et al., *Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells.* Cancer Res, 2008. **68**(13): p. 5439-49.
18. Sakaguchi, S., *Regulatory T cells: key controllers of immunologic self-tolerance.* Cell, 2000. **101**(5): p. 455-8.
19. Onizuka, S., et al., *Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody.* Cancer Res, 1999. **59**(13): p. 3128-33.

Publications in Refereed Journal

A) Published manuscripts

5. Dimpu Gogoi, **Asif A Dar**, Shubhada V Chiplunkar "Involvement of Notch in activation and effector functions of gamma delta T cells" *Journal of Immunology* 2014 Mar; 192(5):2054-62. **doi: 10.4049/jimmunol.1300369**

6. **Asif A Dar**, Rushikesh S patil, Shubhada V Chiplunkar "Insights into the relationship between toll like receptors and gamma delta T cell responses" *Frontiers in Immunology* 2014 Jul; 5:366. **doi: 10.3389/fimmu.2014.00366**

7. Rushikesh S patil, Sajad A Bhat, **Asif A Dar**, Shubhada V Chiplunkar "The Jekyll and Hyde story of IL17 producing γδT (Tγδ17) cells" *Frontiers in Immunology* 2015 Jan*;* 6:37. **doi: 10.3389/fimmu.2015.00037**

8. **Asif A Dar**, Trupti N Pradhan, Dakshayni P Kulkarni, Sagar Shah, Kanury V Rao, Devendra A Chaukar, Anil K D'Cruz, Shubhada V. Chiplunkar "Extracellular 2'5' Oligoadenylate synthetase 2 mediates TCR CD3-ζ chain downregulation via caspase 3 activation in oral cancer" *Immunology* 2015 Nov 23; **doi: 10.1111/imm.12560**

B) Communicated Manuscript

C) Manuscripts in preparation

1. **Asif A Dar**, Dimpu Gogoi, Abhiram Gokhale, Shubhada V Chiplunkar "Inhibition of Notch signaling alters CD3-ζ chain expression in human $CD3^+$ αβ T-cells by regulating E3 ubiquitin ligases"(Manuscript under Preparation)
2. **Asif A Dar**, Trupti N Pradhan, Devendra A Chaukar, Anil K D'Cruz, Shubhada V Chiplunkar " Crosstalk of myeloid derived suppressor cells and regulatory T cells in oral cancer"(Manuscript under Preparation).
3. **Asif A Dar**, Chinmay Ghoda, Trupti N Pradhan, Devendra A Chaukar, Anil K D'Cruz, Shubhada V Chiplunkar "TNFR-I receptor initiates CD3-ζ chain

downregulation in T cells on chronic TNF- α exposure"(Manuscript under Preparation).

Other Publications / Conference Presentations:

Conference Proceedings:

3. **Asif Dar, Trupti Pradhan, Anil D'Cruz, Devendra Chaukar, Shubhada Chiplunkar** S31 "Mechanism of immune dysfunction mediated by HLA-$DR^{-/low}$ $CD33^{+}$ $CD11b^{+}$ myeloid cells in oral cancer patients" *Journal of Carcinogenesis*. 2015; 14 (Suppl 1): S21–S38. Published online 2015 Feb 10
4. **Asif A Dar**, Anil D'Cruz, Devendra Chaukar, Shubhada Chiplunkar "Targeting Myeloid-derived suppressor cells to rejuvenate the antitumor immunity in oral cancer" *European Journal of Cancer*. Feb 2016; http://dx.doi.org/10.1016/S0959-8049(16)31953-0

Presentations:

1) Presented a Poster titled "Contribution of myeloid derived suppressor cells to immune suppression in oral tumor microenvironment" at **"13th FIMSA Advanced Immunology Course"** organized by PGIMER, Chandigarh , India (March 2016)
2) Presented a Poster titled "Targeting Myeloid-derived suppressor cells (MDSCs) to rejuvenate the antitumor immunity in oral cancer" at International conference on **"New Ideas in cancer challenging the dogmas"** organized by Tata Memorial Centre, India (February 2016)
3) Oral presentation titled "Crosstalk of myeloid derived suppressor cells and Regulatory T cells in oral cancer" at "42nd Annual Convention of Indian Immunology Society (IIS), **"Immunology in 21st century and beyond",** Patna (October 2015)

4. Oral presentation/poster titled "Myeloid derived suppressor cells are key players of immune suppression in oral cancer patients" at "34th Annual Convention of Indian Association for Cancer Research (IACR), **"Cancer Research: from Bench to the Bedside",** Jaipur (February 2015)
5. Presented a Poster titled "Mechanism of immune dysfunction mediated by HLA-DR $^{-/low}$ CD33 $^{+}$ CD11b $^{+}$ myeloid cells in oral cancer patients" at International conference on "**Molecular pathways to therapeutics: Paradigms and challenges in Oncology"** organized by Carcinogenesis foundation, USA (February 2015)
6. Presented a Poster titled "Immune dysfunction in patients with oral cancer: role of myeloid derived suppressor cells and regulatory T cells" at "**Keystone Symposia "Inflammation, Infection and Cancer"**, held at Fairmont Chateau, Whistler, Canada (March 2014)
7. Presented a Poster titled "Immunosuppressive role of myeloid derived suppressor cells and regulatory T cells in patients with oral cancer" at 'IX-DAE BRNS Life Sciences Symposium LSS-2013' on **"Current Advances in Immunobiology and Cancer"**, held at BARC, Mumbai (November 2013)
8. Presented a poster titled "Immune dysfunction in oral cancer patients: Role of tumour microenvironment" at "32nd Annual Convention of Indian Association for Cancer Research (IACR) and "**International symposium: Infection and Cancer"**, held at ACBR, New Delhi (February 2013)

Signature of Student: Asif Amin Dar

Date: 06/04/2016

Doctoral Committee:

S. No.	Name	Designation	Signature	Date
1	Dr. S. N. Dalal	Chairperson		6/4/2016
2	Dr. S. V. Chiplunkar	Guide/Convener		6/4/2016
3	Dr. Manoj Mahimkar	Co-guide		6/4/2016
4	Dr. Ashok Varma	Member		6/4/16

Forwarded through:

Dr. S.V. Chiplunkar
Director, ACTREC
Chairperson,
Academics and Training Program,
ACTREC

Dr. S. V. Chiplunkar
Director
Advanced Centre for Treatment, Research &
Education in Cancer (ACTREC)
Tata Memorial Centre
Kharghar, Navi Mumbai 410210.

Prof. K. Sharma
Director, Academics,
Tata Memorial Centre

PROF. K. S. SHARMA
DIRECTOR (ACADEMICS)
TATA MEMORIAL CENTRE,
PAREL, MUMBAI

List of Figures:

List of Tables:

Abbreviations:

^{3}H-TdR	Tritiated Thymidine
^{51}Cr	^{51}Chromium
ADCC	Antibody dependent cellular cytotoxicity
Ag	Antigen
Agr1	Arginase 1
AMC	7-amino-4-methylcoumarin
APC	Antigen Presenting Cell
APC	Allophycocyanin
APS	Ammonium Persulphate
ATCC	American type culture collection
ATRA	all-*trans* retinoic acid
bp	Base pair
BSA	Bovine serum Albumin
cAMP	cyclic adenosine monophosphate
CBA	Cytometric Bead Array
Cbl-b	Casitas B cell lymphoma-b
CCL	Chemokine Ligand
CCR	Chemokine receptor
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CHISAM	Chloroform and Isoamyl alcohol
CMP	common myeloid progenitors
CNV	copy number variations
COX	Cyclooxygenase
cpm	Counts per minute
CRT	calreticulin
CSL CBF1	Suppressor of Hairless, Lag-1
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte associated protein 4
DAMP	Damage associated molecular patterns
DAPI	4,6-diamidino-2-phenylindole
DC	Dendritic Cell
DEPC	Diethyl Pyrocarbonate
Dll	Delta Like Ligand
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DR5	death receptor 5
DTT	Dithiothreitol
DTX	Deltex
ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diamine tetra acetic acid
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
ELISA	Enzyme Linked Immunosorbent Assay
EMH	Extramedullary haematopoiesis
FACS	*Fluorescence-activated cell sorting*
FasL	Fas ligand
FBS	Fetal bovine serum
FCS	Fetal calf serum
FH	Ficoll Hypaque
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
GAM	Goat anti Mouse
GAPDH	Glyceraldehyde diphosphate dehydrogenase
GMCSF	Granulocyte macrophage colony-stimulating factor
GRAIL	gene related to anergy in lymphocytes
Gr-MDSC	Granulocytic MDSC
GSI-X	γ-secretase inhibitor-X
HERP	Hes-related repressor protein
HES	Hairy enhancer of split
HIF	Hypoxia inducing factor
HIs	Healthy Individuals
HLA-DR	Human leukocyte antigen- D related
HNSCC	head and neck squamous cell carcinoma
HPC	hematopoietic progenitor cells
HPV	Human papillomavirus
hr.	Hours
HRPO	Horse radish peroxidase
HSC	hematopoietic stem cells
HSP	Heat shock proteins
IDO	Indoleamine 2,3-dioxygenase
IFN-α/β	Interferon alpha/beta
IFN-γ	Interferon gamma
iNOS	inducible nitric oxide synthase
ITAMs	immunoreceptor tyrosine based activation motifs
Jag	Jagged
JAK	Janus tyrosine kinase
kDa	Kilo Dalton
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LOH	Loss of heterozygosity
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetically activated cell sorting
MAML	master-mind-like
MAPK	Mitogen Activated Protein Kinase
MCA	methylcholanthrene
MCSF	macrophage colony-stimulating factor
MDSCs	myeloid-derived suppressor cells
mg	microgram
MHC	Major histocompatibility complex
MIC	MHC class I chain-related molecules
min	minutes

ml	millilitre
mM	milli moles
MMP	matrix metalloproteinases
Mo-MDSC	monocytic MDSC
MPP	multipotent progenitor
mRNA	Messenger Ribonucleic acid
N1ICD	Notch 1 intracellular domain
N2ICD	Notch 2 intracellular domain
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B
NICD	Notch intracellular domain
NK	Natural killer
NKG2D	Natural Killer Group 2D
nM	Nano Moles
NRARP	Notch regulated ankyrin repeated protein
NS	Natural Suppressor
OAS	Oligoadenylate Synthetase
OD	Optical Density
OSCC	Oral Squamous Cell Carcinoma
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffer Saline
PCR	Polymerase chain reaction
PD1	programmed death-1
PDL1/2	programmed death ligand 1/2
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
pg.	Pico gram
PGE2	prostaglandin E2
PHA	Phytohaemagglutinin
PI	Propidium Iodide
pmoles	Pico moles
PMSF	Para methyl sulphonyl fluoride
PTK	protein tyrosine kinases
rIL-2	Recombinant Interleukin 2
RNA	Ribonucleic acid
RNAase	Ribonuclease
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RPMI	Roswell Park memorial Institute
RT	Room temperature
RT-PCR	Real Time-Polymerase chain reaction
SCC	Squamous cell carcinoma
SCF	stem cell factor
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error mean
siRNA	small interfering ribonucleic acid
SLAP	Src-like adaptor protein
SLE	systemic lupus erythematosus
SSC	Side scatter

STAT	signal transducer and activator of transcription
TAA	Tumor associated antigen
TAMs	tumor-associated macrophages
TCR	T-cell receptor
TDLNs	tumor draining lymph nodes(
TGF-β	Transforming growth factor beta
Th	T helper
TIL	Tumor infiltrating lymphocytes
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
Tregs	Regulatory T cells
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
αβ	Alpha beta
γδ	Gamma delta
μCi	Micro-Curie
μg	Microgram
μl	Microliter

Chapter 1
Introduction

Introduction

Cancer has emerged as the deadliest disease worldwide. It is the leading cause of death in economically developed countries and the second leading cause of death in developing countries [1]. Incidences of cancer are on the rise worldwide due to increase in the growth and aging of the population, as well as increasing prevalence of conventional risk factors such as smoking, obesity, and physical inactivity [1]. The most common malignancies in India are of the breast, uterine cervix, and oral cavity [2]. India has the highest incidences of oral cancer, accounting for one-third of the total cancers with an estimated 77003 new cases and 52067 deaths annually [1]. Oral cancer has a multifaceted etiology, but tobacco use (smoking or smokeless), alcohol use, and HPV infection are the major risk factors for oral cavity cancer, with a very strong synergistic effect when a person is both a heavy smoker and drinker [3, 4]. The contribution of each of these risk factors to the oral cancer burden varies across regions and in India smokeless tobacco products and betel quid with or without tobacco are the major risk factors [5, 6].

The primary treatment modalities for the management of early and locoregionally advanced oral squamous cell carcinomas (OSCC) are surgery and radiotherapy. The proven efficacy of cisplatin-based chemoradiotherapy has replaced radiotherapy as a standard treatment for patients with locally advanced disease [7]. Chemotherapy, originally used to treat systemic disease is now utilized as a combination therapy in locoregionally advanced oral cancer with adverse features or in the setting of distant metastatic disease [8]. Despite many prospective trials using various combinations of surgery, chemotherapy, and radiotherapy to improve locoregional control, survival rates for advanced carcinogen-associated OSCC remain miserable and unchanged over last 15 years [5, 9]. A significant proportion of patients develop disease recurrence, with up to 60% risk of local failure and up to 30% risk of distant failure even when treated with site-specific multimodality therapies [10, 11]. Given that many

patients with recurrent and/or metastatic OSCC have disease that is no longer responsive to curative therapy, the subsequent morbidity is high and survival is dismal [12].

Immunotherapy is an attractive option for cancer treatment and there are several reasons to believe that immune approaches could be effective in OSCC patients. OSCC is often associated with the infiltration of lymphocytes which are believed to mount an in vivo immune reaction against the tumor cells. The extent of infiltration of lymphocytes is correlated with clinical prognosis, suggesting the relevance of the immune response to tumors [13-15]. Further, anti–programmed death-1 (anti-PD1) based therapy (pembrolizumab) in head and neck squamous cell carcinoma (HNSCC) patients showed remarkable antitumor activity and safety ensuring that immunotherapy will be a major focus of future head and neck cancer research [16]. The effective killing of cancer cells by the immune cells is a stepwise cyclic process (known as cancer-immunity cycle, **Fig. 1.1)**. This cycle is initiated and allowed to progress and expand repeatedly [17, 18]. The process starts with the formation of neoantigens during oncogenesis and the released antigens are captured by dendritic cells (DCs) for processing. The antigens on MHCI and MHCII molecules are presented to T cells resulting in their priming and activation. To yield an anticancer T cell response, the antigen presentation is accompanied by co-stimulatory signals specifying immunity to the tumor antigens. Finally, the activated effector T cells traffic and infiltrate to the tumor bed where they specifically recognize and bind to cancer cells and kill them. Killing of the cancer cell releases more tumor-associated antigens increasing the dimension of this response in subsequent revolutions of the cycle [17, 18]

The aim of cancer immunotherapy is to maintain the self-sustaining cycle of cancer immunity, enabling it to enlarge and propagate, but not to the extent that it generates uncontrolled autoimmune inflammatory responses. However, the tumor microenvironment also includes non-malignant cells, secreted proteins, and blood vessels that surround and

support the growth of the tumor, apart from the malignant cells. Interactions between the various components of the tumor microenvironment are significant and can affect tumor growth by modulating the cancer immunity cycle [18, 19]. The major problem in the functioning of the cancer-immunity cycle is the tumor-induced immune suppression [20, 21]. A greater understanding of the dysregulation and evasion of the immune system in the evolution and progression of OSCC will provide the basis for improved therapies and outcomes for patients.

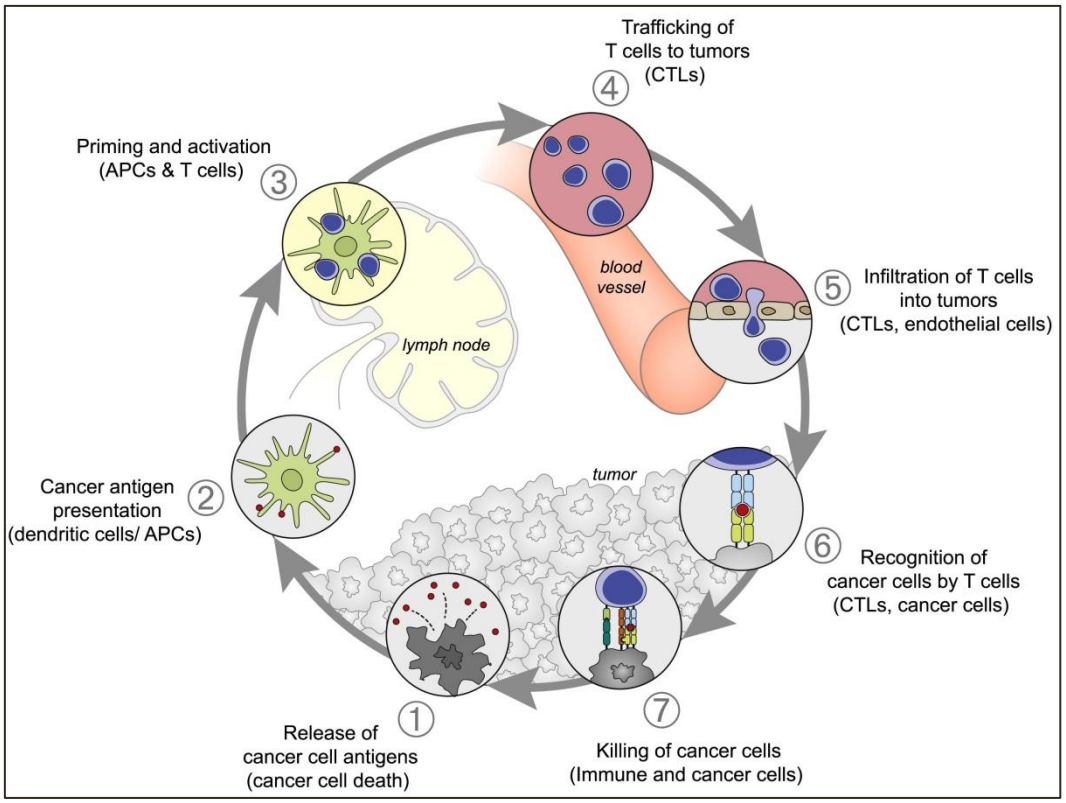

Figure 1.1: The Cancer-Immunity Cycle: The generation of immunity to cancer is a cyclic process with self-propagating potential, leading to an accumulation of immune-stimulatory factors that in principle amplify and broaden T cell responses. (**Source:** Daniel S. Chen, Ira Mellman, **Immunity**, Volume 39, Issue 1, 2013, 1–10)

Most human tumors have evolved sophisticated means of escape from the host immune system via the downregulation of key molecules essential for the immune response to precede and/or deteriorate the functions and survival of immune cells. Moreover, the immune effector cells (especially T cells) obtained from the peripheral blood of cancer patients, including oral

cancer are reported to have a variety of functional abnormalities [22], which may vary in magnitude from patient to patient and may be related to the extent of disease [23]. These include abnormalities in signaling via the T-cell receptor (TCR) [24], poor proliferative responses [25], defects in lytic capacity [26], decreased ability for cytokine production [27] and increased propensity for spontaneous apoptosis [28]. These significant functional defects have been correlated with decreased expression of the CD3-ζ chain, the key T-cell signaling molecule, this phenomenon is also observed in T cells of oral cancer patients [29]. The defect could also be reflected in the inability of T cells to properly home and infiltrate to the tumors. More importantly, the factors present in the tumor microenvironment suppress the generation of tumor antigen specific effector T cells [17, 18].

Many tumors are characterized by the overproduction of a range of immunosuppressive proteins and cells. The detection of immunosuppressive factors from culture supernatants or body fluids has been practically difficult due to the reason that they are present in very small amounts which hampers their isolation and detection. However, few of these substances have been characterized and studied with respect to their biological activity: transforming growth factor-beta (TGF-beta), interleukin 10 (IL-10), prostaglandin E2 (PGE2), vascular endothelial growth factor (VEGF), indoleamine dioxygenase (IDO), galectins, and adenosine [30]. These not only inhibit the host's antitumor immune response but also impede the attempts to augment anticancer immunity through the use of cancer vaccines. Oral cavity of OSCC patients is flooded with microbiota and recently the role of host microbiota during tumor formation and progression has been established [31, 32]. The microbiome and stromal cells in oral cancer might be influencing the neighbouring epithelial cells to enhance the production of factors like IL-6, IL1β and TNF-α which are protagonists of immunomodulation. Thus, characterization and identification of oral tumor derived factors having immunosuppressive

function would be important in developing immunotherapeutic protocol aimed at changing the tumor microenvironment from tumor promoting to tumor rejecting.

T cells control the diverse immune responses through their organized antigen recognizing T cell receptor (TCR). TCR is a multi-subunit receptor containing CD3-ζ chain dimers which has six immunoreceptor tyrosine based activation motifs (ITAMs) [33]. These ITAMs on CD3-ζ chain trigger intracellular signals leading to the proliferation, induction of cytolytic activity and cytokine production [34]. Due to the key role of CD3-ζ chain in the TCR signaling, the biological consequence of its low/absent expression is significant, resulting in the depressed antitumor immunity, poorer prognosis and shorter overall survival. Moreover, a correlation has been shown between CD3-ζ chain expression in T cells accumulating at the tumor site and tumor progression [35]. Therefore, part of this study was carried out to identify the different proteins/cytokines regulating the function of T cells in oral cancer patients with CD3-ζ chain as end point measurement. Different approaches were employed to find out the negative and the positive regulators of T cell signaling in oral cancer patients. For negative regulators, two approaches a) pragmatic approach and b) idealistic approach were used. For the pragmatic approach, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was employed to identify the protein(s) responsible for the downregulation of CD3-ζ chain in T cells. In case of idealistic approach, the role of pro-inflammatory cytokine TNF-α in regulating CD3-ζ chain expression was unravelled. In case of the positive regulator of T cell signaling, Notch signaling was selected. The role of Notch in regulating effector functions of $CD8^+$ T cells have been described and TCR signaling in both $CD4^+$ and $CD8^+$ T cells induces the activation of Notch-1 [36, 37]. However, how Notch regulates the proximal and distal T cell signaling in the human αβ T cells is not well understood.

Importantly, tumors also promote the accumulation of immune cells which are suppressive to T effector cells. The best characterized immunosuppressive cell subsets are $FoxP3^+$ regulatory T cells (Tregs), tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) [38]. MDSCs arise from myeloid progenitor cells that have failed to terminally differentiate into mature granulocytes and macrophages. They suppress $CD4^+$ and $CD8^+$ T cells and have high intracellular arginase-1 levels that deplete arginine, an essential amino acid for T cell activation [39]. MDSCs produced ROS and peroxynitrite inhibit $CD8^+$ T cells by catalysing the nitration of the TCR and thereby preventing T cell-peptide-MHC interactions [40]. MDSCs also indirectly affect T cell activation by inducing T regulatory cells (Tregs), which in turn down-regulate cell-mediated immunity [41]. Naturally occurring $CD4^+$ Tregs are characterized by the expression of the IL-2 receptor α-chain (CD25) and arise naturally in the thymus, and after differentiation are exported to the periphery [42]. Tregs inhibit the proliferative responses of $CD4^+CD25^-$ T cells and the significance of Tregs in the context of the antitumor immune response is highlighted by the demonstration that interfering with the Tregs function stimulates elimination of several types of tumors in animal models [43]. There are also evidence that OSCC is brought about by chronic inflammation induced by persistent chemical, bacterial or viral agents [44]. One of the immuno-inflammatory response is Th17 response which along with MDSCs promote tumourogenesis, and Th17 levels in colorectal cancer has been associated with poor survival [45]. The key in this process is activation of tumor-associated myeloid cells to produce IL-23 by microbial products which induces differentiation of Th17 cells [46]. Thus in context of OSCC progression the dynamic crosstalk between the MDSCs, Tregs and Th17 cells will be of paramount importance to suggest their role in immune dysfunction and hence development and progression of OSCC.

Advances in the understanding of how tumor-cells escape from immunity will transform the treatment of OSCC patients. Considerate validation of the function of immuno-suppressive factors/cells in OSCC patients will provide a rationale in designing immunotherapies which can be used in combination with other therapies to decrease the burden of tumor in the host. Based on these evidences, in the present thesis we aim to address the following aspects:

1) Characterization and identification of tumor derived factor(s) responsible for CD3-ζ chain downregulation in T cells of OSCC patients
2) Defining the role of TNF-α in the downregulation of CD3-ζ chain in T cells
3) Role of Notch signaling in regulating the proximal and distal TCR signaling in human $CD3^+$ αβ T cells
4) Role of myeloid derived suppressor cells, regulatory T cells and TH17 cells in the observed immune dysfunction of OSCC patients

Chapter 2
Review of Literature

2.1 Oral cancer

2.1.1 Oral cancer epidemiology

Globally, oral cancer is the eleventh most common cancer [47]. The incidences vary widely reflecting geographic differences with approximately two-thirds of oral cancer patients in the developing countries of Southeast Asia, Eastern Europe and Latin America. According to World Health Organisation (WHO), 40% of the oral cancers which were diagnosed worldwide occur in southeast Asian countries like India, Srilanka, Bangladesh, and Pakistan [48]. Worldwide, India holds highest cases of oral cancer, accounting for one third of the total cancers. A recent survey of India on cancer mortality shows that oral cavity cancer is the leading cause of mortality in men responsible for 22.9% of cancer-related deaths [49].

Oral cancer is a subgroup of head and neck malignancies which include cancers of the lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx (throat), buccal surfaces and other intra-oral locations [50]. In India, the gingival–buccal complex (gingival–buccal sulcus, alveolar ridge, buccal mucosa) forms the most common subsite for cancer of the oral cavity, compared to western world were cancer of the tongue is more common [51].

2.1.2 Etiology

The habits of tobacco smoking, chewing of tobacco/areca-nut betel-quid and drinking alcohol are established etiological factors worldwide. Other factors associated with the prevalence of oral cancer are dietary factors, occupational activity, socioeconomic status, exposure to external agents, and genetic susceptibility [52]. The most important risk factor for the development of oral cancer is the consumption of tobacco and alcohol [53]. Tobacco is the single most important risk factor for oral cancer. People smoking <15 cigarettes per day has 5.3 times more chances of developing cancer compared to those who never smoke. While people smoking ≥25 cigarettes per day have 14.3 times more chances of developing oral

cavity cancer [54]. In Asian countries, the use of smokeless tobacco products in the form of betel quid (pan), containing areca nut and lime with dried tobacco leaves, is rampant and this form of tobacco is shown to be highly carcinogenic [55, 56]. The high prevalence of gingivo-buccal cancer is because of the "pan" which is placed and retained in the gingival–buccal sulcus for prolonged durations. Although drinking and smoking are independent risk factors, they have a synergistic effect and greatly increase the risk together [57].

In different geographic regions, the role of Human papillomavirus (HPV) in the development of a subset of head and neck squamous cell cancers (HNSCCs) is now established [58, 59]. The role of HPV in the development of OSCC has received tremendous interest, and particular attention has been paid to HPV subtypes 16, 18, and 31. Human papilloma virus 16 and 18 are reported in around 22% and 14% of oropharyngeal tumors, respectively, increasing the risk of OSCC development by 3 to 5-fold [60]. HPV-16 infection is frequently found to be significantly associated with the risk of laryngeal squamous cell carcinoma [61].

OSCC also results from a multistep process characterized by the amassing of genetic and epigenetic alterations [62]. The aberrant pathways in OSCC patients are due to genetic alterations which might be either due to random chance or could be attributed to the lifetime environmental exposure of tobacco, alcohol and HPV infection. Genetic alterations include copy number variations (CNV), gains or losses of heterozygosity (LOH) which may cause the inactivation of tumor suppressor genes and the activation of oncogenes leading to uncontrolled cell growth and metastasis [63, 64].

TP53 is a tumor suppressor gene, recognized as a frequently mutated gene in HNSCC, with modern series estimating 46–73% of HNSCC cases having a mutation [65]. There is homozygous deletion of CDKN2A in nearly 30% of HNSCCs with another 10–20% of the samples harbouring a mutation [66]. Epidermal growth factor receptor (EGFR) is a member of the HER/erbB family of receptor tyrosine-kinases which activate signaling cascades like

RAS/RAF/MAPK, PI3K/AKT/mTOR and/or JAK/STAT [67]. These pathways have a significant role in proliferation, invasion, angiogenesis and metastasis of tumor cells. Over expression of EGFR and transforming growth factor (TGF) has been found to be associated with poor survival of OSCC patients.

2.1.3 Staging

Staging of oral cancer is conventionally performed with the use of the ‘‘tumor, node, metastasis’’ (TNM) classification system and its variant (pTNM), which are respectively based on clinical and pathological assessment of tumor size and lymph node involvement. However, traditional staging is often insufficient and does not always provide correct prognostic information.

2.1.4 Treatment

Currently oral squamous cell carcinoma (OSCC) is treated largely by surgery and/or definitive radiation therapy. Patients with advanced-stage oral cancer (stage III and IV) commonly receive multimodal treatment with surgery and chemoradiotherapy. The best therapeutic approach for the primary tumor depends on the anatomic site [68]. In patients with advanced disease, radiation therapy is preferred [69]. Chemotherapy is used more frequently as a radio-sensitizer for patients with locoregional recurrence or distant metastases. It is also used as a neoadjuvant preoperative treatment with radiotherapy to improve tumor resectability for stage IV patients [12].

Despite advances in surgery, radiotherapy and chemotherapy, the long-term survival for patients with oral cancer remains poor. Standard treatments are increasingly toxic and have failed to impact long-term survival in these patients. Adjuvant therapy is now considered as a potential treatment modality to eradicate local, regional and metastatic microscopic disease [70, 71]. Immunotherapy is emerging as a new option in treating OSCC patients like other malignancies. There is little doubt that our treatment of OSCC patients is likely to be changed

by the rapid advances in the understanding how tumor cells escape from immune system. Initial report on the activity of anti–programmed death-1 therapy, in both HPV positive and negative head and neck cancers suggest that major focus of future head and neck cancer treatment will be on immunotherapy [72].

2.2 Immunotherapy

Enhanced understanding of the underlying mechanisms behind control of the development and progression of malignancies by the immune system has led to the general acceptance of immune-based treatments as being a viable approach to treat cancer and the development of new immunotherapeutic strategies [73]. A plethora of immunotherapies are under active investigation for the treatment of established head and neck malignancies including OSCC. These include vaccine approaches, adoptive T-cell therapy, and the use of targeted agents such as immune checkpoint inhibitors [74]. The goal of cancer immunotherapy is to initiate or reinitiate a self-sustaining cycle of cancer immunity, enabling it to amplify and propagate, but not so much as to generate unrestrained autoimmune inflammatory responses [18]. However, the tumor antigen-specific or nonspecific immunotherapies had failed miserably mainly due to systemic and local broad immunosuppression in the tumor-bearing host. Many recent clinical results suggest that a common rate-limiting step is immunosuppression that occurs in the tumor microenvironment [20]. Cancer immunotherapies must therefore be carefully configured to overcome the negative feedback mechanisms. Checkpoints are built at each step of cancer immunity cycle that oppose continued amplification and can dampen or arrest the antitumor immune response, therefore, the most effective approaches will involve selectively targeting these rate limiting step in any given patient [18]. It is now crucial to make efforts to overcome immune suppression in cancer patients for better responses to immunotherapy which requires understanding the interaction between the growing tumor and immune system.

2.3 Interaction between immune system and cancer

The long-lasting argument about whether the immune system has positive, negative or null effects on tumor development is now over. In 1909, Paul Ehrlich predicted that the immune system can repress the growth of carcinomas that would otherwise occur at greater frequency, thus initiating a contentious debate over immunologic control of neoplasia [75]. Later a concept of "cancer immune surveillance" was proposed by Burnet and Thomas which predicted that the immune system acts as a sentinel in recognizing and eliminating nascent transformed cells [76]. More than 50 years after Burnet proposed his theory, the immunological scientific community remained largely divided with both supporters [77, 78] and rivals [79, 80] of the cancer immunosurveillance hypothesis. Despite subsequent challenges to this hypothesis over the next several decades [81, 82], new studies in the 1990s—fuelled by technologic advances in mouse genetics and monoclonal antibody (mAb) production—strengthened and ultimately validated the cancer immunosurveillance concept [83-85] and expanded it to incorporate the contributions of both innate and adaptive immunity. The theory has been rigorously examined and the accumulation of lymphocytes around a malignant tumor suggest that cancer cells might be recognized by the immune system [86]. However, lately it was revealed that this surveillance function of immunity was only a part of the story which prompted people to refine and extend the concept into a conceptual framework of 'cancer immunoediting' to more accurately describe the many facets of immune system–tumor interactions [87, 88]. This dynamic process of immunoediting not only protects against cancer development but also shapes the character of emerging tumors [89, 90].

2.4 Immunoediting

Contemporary work from many labs has clearly documented that immune system, in fact, facilitate cellular transformation, prevent or control tumor outgrowth and shape the

immunogenicity of tumors. These three apparently paradoxical functions of the immune system are separable based on their temporal occurrence during tumor formation, the nature of the transforming event, the particular components of immunity involved in each process, and in the nature of the tumor specific antigens expressed in the transformed cell. Whereas one body of work has clearly established the capacity of chronic inflammation to initiate and promote cancer [91], a second set of studies from other laboratories has revealed that an intact immune system can prevent/control and shape/promote cancer by a process called as "Cancer Immunoediting" [87, 88]. The evolution of the cancer immunoediting concept from the older and perhaps more controversial 'cancer immunosurveillance' hypothesis has helped to interpret the predictive and prognostic significance of immune infiltrates into tumors. Immunoediting was divided into three phases of Elimination, Equilibrium and Escape to describe the dual nature of the immune system.

2.4.1 Elimination/cancer immunosurveillance

The immune system was believed to monitor and exclude cancer cells in the early phase of carcinogenesis; however, this was quite difficult to reproduce experimentally using animal models for a long period. This aspect of tumor immunology got new life after it was shown that transplanted tumors grew rigorously in mice treated with IFN-γ neutralizing monoclonal antibodies or in immunodeficient mice lacking either INF-γ responsive components or T cell compartment [87, 92, 93]. Later in 2001, Schreiber's group reported that immunodeficient (lymphocyte-deficient) recombinase activator gene 2 $(RAG2)^{-/-}$ mice (lacks ability to produce T, B and natural killer (NK) T lymphocytes) developed carcinogen induced tumors more frequently than wild-type (immunocompetent) mice [87]. Moreover, their study showed that tumors developed in mice lacking intact immune system are more immunogenic than tumors developed in immunocompetent mice as they grew progressively in wild-type mice, while tumors from immunodeficient mice were rejected by wild-type mice [87, 89]. Further, studies

elucidated the importance of both innate and adaptive immune systems which work together to detect the presence of a developing tumor and eradicate it before it becomes clinically apparent. Cells of both innate and adaptive immune system were found to play a critical role in tumor rejection. Apart from the $rag1^{-/-}$ or $rag2^{-/-}$ mice which lack T cells, B cells and NKT cells, mice deficient for either αβ or γδ T cells also displayed increased methylcholanthrene (MCA) induced tumor vulnerability [94, 95]. In continuation, role of immune cells like CD1d-restricted T cells, NKT, NK and eosinophils has also been validated in different studies and it has been found that mice deficient in these cells are more susceptible to MCA-induced sarcoma formation than wild-type mice [96-99].

Subsequent studies in different immunodeficient mice unravelled the role of host effector molecules or their component pathways, such as type I interferons (IFNα/β), type II interferons (IFN-γ), IFNγR1, IFNαR1 or IFNαR2 (components of the type I IFN receptor) perforin, Fas/FasL, TRAIL; NKG2D (recognition molecule); IL-12, TNF-α, and DNAM-1 (DNAX accessory molecule-1). Each of these mice strains displayed enhanced susceptibility to MCA induced sarcoma tumors.

Another interesting study reported that the chromosomal content is also controlled indirectly, by an immunosurveillance mechanism that ensures the elimination of hyperploid cells. This study underscored the relation between immunoselection, hyperploidy, and endoplasmic reticulum (ER) stress, which can induce calreticulin (CRT) exposure on cell surface and increase their cellular immunogenicity [100]. The immunosurveillance/elimination mechanism for control of ploidy was dependent on $CD4^+$ and $CD8^+$ T lymphocytes, as well as both type I and type II interferons [78]. These provoking results unveil an immunosurveillance system that imposes immune elimination of hyperploidy tumors induced either by carcinogen or oncogene.

Accumulating evidence support that immunodeficiency can increase the frequency of tumors in genetic mouse tumor models. IFN-γ-insensitive mice (i.e., IFN-γ receptor-deficient mice) developed tumors more rapidly than wild-type mice when bred onto a p53 null background. IFN-γ-insensitive $p53^{-/-}$ mice also developed a broader spectrum of tumors compared to mice lacking p53 alone. When tumor cells derived from methylcholanthrene-treated IFN-γ-insensitive mice were used, it was found that IFN-γ can act directly on the tumor cell leading to enhanced tumor cell immunogenicity [93]. Immune surveillance by cytotoxic lymphocytes against cancer is dependent on the pore-forming protein perforin (pfp) [101]. It is reported that pfp-deficient mice has increased cancer susceptibility and a high incidence of malignancy in distinct lymphoid cell lineages (T, B, NKT), indicating a specific requirement for pfp in protection against lymphomagenesis. The susceptibility of pfp-deficient mice to lymphoma was further heightened by simultaneous lack of expression of the p53 gene [83]. Similar to $Pfp^{-/-}Trp53^{+/-}$ mice, perforin-deficient mice that are heterozygous for the tumor-suppressor Mlh1 (DNA mismatch repair gene) developed more B cell lymphomas with faster kinetics than mice that are wild-type for perforin and heterozygous for Mlh1 [102]. Role for CD1d-restricted T cells in anti-tumor immune response under genetic background of $p53^{+/-}$ mice established a key role for type I NKT (TCR $J\alpha18^{-/-}$) cells in suppressing the onset of sarcomas and hematopoietic cancers caused by p53 loss [96].

Above outlined evidences revealed that immune components are required for effective elimination of any given tumor. Although the elimination phase is yet to be directly observed *in vivo*, but its existence can be inferred from the earlier onset or greater penetrance of neoplasia in mice lacking certain immune cell subsets, recognition molecules, effector pathways, or cytokines and by studies comparing tumor initiation, growth, and metastases in wild-type versus immunodeficient mice (as reviewed in [103]).

2.4.2 Equilibrium Phase

Equilibrium phase is envisaged to be the longest phase of the cancer immunoediting process—perhaps extending throughout the life of the host. Rare tumor cell variants which survive the elimination phase enter the equilibrium phase. Tumors in the equilibrium phase are a subset of dormant tumors that are specifically controlled by components of the immune system [103]. Equilibrium thus represents a type of tumor dormancy or latent tumor in which outgrowth of occult tumors is specifically controlled by immunity. The first experimental demonstration for presence of equilibrium phase was reported in a study where low dose regimen of the chemical carcinogen, MCA was used to monitor the appearance of progressively growing sarcomas [104]. Treatment of naive wild-type mice with low doses of MCA led to overt tumors in only a low proportion of mice. It was found that dormant cancer cells were present in these lesions together with actively proliferating immune infiltrates in the mice which failed to develop tumors. The components of adaptive immunity namely $CD4^+$ and $CD8^+$ T cells, IFN-γ and IL-12p40 were found to be maintaining the primary undetected cancer lesions in an equilibrium phase [104]. These results support the conclusion that adaptive immunity is responsible for immune-mediated dormancy of fibrosarcoma. Later, a new perspective was added to the equilibrium phase by showing that the length of the equilibrium phase during immune control of MCA induced or p53 mutant cancers were influenced by IL-23 and IL-12 [105]. These two interleukins have critical and opposing roles in maintaining cancer cells in a state of immune-mediated dormancy. Inhibition of IL-23p19 was shown to reduce the malignant potential of lesions established by MCA inoculation, whereas inhibition of IL-12/23p40 enhanced tumor outgrowth. However, other cytokines such as IL-4, IL-17, TNF, and IFNα/β, which are known to play important roles either in MCA tumorigenesis or in the elimination phase of cancer immunoediting, did not play critical roles in maintaining the equilibrium phase [105]. Another study which proved the

existence of equilibrium phase demonstrated that inactivation of the E3 ligase Casitas B cell lymphoma-b (Cbl-b) led to the spontaneous *in vivo* rejection of tumor cells that express human papilloma virus antigens. $CD8^+$ T cells were identified as key players in the spontaneous tumor rejection response and therapeutic transfer of naïve Cbl-$b^{-/-}$ $CD8^+$ T cells also led to the significant reduction in the tumor size. Loss of *Cbl-b* in $CD8^+$ T cells enhanced antitumor reactivity and mechanistically, *cbl-*$b^{-/-}$ $CD8^+$ T cells were resistant to T regulatory cell mediated suppression and exhibited enhanced activation and rapid tumor infiltration [106] . *Cbl-b* thus functions as a negative regulator of antigen-specific T cell activation and is a critical mediator of T cell anergy. This data identified *Cbl-b* as a key signaling molecule, whose inhibition would serve as a novel approach to stimulate long-lasting immunity against cancer.

New insights into $CD4^+$ T cell-mediated control of multistage carcinogenesis was shown in mice that express T antigen (Tag) under the control of the rat insulin promoter (RIP1-Tag2). Tag is known to inhibit the tumor suppressor's p53 and retinoblastoma protein (Rb) [107]. RIP1-Tag2 mice were found to develop islet adenomas with new $\alpha_v\beta_3$ integrin-expressing blood vessels of abnormal configuration in 20% of the islets. Within 6 weeks, two to five of those adenomas progressed into carcinomas [108, 109]. However, on transfer of the Tag-specific $CD4^+$ T cells expressing a T cell receptor (TCR) specific for Tag peptide [110] directly regulated the multistage carcinogenesis through cytokine signals. Tag-specific Th1 cells induced a state of tumor dormancy [111]. Paradoxically, in the absence of either IFN-γ signaling or tumor necrosis factor p55 receptor (TNFR-I) signaling the same Tag-specific Th1 cells strongly promoted multistage carcinogenesis. Later, it was shown that IFN-γ and TNF induce senescence in cancer cells and treatment of cancer cells with IFN-γ and TNF arrested most of cancer cells in G1/G0 within 3 days, reducing S-phase cells to 3% which were more than 25% in untreated cells. Combined stimulation of islets or islet tumors with

IFN-γ and TNF strongly induced p16INK4a which stabilized the p16INK4a–Rb senescence pathway in Tag-expressing β cell cancers (β-cancers). However, STAT1- or TNFRI-deficient β-cancer cells fully resisted senescence induction by IFN-γ and TNF, showing that hypo-phosphorylation of Rb strictly requires the combined action of the STAT1 and TNFRI signaling pathways [112].

In another study, immune signature of the dormant sarcomas, which displayed small, stable masses, and the apparent sarcomas, which exhibited progressive growth was analyzed. The relative balance of monocytic MDSCs and antitumor immunity cells (especially CTLs, NK cells and γδ T cells) were found to be involved in maintaining tumor cells in a state of immune-mediated dormancy. At equilibrium phase, the infiltration of $CD3^+$ T cells into the tumor tissue was significantly increased while in mice with progressing sarcomas, the levels of intratumoral and splenic $FoxP3^+$ Treg cells and splenic PMN-MDSCs were significantly increased [113]. Thus, a promising anticancer therapy will aim to activate patients naturally occurring anticancer immunity or to inhibit the immunosuppressive immunity to enhance the elimination of residual tumor cells or to prolong dormancy in disseminated tumor cells. Such an endogenous immune response plays a significant role in controlling the balance between dormant tumor cells and tumor escape, and restraining metastases.

2.4.3 Escape Phase as a guide for targeted immunotherapies

Escape of dormant lesions from immune system is a critical gateway to malignancy and represents their victory over immune surveillance. Immune escape is a central modifier of clinical outcomes as it affects tumor dormancy versus progression, allowing invasion and metastasis and impacting therapeutic response. Tumor cells use different strategies to avoid recognition and destruction by the immune system [85]. One strategy is to escape immune system recognition via downregulation of human leukocyte antigens (major histocompatibility complex (MHC) class I) which are required to present antigens on

malignant cells to T cells [114], or via apoptosis of circulating T cells, which seems to be mediated at least in part by tumor-derived Fas ligand or by tumor associated B7-H1 [115, 116]. Reduced T-cell function linked to abnormalities in the TCR-associated CD3-ζ chain in T cells is correlated with poor prognosis [117]. Another potential mechanism is secretion of immunosuppressive factors (cytokines such as VEGF, TGF-β; immunoregulatory molecules such as IDO, PD-1/PD-L1, Tim-3/galectin-9, LAG-3) [90]. In the past few years a growing list of new moieties that contribute to tumor-induced immunosuppression, such as CD73, adenosine receptors, and new B7 family check- point molecules including V-domain Ig suppressor of T cell activation (VISTA) and B and T lymphocyte attenuator (BTLA) has been witnessed **(Fig. 2.1)** [90].

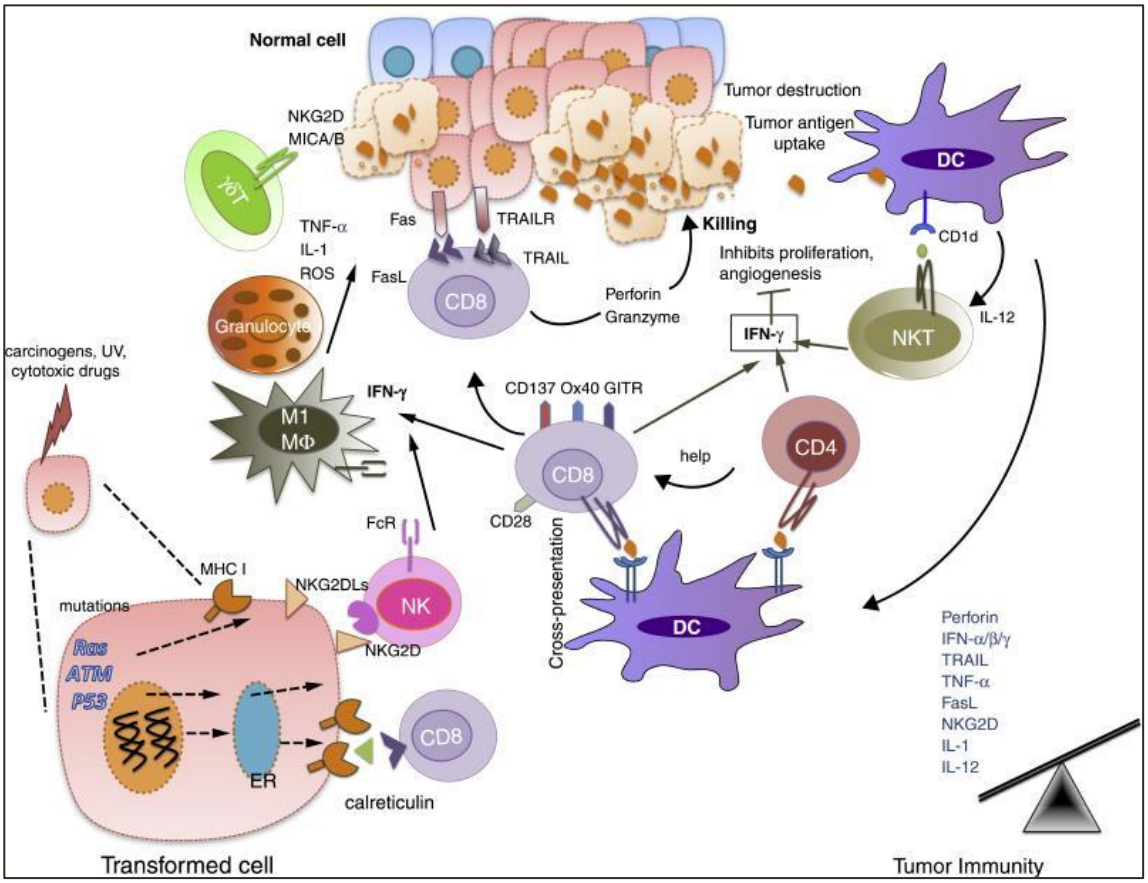

Figure 2.1: Escape phase of cancer immunoediting: The immune system fails to restrict tumor outgrowth and tumor cells emerge causing clinically apparent disease (**Source:** Deepak Mittal, Matthew M Gubin, Robert D Schreiber, Mark J Smyth, **Current Opinion in Immunology,** Volume 27, 2014, 16–25)

Additionally, immune defence can be directly inhibited by immune suppressive cells like myeloid derived suppressor cells (MDSCs), regulatory T cells (Treg) or the activated tumor associated macrophages (M2) [118]. These cells accumulate in the tumor mass and contribute to the formation of the immunosuppressive microenvironment. Ablation or reprogramming of this aberrant microenvironment might dramatically augment cancer therapies, and this strategy is currently being deployed in a variety of clinical trials. A better understanding of the molecular and cellular constituents of tumors and the mechanisms involved in immune evasion may help to guide the next generation of innovative cancer immunotherapies. Hence, in the remaining part of this review following aspects of immune evasion used by tumor cells will be discussed 1) Defects in proximal TCR-mediated signaling; 2) Tumor derived immune suppressive factors, and 3) Induction of immune suppressive cells (Tregs, MDSCs, and TAMs).

2.5 Proximal TCR-mediated signaling and its deficiencies in cancer patients

T cells are key effector cells of the adaptive immune system that play a crucial role in controlling the development of neoplastic lesions *in vivo*. Activated T cells can bind to the antigen peptides presented on major histocompatibility complex class molecules (MHCs) via their T-cell receptor (TCR). Following the recognition of peptides presented by MHC class I molecules present on target cells, activated $CD8^+$ cytotoxic T cells (CTLs) can efficiently destroy them using death cell ligands such as TRAIL (TNF-related apoptosis-inducing ligand) or by execution of the perforin/granzyme pathway [119, 120].

The T-Cell Receptor (TCR) is a complex of integral membrane proteins that recognize peptide fragments presented by MHC molecules and delivers signals that control T cells development, homeostasis, activation, acquisition of effector functions and apoptosis [121, 122]. The TCR is a disulphide-linked membrane-anchored heterodimer normally consisting of the highly variable alpha (α) and beta (β) chains expressed as part of a complex with the

invariant CD3 chain molecules. T cells expressing this receptor are referred to as αβ T cells, though a minority of T cells express an alternate receptor, formed by variable gamma (γ) and delta (δ) chains, referred to as γδ T cells [123].

TCR is associated on the membrane with CD3, to form the TCR-CD3 membrane complex. The CD3 complex participates in signal transduction after the interaction of TCR with the antigen. CD3 is a complex of five invariant polypeptide chains that associate to form three dimers: a heterodimer of gamma and epsilon chains (γε), a heterodimer of delta and epsilon chains (δε), and a homodimer of two zeta chains (ζζ) or a heterodimer of zeta and eta (η) chains (ζη). The cytoplasmic tails of CD3 chains contain a motif called the immunoreceptor tyrosine-based activation motifs (ITAMs), which play an important role in signal transduction. The γ, δ, and ε chains each contain a single copy of ITAM, whereas the ζ and η chains contain three copies [124-127].

TCR initiates signaling by recruiting and activating protein tyrosine kinases (PTK) of the Src, Syk and Tec families [128]. Upon engagement of the TCR by antigenic peptide presented on MHC molecule on antigen presenting cells, a Src family kinase p56Lck is activated. Activated p56Lck phosphorylates the ITAMs on the γ, δ, ε and ζ chains. The phosphorylated ITAMs provide docking sites for another tyrosine kinase ZAP-70, which subsequently gets activated. The main substrates for antigen receptor regulated tyrosine kinase are adaptors such as LAT (linker of activated T cells) or SLP-76 [129, 130]. These adapter proteins provide scaffold to assemble signal transduction molecules in the correct intracellular location for them to execute their effector function either directly or after allosteric regulation by co-assembled regulatory proteins. Tyrosine phosphorylation of adapters links antigen receptors to a cascade of signaling pathways during T-cell activation; the key ones are the activation of Ras and Rho-family GTPases signaling networks [131]. Antigen receptor tyrosine kinases also control inositol phospholipid metabolism which regulates both

intracellular calcium and the activity of diverse serine/threonine kinases, including members of the PKC family and phosphatidyl inositide-3 kinase (PI3K)-controlled serine kinases [121].

2.5.1 Significance of CD3-ζ chain in T cell signaling

Of the TCR subunits, the CD3-ζ chain has a key role in receptor assembly, expression and signaling. Downregulation of CD3-ζ chain expression and impairment of T-cell function have been shown for T cells isolated from hosts with various chronic pathologies, including cancer, autoimmune and infectious diseases [132]. CD3-ζ chain is a 16-kDa transmembrane signaling molecule associated with the TCR-CD3 complex in T cells and Fc receptor for IgG (FcγRIII) in NK cells. Under normal conditions in both T cells and NK cells, the CD3-ζ chain has an important role in the expression and signaling functions of these receptors. The CD3-ζ chain dimers associate with newly synthesized hexameric complex (αβ-γε-δε), and stabilizes the newly formed TCR to be transported to the cell surface for its expression as a complete complex of TCR/CD3 (αβ-γε-δε-ζ_2) [126, 133]. CD3-ζ is synthesized at ~10% the rate of other TCR components, and therefore, the amount of CD3-ζ available in a given T cell is thought to regulate TCR/CD3 expression at the cell surface [134]. CD3-ζ chain is indispensable for coupling antigen recognition by the TCR to diverse signal transduction pathways. To ensure that only receptor complexes with the correct stoichiometry reach the surface of T cells, incomplete complexes are degraded in lysosomes in the case of hexameric αβγεδε or are processed through degradation in pre-golgi compartments in case of components lacking other than CD3-ζ chain.

The biological basis for defective anti-tumor T-cell function has been long sought, and several laboratories have pursued the observation that specific components of the proximal TCR signaling pathway, CD3-ζ chain and p56Lck, are severely reduced in expression in tumor infiltrating or peripheral blood lymphocytes in animal models and in patients [135,

136]. Systemic defect in CD3-ζ chain (and/or p56Lck) expression has been found to be responsible for various phenotypes in anti-tumor T cells including the following: decreased proliferation, decreased cytokine secretion, defective lytic function, and counter intuitively, enhanced apoptosis [137]. The diminished T cell proliferation or cytokine secretion by PBLs has been correlated with decreased CD3-ζ chain expression [138]. For example, IL-2 secretion *in vitro* was shown to be decreased (but not extinguished) in a subset of melanoma patient which were also having reduced levels of CD3-ζ chain expression [23]. These melanoma patients also had diminished protein levels of p56Lck and phospholipase Cγ-1 (PLCγ-1) compared to healthy volunteers [23]. In sum, there is a good possibility that defective anti-tumor T-cell functions may be associated with decreased TCR-ζ chain.

Down-regulation of the CD3-ζ chain has been reported in a number of chronic inflammatory and infectious diseases. Rheumatoid arthritis (RA) is a chronic inflammatory joint disease categorized as an autoimmune disorder in which auto-aggressive T cells are responsible for the local inflammatory response. Synovial T cells in RA patients express reduced levels of CD3-ζ, resulting in a defective TCR-mediated signaling [139]. T cells from patients with systemic lupus erythematosus (SLE) display multiple abnormalities including decreased cytotoxic cell function, increased helper activity, abnormal cytokine production, and the presence of unusual T cell subpopulations. While increased TCR/CD3-initiated protein tyrosine phosphorylation was observed in T cells from SLE patients, diminished expression of the CD3-ζ chain in T cells was also observed [140]. T cells from leprosy patients demonstrated decreased activation and alterations in the molecules mediating signal transduction. These alterations include decreased expression of CD3-ζ and p56Lck, which are accompanied by a diminished ability to mobilize Ca^{2+} in response to activation signals, decreased cytotoxic function, and decreased production of IFN-γ [141]. Even in HIV infected patients, post-translational modification of the T cell signaling molecule CD3-ζ

chain has been implicated in the impairment of TCR [142]. T cells from patients with aplastic anaemia expressed decreased CD3-ζ chain and a corresponding abnormal response in the intracellular calcium following stimulation through the TCR [143].

The decreased expression of CD3-ζ is also reported in different malignancies and the first report showing downregulation of CD3-ζ chain was in $CD8^+$ T cells of murine colon cancer model [144] . Later loss of T-cell receptor (CD3-ζ chain) and p56Lck was reported in the T cells infiltrating human renal cell carcinoma [135]. Loss of CD3-ζ chain has been shown to correlate with both the stage of cancer as well as proximity of T cells to the tumor. Low levels of CD3-ζ chain have been detected in tumor infiltrating lymphocytes (TILs) in colorectal tumors, cervical cancer, ovarian cancer, breast cancer, head and neck cancer, pancreatic cancer and gastric cancer [25, 29, 35, 145, 146]

2.5.2 Factors responsible for impaired CD3-ζ chain expression in T cells

Various mechanisms have been reported that are responsible for the downregulation of CD3-ζ chain expression. Reactive oxygen species (ROS) produced by activated macrophages or neutrophils have been shown to induce downregulation of CD3-ζ chain in T cells. A direct correlation between the extent of T cell hyporesponsiveness and the concentration of ROS produced is reported [147, 148]. Consistent with this, addition of oxidative reagents such as hydrogen peroxide and diamide to T cells induced the downregulation of CD3-ζ chain expression. Heat stress is also known to downregulate the expression of the TCR ζ chain in T cells which was later associated with oxidative stress induced by the heat exposure [149, 150]. Dexamethasone, a potent anti-inflammatory and immunosuppressive agent modulates the expression of TCR CD3-ζ chain and TCR/CD3-induced early signaling events in human T lymphocyte [151]. Trichostatin A (TSA), a potent reversible inhibitor of histone deacetylase, has been shown to suppress the expression of the TCR CD3-ζ chain in T cells [152].

L-arginine availability has been linked to the CD3-ζ chain expression in T cells. Human T cells stimulated and cultured in the absence of L-arginine lose the expression of the TCR CD3-ζ chain and show an impaired proliferation and cytokine production [153]. Increased metabolism of L-arginine in tumor microenvironment has been associated with activated macrophages and MDSCs. These cells produce L-arginine metabolizing enzymes: inducible nitric oxide synthetase (iNOS) and arginase (Arg) [154]. Additional studies have indicated the role of caspase-3 and granzyme B in mediating the CD3-ζ chain downregulation. CD3-ζ chain contains putative cleavage sites for both caspase 3 and granzyme B [155, 156]. NKG2D signaling in human T cells initiates Fas ligand/Fas-mediated caspase-3/-7 activation which results in CD3-ζ chain degradation [157].

Loss of CD3-ζ chain expression has been linked to ubiquitination. Ubiquitination refers to the post translational modification of a protein by the covalent attachment of one or more ubiquitin monomers. Upon TCR engagement, CD3-ζ chain has been shown to undergo ubiquitination and lysosomal degradation [158]. E3-ubiquitn ligases like Casitas B-lineage lymphoma protein (cbl) and gene related to anergy in lymphocytes (GRAIL) can target CD3-ζ chain for ubiquitination and thereby its degradation [159]. Src-like adaptor protein (SLAP) has also been found to recruit c-Cbl to the TCR complex and targeting the TCR CD3-ζ chain for ubiquitination and subsequent degradation [160]. TNF-α is also known to regulate CD3-ζ chain expression in human T lymphocytes via Src-like adaptor protein-dependent proteasomal degradation [161]. Additionally, it has been reported that CD3-ζ chain downregulation is regulated by the CD3-ζ chain inhibitory protein (ZIP) present in the ascetic fluid derived from ovarian cancer patients [146].

Correlations made between clinical findings, pathologic results, and CD3-ζ chain expression in immune cells suggests that low/absent CD3-ζ chain is predictive of poor prognosis and survival in cancer patients. Thus, CD3-ζ chain is emerging as a clinically relevant signaling

molecule, which also seems to predict a favourable response to biologic therapies and could be helpful in a selection of patients for immunotherapy trials. Now, the challenge is to understand how the different signal transduction pathways control T cell behaviour during an immune response in cancer patients. One of the major challenges in this field is to characterize signal transduction events regulating CD3-ζ chain expression in T cells.

2.5.3 Notch as a potential positive regulator of Proximal TCR signaling

The Notch receptor plays a crucial role in embryonic development and specification of cell fates [162]. In mammals, there are four Notch receptors (Notch-1–4) which are activated by ligands that belong to either the Delta-like or jagged family (DLL1, 3, 4, jagged 1 and 2) of proteins [163]. Binding of a Notch ligand to its receptor initiates a series of proteolytic cleavages leading to the release of the Notch intra-cellular domain (NICD) by γ-secretase enzyme. This active, NICD migrates to the nucleus and interacts with the transcriptional repressor, recombination signal binding protein-Jκ (RBP-Jκ). RBP-Jκ is converted to a transcriptional activator on the recruitment of co-activators such as p300 and master-mind-like (MAML), leading to the expression of downstream target genes regulating T cell responses. Intra-cellular domain of Notch also uses an alternative way to exert some of its effects via a RBP-Jκ – independent or "non-canonical" manner [164-166].

Notch signaling is known to be involved in T cell activation. Activation of T cells via TCR accompanied by co-stimulation generates intra-cellular Notch domain in $CD4^+$ T cells and γδ T cells while inhibition of Notch signaling with γ-secretase inhibitors (GSI) decreases T cell activation and proliferation [37, 167, 168]. Upon TCR stimulation, Notch signaling contributes to regulation of $CD4^+$ T cell immune responses by modulating CD25 expression [167]. Signaling through the TCR in both $CD4^+$ and $CD8^+$ T cells induces the activation of Notch-1 [36, 37]. Notch activation has been shown to be important for NF-κB activation and proliferation as well as IFN-γ production by αβ T cells [37]. Notch signaling is involved in

cytolytic effector function in CD8+ T cells by regulating the expression of key effector molecules, perforin and granzyme B [36]. Notch signaling was shown to be required for potent anti-tumor immunity. Notch-2 was found to be essential for mediating anti-tumor immunity by $CD8^+$ T cells [169]. This suggests that TCR and Notch signaling pathways interact with each other to induce proliferation and homeostasis of T cells. However, the point in the TCR signaling cascade at which Notch signaling converges remain to be determined.

Notch acts as a critical regulator of TCR signal strength. Stimulating T cells with increasing concentrations of either anti-CD3 mAb or antigen pulsed APCs, increases the amount of N1ICD in proportion to increasing TCR signal strength [170]. Thus, inhibiting the Notch signaling at suboptimal condition will induce a state of anergy by increasing threshold of TCR stimulation. An anergizing stimulus leads to a sustained increase in intracellular free calcium, which induces dephosphorylation and nuclear translocation of the nuclear factor of activated T cells (NFAT) family of transcription factors. This occurs in a calcineurin dependent fashion in the absence of full activation of activator protein 1 (AP-1) complexes [171-173]. NFAT proteins play a key role in the induction of tolerance in T cells by driving the expression of anergy-inducing genes [173]. The specific expression of these genes is required to inflict a state of functional unresponsiveness through different mechanisms. Anergizing stimuli majorly upregulate the expression of three E3-ubiquitin ligases: the Casitas B-lineage lymphoma b (Cbl-b), the itchy E3 ubiquitin protein ligase homolog (Itch), and the gene related to anergy in lymphocytes (GRAIL) leading to the downregulation of TCR signaling by inactivation or degradation of signaling molecules [174-177]. During anergy, caspase 3 an effector member of the caspase family is also expressed. It recognizes proteins with a common DXXD motif and cleaves after the second aspartic residue [178].

Caspase 3 in non-apoptotic and anergic T cells induces proteolytic inactivation of signaling proteins to block TCR signaling to maintain T cell tolerance [179].

Tumors cause global T cell unresponsiveness or anergy, possibly related to paraneoplastic secretion of immunosuppressive cytokines such as transforming growth factor-β (TGF-β). Unusual defects in TCR-associated signaling mechanisms have been observed in tumor-bearing animals, such as absence of TCR CD3-ζ chain [144]. In part it could be associated with defective Notch signaling in T cells of cancer patients. Downregulation of Notch signaling in T cells has been shown to help tumors to evade the immune recognition. The selective activation of DLL1-Notch signaling pathway in bone marrow precursors to initiate T-cell activation reduced tumor growth [180]. Notch-2 signaling in $CD8^+$ T cells is required for generating potent antitumor CTLs, thus providing a crucial target for augmenting tumor immune responses [169]. However, MDSCs through nitric oxide-dependent mechanisms are reported to block the expression of Notch-1 and Notch-2 in T cells of cancer patients. Rescue of N1ICD expression in T cells was found to render T cells resistant to the tolerogenic effect induced by MDSC *in vivo* and increase the efficacy of T cell based immunotherapies [181]. This suggests a potential role of Notch signaling in regulating the antitumor immunity mediated by the T cells. Understanding the dynamic crosstalk between TCR and Notch signaling will provide new avenues in generating the effective immunotherapeutic protocols.

2.6 Immunosuppressive tumor derived factors

Tumors can evade immune clearance by secretion of immunosuppressive factors which play an important role in immune suppression. Tumor-secreted factors can inhibit differentiation of dendritic cells (DCs), induce expansion of immature myeloid cells and impair T cell activation. Several different tumor-derived factors including VEGF, GM-CSF, IL-10, IL-6, TGF-β, prostaglandins, gangliosides, tumor-derived soluble MHC class 1 chain related (MIC) ligands, Indoleamine 2, 3-dioxygenase **(**IDO), TNF-α have been implicated in these

processes [182-184]. Vascular endothelial growth factor (VEGF) is produced by most tumors and has a crucial role in the development of tumor neovasculature. Increased levels of VEGF in cancer patients are involved in tumor-induced abnormalities of DC differentiation and increase in number of immature myeloid cells [185]. Standard levels of Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) are required for normal myelopoiesis and DC differentiation. However, excessive amounts of this growth factor could exert immunosuppressive effects by the induction of immature myeloid cells [186].

Various tumor cells, tumor-infiltrating lymphocytes (TILs) and tumor associated macrophages (TAMs) express and release Interleukin-10 (IL-10), which is a potent modulator of differentiation and antigen-presenting function of macrophages and dendritic cells [187]. Tumors, through production of IL-1,0 may inhibit a broad range of macrophage functions including tumor cytotoxicity, production of IL-12 and nitric oxide (NO) [188]. IL-10 can also suppress T cell proliferative capacity in response to antigens and to the superantigen staphylococcal enterotoxin B [189]. Macrophage-Colony Stimulating Factor (M-CSF) and IL-6 inhibits the differentiation of myeloid progenitor cells into DCs and promote their commitment to weak antigen presenting cells of monocytic origin [190]. Gangliosides are ubiquitous membrane-associated sialic-acid-containing glycosphingolipid, which are involved in the regulation of cellular proliferation and differentiation. Most tumor cells synthesize and shed large amounts of gangliosides into their microenvironment and, eventually into the circulation. Tumor-derived gangliosides are potent modulators of myeloid cell proliferation and its differentiation into APCs [191].

Tumor-derived TGF-β has pleiotropic consequences and many tumors are known to secrete TGF-β, which is the most potent immunosuppressive cytokine described to date. TGF-β has been described to regulate the immunosuppressive activity of immature myeloid cells [192]. Tumor or stromal cell derived TGF-β also abrogates T cell differentiation, proliferation and

effector function, in part, through the loss of IL-12 production or by altering the cytolytic capability of cytotoxic T cells [193, 194]. The production of IL-4 and IL-10 by TGF-β stimulated cells can also tilt the immune response towards an inappropriate Th2 phenotype [193].

The release of MIC protein from the surface of tumor cells into the circulation is one mechanism hypothesized to prevent NKG2D-mediated tumor destruction [195]. Soluble MIC protein has been identified in many human tumor types including breast, lung, colon, and ovarian carcinoma, glioma, neuroblastoma, leukemia, and melanoma [196]. A correlation between the presence of soluble MHC class 1 chain related A **(**MICA) in the sera of MICA expressing tumors and the level of NKG2D downregulation on tumor infiltrating and peripheral $CD8^+$ T cells has been reported [183]. This downregulation of NKG2D leads to the severe impairment in the responsiveness of tumor-antigen-specific effector T cells promoting tumor immune evasion. IDO is an enzyme that specifically catabolizes tryptophan, an amino acid essential for T cell viability and proliferation [184]. IDO causes immunosuppression through breakdown of tryptophan in the tumor microenvironment and tumor-draining lymph nodes. The depletion of tryptophan and generation of toxic catabolites renders the effector T cells inactive and induces immune-suppressive property in dendritic cells [197].

2.6.1 Tumor necrosis factor-α

Tumor necrosis factor-α (TNFα) is a pleiotropic pro-inflammatory cytokine that plays a central role in inflammation, apoptosis and immune system development. TNF-α is shown to play a dominant role in rheumatoid arthritis (RA), which is a chronic autoimmune inflammatory disorder characterized by inflammation of synovial tissue, leading to progressive damage, erosion of adjacent cartilage and bone and chronic disability [198, 199]. TNF-α has also been implicated in tumor-cell growth and stromal interactions that facilitate tumor invasion and metastasis. Although TNF-α is capable of initiating a tumor apoptotic

response, these pathways are frequently deactivated within tumor cells by manganese superoxide dismutase, which scavenges oxygen free radicals induced by TNF-α [200, 201]. Chronic exposure to TNF-α has been shown to attenuate TCR-signaling and to down-modulate T cell proliferative responses and cytokine production *in vivo* and *in vitro* in patients with RA [202, 203]. In murine models results suggested that the inhibitory effect of TNF-α is due to its ability to regulate the expression of TCR CD3-ζ chain homodimers and reduced surface expression of TCR-CD3 complexes [204]. Elevated concentrations TNF-α are detected in pathologies characterized by chronic inflammation [205]. Administration of etanercept (TNF-α antagonist) during early chronic inflammatory stages reduces MDSCs' suppressive activity and enhances their maturation into DCs and macrophages, resulting in the restoration of *in vivo* immune functions and recovery of CD3-ζ chain expression on T cells [206]. Thus, TNF-α has a fundamental role in promoting an immunosuppressive environment generated during chronic inflammation.

2.7 Induction of immunosuppressive cells

Importantly, tumors also promote the accumulation of immunosuppressive cells which inhibit T cell effector functions. The most common of these include myeloid-derived suppressor cells (MDSCs), tumor associated macrophages (TAMs), regulatory T cells (Tregs) [207]. Elevated levels of MDSC and Tregs have been reported in the blood of cancer patients bearing several types of tumors. Their elimination is thought to be crucial in breaking immune tolerance to self-antigens, and thus clinical strategies aimed at their depletion are currently under investigation in cancer patients [208, 209].

The phenotype and function of MDSCs, TAMs and Tregs as well as the cytokines/growth factors important for their accumulation in the tumor-bearing host will be described thoroughly in the remaining part.

2.7.1 Myeloid derived suppressor cells

Hematopoietic stem cells (HSCs) lead to the generation of all mature blood-cell types. Mature blood cells are majorly classified into two separate lineages: myeloid and lymphoid. The myeloid lineage includes a number of morphologically, phenotypically, and functionally distinct cell types including different subsets of granulocytes (neutrophils, eosinophils, and basophils), monocytes, macrophages, erythrocytes, megakaryocytes, and mast cells. The lymphoid lineage consists of T, B and Natural killer (NK) cells [210]. Dendritic cells (DCs) have a unique developmental program that can be activated from either the myeloid or the lymphoid pathways [211, 212].

In case of myeloid lineage under steady state, myeloid cells develop in a step-wise fashion from long term HSC (LT-HSC) to short-term hematopoietic stem cells (ST-HSC) to multipotent progenitor (MPP) cells, which together compose the Lineage$^-$Sca1$^+$c-Kit$^+$ (LSK) fraction of the BM. MPP mature into common myeloid progenitors (CMP) or lymphoid primed MPP, that further develop through a series of increasingly committed progenitor cells, such as the GMP and CDP, to give rise to mature myeloid cells [210]. During inflammation or immunologic stress, myelopoiesis/haematopoiesis shifts towards emergency haematopoiesis. This process is co-opted by tumors to enhance the generation of tumor-promoting myeloid cells [213]. Cancer-driven myelopoiesis stimulates expansion of tumor promoting myeloid populations, mostly myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs).

Extramedullary haematopoiesis (EMH) and neutrophilia is a characteristic feature of tumor progression which aid in immune evasion and tumor vascularization [214, 215]. Increase in circulating myeloid cells in tumor bearing hosts was originally referred to as reactive neutrophilia/emergency granulopoiesis or emergency haematopoiesis/emergency neutro-monocytopoiesis [216]. Naturally, this occurs within the host macro-environment and is

associated with increased abnormal myeloid cell differentiation. These increased abnormal myeloid cells were earlier described as veto cells, null cells or as natural suppressor (NS) cells and they were later reported to inhibit lymphocyte numbers and cytotoxic T lymphocyte (CTL) functions [217]. In recent years, a concept of myeloid derived suppressor cells was introduced to reflect the immature myeloid cells and abnormal nature of myelopoiesis in cancer.

MDSCs are a heterogeneous population of cells of myeloid origin that comprises myeloid progenitor cells and immature macrophages, immature granulocytes and immature dendritic cells [218]. Features that are common to all MDSCs are their myeloid origin, their immature state and a remarkable ability to suppress T-cell response.

2.7.1.1 Phenotype of myeloid derived suppressor cells

MDSCs consist of a heterogeneous population of myeloid cells arrested at early stages of differentiation bestowed with immunosuppressive activity. They encompass myeloid progenitors and immature myeloid cells, arising in cases of chronic infection, trauma, sepsis, or tumor [218] **(Fig. 2.2**). MDSCs are characterized in mice by the co-expression of the myeloid-cell lineage differentiation antigen Gr1 and CD11b, also known as αM-integrin and by the absence of expression of CD11c, F4/80, and MHC Class II (major histocompatibility complex class II). In humans, however, the cell markers of MDSCs are CD11b and CD33 but lack MHC class II molecule HLA-DR [219]. Currently, additional phenotypic markers including CD66b, CD62L, CD16 , CD14 , CD15, vascular endothelial growth factor receptor 1 (VEGFR1; also known as FLT1) expression and S100A9 expression are further used to classify MDSCs [219, 220]. In both humans and murine models, however, MDSCs can be divided into two major subsets: granulocytic (Gr-MDSC) and monocytic populations (Mo-MDSC). MDSCs present in steady-state conditions have lesser ability to induce suppression of activated T cells and can quickly differentiate into mature granulocytes, macrophages or

dendritic cells (DCs). In healthy individuals, immature myeloid cells (IMCs) or MDSCs constitute ~0.5-1% of peripheral blood mononuclear cells but in the blood of patients with different types of cancer a tenfold increase in MDSC numbers has been detected [221-223]. Though initial observations of MDSCs expansion were documented in cancer bearing hosts, expansion of immunosuppressive MDSCs is now reported in multiple pathological inflammatory conditions. The accumulation of MDSC in cancer has been shown to correlate with an increased clinical cancer stage, increased tumor progression, poor prognosis and resistance to therapy [224].

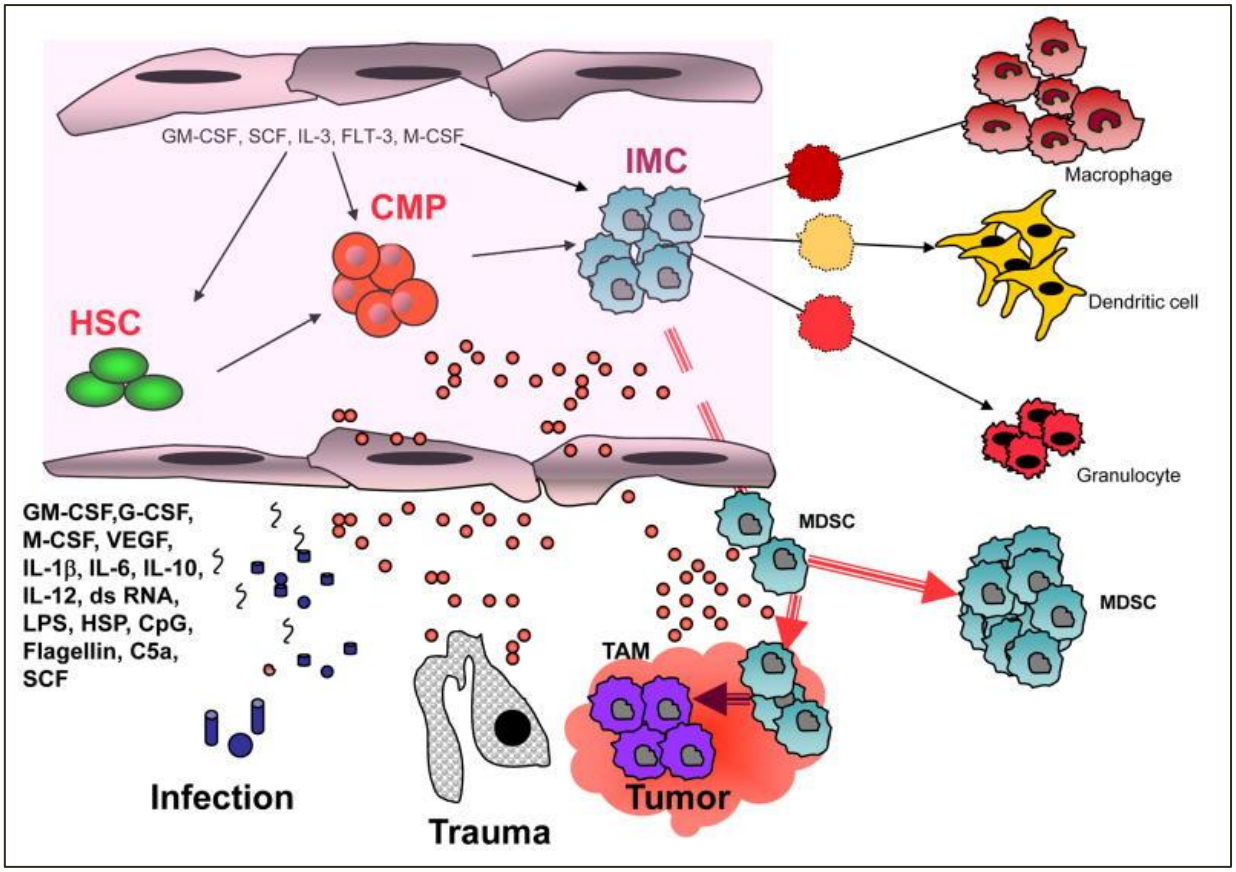

Figure 2.2: The origin of MDSCs: Factors produced in the tumor microenvironment and/or during acute or chronic infections, trauma or sepsis, promote the accumulation of IMCs at these sites, prevent their differentiation and induce their activation. These cells exhibit immunosuppressive functions and are therefore known as myeloid-derived suppressor cells (MDSCs). (**Source:** Dmitry I Gabrilovich and Srinivas Nagaraj**, Nat Rev Immunol,** Volume 9, 2009, 162-174)

2.7.1.2 Expansion of myeloid derived suppressor cells in cancer

MDSCs are expanded in a variety of pathological conditions including infection, trauma, or the presence of a tumor**.** Moreover, MDSC expansion has been shown in various organs including the peripheral blood, spleen, lymph nodes and bone marrow, as well as within the tumor compartment [225, 226]. MDSC expansion in cancer can be attributed to a number of factors but, the basic induction process is the myelopoiesis. The unregulated induction of myelopoiesis in cancer, attributed to the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) by tumor cells, leads to their accumulation. In order for these newly formed myeloid cells to become MDSCs they must be activated. Activation will have two results: first it will ensure that the cells are kept in a state of immaturity, second they will gain suppressive function [227]. Activation of MDSCs can result from exposure to multiple factors, which are produced by T cells, stromal cells, or tumor cells. Some of the factors which are known to cause the activation of MDSCs include stem cell factor (SCF), IL-1β, macrophage colony-stimulating factor (M-CSF), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), IL-10, IL-12, and IL-13 [218].

Most of these cytokines trigger signaling cascades that converge into a common signaling pathway, the Janus tyrosine kinase (JAK) protein family members and signal transducer and activator of transcription 3 (STAT3), which are signaling molecules that are involved in cell survival, proliferation, differentiation and apoptosis [228]. STAT3 is a member of the STAT family of transcription factors comprising of STAT-1,2,3,4,6, and the closely related STAT5A and STAT5B. Engagement of cytokine receptors activates JAKs which subsequently recruit and phosphorylate STAT members [227]. STAT3 is constitutively activated in tumor cells and in diverse tumor-infiltrating immune cells leading to inhibition of proinflammatory cytokine, reduced chemokine production, and enhances release of factors

that downregulate the immune response. Hyper-activation of STAT3 is also observed in MDSCs from tumor-bearing mice and its persistent activation prevents myeloid progenitors from differentiating [229, 230]. An *in vitro* study showed that exposure of hematopoietic progenitor cells (HPCs) to supernatants from tumor-cell cultures results in the accumulation of Gr-1^{+}CD$11b^{+}$ MDSCs and diminution of mature DCs. Inhibition of STAT3 activity in the HPCs restored the ability of MDSCs to differentiate into mature DCs [229]. Thus, hyper-activation of STAT3 in MDSCs promotes their expansion in tumor-bearing animals; however other potential outcomes stemming from STAT3 signaling in MDSC remain to be elucidated.

The list of inflammatory mediators implicated in MDSC expansion includes the complement protein C5a, bioactive lipid PGE_2, and the family of calcium binding proteins S100A8/A9 [231, 232]. A potent inflammatory mediator produced by many tumors and implicated in MDSC expansion are PGE2 molecules. Use of Cox-2 inhibitors to block the PGE_2 pathway in tumor bearing mice decreases the numbers of MDSCs and delays progression of cancer [233]. Myeloid progenitors express receptors for the S100 family members and accumulating evidence confirms their role in MDSC expansion during infection and inflammation [234].

2.7.1.3 MicroRNAs as potential regulators of MDSCs

In the hematopoietic system, microRNAs (miRNAs) have emerged as important regulators of myeloid lineage development and differentiation [235]. Recent studies have begun to unravel the significance of individual miRNAs in MDSC biology. miRNAs are noncoding single-stranded RNAs approximately 22 nucleotides long that regulate gene expression [235]. miRNAs enhance and inhibit MDSC accumulation and suppressive potency. For example, miRNAs 146a and 223 prevent MDSC accumulation [236, 237]. miRNA-146a blocks inflammation, while miRNA-223 is needed for the development of granulocytes. In contrast, miRNAs 494, 155, and 21 facilitate the accumulation of MDSCs. miRNA-494 induces MMPs 2, 13, and 14 which drive MDSC growth and survival signals, and by inhibiting

phosphatase and tensin homolog (PTEN), promote STAT3 activation [238]. miRNAs 155 and 21 promote MDSC accumulation by activating STAT3, which, as previously discussed, drives both MDSC accumulation and suppressive potency [239]. miRNAs also negatively regulate MDSC suppressive function. These include miRNAs 17-5 and 20a which silence STAT3, thereby reducing MDSC production of ROS and hydrogen peroxide (H_2O_2) [240].

2.7.1.4 Mode of action of MDSC and mechanism of T cell suppression

MDSCs use multiple mechanisms to suppress T cell activation which has been extensively studied and proven by many research groups [231, 241]. Factors implicated in MDSCs mediated T cell suppression include ROS [40], regulation of L-arginine metabolism [242], production of IL-10 [243], TGF-β [244], depletion of cysteine [245], down regulation of L-selectin surface protein on T cells [246] and others.

Increased production of ROS by MDSCs is main immunosuppressive mediator and inhibition of ROS generation abrogates the suppressive function of MDSCs *in vitro* [247]. The primary stimuli promoting ROS production in MDSC is interaction with T cells mediated by the integrins CD11b, CD18 and CD29. Blocking of this interaction has significantly reduced the ROS production by MDSCs [248]. The peroxynitrite produced from superoxide and NO results in the nitration of the T-cell receptor and CD8 molecules, altering specific peptide binding and rendering T cells unresponsive to antigen-specific stimulation [40]. Although studies have suggested role of ROS molecules in tumor-mediated immune suppression, the mechanism leading to generation of ROS by MDSC remains to be elucidated.

Metabolism of the amino acid L-arginine has been implicated in the suppressive activity of MDSCs by limiting the expansion and function of T cells [249]. L-arginine serves as a substrate for two distinct but related enzymes arginase 1(Arg1) and inducible nitric oxide synthase (iNOS). Consumption of L-arginine by MDSC produced Arg1 leads to the downregulation of the CD3-ζ chain of the T cell receptor [250]. Arg1 expression is induced

in myeloid cells by exposure to the Th2 cytokines IL-4 or IL-13, TGF-β, and GM-CSF [39, 218, 251]. L-arginine is also the substrate for a family of enzymes known as iNOS. Similar to Arg1, the production of iNOS by monocytic MDSCs also disrupts T cell activation through the suppression of CD3-ζ chain. These enzymes catalyse the reaction between oxygen and L-arginine, generating L-citrulline and NO and NOS inhibitors were found to reverse immune suppression mediated by NO [252, 253]. NO operates through various mechanisms to suppress T cell function, it interferes with the IL-2R signaling pathway by blocking the phosphorylation of signal-transducing pathways coupled to IL-2R and by altering the stability of IL-2 mRNA [254-256].

Additional proposed mechanism utilized by MDSCs to regulate immune function of T cells is deprivation of the amino acid cysteine [245]. Cysteine is essential for T cell activation and T cells depend on APCs during antigen presentation for cysteine. Macrophages and DCs import cystine, a cysteine precursor, from their environment and metabolize it into cysteine. MDSC import cystine at comparable rates as APCs but fail to export cysteine into their surroundings. Thus, through the competition for cystine, MDSCs make their immediate environment cysteine-deficient, because of which T cells are unable to synthesize the necessary proteins for activation [218, 245].

For extravasation, naïve T cells use L-selectin (CD62L) and migrate from the blood and lymphatics to lymph nodes and inflammatory locales, such as tumor microenvironments [257]. However, in cancer patients and animal models, circulating naïve lymphocytes typically express low levels of L-selectin which has been inversely correlated with the levels of MDSCs in tumor bearing mice [246].

2.7.1.5 Depletion strategies for MDSC in cancer

Due to the critical role of MDSC in the process of tumor-induced immune escape, depletion of MDSCs in cancer patients has become an increasingly attractive strategy. The suppression

of MDSC through therapeutic manipulation may promote tumor-specific immunity, foster activity of innate immune cells, and most importantly, may improve efficacy of existing therapies. For these reasons multiple depletion strategies have been evaluated including inhibiting MDSC expansion, promoting MDSC differentiation, inhibiting their suppressive function, and directly eliminating MDSC [218] .

Treatment of tumor-bearing mice with Celecoxib, a Cox-2 inhibitor has shown decreased numbers of MDSCs in glioma, mesothelioma, pancreatic adenocarcinoma, and intestinal tumors [258-260]. Cox-2 inhibitors have also shown some efficacy in suppressing the function of MDSC in both breast and lung cancer tumor models [261, 262]. The Cox-2 inhibitor was shown to decrease the production of arginase 1 by MDSCs, reduce ROS and NO production by MDSCs and enhanced T cells anti-tumor immunity [258].

Another strategy utilized to target MDSC is to promote their differentiation into mature myeloid cells which have antigen presenting capability. The goal of this approach is to force MDSC to become pro-inflammatory cells, which can then foster tumor immunity. The most promising compound which has been investigated is the vitamin-A derivative all-*trans* retinoic acid (ATRA) [263]. Administration of ATRA has been shown to induce the differentiation of MDSCs into mature dendritic cells and macrophages both *in vivo* and *in vitro* [223, 264, 265].

Treatment of tumor bearing mice with Sildenafil, a Phosphodiesterase-5 inhibiting agent decreased the expression of Interleukin 4 receptor α (IL-4Rα), Arg1 and NOS2 by MDSC. These molecules are linked to MDSC-mediated T cell suppression and their inhibition led to the increased anti-tumor immunity which was due to both a decreased number of circulating MDSCs as well as impaired suppressive function [252, 266]. The well-known chemotherapeutic drug gemcitabine has been shown to eliminate MDSCs in a selective manner and the observed decrease in MDSCs was followed by an increase in anti-tumor

immunity [267, 268]. Another chemotherapeutic drug, 5-Fluorouracil (5-FU), has also been shown to specifically eliminate MDSCs [269]. Interestingly, the use of the tryrosine kinase inhibitor sunitinib has proven to prevent both the accumulation of MDSC and impede their suppressive function. It is thought that sunitinib decreases the accumulation of MDSC by inhibiting c-Kit ligand stimulation, which is required for MDSC accumulation [270, 271]. It is important to note that not all chemotherapeutic drugs will act to reduce MDSC population. For example, doxorubicin-cyclophosphamide regimens have been shown to increase the number of circulating MDSC [272]. People have also used different strategies to block the mobilization of MDSCs to the tumor site. These include Vemurafenib (inhibitor of $BRAF^{V600E}$), Anti-G-CSF (Granulocyte- colony stimulating factor) and anti-Bv8 (prokineticin-2) antibodies, Anti-CSF-1 receptor (CSF1R) (GW2580), Anti-CCL2 antibody and CXCR2 and CXCR4 antagonists [273]**.** Immune evasion is a hallmark of cancer, and the literature provides substantial evidence that MDSCs are important in the biology of tumor progression and immune evasion. Greater insights into the biology of MDSCs would help in the development of MDSC-targeting therapy with a potential to greatly enhance the effectiveness of antitumor immunity.

2.7.2 Tumor associated macrophages

Macrophages are known as the first line of defense against pathogens and the activation of adaptive immune responses. Macrophages play vital role in many physiopathological processes including 1) phagocytosis and destruction of pathogens or altered cells; 2) modulation of inflammation by acquiring different phenotypes or acting in concert with other immune cells; 3) and antigen processing and presentation [274]. Macrophages are thought to originate from the monocytes derived from bone marrow haematopoietic stem cells (HSCs), in the presence of granulocyte–macrophage-colony stimulating factor (GM-CSF) [275]. Monocytes leave the bone marrow and circulate into the bloodstream. Monocytes are

recruited by different chemo-attractants into various tissues, where they differentiate into different tissue macrophages, for example alveolar macrophages in lung, Kupffer cells in liver, osteoclasts in bone, etc. [276, 277]. However, there are reports indicating that some resident tissue macrophages developed in the embryo before the appearance of HSCs [278-280].

The functions of macrophages are largely dependent on the microenvironments in which they are located. Macrophages are broadly classified into two types- classically activated macrophages (M1 type) and alternatively activated macrophages (M2 type) [281]. The M1 macrophages response to stimulation with the bacterial membrane component lipopolysaccharide (LPS) and Th1 lymphocyte-derived IFN-γ, both of which result in the production of cytotoxic mediators such as nitric oxide (NO) and superoxide ($O2^-$), and inflammatory cytokines and chemokines such as IL-12, IL-23 and IL-1β, which play important roles in pathogen killing and clearance [282]. Generally, it is considered that M1 macrophages mainly drive host immune system to kill pathogens and destroy tumor cells. By contrast, M2 macrophages when stimulated with LPS alone produce low levels of pro-inflammatory cytokines, whereas they respond strongly to IL-4/IL-13 to generate anti-inflammatory cytokines, most notably IL-10, TGF-β, proangiogenic and matrix-remodelling factors such as VEGF, matrix metalloproteinases (MMPs) and arginase 1 [283-286]

2.7.2.1 Role of tumor-associated macrophages (TAMs) in cancer

Tumors, in particular solid tumors, are characteristically formed by both tumor and stromal cells, and the latter constitute a dynamic and complex system, termed the tumor microenvironment that functions to foster tumor cells, and is comprised of endothelial cells of the blood and lymphatic vessels, fibroblasts and a diverse selection of immune cells including neutrophils, basophils mast cells, macrophages, dendritic cells, and MDSCs, etc. [287, 288]. Macrophages present in tumor tissue, termed tumor-associated macrophages, are generally

the most abundant immune cells present in the tumor microenvironment. Although earlier studies showed that optimally activated macrophages could kill the cancer cells *in vitro* [289], it is now widely recognized that TAMs with an M2 phenotype possess a variety of tumor-promoting functions that support tumor progression. These include inducing tumor angiogenesis, sustaining the proliferation and survival of cancer cells, facilitating tumor invasion and metastasis, and generating the immunosuppressive microenvironment that favours tumor growth [277, 290, 291]. The emerging approaches to polarize macrophages to M1 phenotype may offer promising and novel strategies for developing cancer immunotherapy.

2.7.3 Regulatory T cells

Regulatory T cells (Tregs) can be described as a T-cell population that functionally suppresses an immune response by manipulating the activity of other immune cells. Gershon was first to report the subset of T cells having suppressive function [292, 293]. However, Sakaguchi and his colleagues led to the rejuvenation of this field after reporting that a small group of T cells co-expressing CD4 and the IL-2 receptor-α chain, CD25 maintain self-tolerance. Breakdown of this tolerance by interfering with Treg function could lead to autoimmune disease [294, 295]. These $CD4^+CD25^+$ T cells were named “regulatory T cells” which were earlier termed as “suppressor T cells”. These two terms are used interchangeably but the term "regulatory T cells" is favoured. The field of Treg cell biology was greatly enhanced by the unearthing and characterization of the Treg-specific gene, FoxP3 [296]. FoxP3, a key intracellular marker is also crucial for the development and functioning of $CD4^+CD25^+$ regulatory T cells [296-298]. In humans, the CD127, the α-chain of the interleukin-7 receptor, was used in combination with $CD25^+$, $FoxP3^+$ to better define the Treg cell population with suppressive function. CD127 expression is reported to be inversely

correlated with expression of FoxP3 and the suppressive potential of $CD4^+$ Treg cells [299, 300].

In the periphery, naturally occurring Tregs represent around 6–10% of total $CD4^+$ T-cell population. In order to be sustained, Tregs need uninterrupted TCR triggering and co-stimulation in the presence of IL-2 [301, 302]. Naturally occurring $CD4^+CD25^+$ Treg cells have been expansively studied in mice and humans and until now, there are at least three recognized subsets of $CD4^+CD25^+$ Treg cells involved in the negative regulation of immune response, these include $CD4^+CD25^+FoxP3^+$, type 1 Treg cells (Tγ1) secreting IL-10 and Th3 cells secreting TGFβ [303].

2.7.3.1 Tregs in tumor microenvironment

The primary function of Treg cells was originally defined as to avoid autoimmune diseases by maintaining self-tolerance [294]. Regulatory T cells are now associated with the development of allergy, rejection of organ transplants, and the suppression of immune responses to cancer [304, 305]. There is an increased prevalence of $CD4^+CD25^+$ T cells in a wide spectrum of human malignancies like skin, lung, head and neck, ovarian, and gastrointestinal. These cells are found in relatively higher numbers in blood, ascites, within the tumor draining lymph nodes and tumor tissue of cancer patients [304, 306]. Importantly, the numbers of $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs present in tumors and, in particular, decreased ratios of $CD8^+$ T cells to $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs in tumors, correlate with poor prognosis in breast cancer [307], gastric cancer [308] , head and neck cancer [309] and ovarian cancer patients [310]. In general, Tregs identified from most human cancers possess potent ability to suppress immune responses in *in vitro* co-culture assays.

Three different, but not mutually exclusive, modes of Treg accumulation within the tumor bed can be envisaged: (i) increased trafficking, (ii) preferential Treg expansion and (iii) *de-novo* differentiation, where the latter two can occur either locally within the tumor

microenvironment. Tregs show an enhanced capacity for infiltration, and accumulation within the tumor in comparison to T effector cells (Teffs) [311]. The recruitment has been observed in many malignancies and has been shown to be dependent on chemokine-driven mechanisms, and several chemokines and their cognate receptors have been associated [312]. The chemokine receptors CCR4 and CCR8 are expressed by Tregs and the CCR4 ligand CCL22 has been shown to be produced by both tumor cells and tumor-infiltrating macrophages [313, 314]. Targeting these chemokine receptors may inhibit T_{reg} accumulation within tumors, however due to the receptor and ligand redundancy and promiscuity within the chemokine–chemokine receptor system provides a significant hurdle to immunotherapy. Other possibility is that the large amount of self-antigens provided by proliferating and dying tumor cells are recognized by Tregs which aid in their recruitment [315]. An inflammatory condition in tumors also recruits Tregs in CCL20-CCR6 dependent fashion [316]. A second mechanism could be through expansion of Tregs in the presence of IL-2 and TGF-β within the tumor mass or in the tumor draining lymph nodes (TDLNs). Presence of IL-2 and TGF-β within the tumor mass could promote Treg proliferation, development and homeostasis [317-319]. A third mechanism involving *de-novo* conversion of $FoxP3^{-}$ T cells into Tregs may play an important role in Treg accumulation in tumors [320]. The role of TGF-β in the induction of Tregs is well established and tumor cell-derived TGF-β can contribute to the induction of Tregs [321, 322].

A favourable state for effective immunotherapy will be produced in cancer patients after depleting Tregs. Modulating the function of Tregs represents one aspect to potentially enhance immunosurveillance. A number of agents affect Tregs in a clinical context, some of which are designed specifically to target known receptors on Tregs (like anti-CD25 or anti-CTLA4 antibodies). The indirect method includes conventional chemotherapeutic drugs like

low-dose cyclophosphamide or gemcitabine which have modulatory effects on Tregs, but the exact mode of action is often unknown [323, 324].

2.7.3.2 Mechanism of Treg function

A myriad of mechanisms has been proposed for Treg mediated immune suppression **(Fig. 2.3)**. Tregs majorly utilize the inhibitory cytokines like IL-10, TGF-β, and IL-35 to directly induce immune suppression [325]. Tregs can also use perforin/granzyme-mediated cytotoxicity as a mechanism to suppress the function of effector T cells [326]. Tregs isolated from cancer were found to express Fas ligand (FasL) and mediate killing of $CD8^+$ T cells on activation via TCR ligation and high-dose IL-2 [327]. Another study suggests that *in vitro*-activated mouse Tregs can induce apoptosis of $CD4^+$ effector T cells in a TRAIL/DR5 (tumor necrosis factor-related apoptosis inducing ligand/death receptor 5) dependent fashion [328].

In addition to directly inhibiting effector lymphocytes through inhibitory cytokines or cytotoxic molecules, Tregs may also suppress immune responses by modulating APCs. FoxP3 regulated cytotoxic T lymphocyte antigen 4 (CTLA-4) expression on Tregs can downregulate the costimulatory molecules (CD80, CD86) on antigen-presenting cells to suppress T cell activation [329, 330]. Besides suppressing the ability of APC to activate other T cells, CTLA-4 can induce the production of IDO in APCs, which can degrade the essential amino acid tryptophan leading to an inhibition of T cell proliferation [331]. In addition, IDO-mediated degradation of tryptophan can generate toxic metabolites that induce apoptosis in lymphocytes [332, 333]

Ectoenzymes like CD39 and CD73 preferentially expressed on the surfaces of Treg cells can catalyse the generation of adenosine from the extracellular nucleotide ATP or ADP [334]. Adenosine inhibits effector T cell function through activation of the adenosine receptor 2A [335]. Tregs harbour high levels of cyclic adenosine monophosphate (cAMP) which can potently inhibit IL-2 synthesis and T cell proliferation [336].

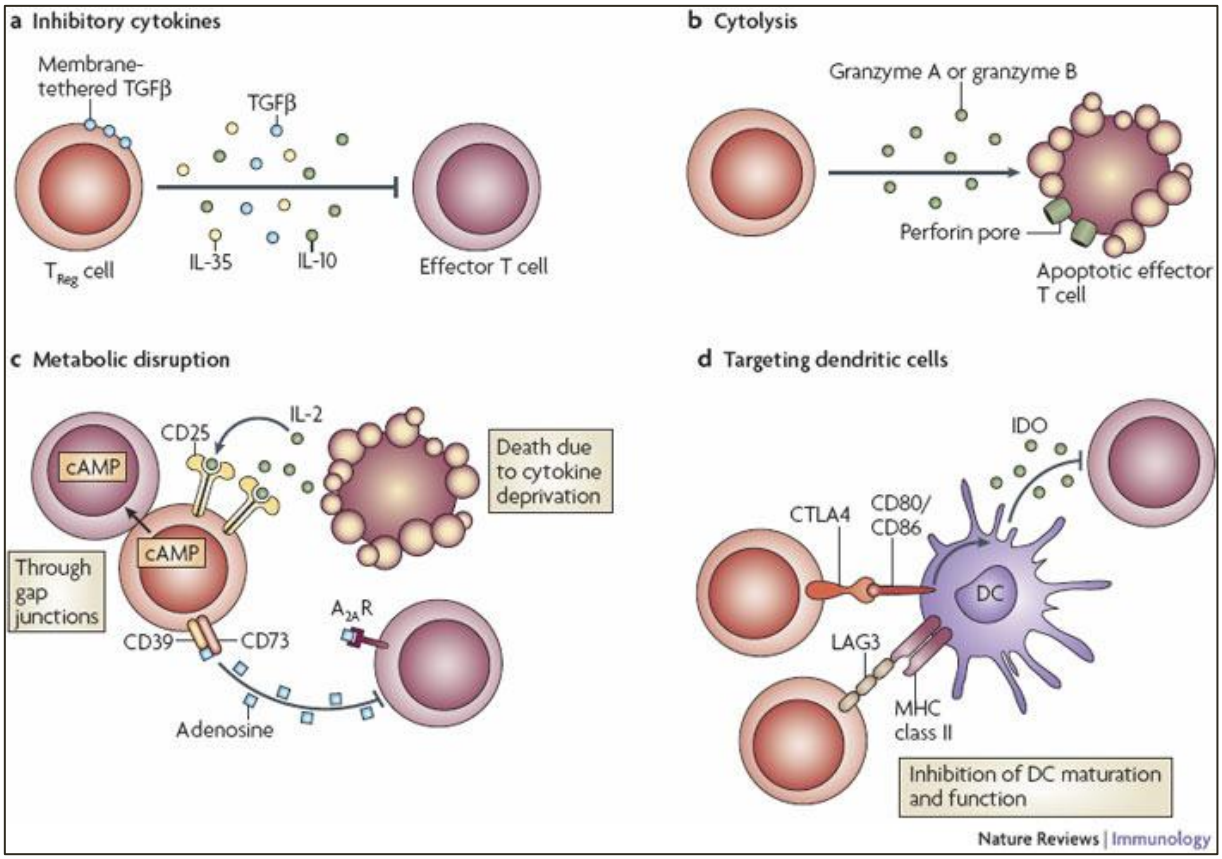

Figure 2.3: Basic mechanisms used by Treg cells: The various mechanisms used by regulatory T cell are centred on four basic modes of action **a)** Inhibitory cytokines (IL-10, TGF-β and IL-35), **b)** cytolysis, **c)** metabolic disruption and **d)** targeting dendritic cells. (**Source:** Dario Vignali, Lauren Collison and Creg Workman**, Nat. Rev. Immunol,** Volume 8, 2008, 523-532)

2.7.3.3 Therapeutic targeting of Tregs

Current strategies to target Tregs in cancer include Treg depletion as well as a blockade of the Treg immunosuppressive activity and migration. Treg depletion has been widely used to restore the anti-tumor immunity and to improve the efficacy of vaccination and adoptive cellular immunotherapy in cancer patients and tumor-bearing mice [337, 338]. Depletion agents used in clinical and preclinical studies include low-dose chemotherapeutics and anti-CD25 antibody (daclizumab) [339, 340]. However, current depletion strategies do not allow a specific Treg targeting since CD25 is also expressed on activated conventional T cells (Tcons) [339]. However, these approaches proved instrumental when Tregs significantly

outnumber activated Tcons in the tumor microenvironment. Therefore, Treg depletion might restore the activation, migration, proliferation and activity of remaining Tcons [337].

The high proliferation rate of Tregs makes them more sensitive to chemotherapy than effector T cells [339]. Therefore, certain chemotherapeutic regimens have led to a selective Treg depletion. Metronomic (low-dose) cyclophosphamide (CTX) administration has shown to improve anti-tumor immunity and vaccination efficacy through Treg depletion [341]. Several groups showed that prophylactic Treg depletion with anti-CD25 mAb in mice triggered the rejection of syngeneic tumors, although such treatment after the tumor inoculation was less effective. However, tumor rejection was substantially facilitated when the anti-CD25 mAb administration was combined with the DC vaccination, CTLA-4 blockade or glucocorticoid-induced TNFR-related protein (GITR) stimulation [340]. Since CD25 is expressed not only on Tregs but also on activated CD4 and CD8 T cells, anti-CD25 therapy may entail concomitant deletion of effector T cells.

FoxP3 is a "master regulator" transcription factor of Treg development and function, the vaccination against FoxP3 might lead to efficient Treg depletion, representing an interesting approach to neutralize the tumor-associated immune suppression [342]. Similar to many other markers, CTLA-4 expression is shared by regulatory and activated T cells [343]. There have been some conflicting data on the specific role of CTLA-4 expression in either subset. Mice with Treg-specific CTLA-4 knockout succumb to lympho-proliferation and autoimmune diseases similar to FoxP3 deficiency [344]. Nude mice reconstituted with splenocytes with CTLA-4-deficient Tregs showed an enhanced survival and decreased tumor growth as compared to the recipients of control splenocytes [344]. On the other hand, it has been demonstrated that although antibody-mediated CTLA-4 blockade on T effector cells significantly improved the survival of mice with B16 melanoma, a specific blockade of CTLA-4 expressed on Tregs alone had no antitumor effects [345]. The maximal prolongation

of survival was achieved by concomitant CTLA-4 blockade in both regulatory and effector T cells. In stage IV melanoma patients, the therapy with the anti-CTLA-4 antibody ipilimumab reduced Treg numbers and restored TCR-dependent proliferation of effector T cells [346].

In mice, Tregs coexpress ectoenzymes CD39 and CD73 producing immunosuppressive adenosine [347]. Over the last years, multiple studies implicated the adenosine production by Treg in their inhibitory activity, and showed that targeting adenosine synthesis in vivo was a promising strategy to diminish tumor-mediated immunosuppression [348, 349]. It has been well documented that the deficiency or inhibition of adenosine production and signaling significantly reduces the tumor growth by improving the anti-tumor immunity [348-352]. Thus, targeting adenosine production and signaling represents a promising strategy to restore spontaneous T cell-mediated anti-tumor reactivity and to improve the efficacy of adoptive cellular immunotherapy in cancer patients.

Chapter 3
Material and methods

3.1 Cell culture media

The cell culture media includes products that support the growth and maintenance of mammalian cells and cell lines. Plain media was prepared by dissolving powdered RPMI-1640 or IMDM or DMEM medium (Invitrogen Life-Technologies, Grand Island, N.Y.) along with sodium bicarbonate (Sarabhai Chemicals, India) and if required HEPES buffer (Sigma St. Louis, USA) in deionized water as per manufacturer's instructions. The plain medium was filtered by membrane filtration (0.45 μm, Millipore Co, USA), and was monitored for sterility.

To prepare complete medium, plain RPMI/IMDM medium was supplemented with 10% heat inactivated fetal bovine serum (FBS; Invitrogen Life Technologies, USA), penicillin (100 IU/ml; Alembic Chemicals India), streptomycin (100 mg/ml; Alembic chemicals India), mycostatin (5 mg/ml; Sigma, USA), gentamycin (40mg/ml; Schering Corpn, India), L-Glutamine (2mM, Hi-Media, India).

3.2 Maintenance of mammalian cells and cell lines

3.2.1 Passaging suspension cultures

For passaging suspension cells, the flasks were removed from the incubator. A small volume of evenly distributed cells in the medium was taken from the culture flask to determine the total number of cells and percent viability. According to the number of cells, volume of media was calculated and added to dilute the culture according to recommended seeding density. If needed, cell cultures were split into multiple flasks.

3.2.2. Passaging adherent cultures

The adherent cell lines were sub-cultured at 65-75% confluency. The cells were removed from tissue culture flasks or plates by dissociation reagent PBS-trypsin (Sigma, USA; 0.3% trypsin in 0.01M PBS pH 7.5, containing 0.02% EDTA, sterilized by Millipore filtration). Briefly, cells were washed using plain medium gently without disturbing the cell layer. Wash

solution was removed from the culture vessel and pre-warmed dissociation reagent was added to it. When 90% cells have detached from the flask, cells were collected and washed with plain medium before use. Cells were seeded at a density of $0.5x10^6$ cells per 5 ml of complete medium (containing 10% FCS) in 25 cm^2 flasks or 60 mm plates.

Table 3.1 List of Cell lines:

Name	Type	Origin	Medium
AW13516	Adherent	Tongue	IMDM+10%FCS
AW8507	Adherent	Tongue	IMDM+10%FCS
AW9803	Adherent	Retromolar	IMDM+10%FCS
SSC40	Adherent	Tongue	α-MEM+10%FCS
SSC29B	Adherent	Buccal Mucosa	α-MEM+10%FCS
HEK293	Adherent	Human Embryonic Kidney Cells	DMEM+10%FCS
Jurkat	Non Adherent	T lymphocyte	RMPI+10%FCS

3.2.3. Cryopreservation of cell lines

Cultured cells in log-phase of growth with at least 90% viability were pelleted by centrifugation at 1000 rpm. Cell pellet was resuspended in cold freezing medium of 10% Dimethyl sulphoxide (DMSO) + 90% FBS. The Freezing mixture was added slowly and drop-wise under constant mixing. $2\text{-}3x10^6$ cells/ml of freezing mixture were dispensed into sterile cryogenic storage vials (Nunc, Denmark) and stored for long term in liquid nitrogen.

3.2.4 Reviving of cells

Cryovials containing the frozen cells were removed from liquid nitrogen and immediately placed in water bath at 37ºC. Cells were quickly thawed by gently swirling the vial in 37ºC water bath. Cells were transferred to a centrifuge tube and pre warmed complete growth medium was added drop-wise into the centrifuge tube containing thawed cells. Cell suspension was washed twice and cell viability was checked using the vital dye trypan blue

(Filter sterilized 0.4% trypan blue (Fluke AG, Buchs SG, Switzerland) in normal saline with 0.01% thiomersal (BDH Lab. Reagents, U.K)).

3.3 Antibodies and Conjugates

Table 3.2: List of Purified Antibodies:

Sr No.	Purified antibody	Clone/catalog No.	Source
1	CD3-ζ chain	6B10.2	Santa Cruz Biotechnology,USA
2	CD3-ε	UCH-T1	Santa Cruz Biotechnology, USA
3	Caspase 3	8G10	Cell signaling technology, USA
4	OAS-2	W-16	Santa Cruz Biotechnology, USA
5	B-Actin	AC74	Sigma-Aldrich, USA
6	TNFR- I	F1A	R&D systems, USA
7	TNFR-II	1B	R&D systems, USA
8	NF-κB	D40E12	Cell signaling technology, USA
9	pNF-κB	93H1	Cell signaling technology, USA
10	p53	SC-55476	Santa Cruz Biotechnology,USA
11	DR5	D4E9	Cell signaling technology, USA
12	HSP 60	K19	Santa Cruz Biotechnology, USA
13	HSP 70	5G10	Santa Cruz Biotechnology, USA
14	HSP 90	68/Hsp90	BD Biosciences, USA
15	pSTAT3	4/P-STAT3	Cell signaling technology, USA
16	ZAP 70	LR	Santa Cruz Biotechnology, USA
17	Notch 1 intracellular domain	D1E11	Cell signaling technology, USA
18	Notch 2 intracellular domain	D76A6	Cell signaling technology, USA
19	SHC	ab15039	Abcam, USA
20	C/EBP alpha	D56F10	Cell signaling technology, USA
21	C/EBP beta	3092	Cell signaling technology, USA
22	Cox-2	N-20	Santa Cruz Biotechnology,USA
23	PDL-1	28-8	Abcam, USA
24	HIF 1 alpha	D2U3T	Cell signaling technology, USA
25	SDF 1 alpha	AF-310-NA	R & D Systems, USA

26	MIP 3 alpha	AF30	R & D Systems, USA
27	IDO	D5J4E	Cell signaling technology, USA
28	GRAIL	M-17	Santa Cruz Biotechnology, USA
29	p56Lck	2102	Santa Cruz Biotechnology, USA
30	CD3	UCHT1	BD Pharmingen, USA
31	CD28	CD28.2	BD Pharmingen, USA

Table 3.3: List of conjugated Antibodies:

Sr No.	Conjugated antibody	Clone/catalog No.	Source
1	CD3-PE	HIT3a	B D Biosciences, USA
2	CD3-FITC	HIT3a	B D Biosciences, USA
3	CD3-PECF594	UCHT1	B D Biosciences, USA
4	CD3-PerCp5.5	UCHT1	B D Biosciences, USA
5	CD3-PE Cy7	UCHT1	B D Biosciences, USA
6	CD3-APC-H7	SK7	B D Biosciences, USA
7	CD4-PECF594	RPA-T_4	B D Biosciences, USA
8	CD4-APC	RPA-T_4	B D Biosciences, USA
9	CD8-FITC	HIT8a	B D Biosciences, USA
10	CD8-PB	HIT8a	B D Biosciences, USA
11	CD11b-AF488	ICRF44	B D Biosciences, USA
12	CD14-PerCp	M5E2	B D Biosciences, USA
13	CD15-PB	HI98	B D Biosciences, USA
14	CD16-PE	3G8	B D Biosciences, USA
15	CD25-PE	2A3	B D Biosciences, USA
16	CD25-PerCp5.5	M-A251	B D Biosciences, USA
17	CD25- PECy7	M-A251	B D Biosciences, USA
18	CD33-PECF594	WM53	B D Biosciences, USA
19	CD34-PE	581	B D Biosciences, USA
20	CD38-APC	HIT2	B D Biosciences, USA
21	CD68- APC	Y1/82A	B D Biosciences, USA

22	CD66b-AF647	G10F5	B D Biosciences, USA
23	CD80-FITC	1610A1	B D Biosciences, USA
24	CD80-PE	1610A1	B D Biosciences, USA
25	CD124-PE	hIL4R-M57	B D Biosciences, USA
26	CD163-PE	GH1/61	B D Biosciences, USA
27	CD45RA-PECy5	HI100	B D Biosciences, USA
28	CD27-APC	M-T271	B D Biosciences, USA
29	GD-APC	B1	B D Biosciences, USA
30	GD-FITC	B1	B D Biosciences, USA
31	CD3-ζ-PE	6B10.2	Santa Cruz Biotechnology, USA
32	CD62L-APC	DREG-56	B D Biosciences, USA
33	pSTAT3- AF647	4/P-STAT34	B D Biosciences, USA
34	IL10-APC	JES3-19F1	B D Biosciences, USA
35	IL12-PE	20C2	B D Biosciences, USA
36	PDL1-PE	MIH1	B D Biosciences, USA
37	PDL2-APC	MIH18	B D Biosciences, USA
38	FOXP3-AF488	259D/C7	B D Biosciences, USA
39	CD127-V450	HIL-7R-M21	B D Biosciences, USA
40	CD127-APC	M-T271	B D Biosciences, USA
41	IL-17A-APC	N49-653	B D Biosciences, USA
42	IFN-γ-PECy7	4S.B3	B D Biosciences, USA
43	Perforin-FITC	δ-G9	B D Biosciences, USA
44	Granzyme-PE	GB11	B D Biosciences, USA
45	FasL-PE	NOK-1	Bio Legend, USA
46	CTLA 4-APC	L3D10	Bio Legend, USA
47	HLADR-PE	G46-6	B D Biosciences, USA
48	HLADR-APCH7	G46-6	B D Biosciences, USA

3.4 Study group

The study was approved by the Institutional Ethics Committee (ACTREC-IEC). As per protocol, 5-15 ml peripheral blood from oral cancer patients (n=84) was collected in EDTA-vacutainer prior to surgery. American cancer society TNM classification was used to classify

patients and patients with Stage I- IV were included in the study. Surgically resected tumor tissues (n=38) were obtained from oral cancer patients. Histopathological confirmation of the Tumor stage /grade was done at the pathology department of ACTREC**.** As controls, peripheral blood was collected from healthy individuals (n=65) in EDTA-Vacutainer. The biological samples were collected after obtaining informed consent from the patients and healthy individuals.

Table 3.4 Patient demographics:

Clinical Parameters	**Cases**
Gender	
Male	72
Female	12
Age (Yrs.)	
Range (Mean)	26-81 Yrs. (48.1)
Male	41-81 Yrs. (54.9)
Female	26-76 Yrs. (47.04)
TNM Stage	
I	07
II	14
III	09
IV	54
Subsite	
Buccal Mucosa	47
Tongue	17
Mandible	09
Others (Lip, Floor of mouth, Alveolus, Retromolar)	11
Habit	
No-Habit	07
Habit	73
NA(Details Not available)	04
Total	84

3.5 Separation of peripheral blood mononuclear cells (PBMCs)

Mononuclear cells were separated from heparinized venous peripheral blood by Ficoll-Hypaque (FH, Sigma, U.S.A.) density gradient centrifugation. Briefly, peripheral blood collected in EDTA-vacutainers (BD-bioscience) was diluted with equal volume of normal

saline (0.85% NaCl in double distilled (dd) water). Diluted blood was loaded on Ficoll-Hypaque [24 parts of 9% Ficoll 400 (Sigma, USA) + 10 parts 33.3% sodium diatrizoate (Sigma, USA), specific gravity adjusted to 1.077 ± 0.001] at 1:3 ratio. Culture tubes containing FH with overlaid blood was centrifuged at 1,500 rpm for 20 mins at room temperature (RT) using a swing-out rotor in Beckman centrifuge. Mononuclear cells were collected from the interface between FH and plasma; cells were washed thrice with sterile normal saline and viability was checked using trypan blue dye.

3.6 Separation of tumor infiltrating immune cells from surgically excised oral tumors

Tumors were collected in sterile plain RPMI (Invitrogen Life-Technology) medium supplemented with antibiotics (8% PSGM). The necrotic, haemorrhagic and fatty tissues were removed and tumor tissues were thoroughly washed with antibiotic containing plain RPMI. The tumor tissues were minced finely and incubated in plain RPMI containing enzyme mixture (0.05% collagenase, 0.02% DNase and 5U/ml hyaluronidase) (Sigma Aldrich), at 37ºC for 2 hrs with intermittent shaking. The tumor tissue was then passed through a 200-gauge wire mesh. The cells were washed with plain RPMI medium and were tested for cell viability. Cells with more than 90% viability were used to carry out further experiments.

3.7 Multi-colour flow cytometry

Flow cytometry was used to assess not only the frequency of cells present in complex mixtures but also quantitate expression of cell-surface proteins, intracellular phosphoproteins and cytokines as well as other functional readouts.

3.7.1 Staining protocol

PBMCs or tumor infiltrates or cultured cells ($0.5\text{-}1x10^6$) were rinsed in cold phosphate buffer saline (PBS) and cold-fixed in 1% paraformaldehyde in PBS for 15 mins at 4 ^oC. The cells were then washed and permeabilized for 15 mins with 0.1% saponin in PBS. $0.5\text{-}1x10^6$ cells

from peripheral blood/tumor tissue/culture cells were washed and resuspended in 50 µl of 0.1% saponin buffer and appropriate antibodies were added. Cells were further incubated for 30 min at 4°C in the dark. When purified/unconjugated antibodies were used, cells were washed with FACS buffer and incubated with conjugated secondary antibody for an additional 30 min at 4°C. After staining, all samples were washed in FACS buffer to remove unbound antibodies and resuspended in FACS buffer. Samples were acquired on FACSAria® or FACS Calibur flow cytometers (Becton Dickinson) and data was analysed using FlowJo software.

3.7.2 Immunophenotyping of immune cell subsets

Frequencies of T helper, cytotoxic T, gamma delta T, regulatory T and myeloid derived suppressor cells in peripheral blood mononuclear cells were compared between healthy individuals and oral cancer patients. Also extent of infiltration of these cell types into the tumor site was analysed using flow cytometry. Range of antibodies was used in multicolour flow cytometry to characterize these cell types in peripheral blood and tumor tissue of oral cancer patients. Following are the antibodies used to characterize different cell subsets. After Staining cells were acquired using FACSAria flow cytometry (Becton Dickinson), and 50,000-100,000 live cells (events) were collected to obtain reliable data.

In case of human αβ T cells, cells were gated on the basis of their forward and side scatter characteristics and the fluorescence intensity was measured. Purified αβ T cells which were either unstimulated or stimulated with α-CD3/CD28 mAb in presence or absence of GSI-X were stained. At different time points, cells were harvested and rinsed with cold phosphate buffer saline (PBS) and cold-fixed in 1% paraformaldehyde in PBS for 10 mins at 4 ^{o}C. The cells were then washed and permeabilized for 15 mins with 0.1% saponin in PBS and were stained with CD3-ζ chain, CD25, CD69, CD71 and phospho-tyrosine (pY) antibodies (BD Bioscience) for 45 min at 4 ^{o}C. In the case of Notch-1 intracellular domain (N1ICD) and

Notch-2 intracellular domain (N2ICD) expression cells were stained with sheep α-human N1ICD α-body and sheep α-human N2ICD (R&D Systems, Minneapolis, MN) for 45 min at 4 ^{o}C. Thereafter, cells were washed and incubated with FITC-labelled donkey α-sheep IgG for another 45 min at 4 ^{o}C (Sigma-Aldrich). The data was acquired on the FACSAria flow cytometer (BD Biosciences) with 50,000 events. Cells were analyzed using FlowJo software (Tree Star, Ashland, OR) and expressed as median fluorescence intensity (MFI).

The different immune cell subsets were also analyzed for diverse functional markers like CD3-ζ chain, perforin, granzyme B, PDL1, PDL2, CD124, CD34, CD38, IL-10, IL-12, pSTAT3, CD27, CD45RA, CD62L, CCR4, CCR6, CCR7, CXCR4, TNFRI, TNFRII

Table 3.5 Antibody panel for Flow cytometry

Subset	Antibodies with fluorochromes
T Cells subsets	CD3-FITC, CD4-PECF594, CD8-PB, GD-APC
Regulatory T cells	CD4-PECF594, CD25-PE, CD127-APC, FoxP3-AF488
Myeloid-derived Suppressor Cells	CD33-PECF594, CD11b-AF488, HLADR-APC-H7, CD66b-AF647, CD15-PB, CD14-PerCP, CD16-PE
Tumor-associated Macrophages	HLADR-APC-H7, CD80-FITC, CD68-APC, CD163- PE
Th17 Cells	CD3-PE, CD4-PECF594, IL17-APC, IFN-γ-PECy5
Tc17 Cells	CD3-PE, CD8-FITC, IL17-APC, IFN-γ-PECy5

FITC: Fluorescein isothiocyanate; **PE**: Phycoerythrin;
PerCP: Peridinin Chlorophyll Protein; **APC**: Allophycocyanin;
AF488: Alexa fluor 488; **AF647**: Alexa Fluor 647,
APC-Cy7: Tandem conjugate that combines APC and a cyanine dye (Cy7):
PE-Cy5: Tandem conjugate that combines phycoerythrin and a cyanine dye.
PerCP-Cy™5.5: Tandem conjugate that combines PerCP with a cyanine dye;

PE-Cy7: Tandem conjugate that combines phycoerythrin and a cyanine dye (Cy7):
APC-H7: APC-cyanine tandem fluorochrome, which uses an analog of Cy7 and has similar spectral properties to APC-Cy7;
PE-CF594: Tandem conjugate of PE and CF594, **PB:** Pacific Blue

3.8 Cell cycle analysis

For cell cycle analysis, a total of $1X10^6$ αβ T cells were incubated in the 24-well culture plate with α-CD3/CD28 mAb (5µg/ml, BD Biosciences) and recombinant IL-2 (rIL2, 10U/ml, PeproTech, Rocky Hill, NJ) for 72 hrs. For experiments using GSI-X, cells were pre-treated for 30 min at 37 ^oC with γ-secretase inhibitor-X, L-685,458 (GSI-X) (Calbiochem, La Jolla, CA) at a concentration of 10µM. Similarly, 1 x 10^6 of αβ T cells were left in the medium alone served as an unstimulated control. After 72 hrs, cells were harvested and fixed by adding chilled 70% ethanol dropwise under constant vortexing. Cells were washed and resuspended in 500 µl of PBS followed by addition of propidium iodide (PI) and RNase at a concentration of 40 µg/ml and 10 µg/ml respectively. Cells were incubated at room temperature for 30 mins and cell cycle was assessed on FACS Calibur flow cytometer and analyzed using Modfit software.

3.9 Detection of apoptosis by Annexin V/PI staining

PBMCs ($1x10^5$) were left untreated or were stimulated with rh-OAS2 at different concentration (6, 12, 24 ng/ml) for 24 hr at 37 °C in round bottomed 96 well plate (Nunc). After 24 hr cells were harvested and washed with cold PBS and resuspended in 1X annexin binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.25 mM $CaCl_2$). Cells were then stained with FITC conjugated Annexin V and propidium iodide (PI) for 15 mins at room temperature in the dark. After incubation, 400 µl of annexin binding buffer was added and acquired on FACS Calibur (BD Biosciences, USA).

The turnover of MDSCs was analyzed by staining the sorted cells with annexin V. Briefly, sorted MDSCs were cultured in C/RPMI for 24 hr after that cells were harvested and washed

with cold PBS and resuspended in 1X annexin binding buffer. Cells were then stained with FITC conjugated Annexin V for 15 mins at room temperature in the dark. After incubation, 400µl of annexin binding buffer was added and acquired on FACS Calibur (BD Biosciences, USA).

3.10 Estimation of intracellular ROS

The detection of ROS in cells was carried out using 2´,7´-dichlorofluorescein diacetate (H_2DCF-DA) dye. Briefly, H_2DCF is a reduced form of fluorescein permeable H_2DCF-DA which passively diffuses into cells and upon cleavage of its diacetate moiety by intracellular esterase is retained within the cytoplasm. Upon oxidation by ROS such as hydrogen peroxide, hydroxyl radicals, and peroxy-nitrite, the non-fluorescent H_2DCF is converted to highly fluorescent 2´,7´-dichlorofluorescein (DCF), which can be detected by flow cytometry.

PBMCs ($1x10^6$) were washed with PBS and incubated with 4 µM H_2DCF-DA at 37 ^oC and 5% CO_2 for 30 mins. Cells were then washed and stimulated with TNF-α (20 ng/ml and 40ng/ml). Cells were acquired on FACS Calibur (BD, Biosciences, USA) at different time points: 0, 3, 5, 10, 15, 20, 25, 30, 45, 60, and 90 mins. The data obtained was analyzed on FlowJo software (Tree Star Inc., USA). In case of MDSCs, cells were sorted and were then analyzed for the baseline ROS.

3.11 Intracellular Calcium release

Intracellular calcium release was estimated by flow cytometry using the fluorescent dye Fluo-3-AM. It is an acetoxymethyl (AM) ester derivative, which is cleaved by non-specific intracellular esterase, thus trapping Fluo-3 inside the cell. Fluo-3-AM is a non-fluorescent compound, but upon binding to Ca^{2+} its fluorescence increases sharply and can be detected by flow cytometry.

PBMCs obtained by FH density gradient centrifugation were washed with 1X PBS and incubated with 5 µM fluo-3-AM for 30 mins at 37 ^oC with 5% CO_2. Cells were washed with

calcium estimation buffer (137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 5 mM Glucose, 0.5 mM $MgCl_2$, 10 mM HEPES, 1 mM $CaCl_2$, 1 g/L BSA). Baseline fluorescence was analyzed after which 5μg/ml of anti-CD3 mAb and 20 ng/ml TNF-α was added to the cells. Cells stimulated with only anti-CD3 mAb were used as control for this experiment. Cells were acquired on FACS Aria III (BD Biosciences, USA) for 5 mins and the data was analysed using FlowJo (Tree Star Inc, USA). Separately, PBMCs obtained by FH density gradient centrifugation were stimulated with 0.1% PHA at 37 ^oC and 5% CO_2 for 2 hrs. Cells were then washed and treated with TNF-α (20 ng/ml) at 37 ^oC and 5% CO_2 for 24 hr. These cells were then analyzed as described above.

In case of the αβ T cells, cells (1×10^6 cells/ml PBS) were loaded with 5 μM Fluo-3-AM (Sigma–Aldrich, St. Louis, MO, USA) for 30 min at 37 °C. Cells were washed with calcium estimation buffer and acquired on the flow cytometer (FACSAria, BD Biosciences) for 30 s to determine the baseline fluorescence intensity in gamma secretase X (GSI-X) or untreated cells. Soluble α-CD3/CD28 mAb (5 μg/ml) was used as stimulants and Fluo-3 fluorescence intensity was measured immediately for up to 10 min in a continuous manner. Changes in Fluo-3 intensity were analyzed by FlowJo software (Tree Star, Ashland, OR).

3.12 PHA stimulation of lymphocytes

The peripheral blood mononuclear cells (PBMCs) obtained from normal volunteer donors have comparable levels of TNFR-I and TNFR-II. To simulate the *in vivo* situation of OSCC patients, PBMCs were stimulated with 1% PHA for 2 hrs at 37^oC to induce TNFR-I expression. In some experiments the induction of TNFR-I by PHA was observed by staining the cells with TNFR-I and TNFR-II antibodies. The cells with appropriate induction of TNFR-I were used for further experiments to study the effect of TNF-α.

3.13 Blocking TNF receptors

To study the role of TNF receptors in TNF-α mediated alteration in T cell signaling, blocking experiments were carried out. The TNFR-I and TNFR-II receptors were blocked using 4 μg/ml of TNFR-I and TNFR-II antibodies respectively. The cells were then stimulated with TNF-α (20 ng/ml) in presence of these respective antibodies for 24 hr. After 24 hr functional readout of decreased CD3-ζ chain was observed using flow cytometry.

3.14 Cell separation

3.14.1 Immuno-magnetic purification of $CD3^+$ T cells from PBMCs

PBMCs were isolated by differential density gradient centrifugation using Ficoll Hypaque method (Sigma-Aldrich, St. Louis, MO). $CD3^+$ T cells were purified from PBMCs using CD3 MicroBeads (Miltenyi Biotec, Bergish Gladbach Germany) by positive selection. The separation procedure was conducted according to the manufacturer's instructions.

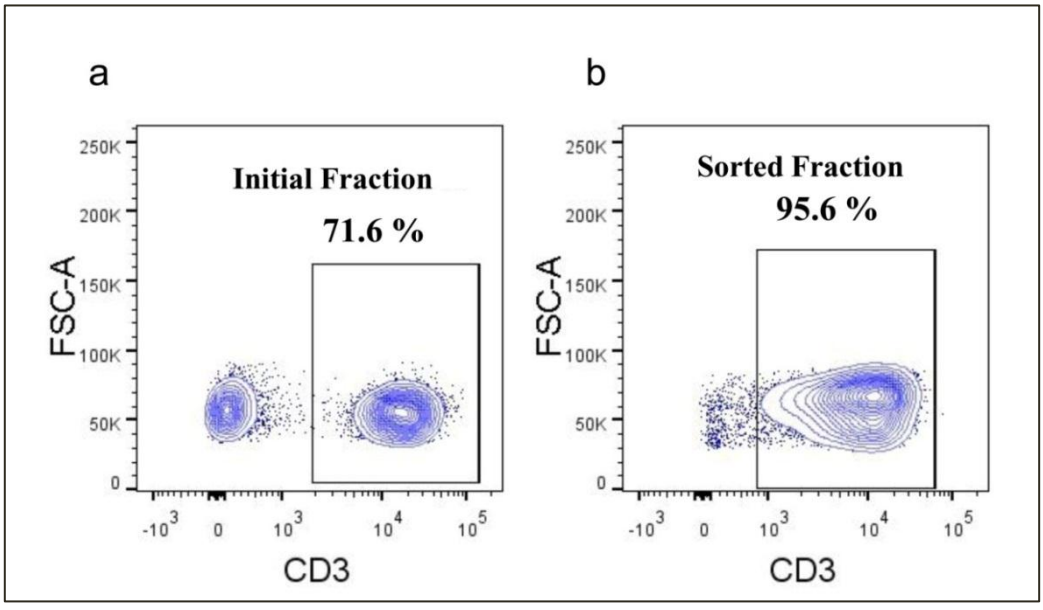

Figure 3.1: Purity of sorted $CD3^+$ T cells: a) shows the initial cell fraction that was used to sort $CD3^+$ T cells. The initial fraction of gated PBMCs was 71.6% positive for $CD3^+$ T cells **b)** Depicting the purity of $CD3^+$ T cells isolated from the PBMCs. The enriched fraction shows that the purity of sorted fraction 95.9%.

Briefly, cells were washed with buffer containing degassed PBS with 0.5% BSA and 2 mM EDTA. Supernatant was removed and cells were suspended in 80µl of buffer per total 10^7 cells. 20 µl of anti-CD3 microbeads were added per 10^7 total cells. Cells were mixed and incubated for 15 min at 4 ºC. Cells were then washed by adding 10-20 times labelling volume of buffer and centrifuged at 1000 rpm for 10 min. Supernatant was removed and cells were suspended in 500 µl buffer for upto 10^8 cells. MS MACS column (for 10^7 cells) was placed in magnetic field of MACS separator and pre-wetted thrice with 500µl of wash buffer. 500µl of cell suspension was applied onto the column. Column was removed from the separator and positively selected cells ($CD3^+$ T cells) were collected by flushing the cells from the column into 1 ml of buffer. The purity of separated cells was >95%, as determined by flow cytometry **(Fig. 3.1)**.

3.14.2 Immuno-magnetic purification of αβ T cells from peripheral blood mononuclear cells

Heparinized peripheral blood samples were collected from healthy individuals. PBMCs were isolated by differential density gradient centrifugation using Ficoll Hypaque method (Sigma-Aldrich, St. Louis, MO). γδ T cells were purified from PBMCs using MicroBeads (Miltenyi Biotec, Bergish Gladbach, Germany) by positive selection. The negative fraction was used to purify the $CD3^+$ αβ T cells as described above. The separation procedure was conducted according to the manufacturer's instructions. The purity of separated cells was >95%, as determined by flow cytometry **(Fig. 3.2)**.

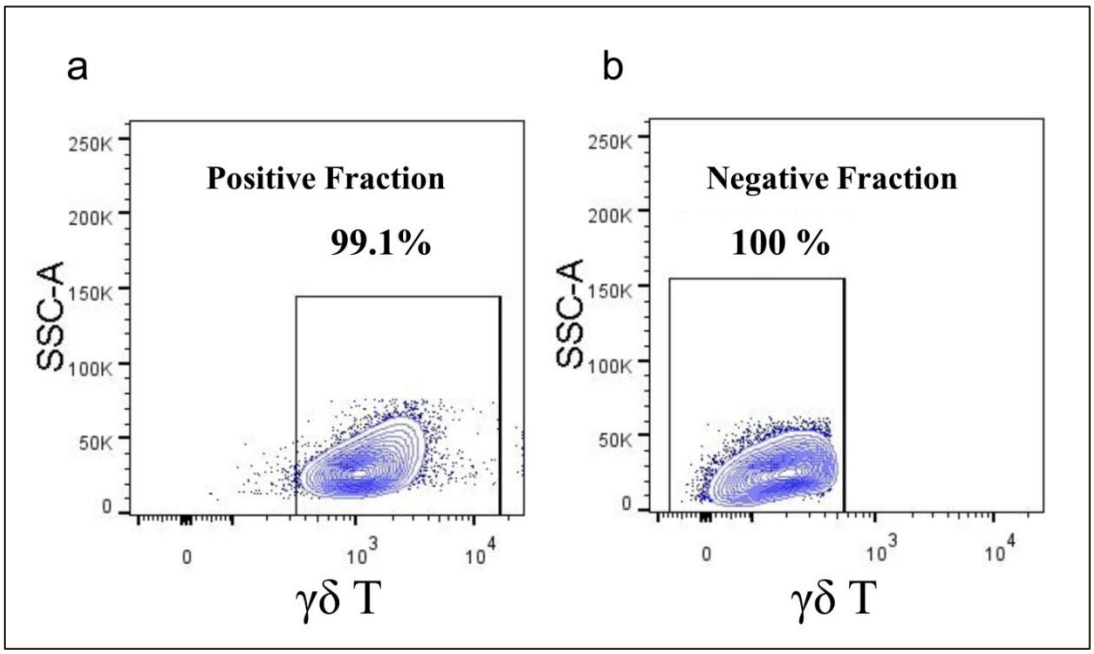

Figure 3.2: Purity of αβ T cells isolated from PBMCs: The γδ T cells were positively selected from the PBMCs and the negative fraction or flow through was used to separate the αβ T cells. **a)** The fraction that is positively selected is enriched by 99.2 % of γδ T cells while **b)** shows that the negative fraction that is depleted of γδ T cells and almost 100% cells are negative for γδ TCR. The $CD3^+$ T cells (representing αβ T cells) were isolated from the negative fraction (as described in section 3.14.1.

3.14.3 Immuno-magnetic purification of Naïve $CD4^+$ T cells from PBMCs

Naïve $CD4^+$ T cells were isolated from PBMCs of healthy individuals using Naïve $CD4^+$ T Cell Isolation Kit II human (Miltenyi Biotec). The naïve $CD4^+$ T cells are isolated by depletion of non-T helper cells and memory $CD4^+$ T cells. In first step the CD45RO+ memory cells and non-$CD4^+$ T cells are indirectly magnetically labelled using a cocktail of biotin conjugated antibodies against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγδ, HLADR and CD235a. In second step, the anti-biotin microbeads are added to the mixture. The labelled cells are subsequently depleted by separation over a MACS® Column, which is placed in the magnetic field of a MACS separator. The magnetically labelled non T helper cells and memory $CD4^+$ T cells are depleted by retaining them on a MACS column while the unlabelled naïve $CD4^+$ T cells pass

through the column. The purity of naïve $CD4^+$ T cells was confirmed using flow cytometry. The purity of separated cells was 95±5 % as determined by flow cytometry **(Fig. 3.3).**

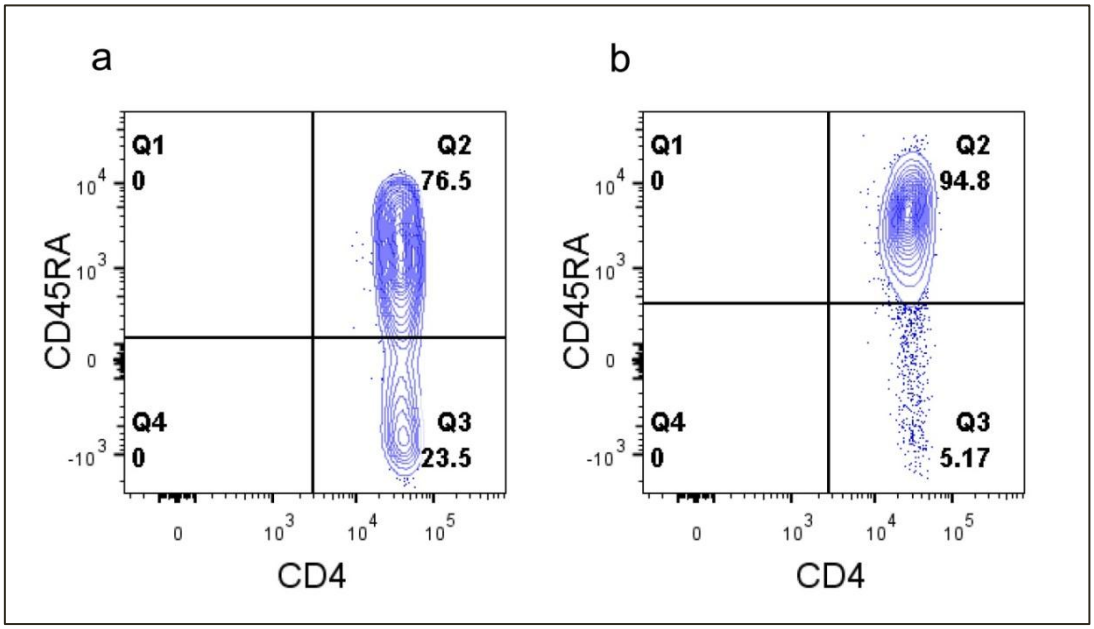

Figure 3.3: Purity of naïve $CD4^+$ T cells. The initial and sorted fractions of cells were stained with CD4-PECF594 and CD45RA-PECy5 antibodies. The cells were gated on the basis of forward and side scatter **a)** shows the initial population of $CD4^+$ T (gated on $CD4^+$ T cells) was mixture of CD45RA Positive and negative cells. **b)** Shows that the isolated $CD4^+$ T cells are 94.5% CD45RA positive which is a marker for naïve $CD4^+$ T cells.

3.14.4 Immuno-magnetic purification of Tregs from PBMCs

Regulatory T cells were isolated from PBMCs of healthy individuals using $CD4^+$ $CD25^+$ $CD127^{dim/–}$ Regulatory T Cell Isolation Kit II human (Miltenyi Biotec). The isolation of $CD4^+$ $CD25^+$ $CD127^{dim/–}$ regulatory T cells is a two-step procedure. First, the non-$CD4^+$ and $CD127^{high}$ cells are indirectly magnetically labelled with a cocktail of biotin-conjugated antibodies, as primary labelling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labelling reagent. The labelled cells are subsequently depleted by separation over a MACS® column, which is placed in the magnetic field of a MACS Separator. In the second step, $CD4^+$ $CD25^+CD127^{dim/–}$ regulatory T cells are directly labelled with CD25 MicroBeads II and isolated by positive selection from the pre-enriched $CD4^+$ T

cell fraction by separation over a MACS column, which is placed in the magnetic field of a MACS Separator. After removing the column from the magnetic field, the magnetically retained $CD4^+$ $CD25^+$ $CD127^{dim/-}$ regulatory T cells can be eluted as the positively selected cell fraction. The purity of regulatory T cells was confirmed using flow cytometry and the purity of separated cells was >95% **(Fig. 3.4)**.

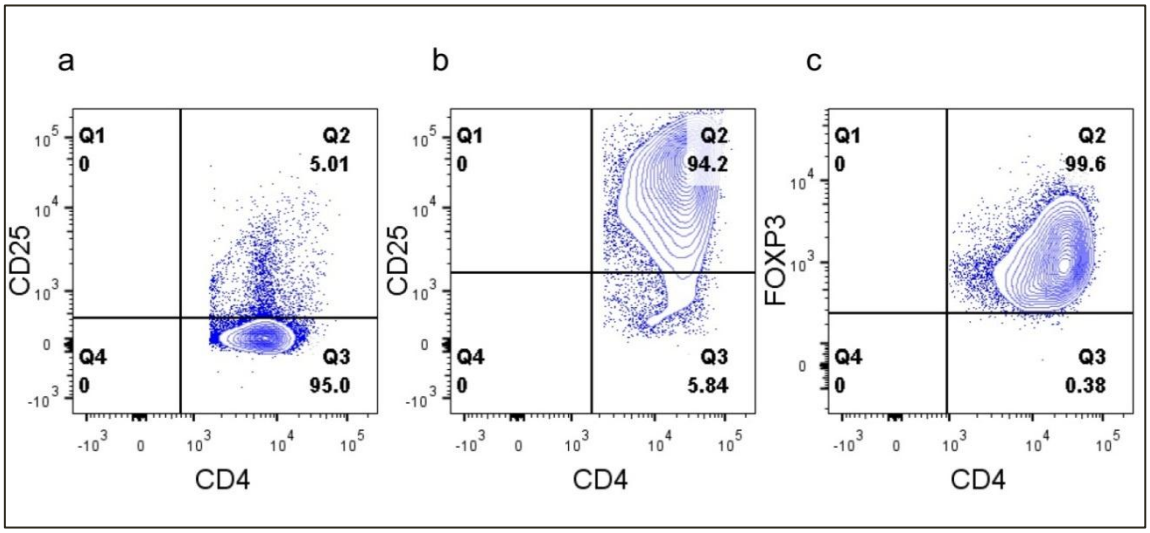

Figure 3.4: Purification of Tregs from human peripheral blood. Tregs were isolated using the $CD4^+CD25^+CD127^-$ Treg isolation kit II magnetic Microbeads method (Miltenyi Biotec). The purity of human Tregs and phenotype expression was analyzed as follows: $CD4^+$ T cells were gated and analyzed for surface expression IL-2 receptor α chain (CD25) and for intracellular expression of FoxP3. **a)** Typical FACS plot displaying the percentage of $CD4^+$ $CD25^+$ T cells (5.01%) in the pre-sort fraction of cells while **b)** shows the fraction of $CD4^+CD25^+$ T cells after sorting and FACS plot confirms that purity of enriched cells is 94.2% **c)** shows the expression of FoxP3 in the sorted $CD4^+$ T cells

3.14.5 Immuno-magnetic purification of MDSCs from PBMCs

MDSCs cells were purified from PBMCs using anti-HLADR microbeads and anti-CD33 microbeads (Miltenyi Biotech, Germany).

Magnetic labelling of cells with anti- HLADR microbeads; Cells were washed with buffer containing degassed PBS with 0.5% BSA and 2 mM EDTA. Supernatant was removed and cells were suspended in 80 µl of buffer per total 10^7 cells. 20 µl of anti- HLADR microbeads were added per 10^7 total cells. Cells were mixed and incubated for 15 min at 4 ºC. Cells were then washed by adding 10-20 times labelling volume of buffer and centrifuged at 1000 rpm

for 10 min. Supernatant was removed and cells were suspended in 500 µl buffer for upto 10^8 cells.

Magnetic separation of positively selected cells: MS MACS column (for 10^7 cells) was placed in magnetic field of MACS separator and pre-wetted thrice with 500 µl of wash buffer. 500 µl of cell suspension was applied onto the wet column. Negatively selected cells ($HLADR^-$ Cells) were collected separately as negative fraction. Column was removed from the separator and positively selected cells ($HLADR^+$ cells) were collected by flushing the cells from the column into 1 ml of buffer.

Magnetic labelling of cells with anti-CD33 microbeads: The $HLADR^-$ cells collected above were washed with buffer containing degassed PBS with 0.5% BSA and 2 mM EDTA. Supernatant was removed and cells were suspended in 80 µl of buffer per total 10^7 cells. 20 µl of anti- CD33 microbeads were added per 10^7 total cells. Cells were mixed and incubated for 15 min at 4 ºC. Cells were then washed by adding 10-20 times labelling volume of buffer and centrifuged at 1000 rpm for 10 min. Supernatant was removed and cells were suspended in 500 µl buffer for upto 10^8 cells.

Magnetic separation of positively selected cells: MS MACS column (for 10^7 cells) was placed in magnetic field of MACS separator and pre-wetted thrice with 500µl of wash buffer. 500µl of cell suspension was applied onto the column. Negatively selected cells ($CD33^-$ cells) were collected separately as negative fraction. Column was removed from the separator and positively selected cells ($CD33^+$ cells or $HLADR^-CD33^+$) were collected by flushing the cells from the column into 1 ml of buffer. The Purity of $HLADR^-CD33^+$ was determined by flow cytometry and the purity of cells was 90±5% **(Fig. 3.5).**

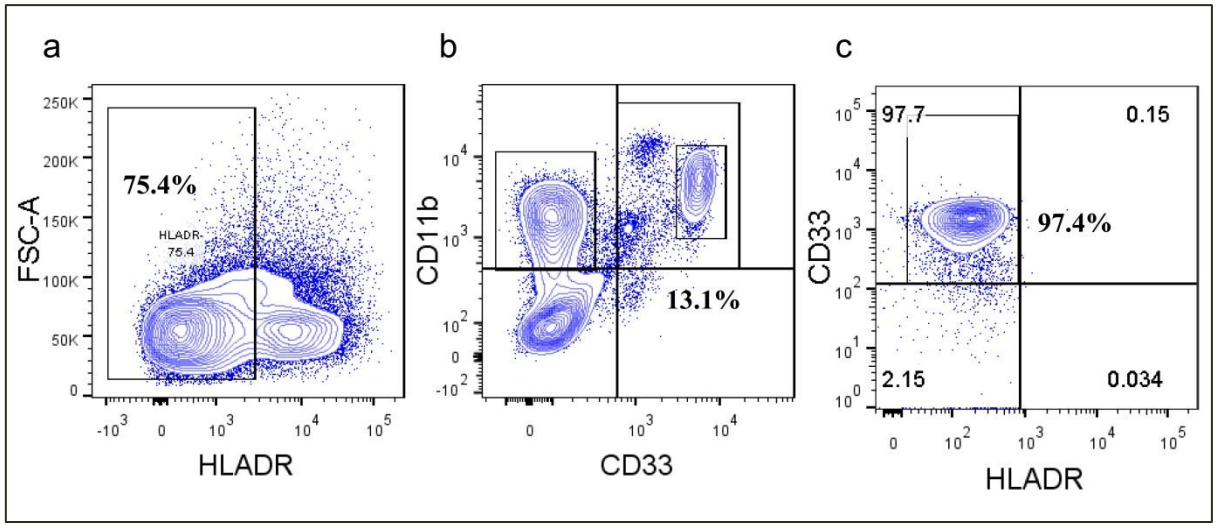

Figure 3.5: **Strategy to isolate the MDSCs from the PBMCs:** The HLADR$^-$ cells are constituted of three populations when plotted for the CD33 and CD11b fluorescence i.e.; HLADR$^-$CD33$^-$CD11b$^-$(LL quadrant), HLADR$^-$CD11b$^+$CD33$^-$ (UL quadrant) and HLADR$^-$ CD33$^+$CD11b$^+$ (UR quadrant). CD11b marker is either present alone or is co-expressed with CD33 generating two different populations. Hence CD33 was used to sort HLADR$^-$ CD33$^+$CD11b$^+$; representing MDSCs. **a)** Shows the distribution of HLADR expression in PBMCs while **b)** shows the initial population of HLADR$^-$CD11b$^+$CD33$^-$ along with other cells representing HLADR$^-$ cells. **c)** Shows the purity of sorted cells which are CD33 positive but HLADR negative. The graph shows the purity of cells (97.7%).

3.14.5.1 Wright stain and microscopy

For wright stain, the immuno-magnetically sorted MDSCs **(as described in section 3.14.5)** were streaked across a sterile slide using cytospin centrifuge. The concentrated cells were fixed by chilled acetone and air dried. Then cells were smeared with 1.0 ml of the Wright stain solution for 1–3 mins. After staining, cells were washed with 2.0 ml distilled water or Phosphate buffer pH 6.5 and kept stand ing for 2-6 mins. Stained smear was further washed with water or the Phosphate buffer pH 6.5 until the edges become faintly pinkish-red. Morphology of the stained cells was analyzed under inverted microscope **(Fig. 3.6)**.

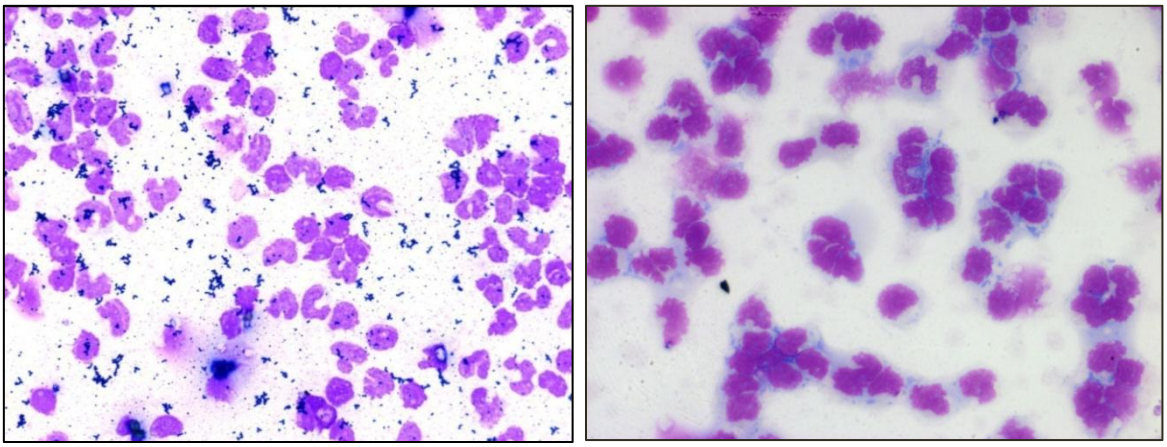

Figure 3.6: Morphology of immuno-magnetically sorted HLADR^{-}CD33^{+}CD11b^{+} MDSCs: The sorted MDSCs were stained with wright stain and analyzed under inverted Microscope. The cells appeared as large polymorphonuclear cells suggestive of their promyelocyte nature. The morphology of MDSCs isolated from two different individuals is shown (10X).

3.14.5.2 Image stream flow cytometry

The immuno-magnetically sorted MDSCs were stained for the HLADR, CD33 and CD11b antibodies and analyzed under Image stream flow cytometry for their purity. Briefly, 2x10^{5} cells were washed and incubated with the cocktail of antibodies at 4 ^{o}C for 45 mins. After that, cells were washed and resuspended in 50 µl of buffer in an eppendorf tube and analyzed by Image Stream flow cytometry using INSPIRE software **(Fig. 3.7)**.

Figure 3.7: Image Stream Imaging showing the purity of sorted MDSCs: Magnetic sorted MDSCs were stained with HLADR-PE, CD33-PECF594 and CD11b-FITC. Images of stained cells were captured using ImageStream$^{®X}$ Mark II Imaging Flow cytometer

3.15 Proliferation assays

3.15.1 Lymphocyte proliferation assay by dye dilution method

Carboxyfluorescein succinimidyl ester (CFSE) staining of PBMC was conducted according to the manufacturer's instructions (Invitrogen). Briefly, 5 X 10^6 PBMCs were incubated in IX PBS containing 5% FBS with 4 μM CSFE for 15 mins at room temperature and then washed 2 times with RPMI medium containing 10% FBS. Labelled cells ($5x10^4$ cells/well) were incubated in the presence or absence of 5 μg/mL anti-CD3 antibody and 5 μg/mL anti-CD28 antibody (eBioscience) in a 96-well flat bottom plate. After 5 days of stimulation, harvested cells were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD3-ζ chain, anti-CD62L antibodies.

3.15.2 Lymphocyte proliferation using Tritiated thymidine incorporation assay

Proliferation of PBMCs (OSCC patients and HIs) and αβ T cells were assayed using ^{3}H-thymidine (3H TdR) uptake. For PBMCs, a total of $1X10^5$ cells were incubated in round-bottom 96-well tissue culture plates with 1% PHA or 5 μg/ml α-CD3 and rIL-2 (10 IU/ml, PeproTech) for 72 hrs. The cultures were pulsed with 0.5 μCi [^{3}H] thymidine/well (Board of Radiation and Isotope Technology, Mumbai) during the last 18 hrs of the assay.

For monitoring the proliferation of αβ T cells using ^{3}H-Thymidine (^{3}H TdR) uptake assay, a total of $5X10^4$ αβ T cells were incubated in round-bottom 96-well tissue culture plates with α-CD3 and CD28 mAb (1 μg/ml, BD Biosciences) and rIL2 (10 U/ml, PeproTech) for 72 h. These cells were pre-treated for 30 min at 37 °C with γ-secretase inhibitor-X, L-685,458 (GSI-X) (Calbiochem, La Jolla, CA) at a concentration of 10 μM, 5 μM, 2.5 μM, and 1.25 μM or left untreated, before stimulation. The cultures were pulsed with 0.5 μCi [^{3}H] thymidine (Board of Radiation and Isotope Technology, Mumbai) during the last 18 hrs of the assay. After 72 hrs in culture, were harvested onto glass-fiber filter paper (Titertek, Norway) using a cell harvester (Titertek, Norway). The filter paper was dried at 50 ºC and

each disc corresponding to a single well was placed in 3 ml of scintillation fluid (0.5 g PPO, 7 g POPOP in 1 litre Toluene). The radioactivity incorporated in the DNA was measured in a liquid scintillation counter (Packard, Meriden, CT) as counts per minute (cpm).

3.16 Mass spectroscopy (LC-MS/MS)

The cell free supernatant (1 ml) from surgically excised oral tumors, oral cancer cells lines AW8501, AW13516 [353] and medium alone (control) were lyophilized. The dried samples were dissolved in 0.5 ml of ammonium bicarbonate buffer (0.1 M, pH 8.5) and the samples were concentrated on a centricon (3kDa cut-off membrane). Samples were dried in a lyophiliser overnight and the dried samples were dissolved in dissolution buffer supplied with i-TRAQ (Applied biosystem) kit. Samples were digested with trypsin according to the manufacturer's protocol and the samples were labelled with the i-TRAQ reagents 114, 115, 116, and 117. Tryptic peptides of media control were labelled with 114, AW8501 peptides with 115, AW13516 peptides with 116 and finally tumor supernatants tryptic peptides with 117. Samples were pooled and dried in speed-vac. The dried samples were fractionated on strong cat-ion exchange chromatography and fractions were collected using salt gradient. Each of these fractions were analysed using LC/MS/MS on QSTAR XL mass spectrometer. By differential labelling, peptides labelled with isotope 117 (3-fold excess) were selected and a Mascot analysis on them was done to identify the peptide and the protein from which the peptide was derived (this work was done in the Laboratory of Dr. Kanury Rao, ICGEB, New Delhi as part of a collaborative project).

3.17 Caspase 3 detection by fluorimetry

The caspase 3 fluorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety. The excitation and emission wavelengths of AMC are 360 nm and 460 nm, respectively.

PBMCs (1x 10^6) were stimulated with different concentrations of rh-OAS2 (6 and 12 ng/ml) or tumor supernatant (1:1) or hydrogen peroxide ($H_20_{2)}$ for 24 hr. After 24 hr, cells were washed in PBS, lysed in ice cold caspase lysis buffer for 30 mins and the lysate was collected after centrifugation. In another set of experiment, PBMCs from HI obtained by FH density gradient centrifugation were stimulated with 0.1% PHA at 37 ^oC and 5% CO_2 for 2 hrs. Cells were then washed and treated with TNF-α (40 ng/ml) at 37^oC and 5% CO_2 for 24 hr and 48 hr. Untreated cells were used as a control for this experiment. The cells were lysed by using the lysis buffer supplied in the kit and protein lysate obtained was used to quantitate the caspase activation induced by different treatments. The caspase enzymatic reaction was carried according to the manufacturer's instruction (Caspase-3 assay kit, Fluorimetric CASP3F, sigma, USA). The reaction was set up as follows:

Table 3.6: Caspase 3 enzymatic reaction

Tubes	**1X assay buffer**	**Caspase 3 (0.5µg/ml)**	**Cell lysate**	**Reaction Mixture**
Reagent Blank	5 µl	-	-	200 µl
Caspase 3 positive control	-	5 µl	-	200 µl
Untreated cells	-	-	5 µl	200 µl
TNF-α treated cells	-	-	5 µl	200 µl

The rate of fluorescence generated was measured using a Synergy II microplate reader (Bio-Tek Instruments, Winooski, VT, USA) and caspase 3 activity in nmoles of AMC released per minute per ml of cell lysate was calculated based on the formula:

Activity, nmol AMC/min/ml= nmol AMC x d / TxV

Where: V=Volume of sample in ml, d= dilution factor and T=reaction time in mins.

The concentration of the AMC released was calculated from a standard curve determined with defined AMC solutions

3.18 Western blotting

3.18.1. Key reagents for western blotting

Composition of 2X SDS buffer (10 ml)

1M Tris HCL (pH 6.8)	1.6 ml
10% SDS	4 ml
Glycerol	2 ml
β-mercaptoethanol (2ME)	1 ml
Bromophenol blue	4 mg
Double distilled water	1.4 ml

SDS Lysis Buffer (1 ml)

2X SDS buffer:	500 µl
Double distilled water	390 µl
1M dithiothritol (DTT)	50 µl
Phenylmethylsulfonyl fluoride (PMSF) (50 mM)	10 µl
Sodium flouride (0.5 M)	10 µl
Sodium Orthovanadate (0.5 M)	10 µl
Aprotinin (2 mg/ml)	10 µl
Leupeptin (2 mg/ml)	10 µl
Pepstatin (2 mg/ml)	10 µl

Lysis buffer was aliquoted and stored at -20°C for up to a year

Running Buffer: Tris/Glycine/SDS (1000 ml)

25mM Tris	3.0285 gm
190mM glycine	14.26 gm
0.1%SDS	1 gm

Transfer Buffer: 1000 ml

25mM Tris	3.0285 gm
190mM glycine	14.26 gm
20% methanol	200 ml

Ponceau S Staining Buffer (100 ml)

0.2% (W/V) Ponceau S	200 mg
5% glacial acetic acid	5 ml
Double distilled Water	95 ml

Tris-buffered saline with Tween 20 (TBST) buffer (1000 ml)

20mM Tris pH 7.5	2.4228 gm
150mM NaCl	1.1688 gm
0.1% Tween	1 ml

Blocking buffer

5% non-fat dried milk in TBST or 5% bovine serum albumin (BSA) in TBST

Nitrocellulose Membrane

Stripping Buffer

2% SDS (10% SDS)	20 ml
0.5 M Tris-HCl	12.5 ml
Double distilled water	67.5 ml
Beta-mercaptoethanol	0.8 ml

Table 3.7: Resolving and Stacking gel components

	10% Resolving gel 8ml	5% Stacking gel 5ml
Double distilled water	3.2 ml	2.6 ml
30% Bis-acrylamide mixture	2.67 ml	1 ml
1.5M Tris-HCl (pH 8.8)	2 ml	-
0.5M Tris-HCl (pH6.8)	-	1.25 ml
10% SDS	80 µl	50 µl
10% ammonium per sulfate	80 µl	50 µl
TEMED	08 µl	05 µl

3.20.2 Cell lysate preparation

Cells were washed with ice cold 0.01 M PBS (PH 7.5) and lysed in SDS lysis buffer. Briefly, to the cell pellet of 1x 10^6 cells, 30 µl of SDS lysis buffer was added; cells were resuspended by vortexing and boiled for 10 mins at 100 ^oC. The lysates were loaded immediately or stored at 80 ºC. In case of frozen lysate, the samples were boiled once again at 100 ºC for 5 mins and then loaded.

3.18.3 Protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Equal amounts of protein lysate were separated on SDS PAGE and the gel was run at 120 V for 1-2 hrs. The selection of gel percentage was dependent on the size of protein of interest. 8-15% gels were used to separate the protein of interests.

3.18.4 Transfer of proteins from the gel to the membrane

Gel was placed in 1x transfer buffer for 3-5 mins after separation of proteins under reducing conditions. The transfer sandwich was assembled with blot on the cathode and gel on the anode. The separated proteins were electrophoretically transferred onto mdi nitrocellulose membrane (Advanced microdevices Pvt. Ltd., India) at RT for 1-2 hrs at a voltage of 100V using the vertical transfer apparatus (Biorad). The membrane containing the standard molecular weight marker along with protein lysates was stained with Ponceau S stain to visualize the protein bands and transfer quality. Membrane was washed with 1X TBST and blocked with 5% skimmed milk or BSA prepared in 1X TBST for 1hr at RT. The membranes were then probed with the appropriate concentration of the primary antibody (dilution buffer used was 3% BSA) and incubated overnight at 4 ºC. After washings, the membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (HRPO) diluted in the dilution buffer for 1 hr at RT. After washing off the excess of the secondary antibody, the protein bands were detected by enhanced chemiluminescence (ECL Plus; Amersham, U.K.) detection kit.

3.19 Real time polymerase chain reaction (RT-PCR)

3.19.1 Extraction of RNA

Oral tumor tissue or oral tumor derived cell lines or myeloid derived suppressor cells were stored at –80 ºC in TRIzol (Invitrogen Life-Technologies, N.Y.) in a ratio of $1X10^6$ cells/100 µl TRIzol solution until further use. At the time of RNA extraction, chloroform was added to make 1:5 ratio of chloroform: TRIzol (e.g. 20 µl choloroform in 100 µl TRIzol) mixed and centrifuged at 10,000 rpm for 15 mins at 4 ^oC and the upper aqueous phase was collected and treated with chilled isopropyl alcohol (Qualigens, India) half the volume of TRIzol, mixed gently for 10 m and centrifuged at 10,000 rpm for 10 mins at 4 ^oC. The pellet obtained was washed with 75% ethanol and centrifuged at 10,000 rpm for 5 mins. The pellet was air dried

and dissolved in appropriate volume of DEPC (Sigma)-treated water. Optical density (O.D.) readings were taken for quantitation of RNA by NanoDrop spectrophotometer (Thermo Scientific, DE). The RNA was run on a 1.5% agarose gel containing ethidium bromide to confirm its purity and integrity. In case of surgically excised oral tumors, tumors were collected and minced in double strength medium. Minced tumor tissues were weighed and collected in a tube containing TRIzol reagent (approx. 100 mg in 1 ml of the reagent). This was followed by homogenization and RNA was extracted as described above.

3.19.2 Complementary DNA (cDNA) synthesis by reverse transcription

Total RNA from the cells was used for first strand cDNA synthesis using oligo dT primers or cDNA synthesis kit (Invitrogen Life-Technologies, N.Y.). 2 µg of RNA with its volume adjusted to 10 µl using DEPC treated water was reverse transcribed. Oligo dT primers (1 µl) and dNTPs (1µl 10 mM) were added to RNA and heated at 65 ºC for 10 min and then chilled on ice for 10 min. To 1st strand mixture, the following components were added on ice.

Component	**Volume**
5X 1st strand buffer	4 µl
0.1 M DTT	2 µl
RNase inhibitor	1 µl
Murine Moloney Leukemia Virus reverse transcriptase enzyme (200 U/µl)	1 µl
RNAase Inhibitor	1 µl

This total mixture was gently mixed and incubated at 37 ºC for 60 min in PTC-100™ Programmable Thermal Controller (MJ Research Inc.) and the reaction was terminated by heating the mixture at 70 ºC for 15 mins. The cDNA was stored at -20 ^{o}C until for further use.

3.19.3 Semi-quantitative PCR

RNA extracted from oral tumor, cell lines or αβ T cells was reverse transcribed into cDNA. This cDNA from oral tumor was used to measure oligoadenylate synthetase 2 (OAS2) and GAPDH (loading control) mRNA expression. In case of αβ T cells, cDNA was used to measure the levels of CD3-ζ chain, ELF1 and β-actin (loading control) mRNA expression.

100 ng of cDNA template was used and amplified using 50 mM $MgCl_2$, 1X PCR Buffer, 10 mM dNTPs, 2.5 units of Taq polymerase and 20 Pico-moles of each primer (Sweden).

Table 3.8: List and Sequence of the primers used

Gene Name	**Primers**
OAS2 Forward	5′-TTAAATGATAATCCCAGCCCC-3′
OAS2 Reverse	5′-TCAGCGAGGCCAGTAATCTT-3′
ELF1 Forward	5-ATGGCTGCTGTTGTCCAAC-3’
ELF1 Reverse	5’-CCTGAGTGCTCTTCCCAT-3’
CD3-ζ chain Forward	5’-TCAGCCTCTGCCTCCCAGCCTCTTTCT-3’
CD3-ζ chain Reverse	5’-ATGCTTCATCCTGTGTCTCATAATCTG-3’
GAPDH Forward	5’-CATCTCTGCCCCCTCTGCTGA-3’
GAPDH Reverse	5’-TCCGACACCCGTTCCAGTAGG-3’
β-actin Forward	5’-ACACTGTCCCATCTACGAGG-3’
β-actin Reverse	5’-AGGGGCCGGACTCGTCATACT-3’

3.19.4 Real Time PCR

RNA was extracted from immuno-magnetically purified αβ T cells and MDSCs using Trizol reagent (Invitrogen Life Technologies, N.Y) and reverse transcribed to cDNA as per company’s instructions. The cDNA of αβ T cells and MDSCs was used to quantitate different target genes using RT-PCR. Reaction mixture used for Real time PCR is as:

Component	**Volume**
10 ng cDNA+ DEPC water	2.25 µl
Primer probe mixture	0.25 µl
Master-mix	2.5 µl

Quantitative RT-PCR for different genes was performed with PRISM 7700 (PE Applied Biosystems, Foster City, CA). Samples were analyzed using TaqMan primer sets purchased from Applied Biosystems (TLR2 Hs00152932_m1, TLR3 Hs00152933_m1, TLR4 Hs00370853_m1, TLR9 Hs00152973_m1, TGF-β2 Hs00234244_m1, IL6 Hs00985639, IL23A Hs00372324_m1, IL1β Hs01555410_m1, RORC Hs01076112_m1, STAT3

Hs01047580_m1, Cox-2 Hs00153133_m1 Arg Hs00968979_m1, iNOS Hs01075529_m1, NOTCH-1 Hs01062011_m1, NOTCH-2 Hs01050719_m1, NOTCH-3 Hs01128541_m1, NOTCH-4 Hs00270200_m1, DLL1 Hs00194509_m1, DLL3 Hs00213561_m1, DLL4 Hs00184092_m1, JAG1 Hs01070036_m1, JAG2 Hs00171432_m1, HES1 Hs00172878_m1, NF-κB Hs00765730_m1, DTX1 Hs00269995_m1, NRARP Hs01104102_m1, ACTB [β-actin] Hs99999903_m1). All values were normalized to the expression of the housekeeping gene β-actin.

3.20 Confocal microscopy

AW13516 and SSC29B tumor cells were plated on a glass slide; these cells were rinsed in cold phosphate buffered saline (PBS) and fixed in 1% paraformaldehyde in PBS for 10 min at 4 ºC. The cells were washed and permeabilized for 15 min with 0.1% saponin in PBS. Cells were stained with mouse goat anti-human MIP-3α (3μg) and SDF-1α (3μg) antibodies for 45 m at 4 °C. Thereafter, cells were washed and incubated with AF488-labeled Rabbit anti-goat IgG for another 45 mins at 4°C. For nuclear staining, 6'4' diamino-2-phenylindole (DAPI) (Sigma Aldrich) was used. The samples were visualized on Laser-Scanning Microscope and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD).

2.21 Small interfering RNA

PBMCs or $CD3^+$ T cells isolated by MACS column **(as described above in section 3.14.1)** were transfected with small interfering RNA (siRNA) specific for Caspase-3 gene and fluorescent oligonucleotide control siRNA (transfection indicator) (Cell signaling Technology,USA), Waltan, USA). siRNA oligos were transfected at a concentration of 100 nM using X-treme GENE HP transfection reagent (Roche Diagnostics, Indianapolis, IN). The inhibition of caspase-3 expression was assessed at 60 hrs post transfection. Briefly $2x10^5$ cells per well were seeded in 24 well plate in 400 μL media supplemented with 10% FBS. Then siRNA duplex was diluted in the 50 μL of transfection media (Opti-MEM® I reduced

serum medium) (Invitrogen Life Technologies, Inc., Gaithersburg, MD) such that each well gets the optimal level of siRNA (3 µl corresponding to 100 nM). 6 µL of transfection reagent was gently mixed with 50 µL of transfection media (Opti-MEM) and incubated for 5 mins. After 5 mins both the solutions were combined and incubated at room temperature for 30 mins to allow the complex to form. Then mixed gently and overlaid the mixture onto the cells. Cells were incubated for 48 hr and monitored for siGLO by flow-cytometry for transfection efficiency (70±5%).

3.22 Cloning and Transfection

To establish a HEK293 cell line with overexpressed OAS2, OAS2 coding sequence (CDS) present in pCMV-sports 6.1 vector was amplified by PCR using forward primer containing Pme1 restriction site and reverse primer containing Sal1 restriction site **(Fig. 3.8a)**. The PCR product (2 kb) was gel purified and by TA cloning inserted into pTZ57R/T vector. The pTZ57R/t vector was digested with EcoR1 and Sal1 to release the OAS2 CDS which was purified and cloned into EcoR1 and Sal1 digested pEGFP-N2 vector.

The pEGF-N2 vector containing OAS2 was probed for OAS2 addition using OAS2 forward primer and GFP reverse primer. The PCR product indicates that certain colonies are positive for OAS2 **(Fig. 3.8b)**. Lane 5 vector was sequenced and sequence confirmed the inframe addition of OAS2 gene with GFP present in pEGFP-N2 vector **(Fig. 3.9)**. Further overexpression of OAS2 gene in HEK293 cells was confirmed by semi-quantitative PCR, western blotting and flow cytometry. The OAS2 encoding Vector was a transfected into HEK293 X-treme GENE HP transfection reagent (Roche Diagnostics, Indianapolis, IN). After 24 hr the culture media was removed and were supplemented with fresh medium. After 72 hrs post transfection, the cell free supernatant was collected which was later used to treat the PBMCs to monitor the CD3-ζ chain downregulation.

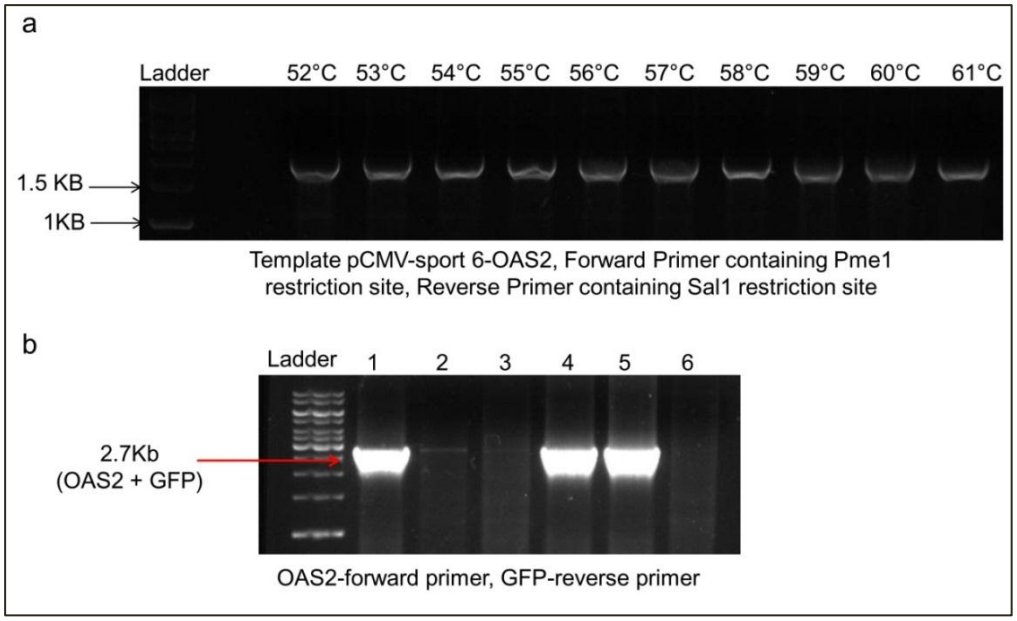

Figure 3.8: Cloning of OAS2 CDS into the GFP expressing Vector: a) Shows the PCR product of OAS2 (2 kb) amplified from pCMV-sport 6 vector encoding OAS2. The OAS2 gene was amplified at different annealing temperatures (52-61^{o}C). The OAS2 PCR product was gel purified and subsequently cloned into pTZ57R/T vector. This vector was released by restriction digestion and ligated in the pEGF-N2 vector. **b)** Shows the PCR product of OAS2 and GFP amplified using OAS2 forward primer and GFP reverse primer from vectors obtained from different bacterial colonies. Lane 1, 4, and 5 shows the inframe addition of OAS2 with GFP

>ref|NT_009775.17| Homo sapiens chromosome 12 genomic contig, GRCh37.p5 Primary Assembly
Length=13107153

Sort alignments for this subject sequence by:
E value Score Percent identity
Query start position Subject start position

Features in this part of subject sequence:
2'-5'-oligoadenylate synthase 2 isoform 3
2'-5'-oligoadenylate synthase 2 isoform 2

Score = 503 bits (272), Expect = 2e-139
Identities = 272/272 (100%), Gaps = 0/272 (0%)
Strand=Plus/Plus

```
Query  158      GGTGGCTCCTATGGACGGAAAACAGTCTTAAGAGGCAACTCCGATGGTACCCTTGTCCTC  217
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4001373  GGTGGCTCCTATGGACGGAAAACAGTCTTAAGAGGCAACTCCGATGGTACCCTTGTCCTC  4001432

Query  218      TTCTTCAGTGACTTAAAACAATTCCAGGATCAGAAGAGAAGCCAACGTGACATCCTCGAT  277
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4001433  TTCTTCAGTGACTTAAAACAATTCCAGGATCAGAAGAGAAGCCAACGTGACATCCTCGAT  4001492

Query  278      AAAACTGGGGATAAGCTGAAGTTCTGTCTGTTCACGAAGTGGTTGAAAAACAATTTCGAG  337
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4001493  AAAACTGGGGATAAGCTGAAGTTCTGTCTGTTCACGAAGTGGTTGAAAAACAATTTCGAG  4001552

Query  338      ATCCAGAAGTCCCTTGATGGGTTCACCATCCAGGTGTTCACAAAAAATCAGAGAATCTCT  397
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4001553  ATCCAGAAGTCCCTTGATGGGTTCACCATCCAGGTGTTCACAAAAAATCAGAGAATCTCT  4001612

Query  398      TTCGAGGTGCTGGCCGCCTTCAACGCTCTGAG  429
                ||||||||||||||||||||||||||||||||
Sbjct  4001613  TTCGAGGTGCTGGCCGCCTTCAACGCTCTGAG  4001644
```

Figure 3.9: Sequence of the OAS2 gene inserted in the p-EGF-N2 vector. The Vector from Lane 5 was purified and sequenced to show the presence of OAS2 gene in the vector.

3.23 MDSCs suppression assay using autologous mixed leukocyte reaction

For the T cell–proliferation assay, fresh immuno-magnetically sorted HLA-DR$^-$ CD33$^+$CD11b$^+$ MDSCs were cocultured with autologous CD3$^+$ T cells for 5 days. Briefly, T cells (100,000/well) were labelled with CFSE (Invitrogen, Molecular Probes) at a final concentration of 1 mM for 10 mins at 37°C. Cells were washed three times with ice-cold complete RPMI 1640 and stimulated with anti-CD3/CD28 microbeads (Miltenyi Biotec.), 1 bead per cell in a 96-well round-bottom plate. These CFSE labelled cells were either cultured in absence of MDSCs or at different ratios of MDSCs 50,000 (1:2), 25,000 (1:4), or 12,500 (1:8). After 5 days, cells were harvested, stained with anti-CD3, anti-CD4, anti-CD8, anti-TCR-ζ, anti-CD62L antibodies (all from BD Bioscience). The change in CFSE signal of gated CD3$^+$ T cells was measured as fluorescence intensity by flow cytometry. The expression of CD3-ζ chain and CD62L was also observed on the gated T cell subsets. Cell free supernatant were also collected which were probed for IFN-γ cytokine by ELISA described later.

3.24 Treg induction protocol

The 40-60 ml of blood was withdrawn from the healthy individuals. PBMCs were separated by ficoll hypaque density gradient centrifugation. Cells collected from the interface were washed twice with normal saline and then resuspended in RPMI for counting on a haemocytometer. The naïve CD4$^+$ T cells were obtained as described above (**section 3.14.3)**. For Treg differentiation, naïve CD4$^+$ T cells (1x 10^5 cells) were stimulated with CD3/CD28 microbeads (1 bead per 1 cells), TGF-β (20ng/ml) and IL-2 (100U/ml) for 4 days [354]. On day 4 cells were washed and media was aspirated. Cells were resuspended in FACS buffer and cocktail of antibodies (CD4, CD25, and FoxP3) was added to visualize the Treg differentiation.

3.25 Myeloid derived suppressor cells and Naïve $CD4^+$ T cells co-culture in Treg polarizing condition

The MDSC and naïve $CD4^+$ T cells were sorted as described above **(section 3.14.5 and 3.14.3 respectively)**. $1x10^5$ naïve $CD4^+$ T cells were cultured in complete RPMI supplemented with 10% human AB-serum and activated by T-cell activation/expansion beads (Miltenyi Biotech), TGF-β (20 ng/ml) and IL-2 (100 U/ml). MDSCs were added at different ratios (1:1 and 1:2) to these naïve $CD4^+$ T cells. After 4 days of co-culturing, the cells were stained for CD4, CD25, and FoxP3 to monitor Treg differentiation in presence of different ratios of MDSCs.

3.26 Th17 differentiation Protocol

For Th17 differentiation naïve $CD4^+$ T cells were stimulated with CD3/CD28 microbeads (1 bead per 1 cells), IL-6 (50 ng/ml), IL-1β (20 ng/ml), IL-23 (10 ng/ml) for 7 days [355, 356]. On day 7, cells were washed for 10 mins and media was aspirated. Cells were resuspended in FACS buffer and cocktail of antibodies (CD3, CD4, IL17 and IFN-γ) was added to visualize the TH17 differentiation **(Fig. 3.10).**

3.27 Myeloid derived suppressor cells and Naïve $CD4^+$ T cells co-culture in presence of α-CD3/CD28 microbeads

The MDSCs and naïve $CD4^+$ T cells were sorted as immuno-magnetically as described above **(section 3.14.5 and 3.14.3 respectively)**. $1x10^5$ Naïve $CD4^+$ T cells were cultured in complete RPMI supplemented with 10% human AB-serum and activated by T-cell activation/expansion beads (1 bead per 1 cell, Miltenyi Biotec.), without TGF-β and IL-2. MDSCs were added at different ratios (1:1 and 1:2) to these naïve $CD4^+$ T cells. After 7 days of co-culturing, the cells were stained for CD3, CD4, IL17 and IFN-γ to monitor TH1 and Th17 differentiation in presence of MDSCs at different ratios.

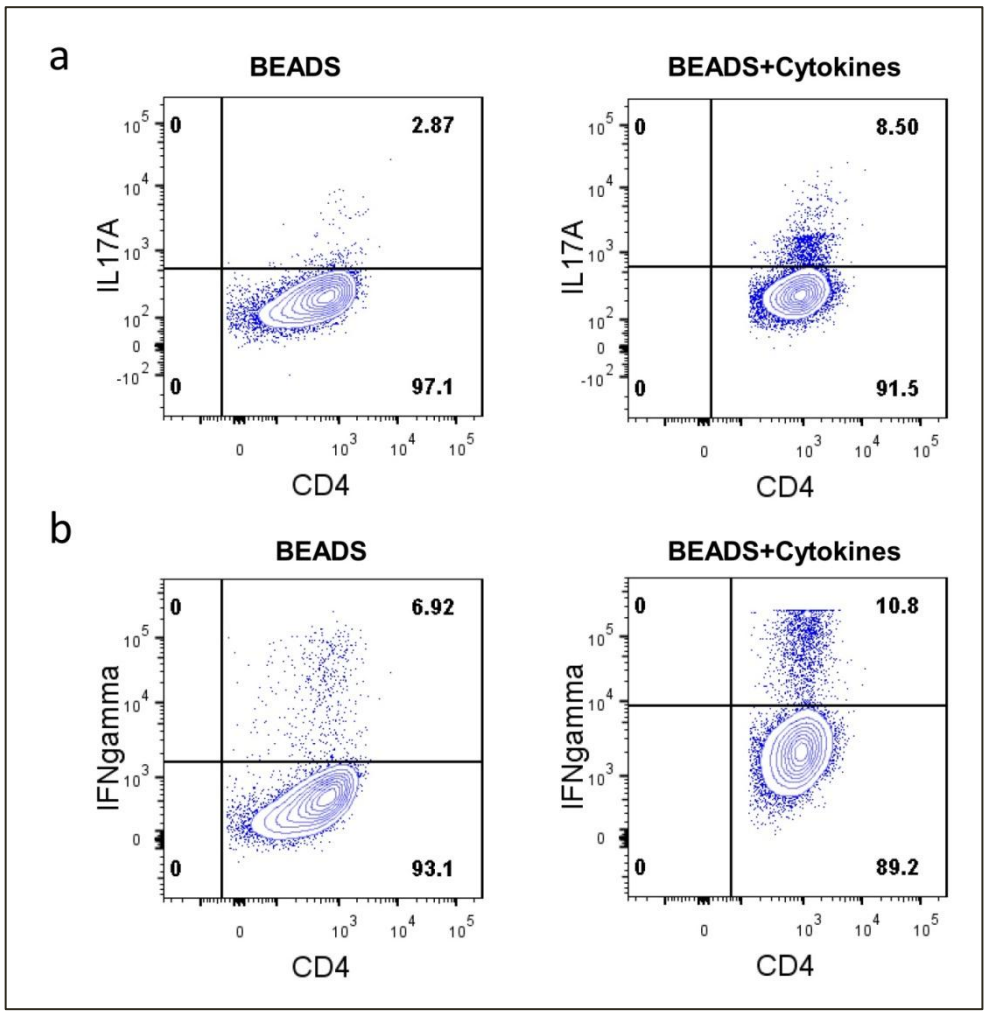

Figure 3.10: TH17 differentiation in presence of IL-6, IL-1β and IL-23. a) Naïve $CD4^+$ T cells were isolated and cultured for 7 days with α-CD3/CD28 microbeads in the presence or absence cytokines (IL-6 50 ng/ml, IL-1β 20 ng/ml and IL-23 10ng/ml). The frequency of $CD4^+$ $IL17A^+$ T cells was determined after 7^{th} day. Representative contour plots depict that in presence of cytokines generation of $CD4^+IL17A^+$ T cells is high while **b)** shows the effect of same cytokines on the generation of $CD4^+IFN\text{-}\gamma^+$ T cells.

3.28 Stimulation of MDSC with recombinant human interleukin 6 (IL-6)

MDSCs were sorted as described above and the sorted MDSCs ($1x10^6$) were stimulated with IL-6 (25 and 50ng/ml) for 24 hr in a 24 well plate. After 24 hr cells were harvested, protein lysate was prepared as described above **(section 3.18.2)**. The protein lysate was later probed using antibodies for PDL1, Cox-2, C/EBPα, C/EBPβ, IL-10, pSTAT3 and β-actin (loading control). The unstimulated MDSCs served as control for the experiment

3.29 *In vitro* migration of Tregs

The Tregs were isolated as described above **(section 3.14.4)** and were stimulated with α-CD3/CD28 (5 μg/ml) and TGF-β (20 ng/ml) for 24 hr to induce the expression of CCR6 and CXCR4 to mimic the *in vivo* situation. Migration of Tregs with induced CCR6 and CXCR4 cells was assayed using 24 transwell well plate (Costar) carrying 8.0 μm millicell transwell inserts. Tregs ($5x10^4$), cultured in 24 well plates were washed and placed in the transwell filter with 100 μl medium in the upper chamber. In the lower chamber of the plate 600μl RPMI (without serum) was added. Recombinant MIP-3α (200 ng/ml) or SDF-1α (200 ng/ml) was added to few well in the lower chamber. After 6 hrs of incubation, migrated cells from lower chamber were counted using haemocytometer. For blocking experiments, neutralizing antibodies were used at a concentration of 10 μg/ml. Number of cells migrated was calculated and were represented as chemotactic index. Chemotactic index represents the ratio of cells migrated in the presence of chemokines to the cells migrated spontaneously with medium alone. Assays were performed in triplicates.

3.30 Estimation of cytokines

The cytokines were estimated in the cell free supernatant and serum samples. Serum samples were collected from HI and OC patients and stored at -80 ºC. While cell free supernatants were collected either from the coculture assays or from the sorted MDSCs cultured for 24 hr in medium. Like serum samples, the cell free supernatants were stored at -80 ºC. Cytokines levels were measured by the following two ways.

3.30.1 Cytometric bead array (CBA)

Cytokines (TH1/TH2/TH17 and inflammatory) and chemokines were quantitated in the serum samples and supernatants of MDSCs by Cytometric bead array kit (BD Pharmingen). Cytokines were determined in the test samples according to the manufacturer's instructions. Briefly, test samples (50 μl) and PE detection antibody were incubated with capture bead

reagent for 3 hrs in the dark at room temperature. All unbound antibodies are washed (1.0 ml wash buffer), re-suspended in 300 µl of buffer before acquisition on BD FACS Aria cytometer (BD Bioscience, San Jose, CA, USA). Set-up bead was prepared by adding 50 µl of setup bead antibody and 450 µl of wash buffer. Samples were vortexed and acquired. The CBA data were analyzed using FCAP Array software version 1.0 (BD Biosciences).

3.30.2 Sandwich enzyme-linked immunosorbent assay (ELISA)

Levels of PGE2, IL-23, VEGF, and TGF-β were quantitated in serum samples by sandwich ELISA using commercially available kits. IFN-γ levels were quantitated in the cell free supernatant using commercially available kit (Opt EIA human IFN-γ, BD Pharmingen, CA) according to manufacturer's instructions.

3.31 Apoptosis multi-target sandwich ELISA

The sorted MDSCs and its mature counterpart i.e; $HLADR^+$ cells were analyzed for different apoptosis related proteins using PathScan® apoptosis multi-target sandwich ELISA kit (Cell signaling. Technology, USA). The kit contains the reagents necessary to detect endogenous levels of total p53 protein, phospho-p53 protein (Ser15), cleaved caspase-3 (D175), cleaved PARP (D214), total Bad and phospho-Bad molecules which are key signaling proteins in survival and apoptosis pathways.

Briefly, $2x10^6$ immuno-magnetically sorted MDSCs ($HLADR^-CD33^+CD11b^+$) and $HLADR^+$ cells were lysed using the 1X lysis buffer (200 µL) provided in the kit followed by sonication on ice. The protein lysate was collected after centrifugation for 10 min (x14, 000 rpm) at 4°C. The protein lysate was quantitated using Bradford reagent and stored at -80 °C in single-use aliquots.

3.31.1 Test procedure

The microwell strips and reagents provided in the kit were brought to room temperature. The sample were diluted with diluent in such a way that 100µl of sample contains 30µg of total

protein. From this diluted cell lysates, 100 µl of each cell lysate was added to appropriate wells and incubated overnight at 4 °C. After incubation, the contents were discarded and wells were washed 4 times by adding 200 µl 1X wash buffer each time for each well. After every wash, the plates were struck hard enough on a dry filter paper to remove the residual solution in each well. After washing, 100 µl of detection antibody (green coloured solution) was added to each well and plate was incubated for 1 hr at 37 °C. After 1 hr, washing steps were repeated as mentioned above and 100 µl HRP-linked secondary antibodies were added to each well. The plate was sealed and incubated for 30 mins at 37 °C. The washing steps were repeated and 100 µl TMB Substrate was added to each plate and incubated for 10 mins at 37 °C. Then, 100 µl stop solution was added to each well. The readings were obtained at optical density of 450 nm within 30 mins after addition of STOP solution by using ELISA plate reader.

3.32 Statistical analysis

Results are expressed as the mean ± standard error of mean (SEM). Statistical analysis was performed using Prism software (Prism Software, Lake Forest, CA) and p-value was calculated using Mann-Whitney test or Student's *t* test. Two sided *p*-values < 0.05 were considered statistically significant. Densitometry analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Chapter 4
Characterization and identification of tumor derived factor(s) responsible for CD3-ζ chain downregulation in T cells of OSCC patients

4.1 Introduction

The concept of cancer immune surveillance, originally hypothesized by Burnet [357] and Thomas [358], indicates that immune cells recognize and eliminate continuously arising nascent transformed cells [77]. However, tumors evade immune surveillance by various means such as loss or down-regulation of tumor antigens and/or HLA class I antigens, defective death receptor signaling, lack of costimulatory molecules, production of immunosuppressive cytokines, and induction of immunosuppressive T cells and other suppressor cells [359-361]. Eventually, tumor cells acquire metastatic ability. Later this concept was expanded to the cancer immunoediting, which involves immune selection pressure modifying the immunogenic phenotype and formation of tumors in immunocompetent hosts. Cancer immunoediting hypothesis stresses the dual role of the immune system; host-protecting and tumor shaping. Immune system apart from eliminating the nascent malignant cells, also shapes the tumor through equilibrium and escape phase[89]. The ability of tumor cells to escape obliteration by immune cells is because of the plethora of strategies and one of these is represented by the production of soluble immunosuppressive factors that may prevent the proinflammatory effects and promote T-cell dysfunction in the tumor microenvironment.

Immune dysfunction appears to be more frequent and profound in patients with cancer. Immune effector cells obtained from the peripheral blood of cancer patients, including oral cancer have been reported to have a variety of functional abnormalities, which may vary in magnitude from patient to patient and may be related to the extent of the disease [22, 23]. These abnormalities include defects in T cell signaling via the T-cell receptor (TCR), decreased tyrosine kinase activity following triggering with anti-CD3 monoclonal antibodies, poor lymphocytic proliferative responses, defects in lytic capacity, and decreased ability for cytokine production [23, 25, 26, 362].The immune dysfunction is also reflected at the

downregulation of expression of the T-cell receptor (TCR)-ζ chain (CD3-ζ) or of intracellular protein kinases in cancer-bearing hosts [144]. Decreased expression of the CD3-ζ chain has been reported in several autoimmune, inflammatory and malignant diseases. It has been reported that cancer cells produce several ligands which function to prevent optimal T-cell activation through CD3-ζ chain downregulation and induces either T cell anergy or apoptosis [89, 91]. Studies from our laboratory have shown that posttranslational downregulation is primarily responsible for decreased CD3-ζ chain expression in the peripheral blood of oral cancer patients while a dominant transcriptional defect is observed in the tumor compartment. The down-regulation of CD3-ζ chain culminates into impaired lymphocyte responses in these patients [29].

Immune dysfunction is a hallmark of patients with oral cancer and the loss of CD3-ζ chain contributes majorly to the observed immune dysfunction in these patients [29]. However, the mechanism responsible for decreased expression of CD3-ζ chain in these patients is not clear. Understanding the role of tumor derived factors in modulating the CD3-ζ chain levels, might ultimately provide a therapeutic targets to generate effective antitumor immune response.

In the present chapter we aimed to address the following questions:

1) Do oral tumors secrete the soluble factors responsible for CD3-ζ chain downregulation?
2) What is the mechanism by which the identified factor (s) induces CD3-ζ chain downregulation and thereby contributes to the T cell dysfunction?

4.2 Results

4.2.1 Kinetics of CD3-ζ chain downregulation induced by oral tumor supernatant in T cells

Our earlier published data demonstrated that compared to other T cell signaling molecules, (p56Lck, Zap70) CD3-ζ chain is markedly decreased in peripheral blood T cells of OSCC patients [29]. This study was proposed to investigate the role of oral tumor derived factor(s) in regulating CD3-ζ chain expression. Single cell suspension of oral tumors was prepared by enzyme digestion (as described in material and methods). Oral tumor cells were cultured in serum free media and cell free supernatant (referred to as tumor supernatant) was collected after 72 hrs in culture. The oral tumor supernatant (stage III patient) was added to the PBMCs of HI and incubated for different time intervals (24-96 hrs). The expression of CD3-ζ chain was monitored in HI PBMCs at defined time intervals after incubation with tumor supernatant **(Fig. 4.1a).** Time kinetics demonstrated that in HI PMBCs, on incubation with tumor supernatant, a progressive decrease in CD3-ζ chain expression was observed compared to PBMCs incubated with medium alone (control) **(Fig. 4.1a)**.

The effect of tumor supernatant collected from oral cancer patients of different stages (I-IV) on CD3-ζ chain expression was also studied. The HI PBMCs were incubated with the oral tumor supernatant for 72 hrs in culture. After 72 hrs PBMCs were stained and analyzed for CD3-ζ chain expression by flow cytometry. The supernatants collected from the patients (Stage I-IV) led to the marked decrease in CD3-ζ chain expression. Interestingly, the effect on the CD3-ζ chain levels correlated inversely with stage of oral cancer patients **(Fig. 4.1b).** The data confirmed that the oral tumors secrete immunosuppressive factors which may alter the T cell signaling by decreasing CD3-ζ chain expression.

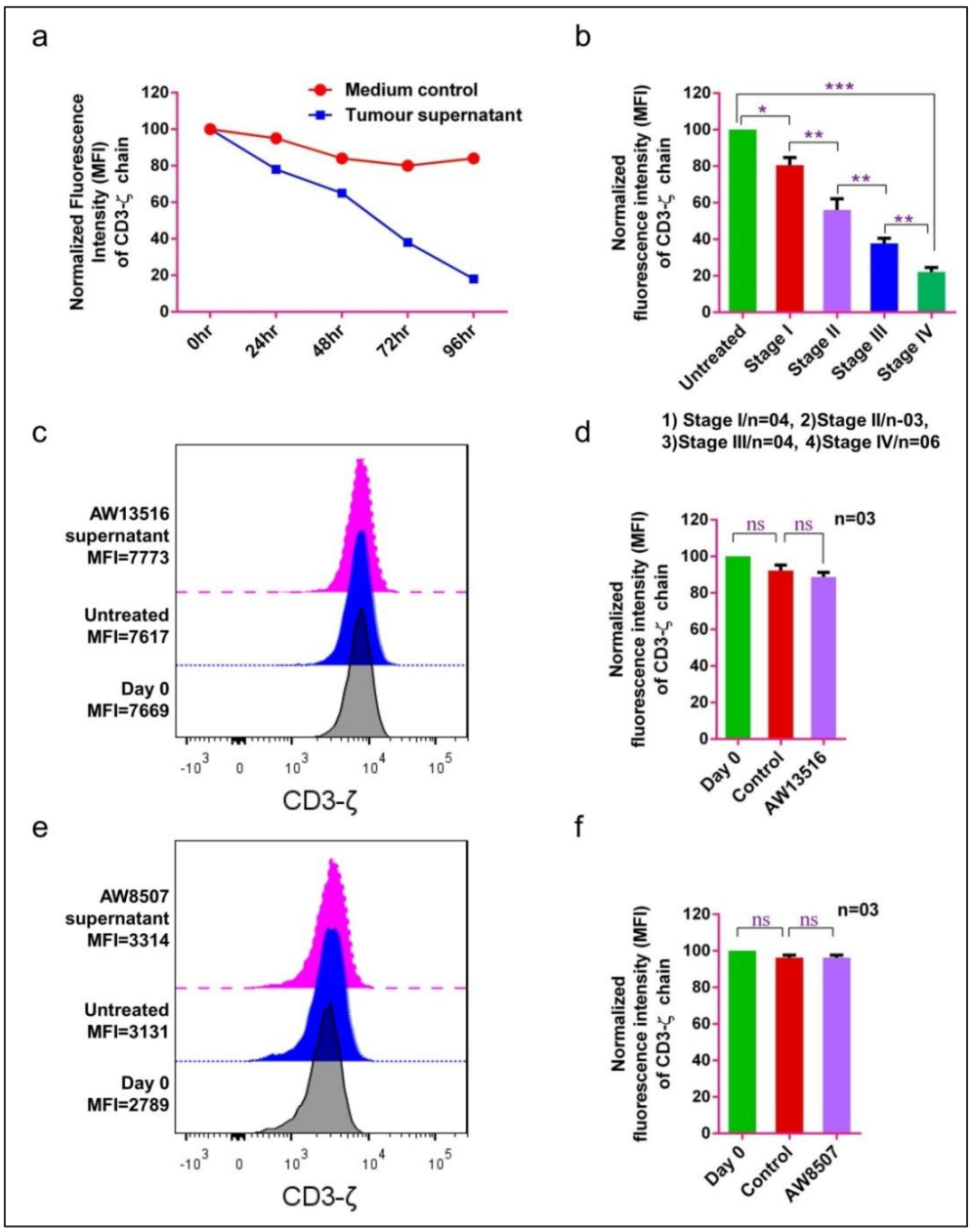

Figure 4.1: Healthy individual PBMCs were cultured in the presence of tumor supernatant to monitor the expression of CD3-ζ chain in T cells. **a)** Representative figure showing the expression of CD3-ζ chain on gated $CD3^+$ T cells in HI PBMCs at different time points (24, 48, 72 and 96 hrs) after incubation with cell-free supernatant (diluted 1:1) obtained from oral cancer patients (Stage III patient) and medium (control). **b)** Oral tumor supernatant were collected after 72 hrs in plain RPMI from different tumor stages (Stage 1(n=4), stage II (n=3) stage III (n=4) and stage IV (n=6)). Oral tumor supernatant were added to the HI PBMCs at 1:1 ratio and after 72 hrs effect of tumor supernatant on CD3-ζ chain was analyzed. The graph indicates the expression (Normalized median fluorescence intensity) of CD3-ζ chain. Fluorescence intensity was adjusted to 100 for control. Accordingly intensity for the test was calculated and represented as normalized fluorescence intensity. (*** represents $p<0.0005$, ** represents $p<0.005$, * represents $p<0.05$) **c-f)** Cell-free supernatants collected from oral tumor-derived cell lines (AW13516 and AW8507) did not cause any change in CD3-ζ chain expression after incubating with HI PBMCs for 72 hrs. The graph

shown is representative of three independent experiments ("ns" represents non-significant with $p>0.05$)

The supernatants from two oral tumor derived cell lines AW8507 and AW13516 was also used to analyze their effect on CD3-ζ chain expression. The cell lines were cultured in the serum free medium for 72 hrs and after that cell free supernatant was collected. The PBMCs from HI were incubated with these supernatants for 72 hrs. After 72 hrs cells were stained and analyzed for CD3-ζ chain expression. However, these supernatants were not able to decrease the CD3-ζ chain expression on gated $CD3^+$ T cells **(Fig. 4.1c-f).** The difference observed with the supernatants of freshly isolated tumors and cell lines could be attributed to the heterogeneous cell types present in the tumor microenvironment of freshly isolated tumors.

4.2.2 Effect of tumor derived factors from oral tumors on other T cell signaling molecules

TCR activation promotes a number of signaling cascades that ultimately determine cell fate through regulating cytokine production, cell survival, proliferation, and differentiation [363]. An early event in TCR activation is phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytosolic side of the CD3-ζ chain by lymphocyte protein tyrosine kinase (p56Lck). The CD3 complex contains a CD3γ chain, a CD3δ chain, and two CD3ε chains. These chains associate with T-cell receptor (TCR) and CD3-ζchain (zeta-chain) to generate activation signal in T lymphocytes. The CD3-ζ chain associated protein kinase (ZAP-70) is recruited to the TCR/CD3 complex where it becomes activated, promoting recruitment and phosphorylation of downstream adaptor or scaffold proteins [364, 365]. The effect of tumor supernatant on other T cell signaling molecules (p56Lck, Zap70 and CD3-ε) was also studied after incubation for 72 hrs. The supernatants collected from the patients (Stage I-III) led to the marked decrease in CD3-ζ chain expression **(Fig. 4.1b)** while

its effect on CD3-ε chain, ZAP-70 and p56Lck expression was marginal **(Fig. 4.2a-c).** However, the tumor supernatant collected from stage IV patients showed a profound broad range effect on all T cell signaling molecules (CD3-ζ chain, CD3-ε, p56Lck, and ZAP-70) **(Fig. 4.2a-c)**.

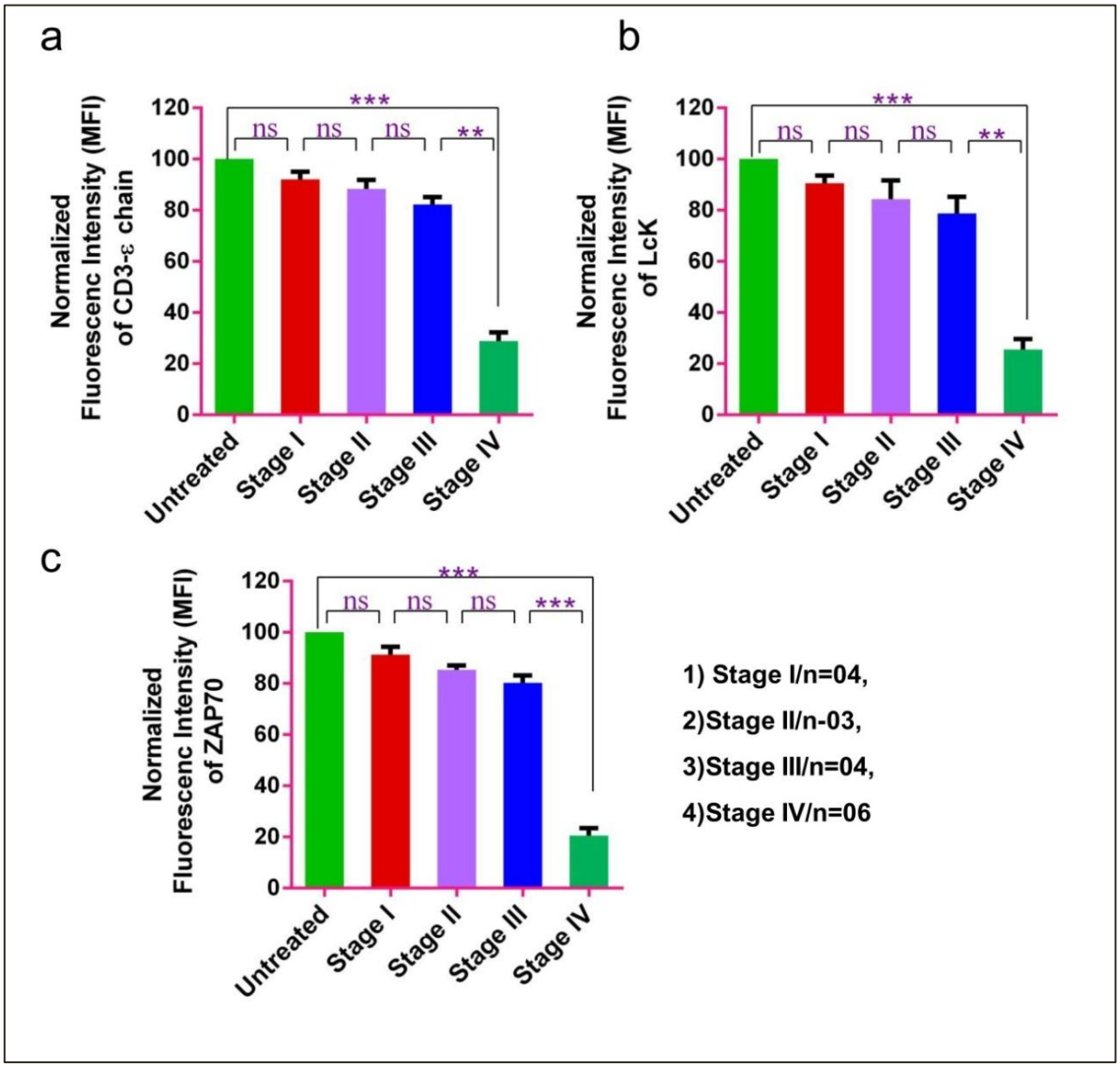

Figure 4.2: Healthy individual PBMCs were cultured in the presence of tumor supernatant to monitor the expression of T cell signaling molecules. **a-c)** Representative figure showing the expression of T cell signaling molecules (CD3-ε, p56Lck, and ZAP70) on gated $CD3^+$ T cells in HI PBMCs after 72 hrs of incubation with cell-free supernatant (diluted 1:1) obtained from different tumor stages of oral cancer patients (Stage 1(n=4), stage II (n=3) stage III (n=4) and stage IV (n=6)). The graph indicates the expression (Normalized median fluorescence intensity) of T cell signaling molecules on incubation with tumor supernatants obtained from different stage tumors. Fluorescence intensity was adjusted to 100 for control. Accordingly intensity for the test was calculated and represented as normalized fluorescence intensity. (*** represents $p<0.0005$, ** represents $p<0.005$ and ns represents non-significant with $p>0.05$)

4.2.3 Proteomic approach to identify the possible factors present in oral tumors that regulate CD3-ζ chain expression

The oral tumor supernatant was capable of altering the CD3-ζ chain expression in the T cells while the supernatants from the oral tumor derived cells (AW8507 and AW13516) were not. In order to identify the oral tumor cell derived factors that degrade CD3-ζ chain, a pragmatic approach was adapted. The cell free supernatants derived from oral tumors and cell lines (AW8507 and AW13516) were subjected to LC-MS/MS. The identities of proteins observed exclusively in cell free tumor supernatants were shortlisted **(Table 4.1)**. The common proteins present in the tumor supernatant and supernatants of the oral tumor cell lines were excluded. Twenty one proteins were exclusively present in the tumor supernatant and among them the proteins like *Nuclear migration protein nudC, SET-binding protein (SEB), Pleckstrin homology-like domain family B member 2, Kinesin-like protein KIF14, 2'5' Oligoadenylate Synthetase (OAS2), Protocadherin beta 16 precursor* showed a higher score (>23) in *ms/ms* analysis. OAS2 belonging to 2′5′ Oligoadenylate synthetase family of antiviral proteins comprising of OAS1, OAS2, OAS3 and OASL proteins was taken for further analysis [366]. It is known to control cellular apoptosis [367]; which may alter the expression of caspase-3, thereby regulating CD3-ζ chain expression. OAS2 is also characterized by different subcellular locations and enzymatic parameters suggesting that this protein might have distinct roles [368]. Hence, based on LC-MS/MS data and previously documented literature, OAS2 was shortlisted as the key molecule present in the cell free supernatants of the oral tumors that may play a role in CD3-ζ chain downregulation.

4.2.4 Expression of Oligoadenylate synthetase 2 in oral tumors and tumor derived cell lines

In order to show the presence of OAS2 in tumor microenvironment, the expression of OAS2 at both protein and mRNA level was analyzed in oral tumors.

Table 4.1: Screening of proteins present exclusively in tumor supernatant by Liquid chromatography coupled with mass spectrometry (LC-MS/MS)

DESCRIPTION OF PROTEIN	PEPTIDE SEQUENCE	MASS/ SCORE
Nuclear migration protein nudC	K.SMGLPTSDEQKK.Q	885.1/23
Dedicator of cytokinesis protein 7	R.SYTEDWAIVIRK.Y	885/19
SET-binding protein (SEB)	R.SYEGFGTYREK.D	884.9/23
Dual specificity tyrosine-phosphorylation-regulated kinase 4	R.NLKPQPRPQTLRK.S	860.8/18
Toll-like receptor 6 precursor (CD286 antigen)	K.SIVENIINCIEK.S	860.4/15
Microtubule-associated proteins 1A/1B light chain 3C precursor (Microtubule-associated protein 1 light chain 3 gamma) (MAP1A/MAP1B LC3 C) (MAP1A/1B light chain 3 C) (MAP1 light chain 3-like protein 3) (Autophagy-related protein LC3 C).	K.SLVSMSATMAEIYR.D	860.4/17
U6 snRNA-associated Sm-like protein LSm2	K.SLVGKDVVVELK.N	860.1/19
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1 (EC 3.6.1.-)	K.LNYAIFDEGHMLK.N	855.4/15
Protein GPR89A (Putative MAPK-activating protein PM01)	R.RLLQTMDMIISK.K	590.6/20
Ephexin-1 (Eph-interacting exchange protein)	K.SVNEPLTLNIPWSR.M	590.3/15
Semenogelin-1 precursor	K.EQTSVSGAQK.G	590.3/17
Aryl hydrocarbon receptor nuclear translocator 2 (ARNT protein 2)	R.SGMDFDDEDGEGPSK.F	582.3/22
Pleckstrin homology-like domain family B member 2	R.SGAASMPSSPK.Q	582/23
Kinesin-like protein KIF14	R.SGHLTTKPTQSK.L	534.0/16
Chromatin-modifying protein 4c	K.KHGTQNK.R	478.3/16
PHD finger protein 21B	R.HQNGDLK.K	478.3/16
CD109 antigen precursor (p180)	R.TYTWLK.G	478.3/16
Ankyrin repeat and SOCS box protein 1 (ASB-1)	K.WESLGPESRGR.R	473.3/14
Interleukin-1 family member 8 (IL-1F8)	R.TNIGMPGR.M	502.8/11
Mastermind-like protein 3 (Mam-3)	R.SLQGMPGR.T	502.8/11
Kinesin-like protein KIF14	R.SGHLTTKPTQSK.L	524.9/27
Microtubule-associated proteins 1A/1B light chain 3C precursor	K.SLVSMSATMAEIYR.D	860.4/15
Integrin alpha-L precursor	R.RGLFPGGR.H	503.8/14
Ras-related protein Rab-6B	M.SAGGDFGNPLRK.F	503.3/14
Kelch domain-containing protein 5	R.SNFKLVAVNSK.L	450.8/11
2'-5'-oligoadenylate synthetase 2 (EC 2.7.7.-)	**R.ILNNNSK.R +**	**473.3/23**
Epididymal secretory protein E1 precursor	K.TYSYLNK.L	517.3/19
Pericentrin (Pericentrin B) (Kendrin)	R.SLTEQQGR.L	531.2/22
Ubiquitin-conjugating enzyme E2 J1 (EC 6.3.2.19)	R.GPPDSDFDGGVYHGR.I	573.8/16
Protocadherin beta 16 precursor (PCDH-beta16)	R.VIDINDHSPMFTEKEMILK.	855.1/23

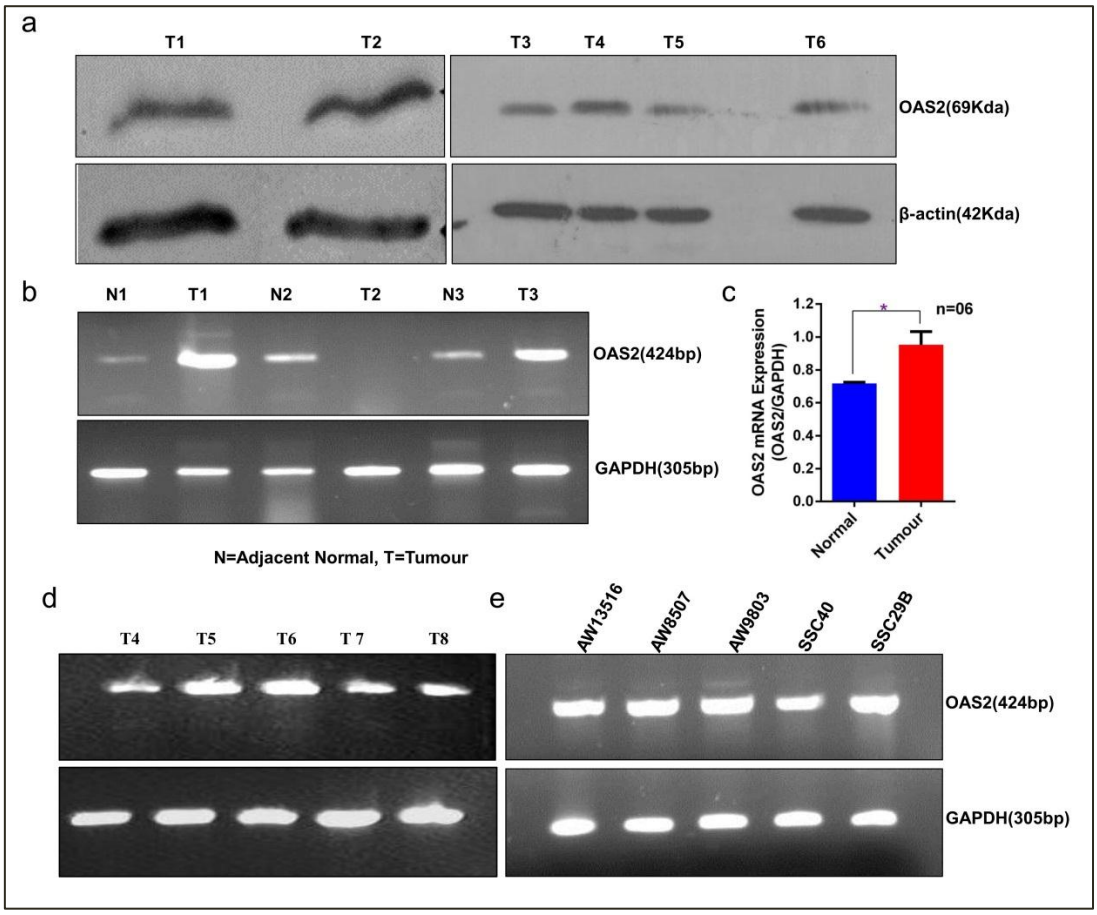

Figure 4.3: Expression of OAS2 in oral tumor cells. **a)** Total protein extract from oral tumors was used to detect the levels of OAS2 and β-Actin by western blotting. The protein extracts (40-60 µg) prepared from oral tumors (n=6) was loaded into the SDS-PAGE and then transferred to the nitrocellulose membrane. The results showed a presence of the OAS2 (p69) in these tumors, β-actin was used as internal control. **b)** Total RNA (150-300 ng/µL) was isolated from the oral tumors and cell lines of oral tumor origin. The isolated RNA was converted into cDNA and cDNA was subjected to PCR, as described in "Materials and methods," to detect *OAS2* and *GAPDH* transcripts. The expression of OAS2 transcript in oral tumors (T) and its adjacent normal tissues (N) is shown**.** The tumors in panel "a" and "b" are different. **c)** Band densitometry illustrating total OAS2 transcript expression is higher in oral tumors compared to the adjacent normal tissue (* represents $p<0.05$)**. d and e)** Shows the expression of OAS2 mRNA in oral tumors and cell lines (AW13516, AW8507, AW9803,SSC40 and SSC29B).

The expression analysis of OAS2 by western blotting showed that oral tumors (Stage II-IV) express OAS2 (69Kda) at protein level **(Fig. 4.3a).** For OAS2 mRNA expression, total RNA was extracted from surgically excised oral tumors and adjacent normal tissue. The complementary DNA (cDNA) was prepared from the total RNA using MuLV RT enzyme. Gene specific PCR was performed for GAPDH and OAS2. The heterogeneous expression of OAS2 mRNA was observed in oral tumors (Stage II and III) **(Fig. 4.3b).** In one of the oral

tumor sample, mRNA expression of OAS2 was not observed while in its adjacent normal OAS2 expression was noted. The overall expression of mRNA for OAS2 remained high in oral tumors compared to the histologically adjacent normal tissues **(Fig. 4.3c).** The expression of OAS2 was also confirmed in additional tumors where adjacent normal tissues were not unavailable **(Fig. 4.3d).** However, in case of oral tumor derived cell lines (AW13516, AW8507, AW9803, SSC29B, and SSC40) OAS2 expression was observed only at mRNA level **(Fig. 4.3e)** but not seen at protein level (data not shown). This may suggest that the tumor microenvironment contributes to OAS2 expression and might be requiring an additional signal (IFNα/β) to trigger its expression in epithelial cells or would be secreted by the immune cells present in the tumor microenvironment.

4.2.5 Secreted 2′5′ Oligoadenylate synthetase 2 mediates CD3-ζ chain downregulation in T cells

To analyze the role of extracellular OAS2 in degrading CD3-ζ chain, the key T cell signaling molecule, a surrogate situation was created by transfecting vector control (pEGFP-N2) or OAS2 encoding pEGFP-N2 vector (pEGFP-N2+OAS2) into HEK293 cells. The coding sequence (CDS) of OAS2 was cloned into the pEGF-N2 vector as described in material and methods. The overexpression of OAS2 in the HEK293 cells was analyzed at both protein as well as RNA levels. The protein was extracted from both vector transfected as well as OAS2 encoding vector cells. The protein was loaded onto 10% SDS PAGE and transferred to the nitrocellulose membrane. The membrane was probed for OAS2 using anti- OAS2 specific antibody. As seen in **Fig. 4.4a**, overexpression of OAS2 was observed in the cells transfected with the OAS2 encoding pEGF-N2 vector. Further total RNA was extracted from transfected cells, and OAS2 mRNA expression level was analyzed by RT-PCR in untransfected, vector control and pEGFP-N2+OAS2 vector transfected HEK293 cells. The OAS2 expression was higher in cells transfected with pEGFP-N2+OAS2 compared to the untransfected or vector

control HEK293 cells **(Fig. 4.4b).** Transfected cells were also analyzed by flow cytometry for GFP positivity which indirectly also confirms overexpression of OAS2 protein as well as the inframe addition of OAS2 with GFP in the pEGF-N2 vector. The flow cytometry data shows that cells transfected with pEGF-N2+OAS2 has higher mean fluorescence intensity (MFI=245) compared to the cells alone (MFI=4.96) **(Fig. 4.4c).**

Supernatants were collected from OAS2 (pEGFP-N2+OAS2) and vector control (pEGFP-N2) transfected HEK293 cells after 48 hr. The collected supernatants from control and OAS2 transfected HEK293 cells were added to the HI PBMCs at 1:1 [supernatant: medium (RPMI)] dilution for different time intervals. The cells were harvested at respective time points and were analyzed for the expression of T cell signaling molecules (CD3-ζ and CD3-ε chains).

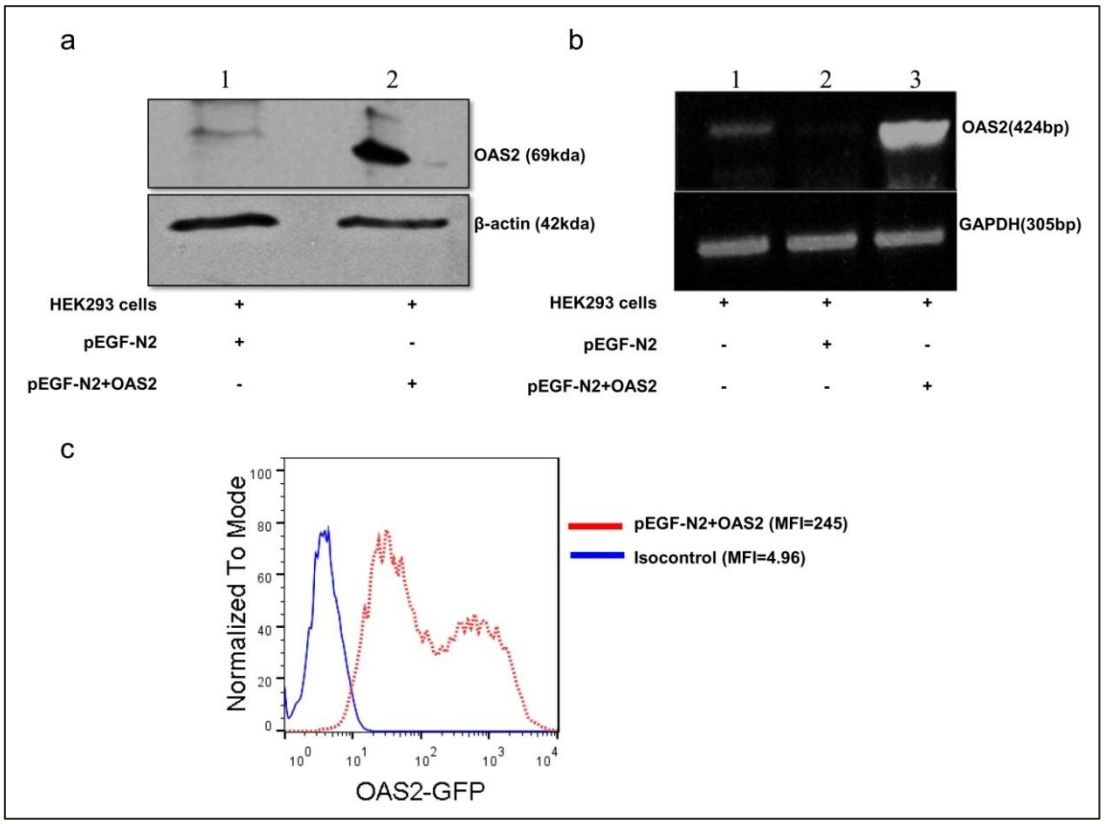

Figure 4.4: Overexpression of oligoadenylate synthetase 2 in HEK293 cells. a) HEK293 cells were transfected with either vector control (pEGFP-N2) or vector encoding the OAS2 cDNA (pEGFP-N2+OAS2), protein lysate was extracted and probed for OAS2 using α-OAS2 mAb (Santa Cruz Biotechnology, 1:500 dilution), β-actin was used as loading control. **b)** HEK293 cells were transfected with either vector control (pEGFP-N2) or vector encoding the OAS2 cDNA (pEGFP-N2+OAS2). Total RNA was extracted from transfected cells, and mRNA expression levels were analyzed by RT-PCR using gene specific primers for OAS2

and GAPDH. Lane 1, 2, and 3 represent untransfected HEK293, vector control transfected HEK293 and pEGFP-N2+OAS2 transfected HEK293 cells respectively. **c)** The pEGFP-N2+OAS2 transfected HEK293 cells were monitored for GFP expression by flow cytometry

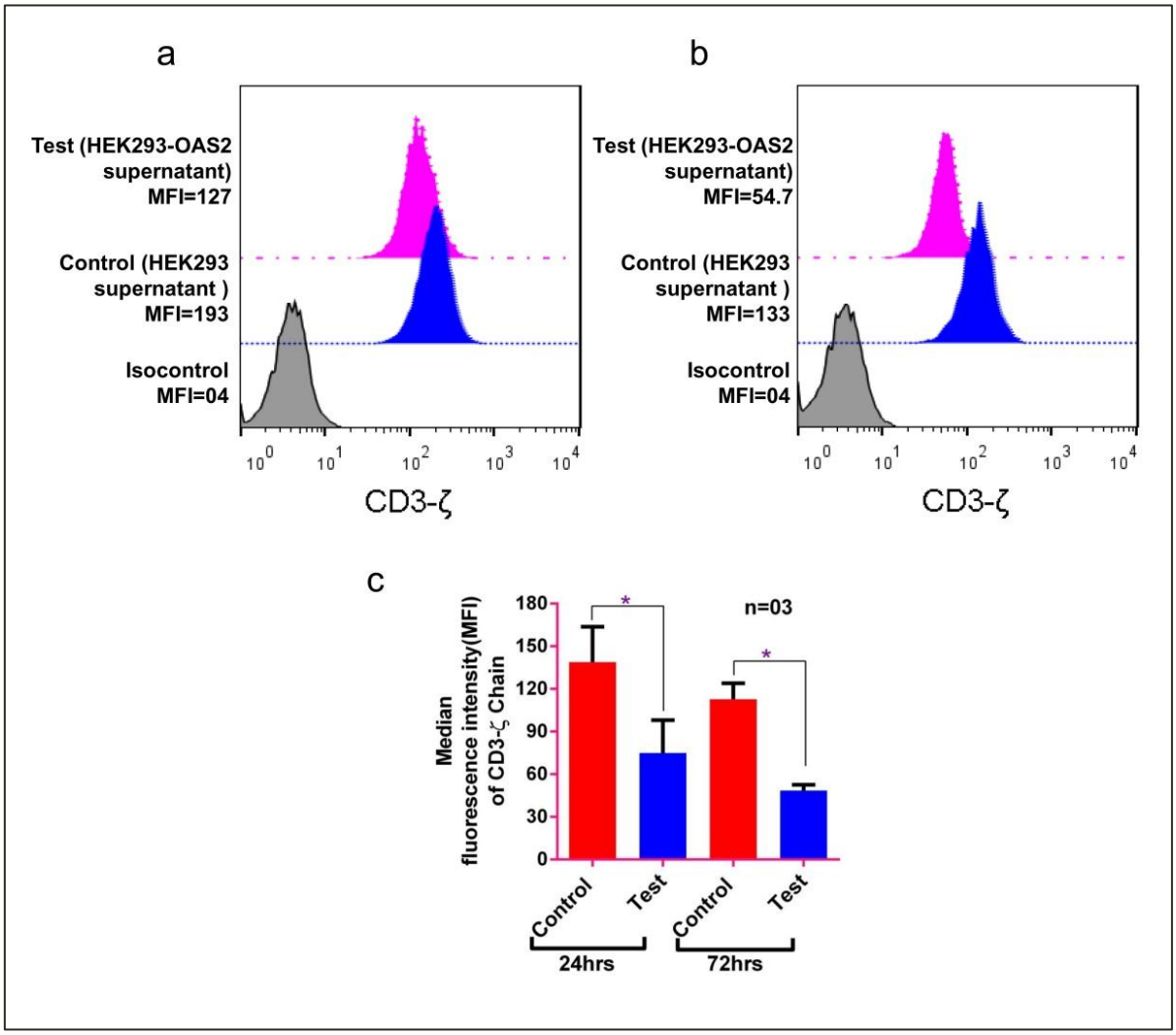

Figure 4.5: Effect of supernatant collected from OAS2 overexpressing HEK293 cells on CD3-ζ chain of T cells. Cell-free supernatant collected from pEGFP-OAS2 transfected HEK293 cell line led to the decrease in expression of CD3-ζ chain on gated $CD3^+$ T cell in HI PBMCs at different time points (24 and 72 hrs) compared to the supernatant collected from vector control transfected cells. **a)** the half offset histogram shows the fluorescence intensity of CD3-ζ at 24 hr while **b)** shows the fluorescence intensity at 72 hrs **c)** The median fluorescence intensity of CD3-ζ chain in HI PBMCs treated with cell-free supernatant of vector control and pEGFP-OAS2 transfected HEK293 at 24 and 72 hrs. The data represented here is the mean of three experiments carried out using supernatants collected from three independent transfections (*represents $p<0.05$).

The results confirmed that at both the time points (24 hr and 72 hrs) there was a decreased expression of CD3-ζ chain when incubated with supernatant collected from OAS2 overexpressing HEK293 cells compared to control HEK293 cells (Vector control transfected)

(Fig. 4.5a-c). On the other hand CD3-ε (epsilon) expression at both the time intervals 24 hr and 72 hrs remained unaltered on incubation with supernatant collected from OAS2 transfected HEK293 cells **(Fig. 4.6a-c).** This confirms that the effect of secreted OAS2 was specific and altered only CD3-ζ chain expression in T cells. The presence of OAS2 in supernatants was confirmed by the immuno dot blot assay **(Fig. 4.7).**

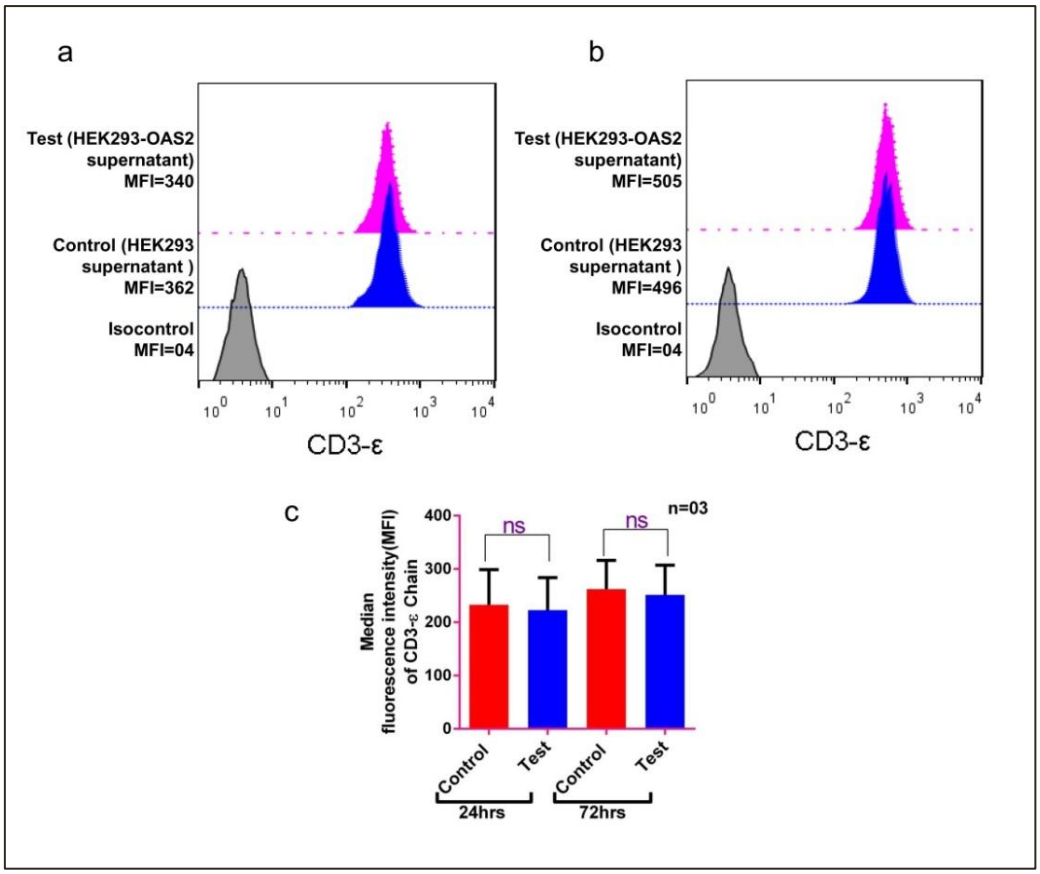

Figure 4.6: Effect of supernatant collected from OAS2 overexpressing HEK293 cells on CD3-ε chain of T cells. Cell-free supernatant collected from pEGFP-OAS2 transfected HEK293 cell line did not alter the expression of CD3-ε chain of T cells. **a and b)** Represent the unaffected fluorescence intensity (median) of CD3-ε chain in HI PBMCs after treatment with cell-free supernatant collected from vector control and pEGFP-OAS2 transfected HEK293 cells at different time intervals 24 and 72 hrs. **a)** The half offset histogram shows the fluorescence intensity of CD3-ε at 24 hr while **b)** shows the fluorescence intensity at 72 hrs **c)** The median fluorescence intensity of CD3-ε in HI PBMCs treated with cell-free supernatant of vector control and pEGFP-OAS2 transfected HEK293 at 24 and 72 hrs. The data represented here is the mean of three experiments carried out using supernatants collected from three independent transfections ("ns" represents non-significant).

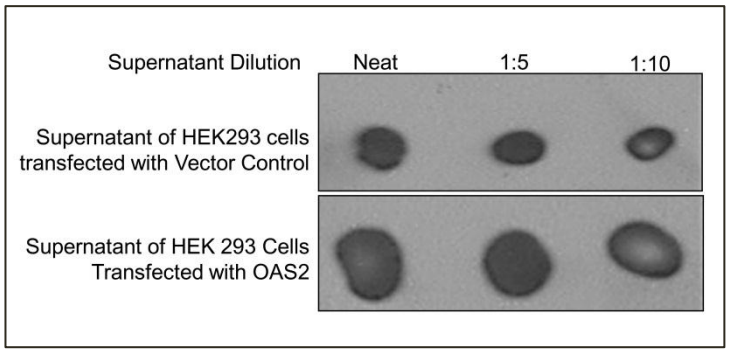

Figure 4.7: Dot blot showing presence of OAS2 in the cell free supernatant. Higher levels of OAS2 were present in the supernatant of HEK 293 cells transfected with OAS2 encoding vector compared to the vector control transfected HEK293 cells. Diluted supernatant (1:5 and 1:10 as well as neat) are analyzed for the presence of OAS2 using OAS2 mAb.

4.2.6 Recombinant human OAS2 (rh-OAS2) protein decreases CD3-ζ chain expression in T cells derived from peripheral blood of healthy individuals

To establish that secreted OAS2 has the ability to reduce the CD3-ζ chain expression, set of in vitro experiments were carried out. To elucidate the role of OAS2 protein in downregulating the CD3-ζ chain expression, HI PBMCs were stimulated with a range of concentrations of rh-OAS2 (3-96ng/ml) for 24 hr. PBMCs were harvested and expression of CD3-ζ chain expression was examined by flow cytometry. The data showed that HI PBMCs stimulated with rh-OAS2 showed decreased expression of CD3-ζ chain expression (median fluorescence intensity) on gated population of $CD3^+$ T cells **(Fig. 4.8a).** Although the dose dependent effect was not evident, the effect was best observed at concentration above 6 ng/ml of rh-OAS2. Hence, the concentrations of 6, 12 and 24 ng/ml were used in further experiments to characterize the role of rh-OAS2. The treatment of PBMCs with rh-OAS2 at 6, 12 and 24 ng/ml concentrations for 24 hr led to the significant decrease in CD3-ζ chain expression **(Fig. 4.8b-c).** However, rh-OAS2 treatment was not able to induce the change in CD3-ε expression in the T cells, confirming the effect of rh-OAS2 was specific in modulating only CD3-ζ chain expression **(Fig. 4.8d).** This is consistent with the earlier observations which clearly indicated that rh-OAS2 was unable to affect the CD3-ε chain expression on

$CD3^+$ T cells. Overall this shows that rh-OAS2 modulates the CD3-ζ chain expression which plays a major role in T cell signaling and remodelling the effector functions of T cells.

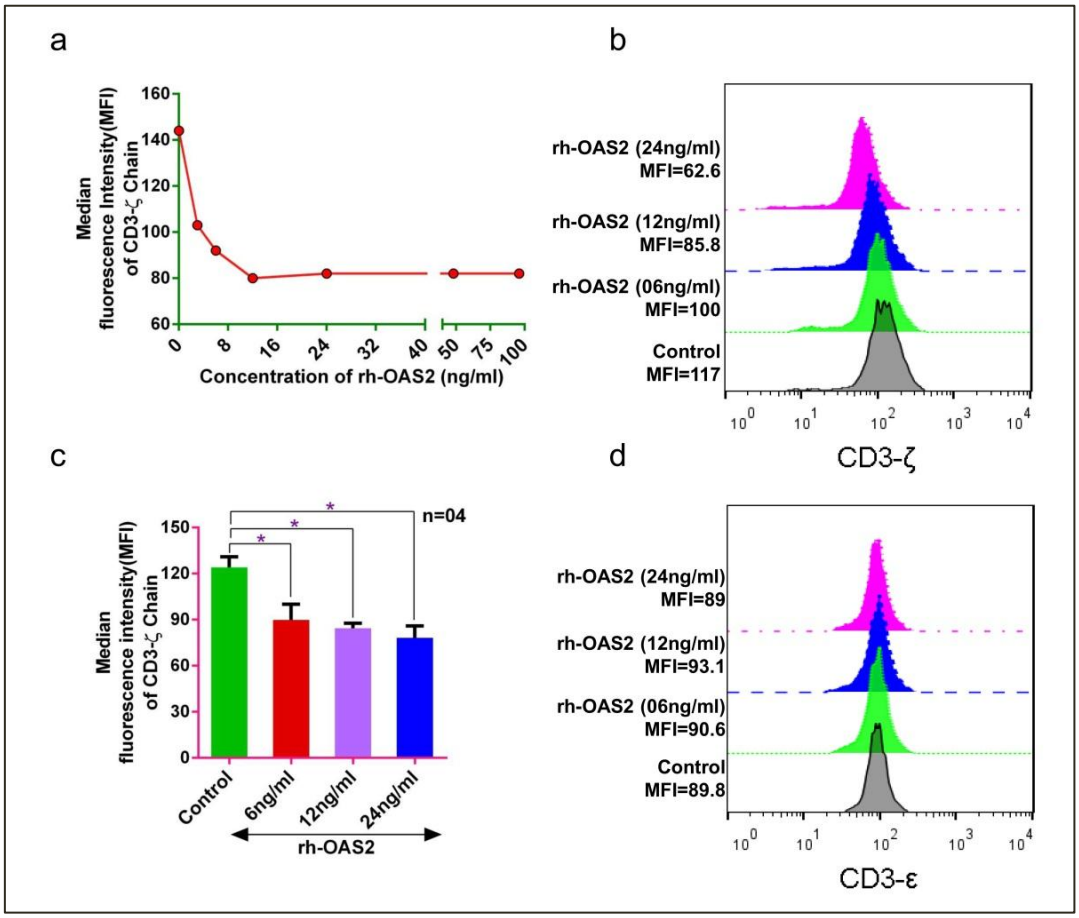

Figure 4.8: Effect of different concentration of rh-OAS2 on the expression of CD3-ζ and CD3-ε chain on $CD3^+$ T cells of HI PBMCs. a) The dose response curve of rh-OAS2 stimulation and CD3-ζ chain downregulation in the HI PBMCs. **b)** The fluorescence intensity (median) of CD3-ζ chain decreased after incubation of HI PBMCs with different concentration of rh-OAS2. **c)** Significant reduction in the expression of CD3-ζ chain was observed on gated $CD3^+$ T cells when treated with 6, 12 and 24 ng/ml of rh-OAS2. The data represented here is the mean of four independent experiments (* represents $p<0.05$). **d)** The half offset histogram shows the fluorescence intensity (median) of CD3-ε after incubation of HI PBMCs with different concentrations of rh-OAS2. The expression of CD3-ε remained unaffected at all the concentration of rh-OAS2 used for stimulation

4.2.7 Effect of recombinant human OAS1 (rh-OAS1) protein on CD3-ζ chain expression in T cells derived from peripheral blood of healthy individuals

OAS2 belongs to 2′5′ Oligoadenylate synthetase family of antiviral proteins comprising of OAS1, OAS2, OAS3 and OASL [366]. To prove whether its ability to downregulate CD3-ζ chain is the function of the whole OAS family or it is associated with only OAS2 protein,

similar experiments were carried out with rh-OAS1 which is closely related to OAS2. To reveal the role of OAS1 protein in downregulating the CD3-ζ chain expression, HI PBMCs were stimulated with range of concentrations of rh-OAS1 (3-96ng/ml) for 24 hr. PBMCs were harvested and the CD3-ζ chain expression was examined by flow cytometry. PBMCs upon stimulation with rh-OAS1 were unable to decrease the CD3-ζ chain expression on gated population of $CD3^+$ T cells **(Fig. 4.9a-b).** Similar to rh-OAS2, rh-OAS1 was also incapable of decreasing the CD3-ε chain expression on T cells **(Fig. 4.9c).** Thus, this data confirmed that OAS2 but not OAS1 was capable of modulating the CD3-ζ chain expression.

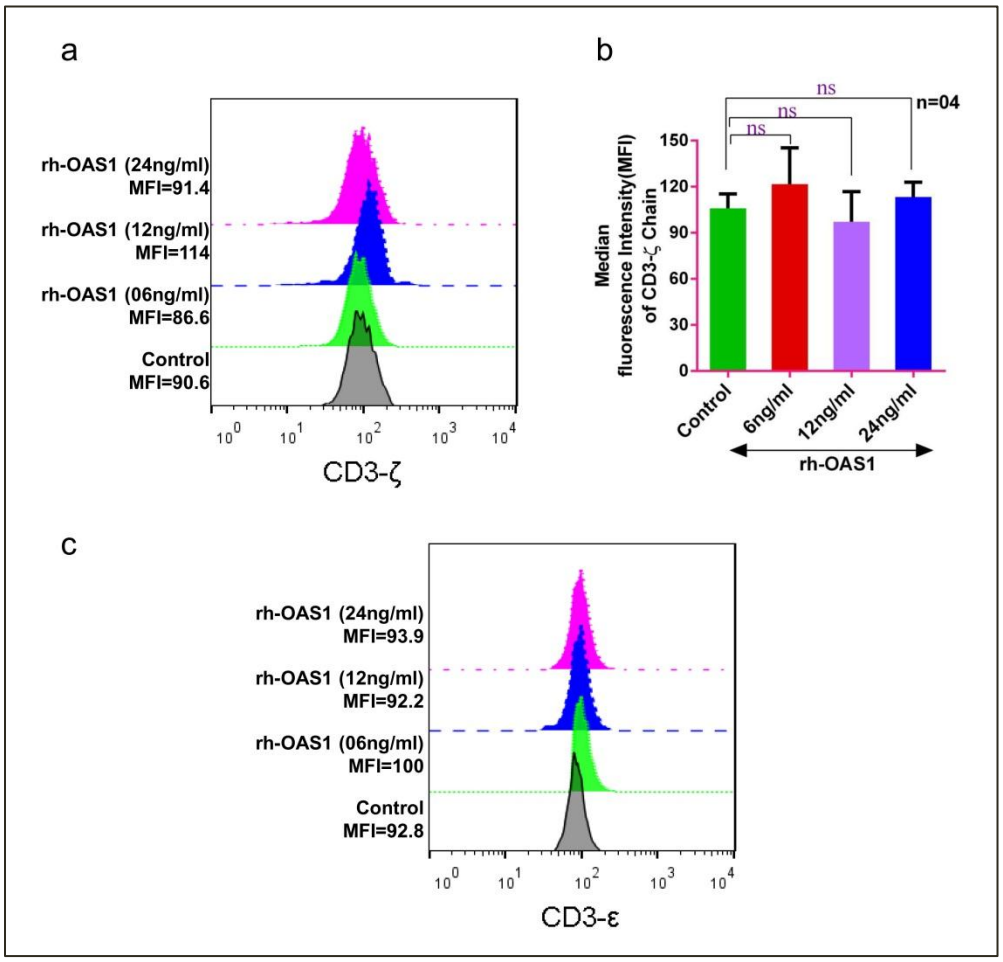

Figure 4.9: Effect of different concentration of rh-OAS1 on the expression of CD3-ζ and CD3-ε chain on $CD3^+$ T cells of HI PBMCs. a) the half offset histogram shows that the fluorescence intensity (median) of CD3-ζ chain remained unchanged after incubation of HI PBMCs with different concentration of rh-OAS1. **c)** No reduction in the expression of CD3-ζ chain was observed on gated $CD3^+$ T cells when treated with 6, 12 and 24 ng/ml of rh-OAS2 compared to the medium control. The data represented here is the mean of four independent experiments ("ns" represents non-significant). **c)** The CD3-ε chain expression also remained

unaffected on $CD3^+$ T cells after treatment with rh-OAS1 at different concentration (6, 12 and 24 ng/ml)**.**

4.2.8 Effect of tumor supernatant and rh-OAS2 on CD3-ζ chain expression in sorted T cells of healthy individuals

PBMCs are a heterogeneous population containing both innate and adaptive immune cells. In order to understand whether oral tumor supernatants or rh-OAS2 directly acted on T cells or whether other immune cell types had an influence on CD3-ζ chain downregulation in T cells, experiment was performed on sorted T cells. $CD3^+$ T cells were purified using Miltenyi microbeads according to the manufacturer's instruction**.** The Purity of $CD3^+$ T cells sorted using CD3 microbeads was ≥ 95% **(Material and Methods).** Tumor supernatants were collected from the oral tumors grown in serum free medium as described above. Tumor supernatants were incubated with purified T cells in a 1:1 ratio for 24 hr. The addition of tumor supernatant to the purified T cells led to the decrease in the expression of CD3-ζ chain in T cells. The decrease in CD3-ζ chain expression observed in purified T cells was comparable to what was observed in gated $CD3^+$ T cells in PBMCs after 24 hr **(Fig. 4.10a-b).** These results demonstrate that tumor supernatant specifically acted on CD3-ζ chain expressed in T cells. In order to understand whether rhOAS2 also acted directly on T cells or whether other immune cell types influenced rh-OAS2 to downregulate CD3-ζ chain in T cells, a similar experiment was carried out. The purified T cells were incubated with different concentrations of rh-OAS2 (6, 12, and 24 ng/ml) for 24 hr. Incubation of T cells with various concentrations of rh-OAS2 (6, 12, 24 ng/ml) also led to the decrease in CD3-ζ chain expression **(Fig. 4.10c).** In case of purified $CD3^+$ T cells a marginal dose response was evident in the CD3-ζ chain downregulation and rh-OAS2 used **(Fig. 4.10d).** However, the data corroborated with the earlier data obtained with PBMCs treated with rh-OAS2. These results demonstrate that tumor supernatant and rh-OAS2 specifically acted on CD3-ζ chain

expressed in T cells and rules out the role of other immune cells in executing the phenomenon of CD3-ζ chain downregulation

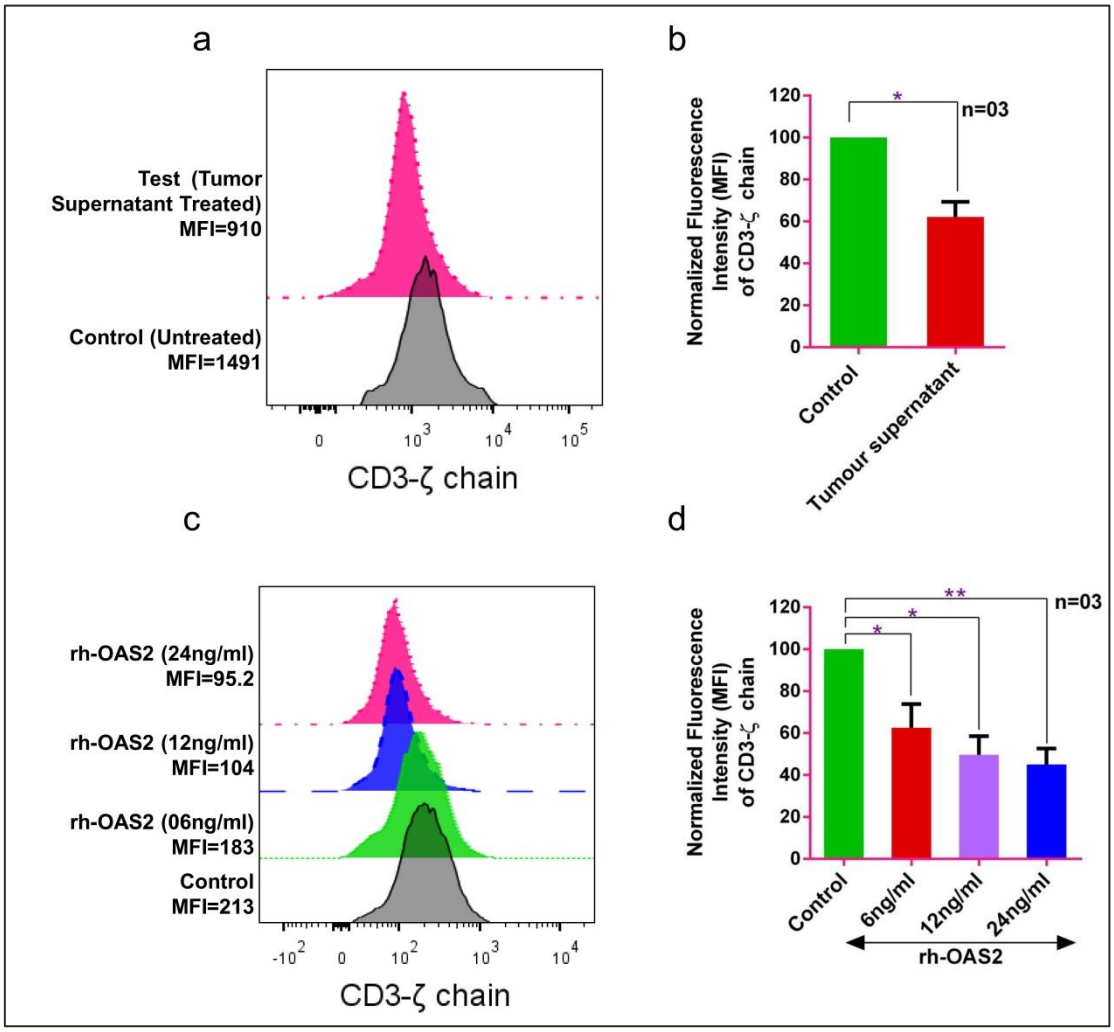

Figure 4.10: Effect of tumor supernatant and rh-OAS2 on the sorted CD3$^+$ T cells. a) Representative figure showing the expression of CD3-ζ chain in CD3$^+$ T cells after incubation with cell-free supernatant (TSN) (diluted 1:1) obtained after culturing oral tumors or with medium (control). **b)** Graph indicates the expression (Normalized median fluorescence intensity) of CD3-ζ chain on incubation with tumor supernatants obtained from oral tumors. The graph shown is representative of three independent experiments and * represents $p<0.05$). **c)** Representative figure showing that fluorescence intensity (median) of CD3-ζ chain decreased after incubation of CD3$^+$ T cells with different concentrations of rh-OAS2. Significant reduction in the expression of CD3-ζ chain (MFI) was observed on CD3$^+$ T cells when treated with 6, 12 and 24 ng/ml of rh-OAS2. **d)** Graph indicates the expression (Normalized median fluorescence intensity) of CD3-ζ chain on incubation with or rh-OAS2. Fluorescence intensity was adjusted to 100 for control. Accordingly, intensity for the test was calculated and represented as normalized fluorescence intensity. The graph shown is representative of three independent experiments (** represents $p<0.005$ and * represents $p<0.05$).

4.2.9 Oral tumor derived supernatants and rh-OAS2 increased caspase-3 levels in PBMCs of healthy individuals

To identify the possible binding partners of OAS2, FuncBase gene function predictive tool was used which confirmed that OAS2 has capability to interact with the ILF2 protein which is a subunit of Nuclear factor of activated T cells (NFAT) protein and is known to regulate caspase 3 and GRAIL expression in T cells undergoing anergy **(Fig. 4.11a).** This could be important as the cytoplasmic domain of CD3-ζ chain has several consensus target sequences for caspases, among which caspase-3 and 7 have been shown to cleave *in vitro* translated CD3-ζ chain [155]. Caspase-3, an effector caspase is expressed during T cell anergy induction and recognizes proteins with a common DXXD motif and cleaves after the second aspartic residue [178, 369].

In order to elucidate that tumor supernatant and rh-OAS2 have the capability to induce the caspase-3 activation and thereby CD3-ζ downregulation in T cells, *in vitro* experiments were carried out. Single cell suspension of oral tumors prepared by enzyme digestion was cultured in serum free medium and cell free tumor supernatants were collected after 72 hrs in culture. The collected tumor supernatants were added to the HI PBMCs in culture. Treatment of HI PBMCs with H_2O_2 served as positive control for caspase-3 induction. The effect of tumor supernatant on caspase-3 levels was observed as it is a key caspase involved in CD3-ζ chain down regulation. A marked increase in caspase-3 levels was observed in HI PBMCs after H_2O_2 treatment (positive control). Addition of the tumor supernatant was also effectively able to induce caspase-3 levels in HI PBMCs confirming that tumor supernatant could be altering CD3-ζ chain by increasing active caspase-3 levels in T cells **(Fig. 4.11b).** To validate this further, HI PBMCs were also incubated with rh-OAS2 to analyze caspase-3 activation. As observed with tumor supernatant, rh-OAS2 also induced caspase-3 activation in the PBMCs of HIs compared to the untreated controls **(Fig. 4.11c).** This data clearly suggests that

caspase-3 might be involved in the regulation of CD3-ζ chain expression in T cells treated with tumor supernatant or rh-OAS2.

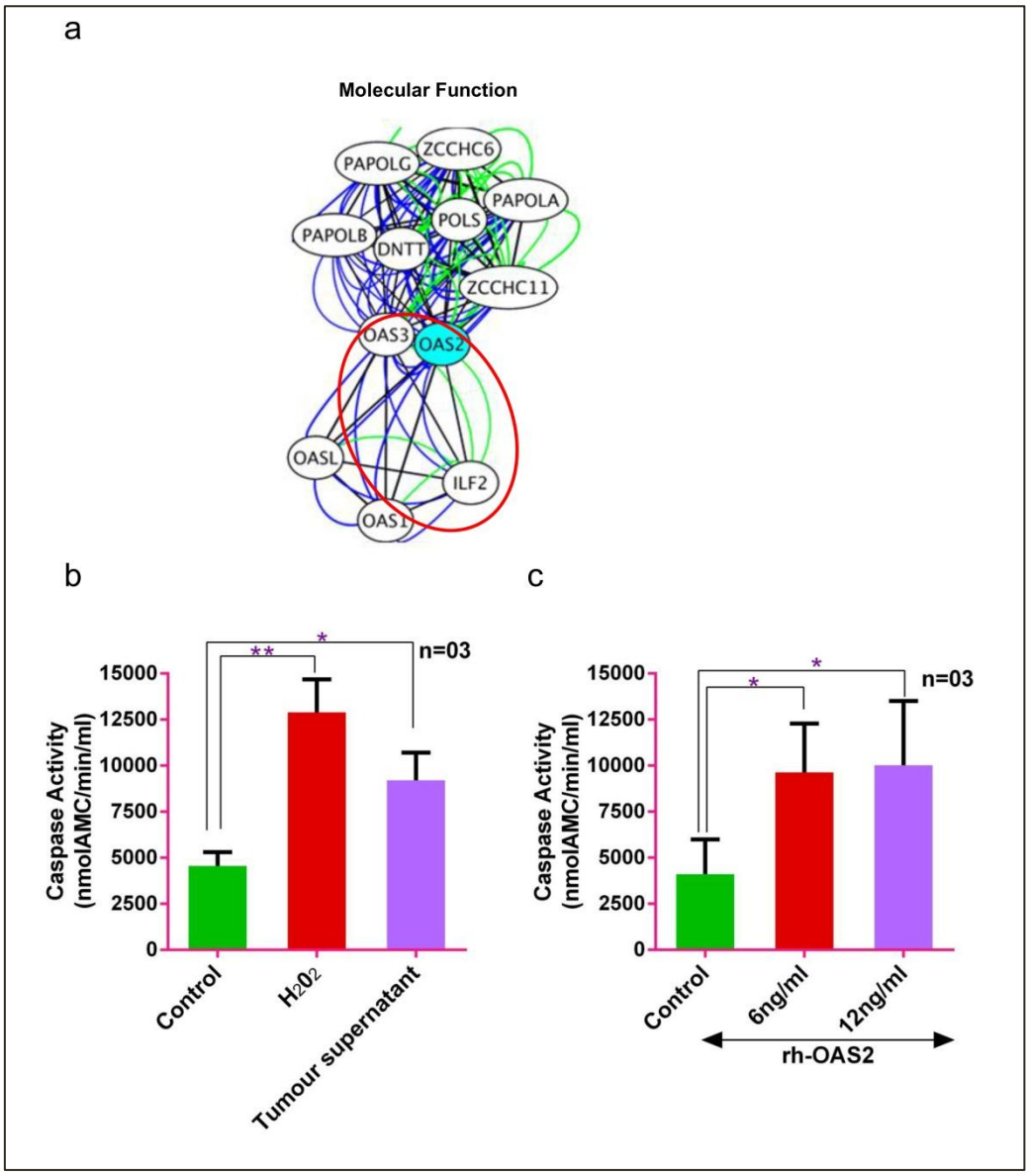

Figure 4.11: Active caspase-3 levels in T cells after treatment with tumor supernatant and rh-OAS2. a) the gene annotation database (FuncBase) predicted that OAS2 has capability of interacting with ILF2 which has capability of inducing T cell anergy under sub optimal TCR stimulation **b)** HI PBMCs were treated with H_2O_2 (positive control for caspase-3 induction) or tumor supernatant (TSN of Stage III patients) or left untreated. H_2O_2 used as positive control to induce caspase-3 levels showed higher levels of caspase-3 activation. The addition of TSN resulted in the increase of active caspase-3 levels in the PBMCs. **c)** Addition of rh-OAS2 (6 and 12ng/ml) on HI PBMCs resulted in the increase in caspase-3 levels. The data represented here is the mean of three independent experiments (** represents $p<0.005$ and * represents $p< 0.05$).

4.2.10 *si*RNA mediated downregulation of caspase 3 prevents rh-OAS2 induced downregulation of CD3-ζ chain in T cells

To validate the role of caspase-3 in decreasing CD3-ζ chain expression, the synthetic siRNA sequences targeting the caspase-3 were transfected into the PBMCs (Fig. 4.12a) or $CD3^+$ T cells (Fig. 4.12b). PBMCs were transfected with 100 nM of control siRNA or caspase 3 specific siRNA duplexes or left untransfected. After 40 hrs post transfection cells were stimulated with rh-OAS2 (24 ng/ml) and cells were harvested at 60 hrs and lysates were prepared. Caspase-3 expression was compared in unstimulated control siRNA transfected PBMCs, PBMCs stimulated with rh-OAS2 and in PBMCs transfected with caspase-3 siRNA but stimulated with rh-OAS2 **(Fig. 4.12a).** Results showed that compared to control siRNA transfected PBMCs (unstimulated, Lane 1), caspase-3 expression in PBMCs treated with rh-OAS2 showed marginal increase (Lane 2). Transfection with caspase-3 siRNA led to the reduction in the expression of active caspase-3 (Lane 3) compared to untransfected PBMCs (Lane 2) when stimulated with rh-OAS2 (24 ng/ml) **(Fig. 4.12a).** Silencing of caspase-3 prevented rh-OAS2 induced downregulation of CD3-ζ chain expression in PBMCs (Lane 3 vs Lane 2) **(Fig. 4.12a).** This clearly demonstrates that expression of CD3-ζ chain was restored and remained comparable to that observed with control siRNA. A similar experiment was performed on purified $CD3^+$ T cells and rh-OAS2 stimulation showed a pronounced effect on caspase-3 activation causing CD3-ζ chain downregulation in them. The downregulation of CD3-ζ chain in purified T cells was rescued by caspase-3 knockdown **(Fig. 4.12b).** Thus, confirming that caspase-3 is the key downstream molecule induced by OAS2 present in the tumor supernatant that may be responsible for CD3-ζ chain downregulation.

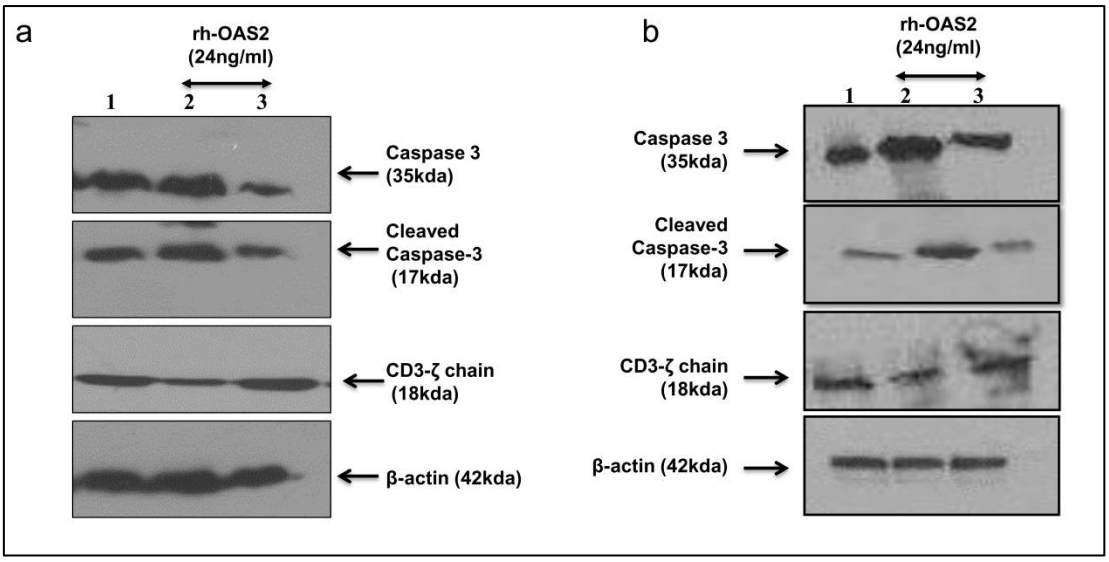

Figure 4.12: Inhibition of caspase 3 activation prevented CD3-ζ chain downregulation. a) Western blotting was carried out on cell lysates prepared from PBMCs transfected with 100 nM control siRNA (Lane 1) or PBMCs transfected with caspase-3 specific siRNA and incubated with rh-OAS2 (Lane 3) or untransfected PBMCs stimulated with rh-OAS2 (Lane 2) using antibodies for caspase-3 (detects total caspase along with cleaved caspase-3), CD3-ζ chain or β-Actin. β-Actin was used as loading control. Stimulation of PBMCs with rh-OAS2 induced caspase-3 activation causing CD3-ζ chain downregulation in them. This downregulation of CD3-ζ chain in PBMCs was rescued by the caspase-3 knockdown. **b)** Western blotting was carried out on cell lysates prepared from purified T cells transfected with 100 nM control siRNA (Lane 1) or T cells transfected with caspase-3 specific siRNA and incubated with rh-OAS2 (Lane 3) or untransfected T cells stimulated with rh-OAS2 (Lane 2) using antibodies for caspase-3, CD3-ζ chain or β-Actin. In purified T cells, a pronounced effect was observed on caspase-3 activation leading to CD3-ζ downregulation on rh-OAS2 stimulation which was recovered back to normal levels on caspase-3 knockdown.

4.2.11 Downregulation of CD3-ζ chain in T cells is not associated with apoptosis

The tumor supernatant or rh-OAS2 induced caspase 3 activation may result in apoptosis of the T cells and thereby decrease CD3-ζ chain expression. To rule out the possibility that CD3-ζ chain downregulation is a result of apoptosis, the effect of rh-OAS2 on apoptotic/necrotic cell death of PBMCs was analyzed. The rh-OAS2 stimulation effect on the frequency of apoptotic cells was compared with unstimulated cells. Results showed no significant differences in the frequency of early apoptotic (annexin V-positive), late apoptotic (annexin V-positive propidium iodide-positive) and necrotic (propidium iodide-positive) cells in untreated HI PBMCs or rh-OAS2 (6, 12, and 24 ng/ml) treated HI PBMCs **(Fig. 4.13a-e)**.

The frequencies of live cells across all the experiment setting remained same (90%±2 annexin V and PI double negative cells) **(Fig. 4.13e).** This shows that caspase-3 activity was not associated with induction of cell death or apoptosis. This caspase 3 activation associated CD3-ζ cleavage is therefore effect of OAS2 signaling in T cells under suboptimal T cell stimulation.

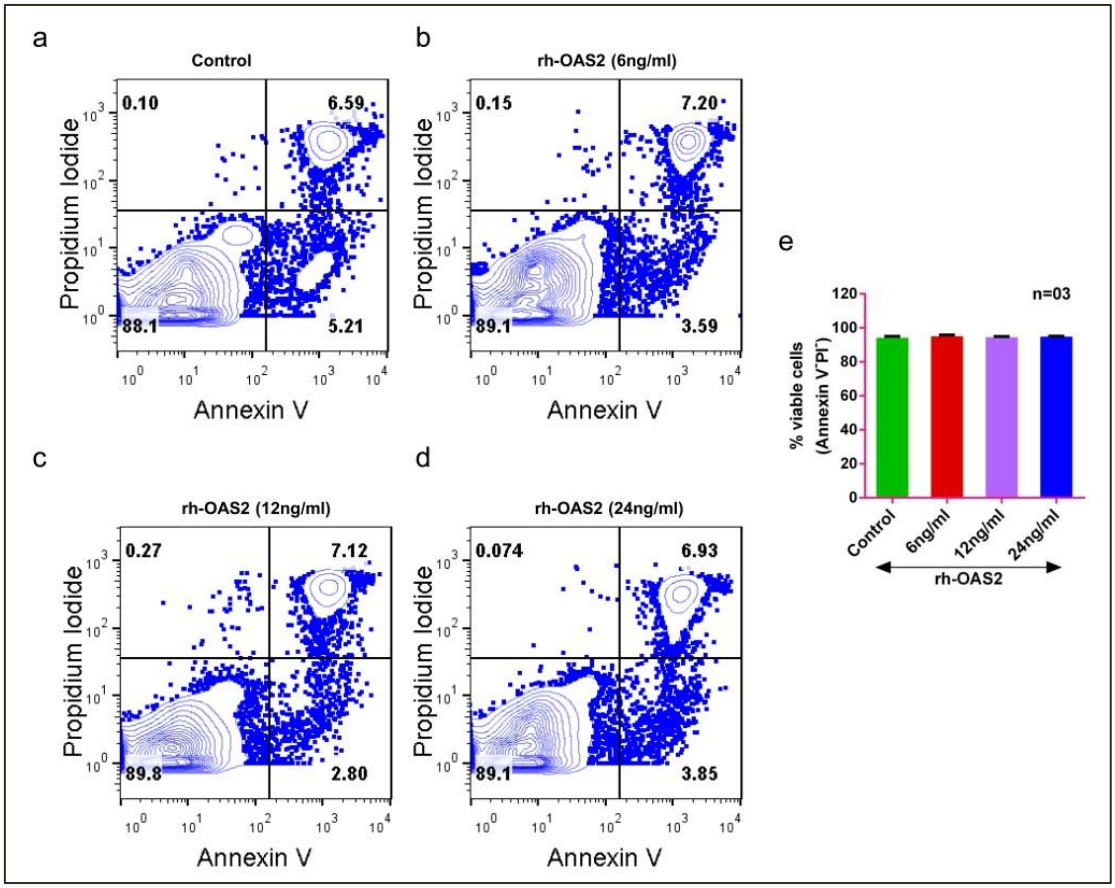

Figure 4.13: **Viability of cells after treatment with rh-OAS2.** The PBMCs of HIs were treated with different concentrations of rh-OAS2 to examine the percentage viable cells (annexin V/PI negative cells) after 24 hr in culture. The percentage of viable cells in control **(a)** was comparable to what was observed in the PBMCs stimulated with 6ng/ml **(b)**, 12ng/ml **(c)** and 24ng/ml **(d)** of rh-OAS2. **e)** The graph shows rh-OAS2 (6, 12 and 24ng/ml) did not alter the viability of PBMCs in culture, indicating that increased caspase-3 levels were not associated with apoptosis. The data is mean of three independent experiments.

4.3 Discussion

Immunosuppression is a hallmark of oral cancer patients, a state in which established tumor escapes immune attack. A number of phenotypic and functional alterations, including downregulation of CD3-ζ chain contributes to immunosuppression [370]. It is well established that downregulation of the CD3-ζ chain limits the favourable Th1 response needed for controlling tumor growth [29, 371]. Decreased or impaired CD3-ζ chain expression was also observed in other malignancies like lymphoma, ovarian cancer, gastric carcinomas leading to ineffectiveness of the anti-tumor immune response against the autologous tumor [35, 146, 372]. Several distinct mechanisms that contribute to a rapid turnover of CD3-ζ chain have been proposed which may be responsible for differences observed in CD3-ζ chain expression in T cells of patients with cancer [157, 161, 373]. In the present study, it was demonstrated that treatment of HI PBMCs with cell-free supernatants derived from oral tumors led to the downregulation of CD3-ζ chain expression in T cells without affecting the other T cell signaling molecules (ZAP70, CD3-ε and p56Lck). The broad range effect of supernatant collected from the advanced stage cancer patients (stage IV) on all the T cell signaling molecules could be attributed to a general state of immunosuppression in these patients contributed by the accumulation of immunosuppressive cells and the secreted immune suppressive factors. LC-MS/MS proteomic approach was therefore adopted and OAS2 (member of OAS gene family) was identified as one of the factors secreted by the oral tumor cells. The transcription of OAS genes is induced by both viral infection and interferons (IFNs) stimulation conferring protective and antiproliferative properties [374, 375]. Among the OAS genes, OAS2 has the highest level of induction by interferons (IFNα and IFNβ). Several studies have reported the presence of OAS2 in the sera of patients with viral infections and even after IFN treatment [376-378]. The success of IFN therapy was also correlated with levels of OAS gene activity in the sera of patients

undergoing IFN therapy [379, 380]. This correlation is interesting and suggests a biological relevance of extracellular OAS.

A variable number of factors, including tumor-secreted 14 KD protein, semaphorin A, NKG2D, are reported to be responsible for CD3-ζ chain downregulation in different malignancies [146, 157, 373]. The present study reported how extrinsic soluble mediator OAS2 secreted by oral tumor cells modulate CD3-ζ chain expression in T cells [381]. The 2′-5′OAS apart from its antiviral action has been demonstrated to be involved in other cellular processes such as cell growth and differentiation, gene regulation and apoptosis [382]. It has been demonstrated that the IFN-β mediated signaling pathways as well as its upstream regulators are up-regulated in oral squamous cell carcinoma (OSCC) lesions [383]. Thus, it appears that IFN-β signaling may act as a key factor responsible for OAS2 expression in oral tumors. IFN-β signaling is also known for its immune regulatory properties, suggesting its importance in tumor progression either directly or through modulation of the immune system through its downstream mediators like OAS2.

The observation of OAS in the sera of patients with infection or undergoing IFN treatment [380] prompted us to look at the role of OAS in modulating the immune system. The differential expression of OAS2 has been reported in prostate and breast cancers [384, 385]. Our study also confirmed the presence of OAS2 in oral tumors at both mRNA and protein level. The significance of OAS2 in tumor microenvironment can be appreciated by the observation that certain tumors exist in an antiviral state and hence are resistant to oncolytic viruses based virotherapy used to obliterate tumor cells. These tumors are reported to have higher expression of JAK/STAT pathway and interferon stimulated genes (ISG) [386]. It was also reported that over-expression of Myxovirus resistance gene A (MxA) and OAS mRNAs were involved in the suppression of HBV replication mediated by IL-17A in a noncytopathic manner [387]. Hence, these tumors with functional IFN responsive pathway and expression

of a downstream gene (OAS2) may be sensitive to IFN stimulation thereby conferring the virus resistant phenotype which shields tumors from virotherapy. The source of IFN in the tumor microenvironment is likely to be either the epithelial cells or the tumor-infiltrating immune cells, such as type 2 dendritic cells and macrophages [383, 388]. The IFN secreted by these cells will activate the interferon stimulated genes (ISG) and will execute their pro-tumor function either intracellularly or in secreted form by modulating the immune system. However, the source of OAS2 in oral tumors needs to be further investigated.

In our study, a surrogate model where OAS2 was overexpressed showed that secreted OAS2 could modulate the expression of CD3-ζ chain in T cells. This was further validated by stimulating HI PBMCs with rh-OAS2 which led to the specific downregulation of CD3-ζ chain expression in T cells. Upregulation of type I IFN response genes has also been reported in peripheral blood cells of patients with autoimmune diseases, like systemic lupus erythematosus (SLE), multiple sclerosis (MS), and, rheumatoid arthritis (RA) and these diseases are associated with decreased CD3-ζ chain expression [389-391]. Type 1 interferons have been shown to have antiproliferative activity on activated $CD4^+$ and $CD8^+$ T cells [392]. Protein and mRNA levels of OAS genes are low in circulating mononuclear cells during clinically active, untreated MS. The IFN-β therapy treatment of MS and experimental autoimmune encephalomyelitis (EAE) patients to decrease the levels of TH17 cells is associated with increased expression of OAS2 protein [380]. Thus, OAS2 might be acting in a paracrine manner in the tumor microenvironment to decrease the expression of CD3-ζ chain in T lymphocytes. However, OAS gene family is comprised of four genes, OAS1, OAS2, OAS3, and OAS-like (OASL), and 10 isoforms, including OAS1 (p42, p44, p46, p48, and p52), OAS2 (p69 and p71), OAS3 (p100), and OASL (p30 and p59) which can be generated by alternative splicing [393] [394-398]. Thus a comprehensive assessment of different

isoforms is required to clearly show that whether it is specific only for OAS2 or could be attributed to the other isoforms as well.

The present data showed that incubation of PBMCs with tumor supernatant or rh-OAS2 leads to increase in caspase-3 levels. Increased caspase-3 levels are observed in T cells under suboptimal T cell stimulation leading to the induction of T cell unresponsiveness without inducing cell death [179]. Under these conditions, the expression of a specific set of anergy-associated genes is activated. Several lines of evidence suggest that nuclear factor of activated T cells (NFAT) proteins may regulate the expression of many of those genes *GRAIL* and *Caspase-3* [399]. The cytoplasmic domain of CD3-ζ chain has several consensus target sequences for caspases, among which caspase-3 and 7 have been shown to cleave *in vitro* translated CD3-ζ chain [155]. Caspase-3, an effector caspase is expressed during T cell anergy induction and recognizes proteins with a common DXXD motif and cleaves after the second aspartic residue [178, 369]. Circumstantial evidence for a physiological involvement of active caspase-3 in generating a CD3-ζ chain deficient T cell phenotype has been described in patients with gastric and liver cancers [400, 401]. T and B cells from $Casp3^{-/-}$ mice show hyperproliferative responses, which have been attributed to reduced activation-induced cell death (AICD) and to alterations of cell cycle regulation in these cells, respectively [402, 403]. Caspase-3 also regulates many non-apoptotic cellular processes, such as cell proliferation, cell-cycle regulation, and cell differentiation [404, 405]. Caspase-3 activity is high in $CD3\zeta^{low/-}$ SLE patient T cells and treatment of these T cells with caspase inhibitors restores CD3-ζ chain expression [406].

2′-5′OASs leads to cytochrome c release into the cytoplasm and then to caspase-3 activation [407]. IFN-α also increases caspase-3 mRNA levels in activated T cells without modulating activation induced cell death [408]. In our study induction of caspase-3 in non-apoptotic cells was observed on stimulation with rh-OAS2. This observation supports that caspase-3-

dependent proteolytic inactivation of CD3-ζ chain is essential to maintain T cell tolerance in cancer patients [408]. The amino acid sequence of translated CD3-ζ chain contains putative caspase 3 cleavage sites DVLD and DTYD (5 in number) [155]. Thus, the selective loss of CD3-ζ chain and not of CD3-ε could be because of the caspase-3 activation as observed upon stimulation with rh-OAS2 or tumor supernatant. However, the downstream consequence of CD3-ζ downregulation on the rh-OAS2 challenge was not found to be dose-dependent but might depend on induction of caspase-3. To alter the expression of CD3-ζ chain on HI PBMCs in a dose-dependent manner, caspases are expected to vary in logarithmic values. In our study, such change in caspase-3 value was not observed upon rh-OAS2 stimulation. It has been shown that exogenously added OAS to cell cultures can be internalized to exert its effect. This exogenous OAS can exert its effect either by binding to the Nucleotide-binding and oligomerization domain-2 (NOD2) to activate RNaseL or independent of this to induce caspase cascade [379, 409]. Thus, the internalization of OAS2 might be a limiting factor in inducing the caspase-3 levels in a dose-dependent manner.

Type I IFNs (IFNα and IFNβ) could exert broad dual effects on the immune system, reflecting both immune-stimulatory and immune-suppressive activities. Immune-stimulatory activities relate to the activation of myeloid dendritic cells, chemokines, chemokine receptors, costimulatory molecules (CD40, CD80, and CD86), and humoral responses. Immune-suppressive effects are reflected by Th2 cell skewing and antiproliferative and pro-apoptotic effects. The role of IFNs in disease may range from beneficial to detrimental. However, it may be postulated that OAS2 plays a major immunoregulatory role based on its expression in oral tumors and autoimmune diseases where CD3-ζ chain expression is downregulated [29, 410]

Type I interferons are promising but incompletely understood anticancer agents. Clinical trials have demonstrated benefit in both haematological and solid tumors, although the

effectiveness is somewhat modest in some cancers [411, 412]. IFN-α therapy has been suggested to be effective in a subgroup of oral cancer patients [413, 414]. Further, it has also been demonstrated that the up-regulation of ISGs (Mx1, OAS3, IFI44, IFI44L, OAS2, USP18, and RSAD2) in the chronic hepatitis C infected liver is related to a poor treatment response to peg-IFN-α therapy [415]. IFN-α may be important in the prevention of carcinogenesis and high expression of OAS2 might be impairing cellular response to IFN-α and promotes oral tumor progression by modulating anti-tumor immune response. However, the role of others ISGs in modulating IFN-α response cannot be ruled out. Identification of molecular biomarkers like OAS2, which can also identify oral cancer patients sensitive to Type I interferon therapy would be very helpful in the clinic.

Thus, the data presented here indicates, for the first time, a potentially important function of tumor-derived OAS2 as a paracrine negative regulator of T-cell functions. Our data highlight OAS2 as a novel molecular target for the manipulation of T-cell– dependent immunity with important implications for cancer immunotherapy.

Chapter 5

Defining the role of TNF-α in the down-regulation of CD3-ζ chain in T cells

5.1 Introduction

Oral cancer patients are associated with chronic inflammation and T cells in them are persistently exposed to cytokines, chemokines, nitrogen and oxygen species resulting in the reduced antitumor immune response [218, 231, 416]. Immune cells in cancer patients are also likely to be exposed to various apoptogenic signals and one of them is the chronic exposure of T cells to tumor necrosis factor-alpha (TNF-α). TNF-α is a pleiotropic cytokine which plays a central role in inducing chronic inflammation. Previous reports have demonstrated that chronic TNF-α treatment impairs mouse T cell activation and downregulates CD3-ζ chain expression [204]. In humans, TNF-α treatment also downregulates CD3-ζ chain on T lymphocytes dose dependently, selectively, and reversibly via SLAP-dependent proteasomal degradation [161]. Chronic exposure of TNF-α has also been shown to attenuate TCR-signaling and down-modulate T cell proliferative responses and cytokine production *in vivo* and *in vitro* in patients with rheumatoid arthritis (RA) [202, 203].

TNF-α is largely produced by activated macrophages, T lymphocytes, and natural killer (NK) cells. Inside cells, TNF-α is synthesized as pro-TNF (26 kDa), which is membrane-bound and released upon cleavage of its pro domain by TNF-converting enzyme (TACE) [417]. Once released, TNF-α interacts with its receptors TNF receptor I (TNFR-I, p60) and TNF receptor II (TNFR-II, p80) and activates several signal transduction pathways, leading to the diverse functions of TNF-α [418]. Despite higher affinity for TNFR-II (five times higher than that for TNFR-I), TNFR-I initiates the majority of the biological activities of TNF-α [419]. TNFR-I is expressed on all cell types, while TNFR-II expression is mainly confined to immune cells [420].

TNFR-I has the ability of inducing apoptosis because of its association with the death domain (DD) which can recruit pro-caspase 8 by the TRADD-FADD complex [421]. Autocatalytic activation of bound pro-caspase 8 releases activated caspase 8, which initiates

apoptosis through cleavage and activation of pro-caspase 3 [422]. In case of immune cells, TNF-α has been shown to trigger cell death of activated $CD8^+$ T in a TNFR-I-dependent manner and blocking TNF-α signaling has been shown to enhance $CD8^+$ T cell-dependent immunity in experimental melanoma. This has been attributed to the accumulation of tumor-infiltrating $CD8^+$ T cells in TNF/TNFR-I signaling deficient animals suggesting that TNFR-I signaling impairs the accumulation of tumor-infiltrating $CD8^+$ T cells [423]. Further, Naive $CD8^+$ T cells which express TNFR-II but not TNFR-I are resistant to TNF-α induced cell death while activated $CD8^+$ T cells expressing both TNFR-1 and TNFR-II are sensitive to exogenous TNF-α [423]. Thus, this insinuates that TNFR-I might be acting as an immune checkpoint. Besides the apoptotic signaling, TNFR-I also has the ability to transduce cell survival signals through NF-κB and AP-1. TNFR-I also mediates TNF-α driven DC maturation and $CD8^+$ T cell response. TNF-α driven maturation could be either through direct binding of TNF-α to TNFR-I on immature DCs or through an indirect method by activating naïve T cells which drive maturation and mobilization of DCs [424].

TNF-α is involved in the modulation of both innate and adaptive immune responses. Conflicting results have been published on the role of TNF-α in the T-cell immune response. On one hand, TNF-α induces cell death of $CD8^+$ T cell via TNFR-I receptor [423, 425]. On the other hand, TNF-α also acts as an effector molecule secreted by $CD8^+$ T cells to trigger cell death of cancer cells [426] and a costimulatory cytokine able to enhance naive $CD8^+$ T-cell proliferation and cytokine secretion [427, 428]. Moreover, TNF-α signaling facilitates stabilization of immune cells via TNFR-II receptor. Increased number of regulatory T (Treg) cells [429-431] and myeloid-derived suppressor cells (MDSC) [206, 432] in cancer patients are associated with TNF-α signaling through TNFR-II. TNFR-II also acts as costimulatory signal during T cell activation, and has a synergistic effect with TCR, and CD28 towards optimal IL-2 induction and T cell survival [433]. However, chronic TNF-α exposure is

known to impair TCR-signaling via TNFR-II and result in down-modulation of T cell activation [434].

These observations indicate that both the TNF receptors can mediate the regulatory effects of TNF-α on T cell activation and function in a spatial and temporal fashion. It would be thus interesting to know which receptor is important in executing the downregulation of CD3-ζ chain in T cells of oral cancer patients. In the present study which TNF receptor significantly contributes to the suppressed T cell effector response during chronic inflammation associated with oral cancer patients was addressed.

In the present chapter, we aimed to address following questions:

1) What is the expression profile of TNFR-I and TNFR-II on T cells from healthy individuals and oral cancer patients?
2) Which TNF receptor is involved in mediating the CD3-ζ chain downregulation on TNF- α exposure?
3) What is the mechanism by which T cell signaling is inhibited by TNF-α stimulation?

5.2 Results

5.2.1 Expression of TNFR-I and TNFR-II on $CD3^+$ T cells obtained from healthy individuals and oral cancer patients

In order to address, whether TNF-α induced alteration in T cell signaling is regulated by the TNF receptors (I and II), first expression of TNFR-I and TNFR-II was analyzed in the freshly isolated $CD3^+$ T cells from healthy individuals (HIs) and oral squamous cell carcinoma (OSCC) patients. The PBMCs were isolated and stained by the antibodies for TNFR-I and TNFR-II. The gated $CD3^+$ T cells from HIs had a comparable expression of TNFR-I and TNFR-II. The expression levels are represented as the median fluorescence intensity (MFI) **(Fig. 5.1a)**. Comparison of MFI values of TNFR-I (Median: 689) and TNFR-II (Median: 822.5) in HIs indicates that there is no significant statistical difference between the two receptors on the $CD3^+$ T cells **(Fig. 5.1b)**. In case of gated $CD3^+$ T cells from OSCC patients, MFI of TNFR-I (10945) was higher as compared to the MFI of TNFR-II (6259) **(Fig. 5.1c)**. Comparison of expression levels of TNFR-I (median: 6049) and TNFR-II (median: 2359) indicate that there is statistically significant difference ($p<0.005$) between the two receptors on $CD3^+$ T cells of OSCC patients **(Fig. 5.1d)**. The $CD3^+$ T cells from OSCC patients expressed significantly higher levels TNFR-I and TNFR-II receptors compared to that expressed by the $CD3^+$ T cells of HIs. However, the drastic change was observed in the expression of TNFR-I than TNFR-II. This suggests that T cells from oral cancer patients are reprogrammed to overexpress TNFR-I which might have an influence on the T cell functioning and survival.

The levels of TNF-α in the serum of the HIs and OSCC patients were analyzed to predict the prevalence of TNF-α signaling in OSCC patients. As evident the levels of TNF-α were elevated in the serum of oral cancer patients compared to the HIs **(Fig. 5.2)**. The levels of

TNF-α were almost 3 fold higher in OSCC patients (Range 2.0-547.6 pg/ml: Median 13.24) compared to the HI (Range 1.310-97.82 pg/ml: Median 4.02) **(Fig. 5.2)**, suggesting that the T cells from oral cancer patients are under the chronic exposure of TNF-α which might have the ability to alter T cell functioning.

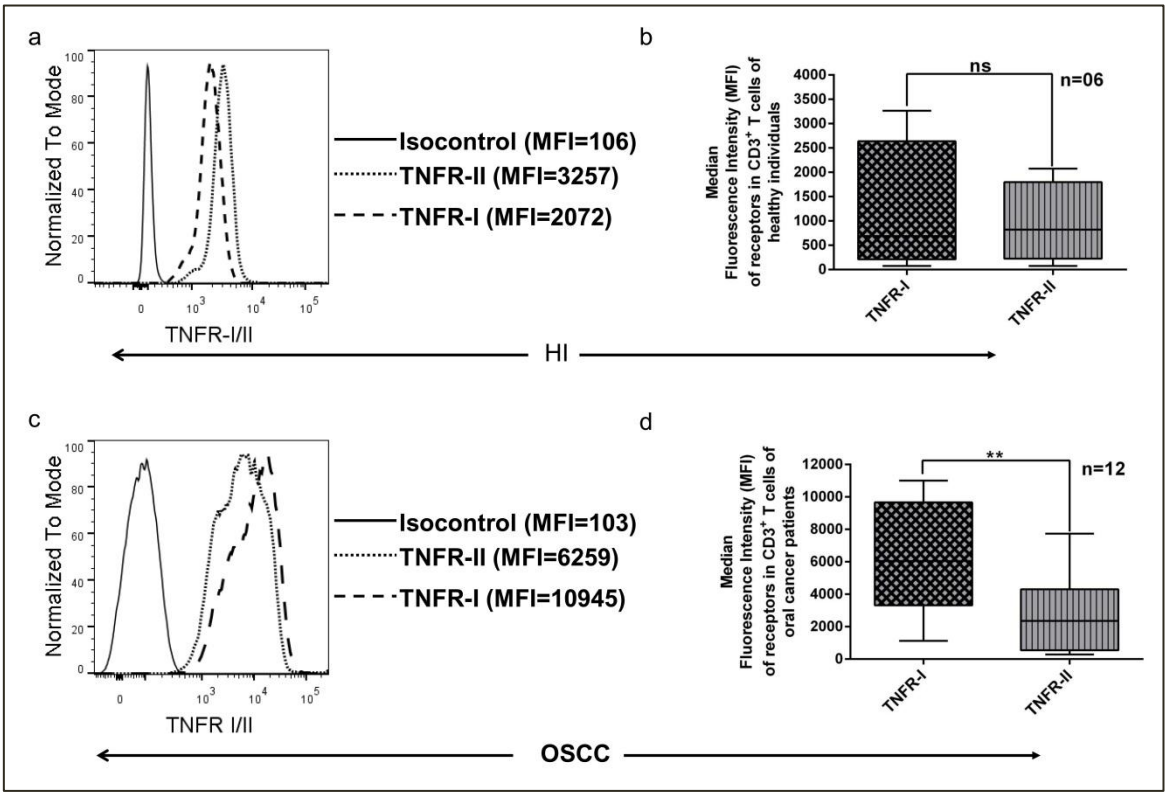

Figure 5.1: Comparison of Expression of TNFR-I and TNFR-II in HIs and OSCC patients. a) Histogram shows the fluorescence intensity of TNFR-I and TNFR-II on $CD3^+$ T cells of healthy individuals. **b)** Graph depicts that the fluorescence intensity of TNFR-I and TNFR-II on $CD3^+$ T cells of healthy individuals is comparable and there is no significant statistical difference (p=0.6905). **c)** Histogram shows the fluorescence intensity of TNFR-I and TNFR-II on $CD3^+$ T cells of oral cancer patients. **d)** Graph shows that median fluorescence intensity (MFI) of TNFR-I on $CD3^+$ T cells of oral cancer patients is higher than that of TNFR-II and there is significant statistical difference (** represents p<0.005, ns represent non-significant data with p>0/05).

5.2.2 Effect of TNF-α treatment on the expression of CD3-ζ chain in Jurkat T cells and $CD3^+$ T cells of healthy individuals

Previous data indicate that TNF-α plays a key role in chronic inflammation induced T cell hyporesponsiveness in cancer and autoimmune disease. The CD3-ζ chain regulates the

TCR/CD3 expression on the T cell surface and thereby controls their activation and functions. The effect of TNF-α on CD3-ζ chain expression was observed in Jurkat T cells and human PBMCs. Jurkat T cells were treated with recombinant human TNF-α (20 and 40 ng/ml) for different time points (24, 48 and 72 hrs). At all-time points, cells were stained for CD3-ζ chain expression. In Jurkat T cells after treatment with TNF-α (20 and 40 ng/ml), downregulation of CD3-ζ chain expression was observed. However, the effect neither appeared to be time dependent or dose dependent. At both the concentrations, CD3-ζ chain protein levels were reduced to almost 40% compared to untreated cells **(Fig. 5.3a-c)**. Unstimulated cells served as the control for this experiment. Comparisons of median values of corrected fluorescence intensity of CD3-ζ chain in TNF-α treated and untreated Jurkat T cells indicated a statistical significant decrease in CD3-ζ chain expression upon treatment with TNF-α **(Fig. 5.3d)**.

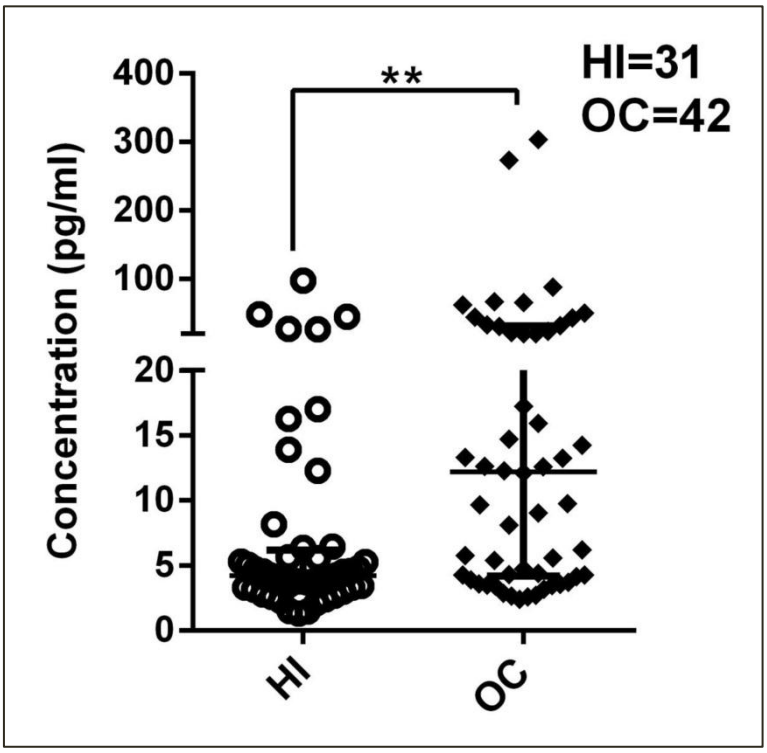

Figure 5.2: Concentration of TNF-α in serum of healthy individuals and oral cancer patients. Serum TNF-α concentration in HI (n=31) and OSCC patients (n=42) was determined by cytometric bead array. Higher levels of TNF-α was observed in OSCC patients as compared to HI. Comparison of serum TNF-α level in HI and OSCC patients indicates that there is significant statistical difference. (** represents $p<0.005$)

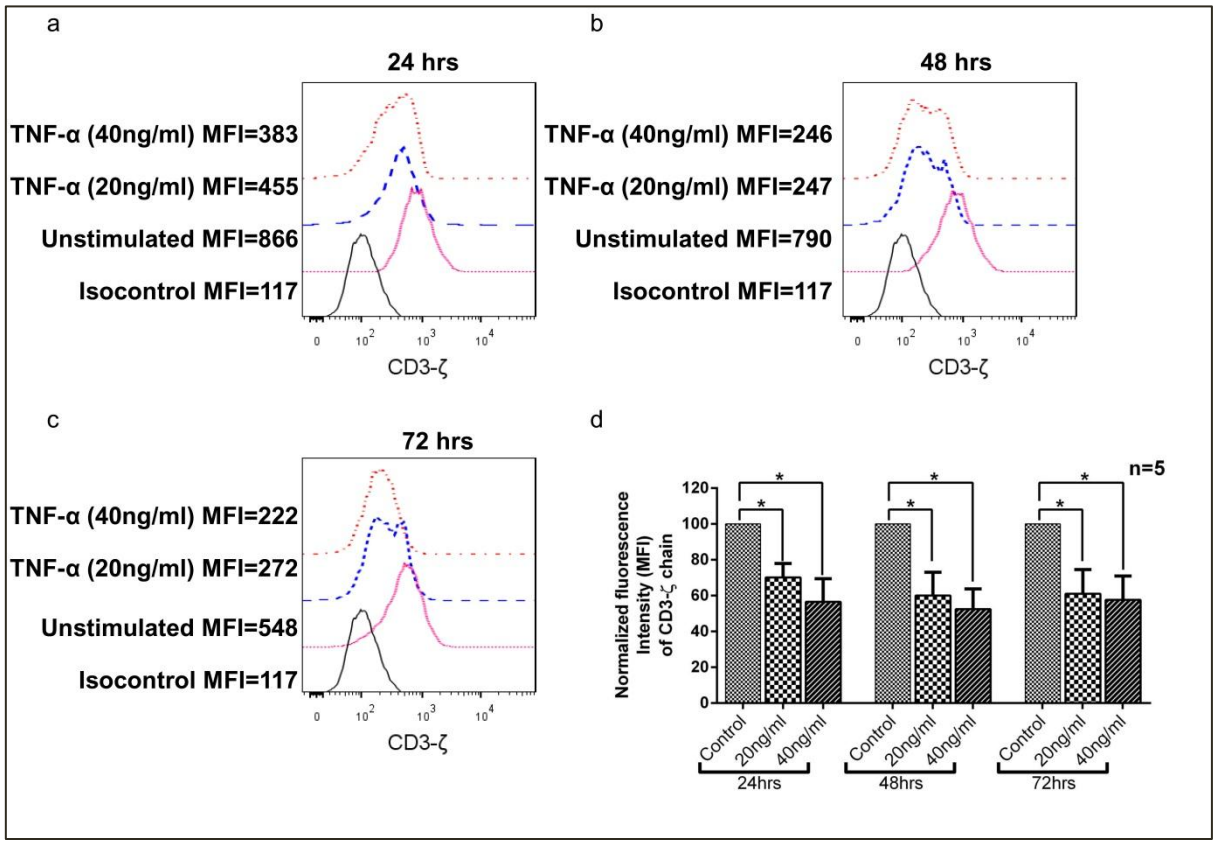

Figure 5.3: Effect of TNF-α on CD3-ζ chain in Jurkat T cells. Jurkat T cells were treated with TNF-α (20 ng/ml or 40 ng/ml) for 24, 48, and 72 hrs and the CD3-ζ chain expression on $CD3^+$ T cells was studied by flow cytometry. The half offset shows the effect of TNF-α on CD3-ζ chain expression at 24 hr **(a)**, 48 hr **(b)** and 72 hrs **(c)**. **(d)** Comparison of corrected fluorescence intensities of CD3-ζ chain of control cells with TNF-α-stimulated cells indicates significant statistical difference at 24, 48 and 72 hrs. Data is represented as mean values of corrected fluorescence intensities. Corrected fluorescence intensity is calculated by correcting the MFI of unstimulated control to 100 and adjusting MFIs of others accordingly. (* represents $p<0.05$)

The TNF-α treatment of freshly isolated PBMCs from HIs was unable to downregulate the levels of CD3-ζ chain on the gated $CD3^+$ T cells (data not shown). To trace the possible reason which led to this inconsistency, the role of TNF receptor expression was anticipated as Jurkat are known to express higher levels of TNFR-I compared to TNFR-II. The expression of TNFRI and TNFR-II was analyzed on Jurkat T cells and results confirmed that these cells expressed higher levels of TNFR-I than TNFR-II **(Fig. 5.4a-b)**. This is in corroboration with our earlier data showing that T cells from OSCC patients have higher levels of TNFR-I than

TNFR-II. The differential expression of TNF receptors on T cells might render them susceptible to TNF-α induced downregulation of CD3-ζ chain.

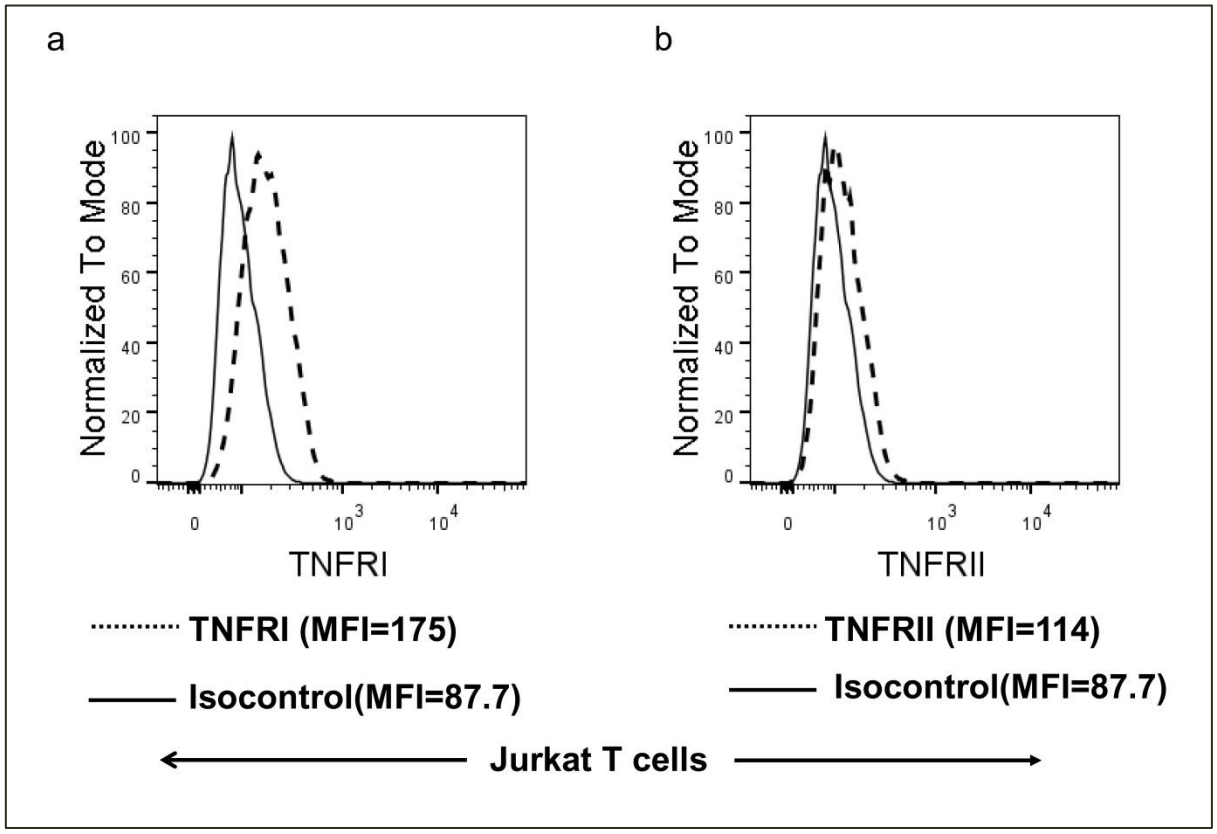

Figure 5.4: Expression of TNFR-I and TNFR-II on Jurkat T cells. Jurkat T cells were stained with TNFR-I and TNFR-II antibodies and analyzed by flow cytometry. The histograms confirm that Jurkat T cells express higher levels of TNFR-I (MFI=175) **(a)** while TNFR-II expression is marginal (MFI=114) **(b)** when compared to Isocontrol (MFI=87.7).

5.2.3 Effect of TNF-α treatment on the expression of CD3-ζ chain in TNFR-I induced $CD3^+$ T cells of healthy individuals

To prove that the differential expression of TNF receptors on T cells marks them susceptible to TNF-α induced downregulation of CD3-ζ chain, PBMCs were pre-treated with PHA (1% PHA for 2h) before the TNF-α treatment. The PHA treatment was carried out to induce the TNFR-I expression on T lymphocytes [161] and mimic the *in vivo* situation of OSCC patients where TNFR-I is higher. The PBMCs from HIs were stimulated with 1% PHA for 2 hrs to induce TNFR-I expression. The results showed that after PHA treatment considerable amount

of TNFR-I was induced on the gated $CD3^+$ T cells without affecting TNFR-II **(Fig. 5.5a-b)**. There was almost one fold increase in the expression of TNFR-I on T cells of HIs after 2 hrs of PHA treatment **(Fig. 5.5c)**. The cells with considerable amount of TNFR-I were used for further experiments to analyze the effect of TNF-α on the T cells.

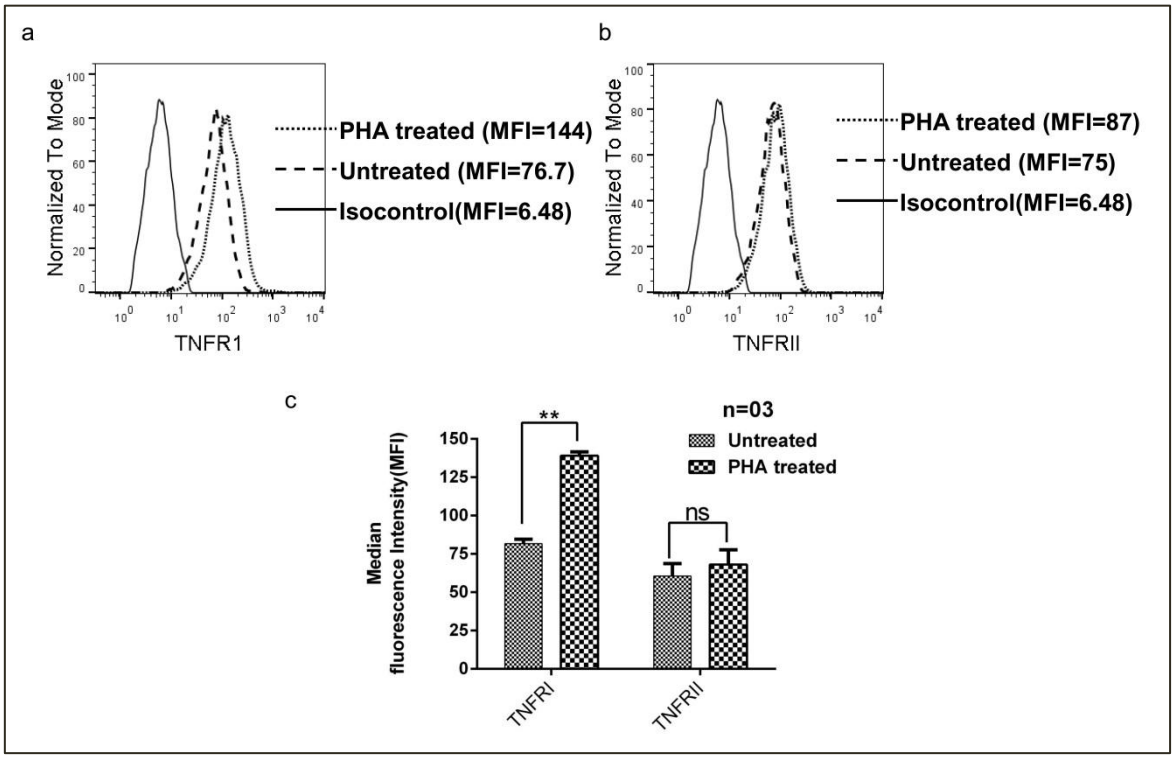

Figure 5.5: Induction of TNFR-I receptor by PHA Stimulation on $CD3^+$ T cells of healthy individuals. PBMCs of HI were obtained by FH density gradient centrifugation. Cells were treated with 1% PHA for 2 hrs and then cells were stained for TNFR-I and TNFR-II using respective antibodies **a)** the histogram shows that PHA stimulation induces TNFR-I expression on gated $CD3^+$ T cells. **b)** Confirms that the expression of TNFR-II remained unaffected on PHA stimulation. **c)** Graph shows the comparison of median fluorescence intensities of TNFR-I and TNFR-II in unstimulated and PHA stimulated cells. Data represented is the mean of three independent experiments. (** represents $p<0.005$ and ns represents non-significant data with $p>0.05$)

PHA stimulated cells were washed and treated with TNF-α (20 and 40ng/ml) for 24, 48, and 72 hrs. At different time points, cells were harvested and the expression of T-cell signaling molecule CD3-ζ chain was analyzed in the gated $CD3^+$ T cells. The effect of TNF-α treatment (TNF-α 20 ng/ml and 40 ng/ml) at different time points (24, 48, and 72 hrs) was

compared with respective untreated cells. A decrease in the CD3-ζ chain expression was observed in T cells treated with TNF-α as compared to untreated T cells **(Fig. 5.6a-c)**. Comparisons of normalized fluorescence intensities of CD3-ζ chain in TNF-α treated and untreated $CD3^+$ T cells at 24, and 48 hr indicated a significant decrease in CD3-ζ chain expression ($p<0.05$) upon treatment with TNF-α **(Fig. 5.6d)**.

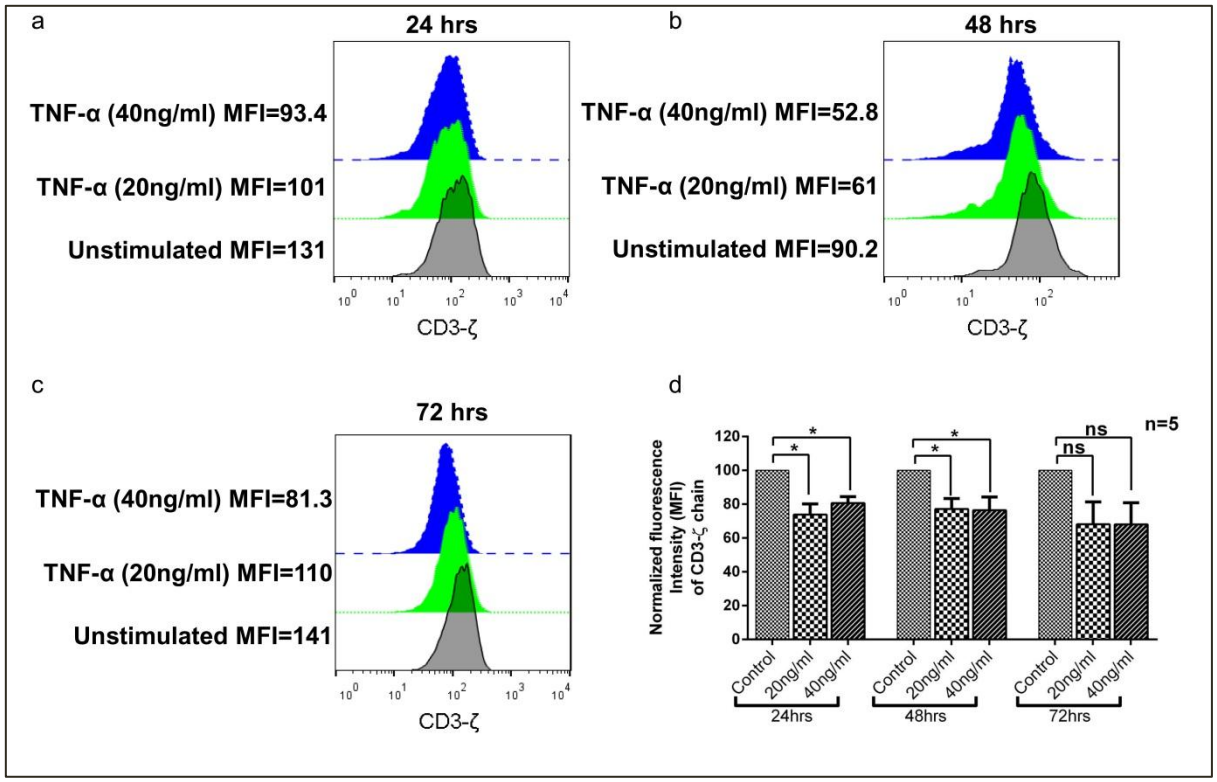

Figure 5.6: Influence of TNF-α on CD3-ζ chain expression in $CD3^+$ T cells of healthy individuals. PBMCs of HI were obtained by FH density gradient centrifugation. Cells were treated with 1% PHA (for 2 hrs) then with TNF-α (20 ng/ml or 40 ng/ml) for 24, 48, and 72 hrs. The half offset histogram shows the effect of TNF-α on CD3-ζ chain expression at 24 hr **(a)**, 48 hr **(b)** and 72 hrs **(c)**. **(d)** Comparison of normalized fluorescence intensities of CD3-ζ chain of control cells with TNF-α-stimulated cells indicates significant statistical difference at 24 and 48 hr. Comparison of normalized fluorescence intensities of control cells with TNF-α-stimulated cells at 72 hrs showed no statistical significance. The normalized fluorescence intensity is calculated by correcting the MFI of unstimulated control to 100 and adjusting MFIs of others accordingly. (* represents $p<0.05$ and "ns" represents non-significant data with $p>0.05$)

The effect of chronic TNF-stimulation on CD3-ζ chain downregulation was also confirmed by western blotting. The protein lysates from T cells treated with TNF-α (20ng/ml and 40ng/ml) for 24, 48 and 72 hrs were probed for CD3-ζ chain using CD3- ζ chain mAb. To

ensure equal loading of proteins, β-actin (molecular weight 42kDa) was used as the loading control. Compared to unstimulated cells, the expression of CD3- ζ chain protein (molecular weight 18kDa) was lower in TNF-α stimulated T cells at all the time points and concentrations used **(Fig. 5.7)**. The results thus confirm that the TNF/TNFR-I signaling is having a differential functional consequence on T cells leading to the downregulation of CD3-ζ chain.

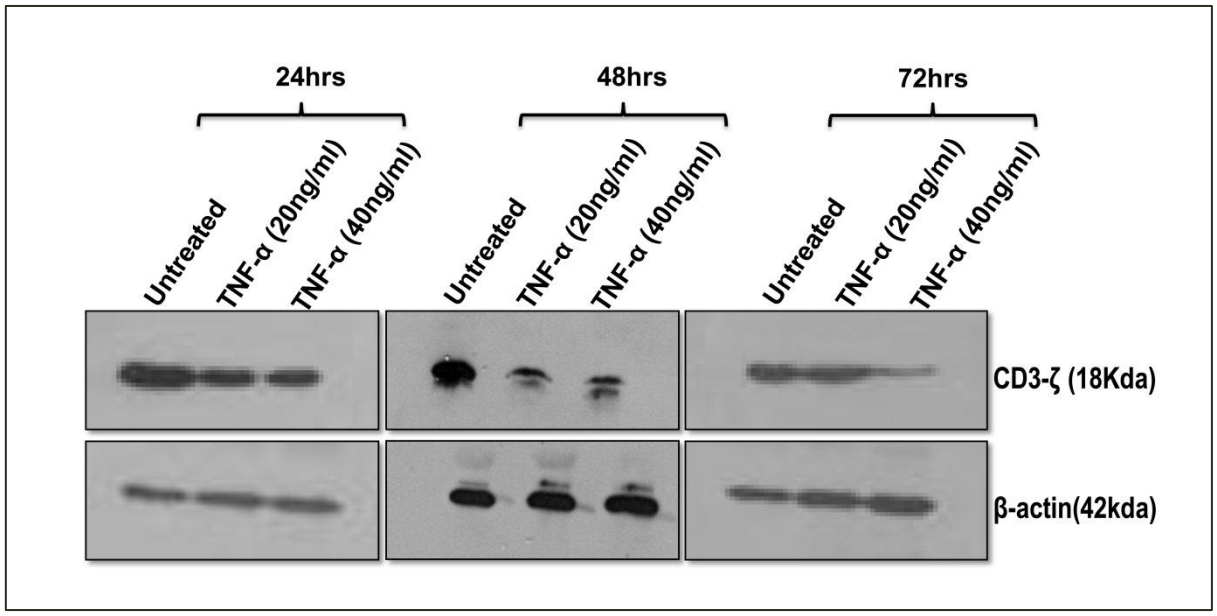

Figure 5.7: **Western blots confirming the effect of TNF-α on CD3-ζ chain expression in CD3$^+$ T cells**. Cells were treated with TNF-α (20 ng/ml or 40 ng/ml) for 24, 48, and 72 hrs and cell lysates were prepared. The lysates were analysed for the expression of CD3-ζ chain by western blotting. The expression of CD3-ζ chain in untreated samples at 24 hr, 48 hr and 72 hrs is higher than the samples stimulated with TNF-α (20ng/ml) for 24 hr, 48 hr and 72 hrs respectively. β-actin is used as loading control to ensure equal loading of proteins. Expression of CD3-ζ chain decreases upon stimulation with TNF-α.

5.2.4 Expression of CD3-ε chain in CD3$^+$ T cells of healthy individuals upon treatment with TNF-α

The CD3-ζ chain can cycle independently from other CD3 subunits, therefore the effect of TNF-α on CD3-ε chain was also studied. PBMCs of HI were treated with 1% PHA for 2 hrs. Cells were washed and incubated with TNF-α (20 ng/ml and 40 ng/ml) for 24, 48, and 72 hrs with unstimulated cells serving as control. The cells were then stained at respective time

points (24, 48, and 72 hrs) and analyzed for the expression of CD3-ε chain in $CD3^+$ T cells upon treatment with TNF-α (20 ng/ml and 40 ng/ml). The results confirmed that TNF-α treatment lacks the ability to alter the expression of CD3-ε chain. No change in CD3-ε MFI was observed in T cells treated with TNF-α as compared to untreated cells at all-time points **(Fig. 5.8a-c)**. Comparisons of normalized fluorescence intensities of CD3-ε in TNF-α treated and untreated T cells at 24, 48, and 72 hrs indicated no significant change in CD3-ε chain expression ($p>0.05$) upon treatment with TNF-α **(Fig. 5.8d)**. This suggests that the effect of TNF-α is specific and affects only CD3-ζ chain in T cells without affecting other key T cell signaling molecules associated with effector functions of T cells.

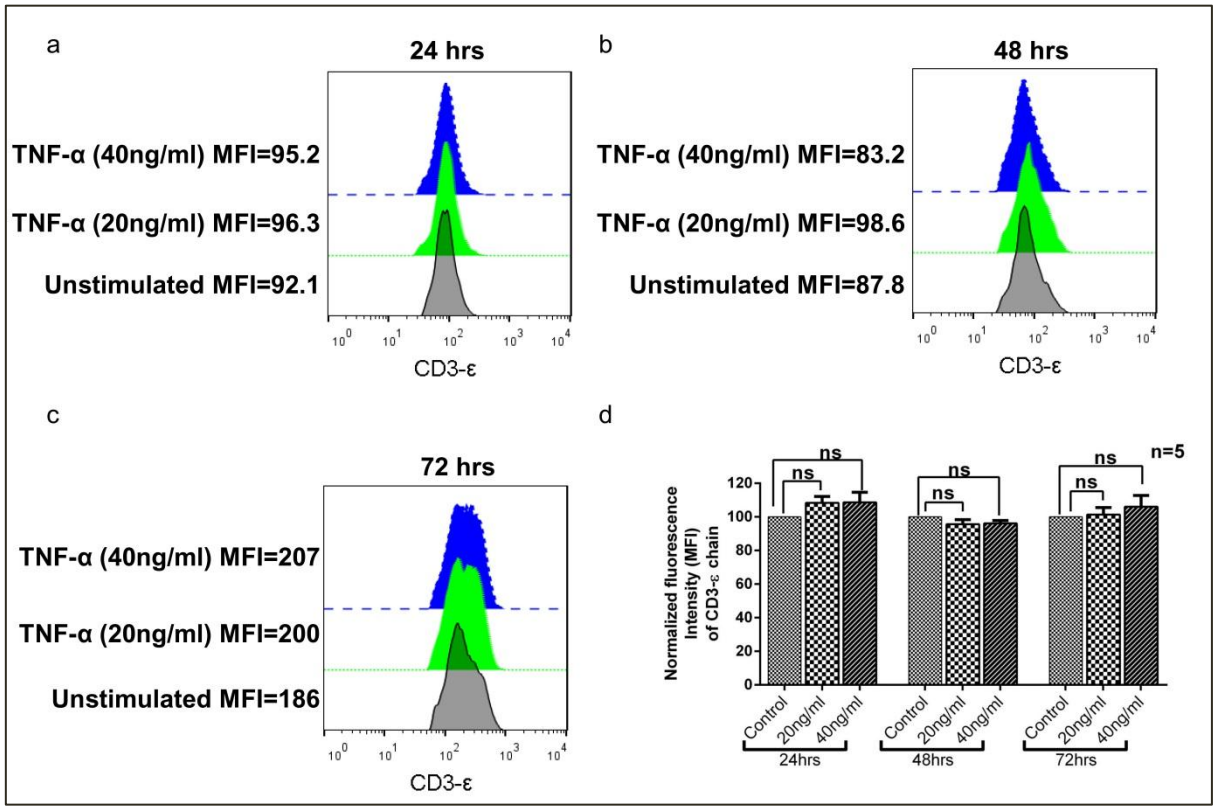

Figure 5.8: Regulation of CD3-ε chain expression in $CD3^+$ T cells of healthy individuals. PBMCs of HI were obtained by FH density gradient centrifugation. Cells were treated with 1% PHA before stimulation with TNF-α (20 ng/ml or 40 ng/ml) for 24, 48, and 72 hrs. The CD3-ε chain expression on $CD3^+$ T cells was studied by flow cytometry. The half offset histogram shows the effect of TNF-α on CD3-ε chain expression at 24 hr **(a)**, 48 hr **(b)** and 72 hrs **(c)**. **d)** Data is represented as mean values of normalized fluorescence intensities. The normalized fluorescence intensity is calculated by correcting the MFI of unstimulated control to 100 and adjusting the MFIs of others accordingly. Comparison of normalized fluorescence

intensities of CD3-ε of control cells with TNF-α-stimulated cells indicates no significant statistical difference at 24, 48 and 72 hrs. (ns represent non-significant data with $p>0.05$)

5.2.5 Blocking of TNFR-I but not TNFR-II reverses TNF-α induced CD3-ζ chain downregulation

Our data confirmed that it is the altered expression of TNFR-I and TNFR-II receptor on T cells which make them susceptible to TNF-α exposure. TNFR-I expression was higher on $CD3^+$ T cells from OSCC patients and induced expression of TNFR-I also made T cells from HI vulnerable to TNF-α induced CD3-ζ chain downregulation. In this context, to verify whether this receptor or both the receptors are involved in TNF-α induced downregulation of CD3-ζ chain, blocking experiments were carried out using respective mAbs (TNFR-I and TNFR-II). $CD3^+$ T cells were stimulated with TNF-α 20 ng/ml with or without TNFR-I and TNFR-II antibodies for 24 hr. After 24 hr cells were stained and analyzed for CD3-ζ chain expression. Results showed that TNF-α is capable of inducing downregulation of CD3-ζ chain and this effect of TNF-α exposure on CD3-ζ chain was reversed by blocking TNFR-I but not TNFR-II **(Fig. 5.9a).** However when TNFR-II was blocked the effect of TNF-α in downregulating showed marginal increase confirming that the TNF-α induced downregulation of CD3-ζ chain is mediated by TNFR-I and variation in their expression is having the differential functional impact on T cell functioning **(Fig. 5.9b).**

5.2.6 Expression of intracellular ROS and caspase 3 in PBMCs of healthy individuals upon treatment with TNF-α

To study the mechanism that could be associated with TNF-α signaling leading to CD3-ζ chain downregulation, levels of ROS and caspase-3 were studied. TCR-signaling pathways are differentially affected by physiological levels of oxidative stress. It is reported that loss of CD3-ζ chain is caused by ROS (in particular by H_2O_2), since addition of catalase restores CD3-ζ chain expression [148]. Superoxide anion can also readily react with nitric oxide (NO)

generating peroxynitrite ($ONOO^-$) which can lead to structural alteration in CD3-ζ chain leading to its degradation.

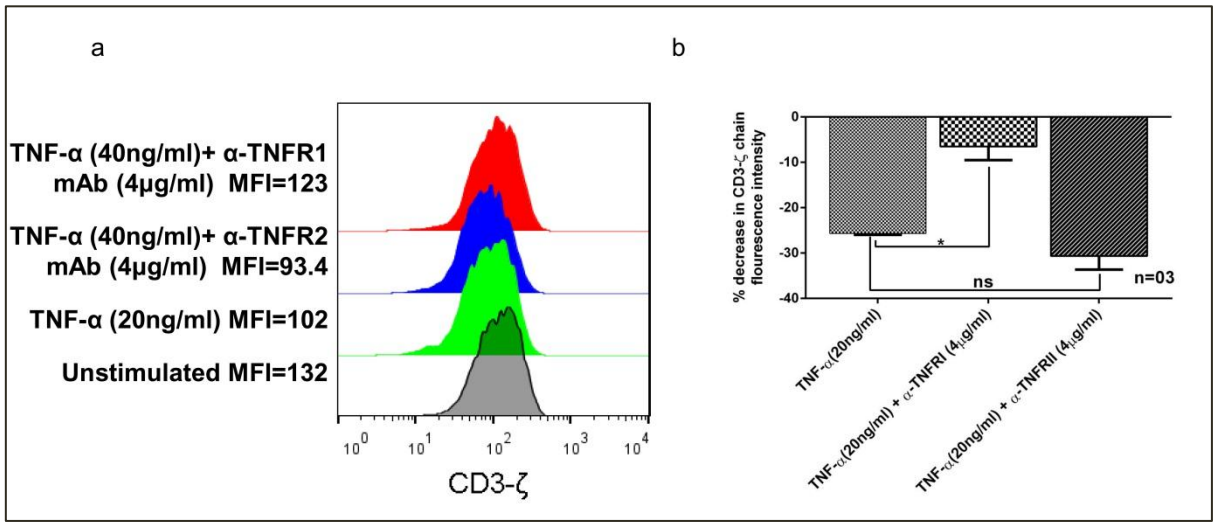

Figure 5.9: Consequential effect of TNF receptor blocking on CD3-ζ chain expression. The PBMCs were isolated using FH density gradient and then stimulated with TNF-α (20 ng/ml) either in absence or presence of TNFR-I and TNFR-II blocking antibodies. **a)** The histogram confirms that on blocking TNFR-I, the TNF-α induced downregulation of CD3-ζ chain is prevented while TNFR-II had no effect on CD3-ζ chain expression. **b)** The data represented is the mean of three independent experiments and is representing the percentage decrease in CD3-ζ chain expression compared to the unstimulated control. (* represents $p<0.05$ and ns represents non-significant data with $p>0.05$)

To prove whether TNF-α can induce ROS, PBMCs were stained with 2',7'-dichlorofluorescin diacetate (H_2DCF-DA) dye and were stimulated with TNF-α (20 ng/ml or 40 ng/ml) and expression of intracellular ROS, at various time points, was analysed by flow cytometry. The fluorescence intensity (MFI) was obtained at different times depicting the generation of ROS. Results confirmed that the ROS generation increases dramatically in PBMCs after stimulation with TNF-α. Intracellular ROS levels in PBMCs increased with time till 45 mins then decreased upon TNF-α stimulation **(Fig. 5.10).** This suggested that TNF-α induced oxidative stress might play a crucial role in the T lymphocyte hyporesponsiveness by regulating CD3-ζ chain expression.

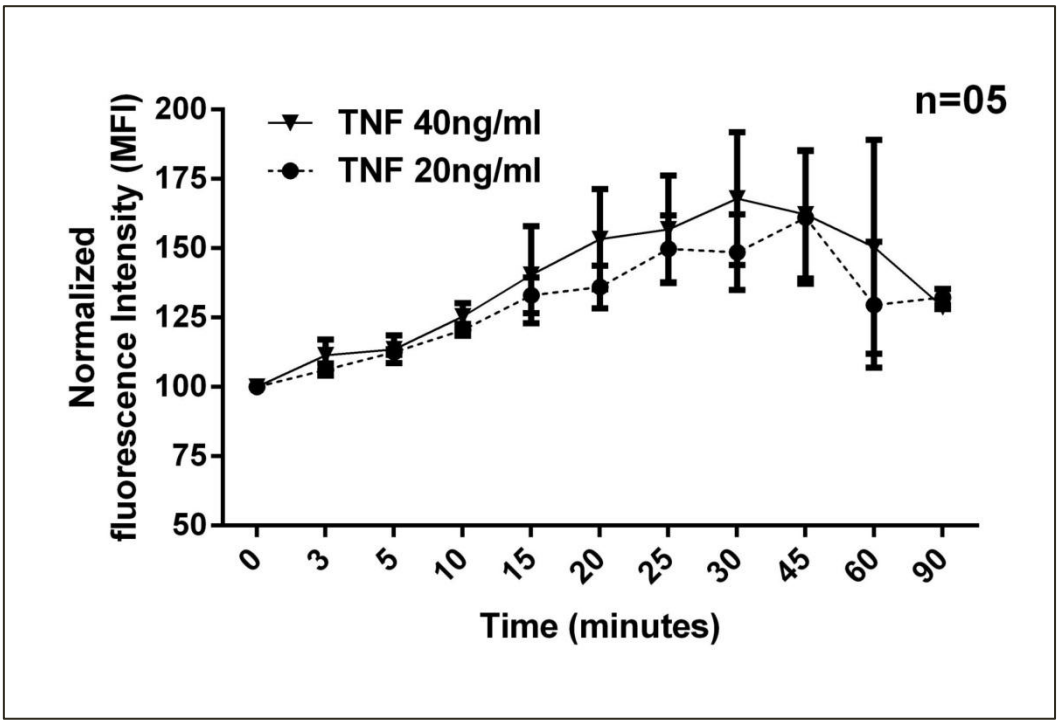

Figure 5.10: Quantitation of ROS in T cells stimulated with TNF-α. PBMCs of HI were obtained by FH density gradient centrifugation and intracellular ROS levels were monitored upon stimulation with TNF-α (20 ng/ml or 40 ng/ml) by flow cytometry. Data is represented as corrected fluorescence intensity plotted versus time. Corrected fluorescence intensity is calculated by correcting the MFI at 0 mins to 100 and adjusting the MFIs of others time points accordingly. Stimulation with TNF-α (20 ng/ml and 40 ng/ml) resulted in an increase in the fluorescence intensity with increase in time till 45 mins, after which it decreased.

Caspase-3 is the other molecule which has the capability to regulate CD3-ζ chain expression in T cells. Hence, the effect of TNF-α on caspase-3 level was observed in PBMCs. PBMCs were stimulated with 1% PHA followed by the treatment with TNF-α (40 ng/ml) for 24 and 48 hr. Protein lysates were prepared and analysed for caspase 3 activity by fluorimetry. Caspase activity in nmole of AMC released per minute per ml of cell lysate was calculated (as described in material and methods). Untreated cells served as a control for this experiment. Caspase activity of TNF-α treated sample was higher as compared to the untreated sample. Comparison of mean caspase activity of treated and untreated samples at 24 hr as well as at 48 hr indicates that there is the significant statistical difference ($p<0.05$) **(Fig 5.11a).** Further as our earlier data has shown that OAS2 **(chapter 4)** also has ability to alter caspase-3 levels, the additive effect of both these proteins was analyzed and data

confirmed that when cells were stimulated with both rh-OAS2 (12 ng/ml) and TNF-α (20 ng/ml) there was an synergistic effect on the induction of caspase-3 levels **(Fig 5.11b).** This data confirmed the possible mechanism that could be utilized by TNF-α signaling to induce T cell hyporesponsiveness in OSCC patients.

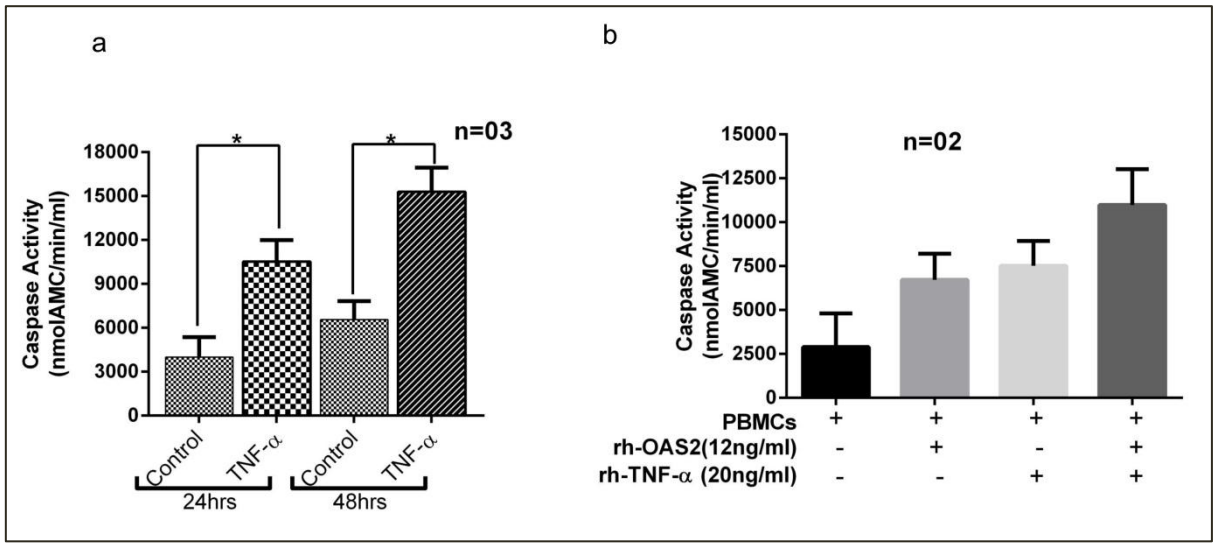

Figure 5.11: Caspase -3 levels in T cells stimulated with TNF-α. PBMCs of HI obtained by FH density gradient centrifugation were stimulated with PHA (1%) for 2 hrs, and then treated with TNF-α (40 ng/ml) for 24 hr and 48 hr. Cell lysates were prepared and analysed for caspase 3 activity by fluorimetry. **a)** Caspase activity in nmole of AMC released per minute per ml of cell lysate was calculated. Cells untreated with TNF-α served as control for this experiment. Comparison of caspase activity of control with TNF-α-stimulated cells indicates a significant statistical difference at 24 and 48 hr. **b)** The histogram shows the additive effect of TNF-α (20 ng/ml) and rh-OAS2 (12 ng/ml) on caspase-3 levels. (* represents<0.05)

5.2.7 Intracellular calcium levels in PBMCs of healthy individuals upon treatment with TNFα

Long-term culture in the presence of TNF-α inhibits subsequent IL-2 mRNA expression in T cells preactivated with anti-CD3 and restimulated via TCR and CD28. But TNF-α signaling via TNFR-II is required for the maintenance of IL-2 levels. So, this inhibition of IL-2 mRNA might be through TNFR-I signaling. Inhibition of IL-2 represents a state of anergy of T cells which have higher Ca_2^+ influx. The level of Ca_2^+ flux in the PBMCs of HI was observed after stimulation with TNF-α. PBMCs were stimulated with 1% PHA for 2 hrs after which cells

were washed and stained with fluo-3-AM. Baseline fluorescence was analysed followed which cells were stimulated with anti-CD3 mAb and TNF-α (20 ng/ml) for 5 mins. Cells stimulated with only anti-CD3 mAb were used as a control for this experiment. Calcium flux in cells stimulated with anti-CD3 mAb and TNF-α was higher than in cells stimulated with only anti-CD3 mAb **(Fig. 5.12a-b).**

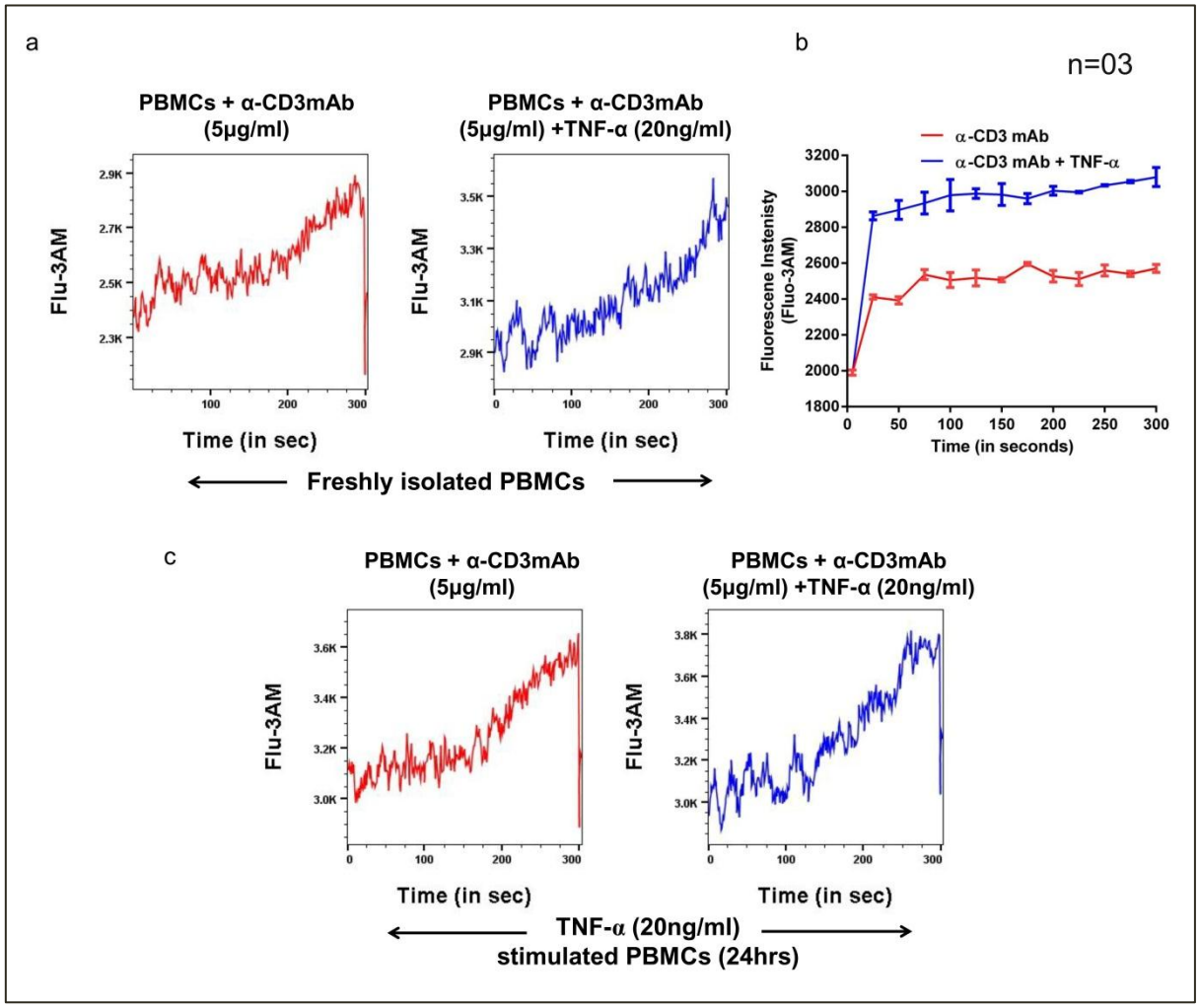

Figure 5.12: Calcium flux in T cells after TNF-α treatment. a) PBMCs of HI treated with PHA for 2 hrs were analysed for calcium flux upon stimulation with anti-CD3 mAb and TNF-α (20 ng/ml). Calcium flux in cells stimulated with anti-CD3 mAb and TNF-α was higher than in the cells stimulated with only anti-CD3 mAb. **b)** Each point on the graph represents the average fluorescence intensity over a range of 25 seconds each. The graph shows the consolidated data of three independent experiments. **c)** In other experiment cells were 1st treated with TNF-α for 24 hr then again stimulated either with anti-CD3 mAb or with anti-CD3 mAb and TNF-α. Cells stimulated with only anti-CD3 mAb served as control for these experiments. Data is represented as increase in calcium flux with time. Calcium flux in cells treated with TNF-α and stimulated with anti-CD3 mAb and TNF-α was higher than in cells treated with TNF-α and stimulated with only anti-CD3 mAb.

Separately, PBMCs were stimulated with 1% PHA and then treated with TNF-α (20ng/ml) for 24 hr. After 24 hr cells, were washed and stained with fluo-3-AM. Baseline fluorescence was analysed after which cells were restimulated with anti-CD3 mAb and TNF-α (20ng/ml) for 5 mins. Cells stimulated with only anti-CD3 mAb were used as the control for this experiment. Calcium flux in cells stimulated with TNF-α after being restimulated with anti-CD3 mAb and TNF-α was higher than in cells treated with TNF-α and restimulated with only anti-CD3 mAb **(Fig. 5.12c).** This confirms that TNF-α is able to alter the stored calcium flux in T cells and signifies its role in altering the T cell signaling. The levels of calcium were lower in the cells stimulated with TNF-α for 24 hr which could be attributed to decreased CD3-ζ chain expression induced by TNF-α stimulation.

5.2.8 Effect of TNF-α on PI3-Kinase independent CD28 signaling in T cells

Incubation of T cell lines with TNF-α have been shown to induce a reduction in the levels of cell surface expression of CD28 [435]. CD28 signaling can occur either through PI3K dependent and independent signaling. PI3K independent pathway depends upon the recruitment of Grb2/sos complex to CD28 by Shc protein [436]. This signaling is important in our context as it can regulate ROS, caspase-3 as well as calcium flux. Therefore, the expression of SHC in the PBMCs of HI treated with TNF-α (20 ng/ml and 40 ng/ml) for 24 hr, 48 hr and 72 hrs was observed. Cell lysates were prepared and the expression of Shc was studied by western blotting. Unstimulated cells served as the control for this experiment. Protein bands of p46Shc and p52Shc were observed. Expression of p46Shc and p52Shc was lower in protein lysates obtained from TNF-α treated cells as compared to untreated cells **(Fig. 5.13)**. Blot stained with Ponceau red, showing all protein bands, was used to ensure equal loading of proteins. This suggests that the CD28 signaling is defective in T cells stimulated with the TNF-α resulting in decreased effector function of T cells.

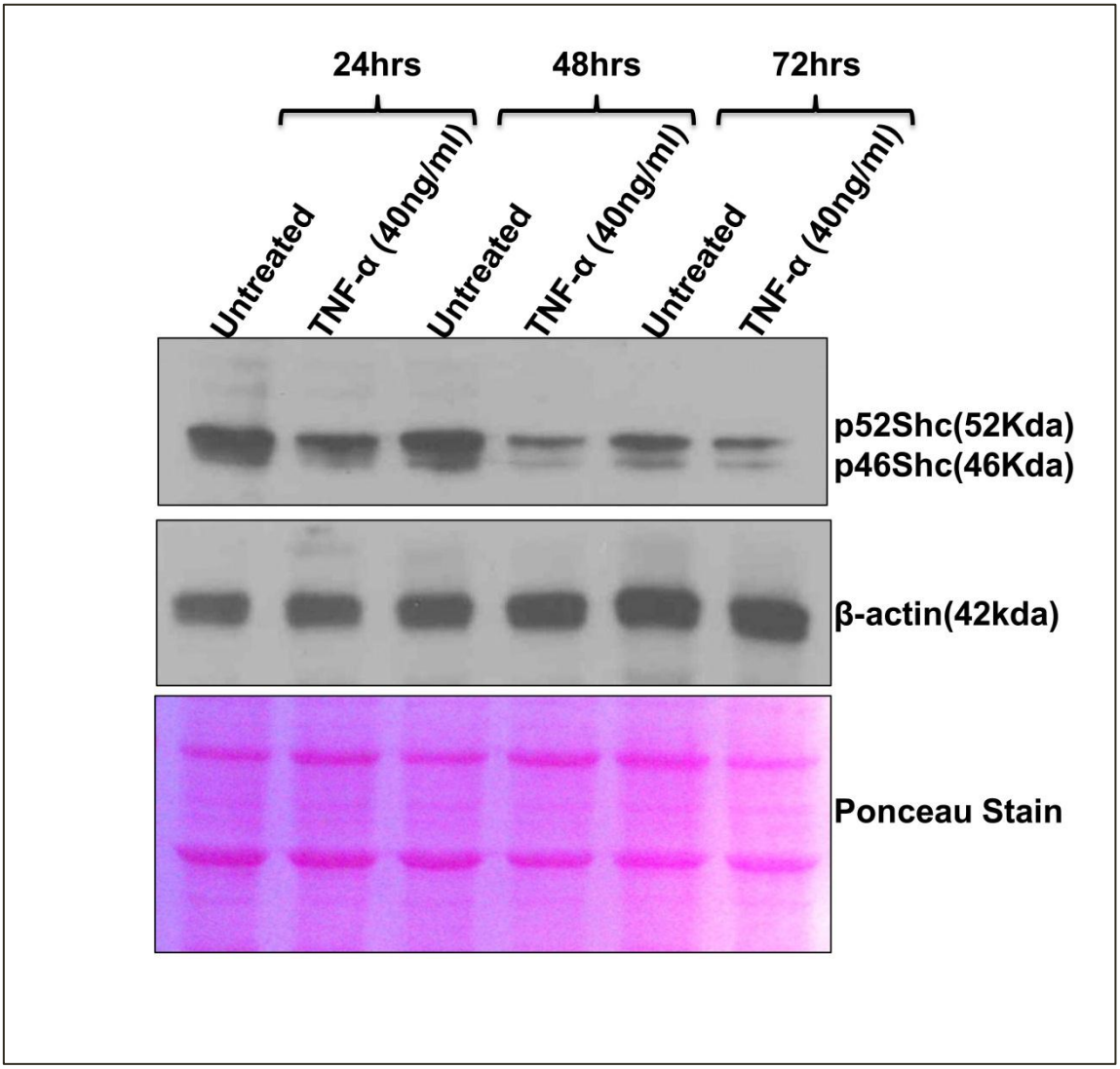

Figure 5.13: Effect of TNF-α on CD28 signaling. PBMCs of HI obtained by FH density gradient centrifugation were stimulated with 1% PHA for 2 hrs and then treated with TNF-α (20 ng/ml and 40 ng/ml) for 24, 48 and 72 hrs. Cell lysates were prepared and the expression of Shc was studied by western blotting. Unstimulated cells served as control for this experiment. The expression of p46 Shc and p52 Shc decreases upon stimulation with TNF-α at all the time points suggesting that CD28 signaling is altered in T cells stimulated with TNF-α. The β-actin and ponceau stained blot served as loading controls.

5.3 Discussion

Under the chronic inflammatory condition, T cells experience a state of hyporesponsiveness on TCR stimulation [437]. Attenuated TCR signaling pathways in T cells derived from sites of inflammation has been reported. TNF-α, a prominent pro-inflammatory cytokine, is involved in the pathogenesis of various pathological conditions (cancer and autoimmune diseases) which are associated with inflammation [438]. Chronic TNF-α exposure has a negative regulatory effect on TCR-mediated T cell activation *in vivo* and *in vitro* as observed in a mouse model [203, 204]. The level of TCR on cell surface expression is critically important in the efficiency and duration of the immune response. The aim of the present study was to show how T cells become susceptible to the TNF-α induced CD3-ζ chain downregulation in inflammatory condition. CD3-ζ chain is considered to be the rate-limiting factor of TCR/CD3 complex formation [161].

The T cells derived from an inflammatory condition like OSCC have differential expression of TNFR-I and TNFR-II. Antigen experienced T cells from the oral cancer patients showed higher expression of TNFR-I compared to TNFR-II. In healthy individuals, the expression of both the receptors was comparable. Overexpression of TNFR-I on T cells might be inducing death of T cells in oral cancer patients and limiting the effector immune response against the growing tumors. This has been also observed in the melanoma tumor model where TNF-α has been shown to induce $CD8^+$ T cell death in a TNFR-I dependent manner. Further, in TNFR-I deficient mice the accumulation of $CD8^+$ T cells in tumor microenvironment was observed [423]. Thus, host TNFR-I dependent TNF-α signaling impairs T-cell–dependent immune response. Despite the fact that TNFR-II has higher binding affinity for TNF-α than TNFR-I, the signaling via TNFR-I is initiated in the oral cancer patients. This signaling will limit the major functions of TNF-α via TNFR-II in modulating both innate and adaptive immune system.

Our in vitro experiments demonstrated that mitogen induced TNFR-I expression on T cells rendered them susceptible to the TNF-α mediated CD3-ζ chain downregulation. This reduction in CD3-ζ chain was thought to be a primary effect which might lead to the impaired TCR/CD3 assembly and expression at the cell surface. The total CD3-ε chain remained unaffected because CD3-ζ chain in known to be regulated independently of other CD3 subunits. Also the rate of synthesis of the CD3-ζ chain is 10% of that of other TCR/CD3 chains; and appears to be the rate limiting step in the regulation of TCR/CD3 internalization and recycling [134, 439]. The decreased expression of the CD3-ζ chain has been reported in several autoimmune (rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)), inflammatory and malignant diseases (oral cancer and others). The TNF-α induced CD3-ζ chain was found to be a reversible phenomenon regulated by Src like adaptor protein (SLAP) dependent proteasomal degradation in human T cells [161]. Our hypothesis was strengthened by the fact that blocking of mitogen induced TNFR-I receptor prevented TNF-α mediated downregulation of CD3-ζ chain expression. Further, the Jurkat T cells which are known to express higher levels of TNFR-I showed conspicuous effect on CD3-ζ chain downregulation.

An earlier study has shown the role of TNFR-II in impairing the TCR signaling by TNF-α [434]. However this study failed to explain how TNFR-II signaling has inhibited IL-2 signaling as TNFR-II signaling is required for the IL-2 induction and T cell survival linked to CD28 co-stimulation [433]. The strength of TCR signaling and duration of antigen encounter are critical parameters for the fate of naive CD8 T cells toward death, anergy, or retainment of naive phenotype [440]. It is, therefore possible that factors influencing TCR signaling would affect developmental fates of T cells after Ag engagement. Collectively, the role of TNF/TNFRs pathways on T cell tolerance relies on differential expression of TNFRs (I and II) and the context of TCR signaling (i.e.; inhibitory or stimulatory). Because TCR-derived signals not only determine the efficacy of T cell immunity but also tolerance induction [440]

and T cell viability [441], it is anticipated that costimulatory molecules can have an influence on these parameters of T cell biology.

The inhibition of TCR signaling will induce anergy in the T cells and anergic T cells are associated with higher calcium flux and caspase-3 activation. The inhibition of TCR signaling will prevent AP-1 activation required for NFAT driven IL-2 secretion. In absence of AP1, NFAT will switch on transcription leading to the synthesis of E3 ubiquitin ligase marking a state of anergy [399]. This will form a feedback loop leading to increased calcium flux. A sustained increase in intracellular calcium was observed when T cells were stimulated with TNF-α marking a state of anergy. T cell activation requires a threshold amount of TCR-mediated signals, an amount that is reduced by signals mediated through costimulatory molecules expressed on the T cell surface. TNFR-II has been shown to act as a putative costimulatory receptor for T cell activation and decrease threshold for TCR stimulation [427]. However, enhanced expression of TNFR-I might increase the threshold for TCR stimulation and at a suboptimal stimulation T cells might fail to respond and show hyporesponsiveness. This was further validated by the prominent caspase 3 activation in the T cells stimulated with TNF-α. The cytoplasmic domain of CD3-ζ chain has several consensus target sequences for caspases, among which caspase-3 and 7 have been shown to cleave *in vitro* translated CD3-ζ chain [155]. Caspase-3, an effector caspase is expressed during T cell anergy induction and recognizes proteins with a common DXXD motif and cleaves after the second aspartic residue [178, 369]. The CD3-ζ chain has various caspase-3 cleavage sites (5 in number) and hence might be involved in active caspase-3 mediated downregulation of CD3-ζ chain in T cell from oral cancer patients.

Attenuation of T cell activation was associated with increased Ca^{2+} flux and could be partly attributed to decreased surface CD3-ζ chain expression or down-regulation of CD28 signaling. CD28 provides a costimulatory signal that results in optimal activation of T cells.

Engagement of CD28 could be either in a phosphatidylinositol 3′-kinase (PI3K) dependent or independent fashion. Association of Grb2 with CD28 via p52*shc* forms the basis for PI3K-independent signaling through CD28 [436]. This PI3K-independent signaling has capability to induce mitochondrial dysfunction associated with increased ROS and caspase 3 activation [442, 443]. We observed that the mitogenic isoform of *Shc* are downregulated after TNF-α stimulation and might be the cause for inducing ROS generation and caspase-3 activation. ROS are known to promote the TNF-α induced programmed cell death via TNFR-I dependent signaling [444]. This might be the possible mechanism associated with TNF-α induced T cell death observed in cancer patients and indicates that intact proximal TCR signaling can be disrupted by chronic TNFR-I signaling. Thus, mitochondrial targeted antioxidants may present a preferred and viable strategy for therapeutic intervention in chronic inflammatory disease like cancer induced by TNF-α. This will enhance the T cell survival and help in generating an immune response against growing tumor.

The resistance of melanoma patients to BRAF V600E inhibitors has been attributed to TNF-α which increase Twist1 levels in these patients [445-447]. This could also be credited to the immune escape mechanism of tumors induced by TNF-α. Many cancers are associated with the upregulation of MDSCs and Tregs. Peripheral accumulation of MDSCs was drastically impaired in $Tnfr^{-/-}$ mice and TNF-α also prevents maturation of immature myeloid cells [206, 432]. Signaling through TNFR-II, but not TNFR-I, promoted MDSC survival in a tumor bearing host [432]. TNFR-II is also critical for the stabilization of the $CD4^{+}FoxP3^{+}$ regulatory T cell phenotype in the inflammatory environment [431]. Taken together, these studies provide the rationale for TNFR-II as a potentially attractive molecular target for in vivo and ex vivo treatment of diverse diseases where MDSCs and Tregs play important roles. However, our study confirmed that blocking TNFR-II has enhanced the TNF-α induced T cell dysfunction. Our findings provide insights that using antagonist ligands aimed at a single

receptor, TNFR-II, might on one hand hold the potential for selectively turning off MDSCs and Tregs for diseases as diverse as allergy, autoimmunity, cancer, transplantation, graft versus host disease (GVHD) and infectious disease but at same time it may also lead to TNF-α induced TCR signaling defect in antigen encountered T cells via TNFR-I. Therefore, these aspects should be taken into account while designing the future therapies using TNF inhibitors.

Chapter 6
Role of Notch signaling in regulating the proximal and distal TCR signaling in human $CD3^+$ αβ-T cells

6.1 Introduction

Notch signaling pathway is involved in cell fate decisions during differentiation and in lineage discrimination [448]. Notch signaling is initiated by the ligand engagement of the Notch receptor. There are four Notch receptors (Notch-1-4) and five Notch ligands, three Delta-like (Dll1, Dll3 and Dll4) and two Jagged (Jag1 and 2) [449, 450]. Notch receptor engagement by its ligand cleaves the extracellular and transmembrane domain of Notch through activation of ADAM proteases and gamma-secretase respectively [451]. The second cleavage by gamma- secretase generates Notch intracellular domain (NICD) that is transported to the nucleus and behaves as a transcriptional activator [452, 453]. NICD binds to cofactors like CSL (CBF-1/suppressor of hairless/Lag1), MAML (mastermind like1) and p300/CBP to regulate the expression of its target genes, e.g., HES1 (hairy and enhancer of split-1), HERP (HES related repressor protein) and NF-κB [454, 455]. The Notch signaling pathway controls the development and activation of a variety of immune cells [456]. Notch has been implicated in the T versus B lymphocyte differentiation from a common lymphocyte precursor [457]. It is also known that Notch signaling is intimately involved in γδ versus αβ lineage decision [458]. The role of Notch in effector functions of T cells has also been reported. Notch is actively involved in peripheral T cell activation and cytokine secretion [37]. Notch signaling is also involved in cytolytic effector functions of $CD8^+$ T cells by regulating the expression of key effector molecules like perforin and granzyme B [36].

For T cells to become activated, myriad of signals integrate together. Ligation of the TCR accompanied by co-stimulation generates intracellular Notch in $CD4^+$ and γδ T cells while inhibition of Notch with γ-secretase inhibitors (GSI) decreases T cell activation and proliferation [37, 167, 168]. However, specifically the point at which TCR and Notch signaling cascade interact with each other remains controversial. Pharmacological inhibition of Notch activation was reported to decrease the distal TCR signaling events such as the

expression of activation markers CD25, CD69, IL-2 and IFN-γ without affecting proximal signaling events such as phosphorylation of Zap70 [170]. On the other hand inhibiting Notch signaling has the potential to induce T cell anergy by increasing the threshold for T cell stimulation [170]. Although anergic T cells show defective cytokine production in response to TCR/CD28 ligation, they still produce meaningful levels of cytokines in response to the pharmacological agents- phorbol 12-myristate 13-acetate (PMA) plus ionomycin [459]. This suggests that the phenomenon of anergy induced by Notch inhibition in T cells might induce a proximal signal transduction defect. Therefore, a detailed evaluation is needed to find precisely the point at which Notch has the capacity to modulate the TCR signaling cascade in human αβ T lymphocytes.

The present study aimed to investigate:

How important is Notch in regulating the proximal and distal TCR-driven signaling in αβ T cells?

6.2 **Results**

6.2.1 Expression of mRNA for Notch receptors and ligands in peripheral human αβ T cells

To test the hypothesis that TCR stimulation regulates Notch signaling and thereby control T cell activity and function, the expression analysis of Notch receptors (Notch-1-4) and their ligands (Dll1, Dll3, Dll4, Jag1, and Jag2) in unstimulated and stimulated αβ T cells was carried out. The mRNA expression of Notch-1 and Notch-2 genes was observed in αβ T cells **(Fig. 6.1a)** while the expression of Notch-3 and Notch-4 was almost undetectable. The TCR-driven activation of αβ T cells led to the marked increase in the expression of Notch-1 and Notch-2 without affecting the Notch-3 and Notch-4 receptors **(Fig. 6.1a).**

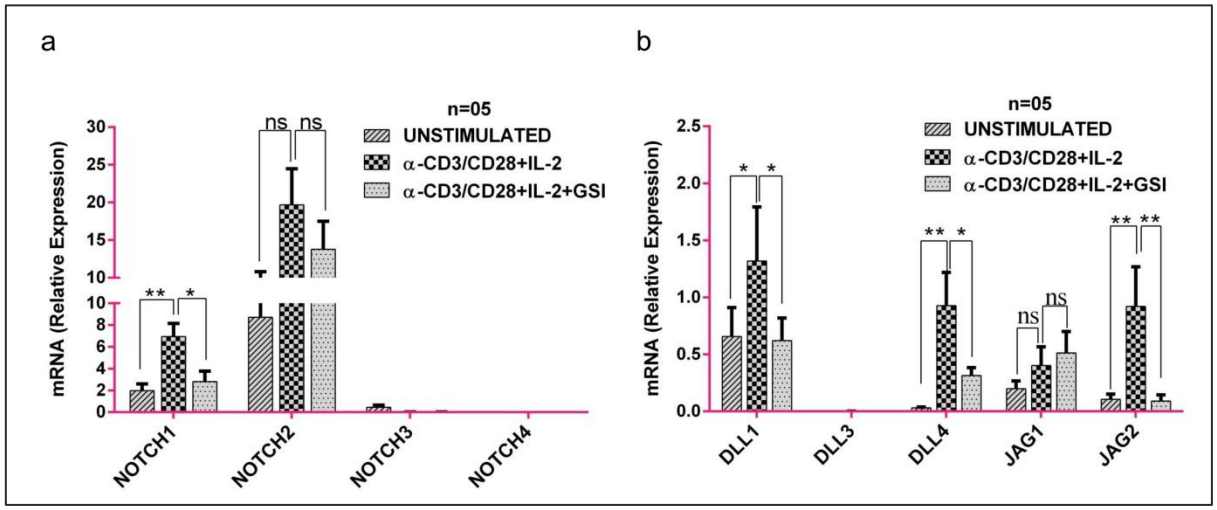

Figure 6.1: Expression of Notch receptor and ligands on human αβ T cells. Human αβ T cells were MACS-purified from the peripheral blood. **a)** Real-time quantitative PCR showed abundant mRNA expression of Notch-1 and Notch-2 in αβ T cells. Activation induces their expression while inhibition of Notch signaling prevents the activation induced overexpression of receptors. The data represents the mean of four independent experiments and data were normalized to expression of β-actin. (* represents $p<0.05$, ** represents $p<0.005$) **b)** Real-time quantitative PCR showed mRNA expression of Dll1, Dll4 JAG1 and JAG2 in unstimulated αβ T cells. Stimulation of T cells induced a drastic increase in the expression of Dll1 Dll4 and JAG2 which was abrogated by inhibition of Notch signaling by GSI-X. The data represent the mean of four independent experiments and data were normalized to expression of β-actin. (* represents $p<0.05$, ** represents $p<0.005$).

Relatively low expression of Notch ligands Dll1 Dll4, Jag1 and Jag2 mRNA was observed in unstimulated αβ T cells which were enhanced accordingly by TCR stimulation suggesting that TCR-driven signaling has the capability to regulate Notch signaling pathway in T cells **(Fig. 6.1b).** Because the dramatic effect was seen in the expression of Notch receptors and ligands on activation, we examined whether inhibition of Notch signaling would have any effect on T cell activation and thereby control the expression of its receptors and ligands. The pharmacological inhibition of Notch receptor using gamma-secretase inhibitor (GSI-X) in TCR-stimulated cells prevented the TCR-driven upregulation of Notch receptors as well as ligands **(Fig. 6.1a-b)**. This suggests that there is a crosstalk between the TCR and Notch signaling pathways whereby they regulate each other.

6.2.2 Expression of Notch target genes in human αβ T cells

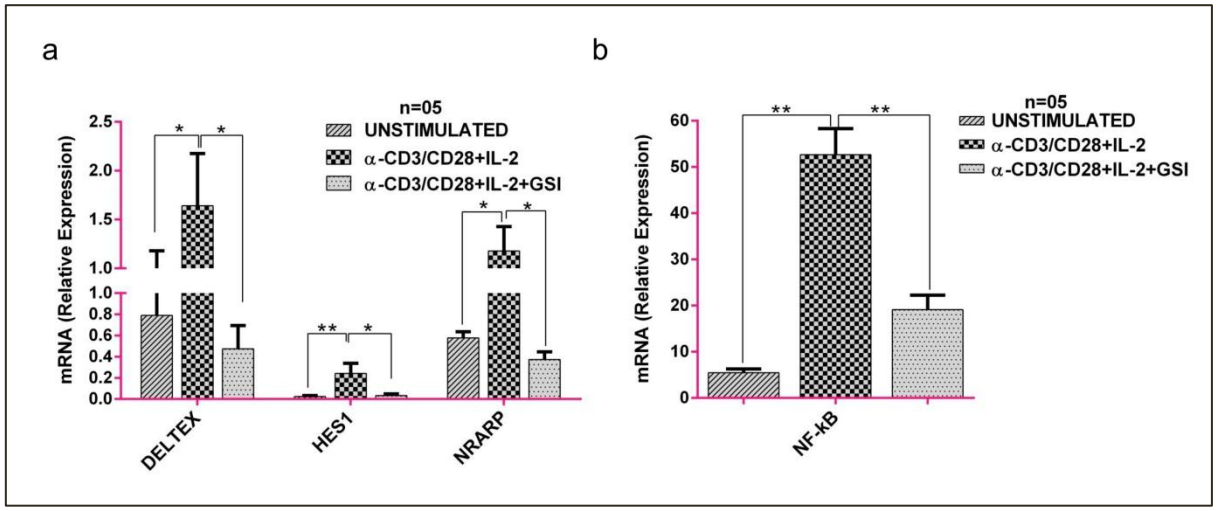

Figure 6.2: Expression of Notch target genes on human αβ T cells. **a and b)** Human αβ T cells were analyzed for target gene expression (HES1, Deltex, NRARP and NF-κB). Like receptors and ligands, target genes were induced by TCR-driven activation and inhibited by inhibition of Notch signaling. The data represented is the mean of four independent experiments and data were normalized to expression of β-actin (* represents $p<0.05$, ** represents $p<0.005$)

To better understand Notch signaling and T cell activity, we focused on two hallmarks of Notch signaling: its dependence on the action of presenilin-dependent γ-secretase activity and

the induction of expression of Notch target genes. Activation of Notch signaling leads to the release of NICD into the nucleus and subsequently regulates its target genes. Following translocation of NICD into the nucleus, it associates with CSL and converts it from a repressor into an activator complex. This step is followed by expression of Notch target genes such as HES1, NF-κB, NRARP (Notch-regulated ankyrin repeat protein) and Deltex [166, 460]. Using real-time PCR, we demonstrated that unstimulated αβ T cells express the Notch target genes at varying degree. However, TCR-driven activation led to drastic increase in the expression of NF-κB, NRARP, Hes1 and Deltex in αβ T cells which was abrogated on pharmacological inhibition of Notch signaling using gamma-secretase inhibitor (GSI-X) **(Fig. 6.2a-b).** Thus, induction of Notch signaling is a consequence of activation induced upregulation of Notch ligands and receptors on T cells.

6.2.3 Expression of Notch intracellular domain (NICD) in human αβ T cells

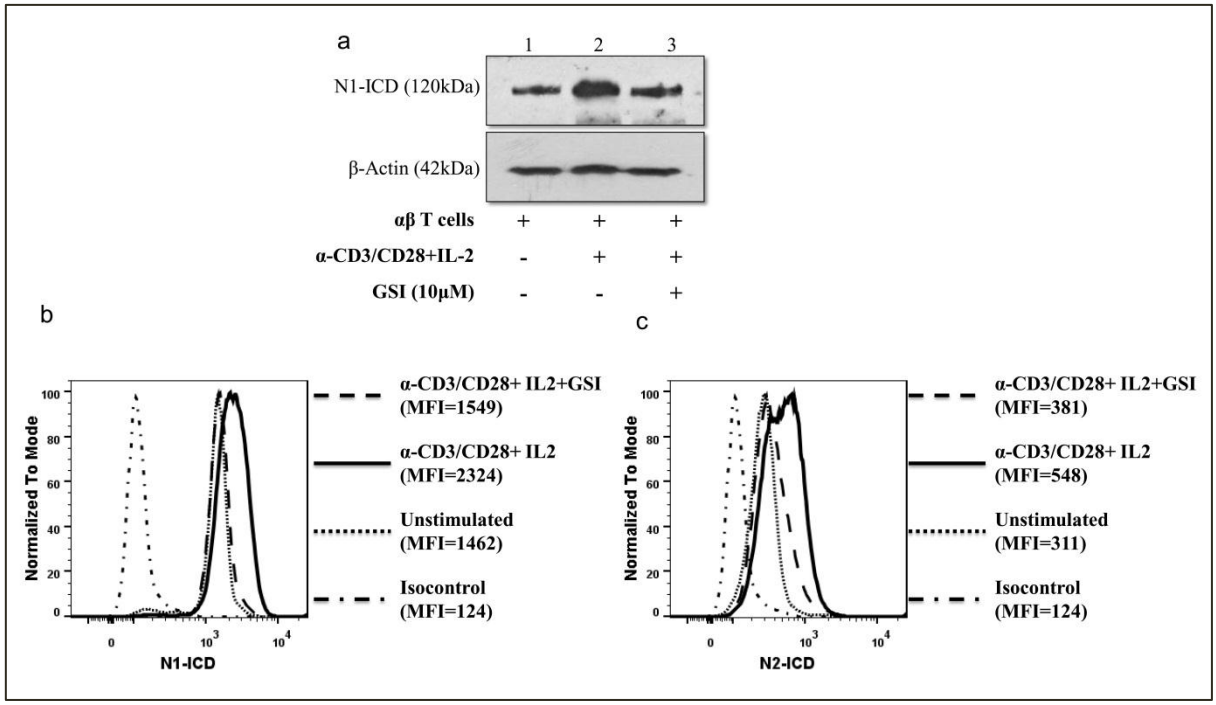

Figure 6.3: TCR driven activation of Notch signaling. a) Detection of 120kDa N1ICD in unstimulated αβ T cells (lane1), α-CD3/CD28 mAb and rIL2 activated (24 h) αβ T cells (lane 2) or in the presence of GSI-X (lane 3), using antibodies that recognize the cleaved active form of Notch-1 (N1ICD). **b)** Flow Cytometric analysis shows the expression of Notch-1 (N1ICD) in unstimulated αβ T cells or in αβ T cells stimulated with α-CD3/CD28 mAb) in presence or absence of GSI-X. The data is represented as median fluorescence intensity

(MFI). **c)** Flow Cytometric analysis shows the expression of Notch-2 (N2ICD) in unstimulated αβ T cells or in αβ T cells stimulated with α-CD3/CD28 mAb) in presence or absence of GSI-X. The data is represented as median fluorescence intensity (MFI).

The basal expression of N1ICD was observed in the unstimulated αβ T cells **(Fig.6. 3a, lane 1)** but the stimulation with α-CD3/CD28 mAb led to the abundant release of N1ICD (120kda) suggesting induction of Notch signaling **(Fig. 6.3a, lane 2)**. Treatment of αβ T cells with GSI-X prevented the α-CD3/CD28 mAb-induced overexpression of N1ICD **(Fig. 6.3a, lane 3)**. The induction of Notch signaling in αβ T cells was also determined using flow cytometry by using N1ICD and N2ICD specific antibodies. The expression of N1ICD (MFI 1462) in steady-state αβ T cells (**Fig. 6.3b)** was higher compared to N2ICD (MFI 311) **(Fig. 6.3c)**. On stimulation with α-CD3/CD28 mAb both N1ICD and N2ICD were induced and in the presence of GSI-X, the expression of N1ICD and N2ICD were markedly reduced.

6.2.4 Notch is required for proximal TCR signaling events in the human $CD3^+$ αβ T cells

Activation of T cells via the TCR accompanied by co-stimulation led to the production of the active, intracellular domain of Notch-1 (N1ICD) and its inhibition via γ-secretase inhibitors (GSI) decreased the activation-induced expression of Notch signaling pathway mediators. While Notch has been demonstrated to influence T cell activation, precisely where Notch exerts its influence downstream of the TCR is obscure. The TCR CD3-ζ chain is an important regulator of T cell function as it contains ITAM. Phosphorylation of the ITAM on CD3-ζ chain renders it capable of binding ZAP70 (Zeta-associated protein), a kinase that is important in the signaling cascade of the T cell [461]. The activation of αβ T cells with α-CD3/CD28 mAb led to the reduction in the expression of CD3-ζ chain which was further abrogated on GSI-X inhibition **(Fig. 6.4a-b)**. The inhibition of Notch signaling by GSI-X was not able to alter the expression of CD3-ζ chain or its regulator ELF1 at mRNA level suggesting that the Notch inhibition induces posttranslational down-regulation of CD3-ζ chain **(Fig. 6.4c).** This was further verified in Jurkat T cells where we found that inhibition of

Notch signaling induced downregulation of CD3-ζ chain **(Fig. 6.4d)**. In Jurkat T cells, inhibition of Notch affected only CD3-ζ chain expression without affecting downstream molecules like ZAP70 and p56Lck **(Fig. 6.5a).** T cell activation is tightly regulated by intracellular signals especially the anergy-related gene (GRAIL) to avoid autoimmunity and maintain tolerance against self-tissues. GRAIL is an E3 ubiquitin ligase associated with T cell tolerance and TCR CD3 degradation [462]. In the current study, we observed that inhibition of Notch signaling induced GRAIL expression both in human αβ T cells as well as Jurkat T cells **(Fig. 6.5a-b).** This indicates that GRAIL might be an essential regulator of T cell tolerance by regulating CD3-ζ chain expression in T cells.

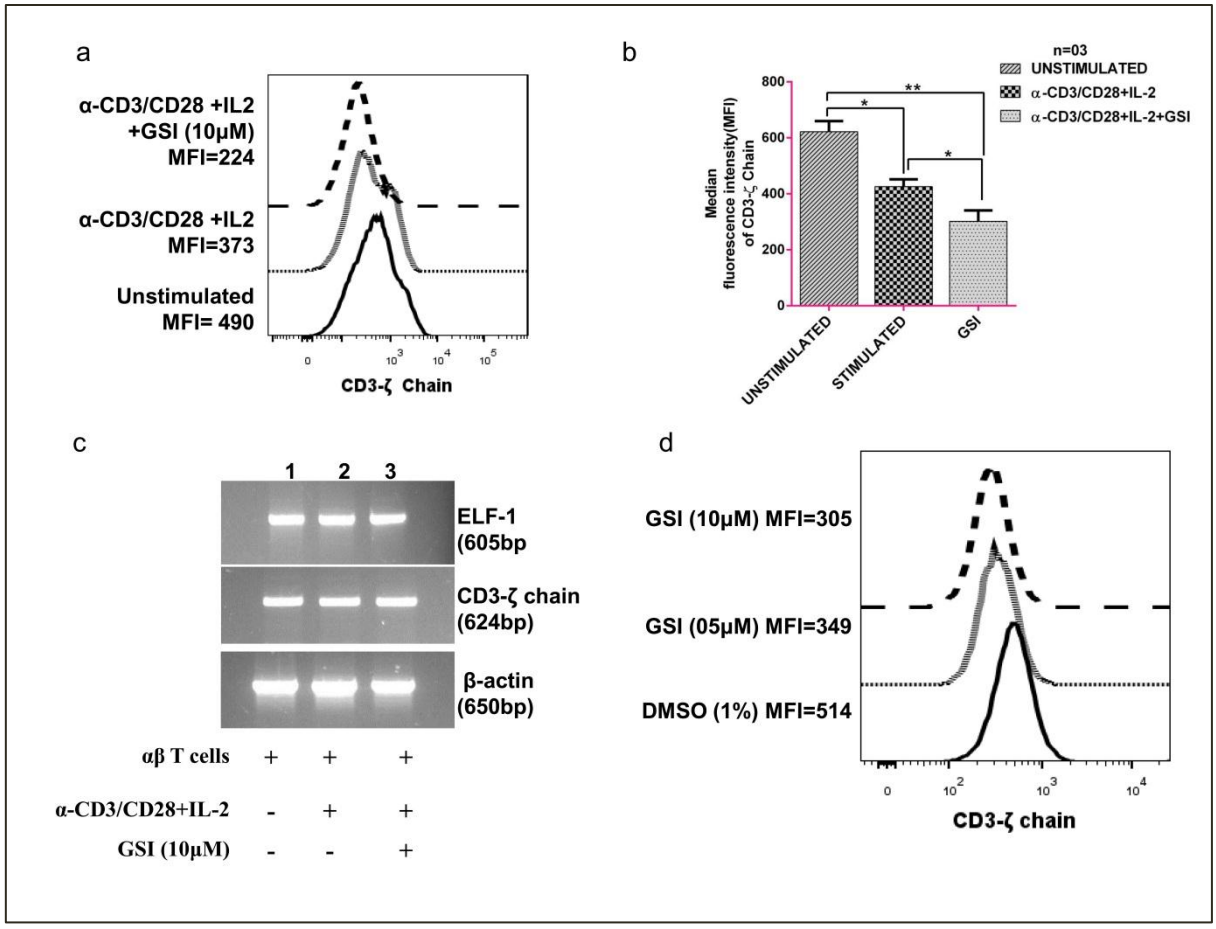

Figure 6.4: Notch regulates CD3-ζ chain expression the human CD3+ αβ T cells and Jurkat T cells. **a)** Inhibition of Notch signaling induced downregulation of CD3-ζ chain expression in the αβ T cells. The half offset histogram shows the median fluorescence intensity of unstimulated αβ T cells (MFI=490) which on stimulation with α-CD3/CD28 mAb decreases (MFI= 373). On inhibition of Notch signaling in αβ T Cells the median

fluorescence intensity of CD3-ζ chain reduces further (MFI= 224). The cells were stimulated for 24 hr in the presence or absence of GSI-X (10μM). **b)** The graph indicates the expression (Normalized median fluorescence intensity) of CD3-ζ chain in unstimulated, stimulated and GSI-X treated αβ T cells. The graph shown is representative of three independent experiments (* represents $p<0.05$). **c)** Total RNA (150-300 ng/μL) was isolated from the αβ T cells (unstimulated, stimulated and GSI-X treated). The isolated RNA was converted into cDNA and cDNA was subjected to PCR, as described in "Materials and methods," to detect CD3-ζ chain, ELF1, and β-actin transcripts. The expression of CD3-ζ chain and ELF-1 remained unaffected across all the αβ T cells i.e.; unstimulated, stimulated and GSI-X treated. **d)** In Jurkat T cells, inhibition of Notch signaling induced downregulation of CD3-ζ chain expression. The half offset histogram shows the median fluorescence intensity of Control (1% DMSO) treated Jurkat T cells (MFI=514) which decrease in a dose-dependent manner after GSI-X treatment. The Jurkat T cells were treated with 5 and 10 μM of GSI-X which reduced the median fluorescence intensity of CD3-ζ chain to 349 (MFI) and 305(MFI) respectively.

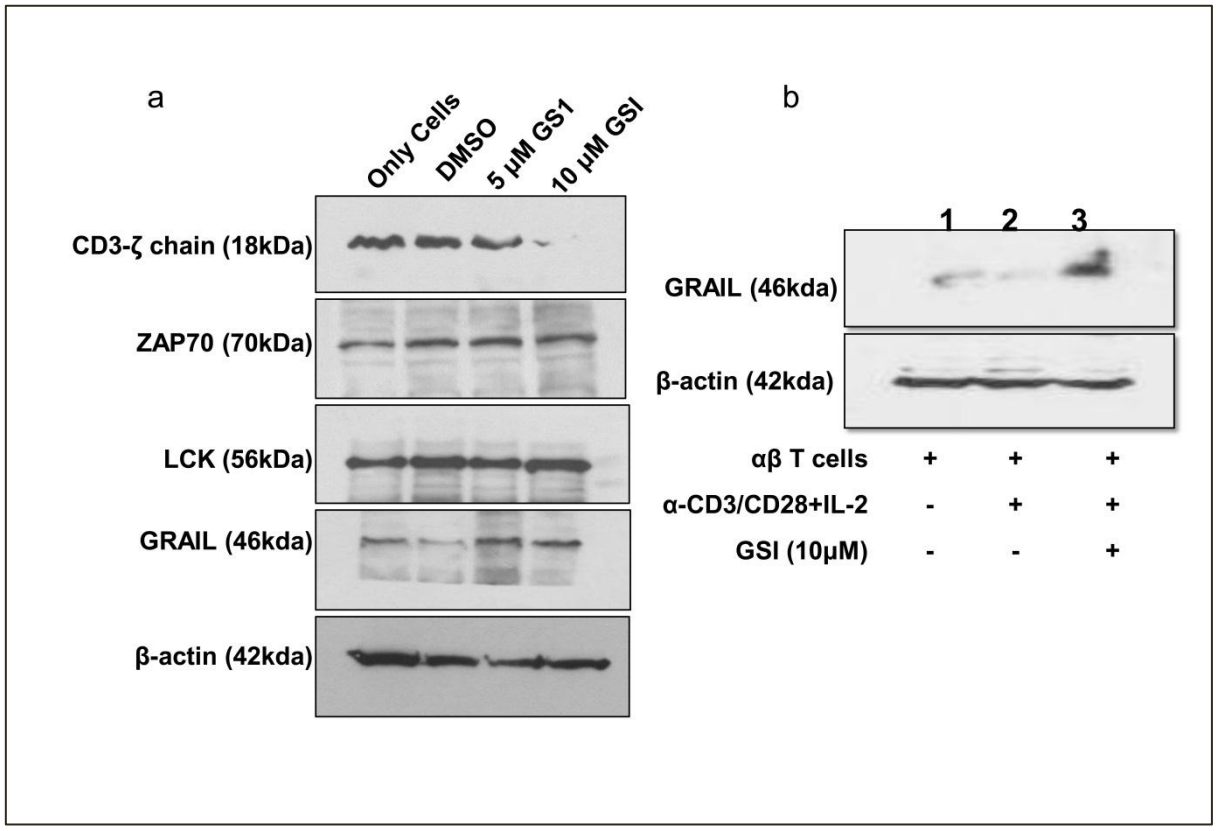

Figure 6.5: Effect of inhibition of Notch signaling on proximal TCR events in the human CD3+ αβ T cells and Jurkat T cells. a) Jurkat T cells were treated with different concentration of GSI-X (5 and 10μM) for 24 hr. After 24 hr total protein extract from these cells was used to detect the levels of CD3-ζ chain, ZAP70, p56Lck, GRAIL and β-Actin by Western blotting. The protein extracts (40-60 μg) prepared from Jurkat T cells was loaded onto the SDS-PAGE and then transferred to the nitrocellulose membrane. The results showed that inhibition of Notch signaling using GSI-X induced GRAIL and decreased CD3-ζ chain expression in Jurkat T cells without affecting other key T cell signaling molecules ZAP70 and p65Lck. β-actin was used as internal control. **b)** Western blotting experiment showing induction of E3 ubiquitin ligase (GRAIL) on inhibition of Notch signaling using GSI-X.

6.2.5 Inhibition of Notch signaling impairs stored calcium flux in human αβ T cells

Calcium signaling plays a central role in the induction of anergy in T cells, which become functionally inactivated and incapable of proliferating and expressing cytokines following antigen re-encounter [463]. Engagement of the TCR leads to rapid increases in the intracellular concentration of calcium. However, suboptimal stimulation of T cells in the presence of GSI-X led to the drastic increase in intracellular calcium flux. The levels of calcium in GSI-X treated cells were maintained at higher levels compared to α-CD3/CD28 mAb stimulated cells **(Fig. 6.6a-b)**. This phenomenon is a characteristic feature of long-lasting anergic T cells suggesting that T cells are intrinsically modified to become tolerant.

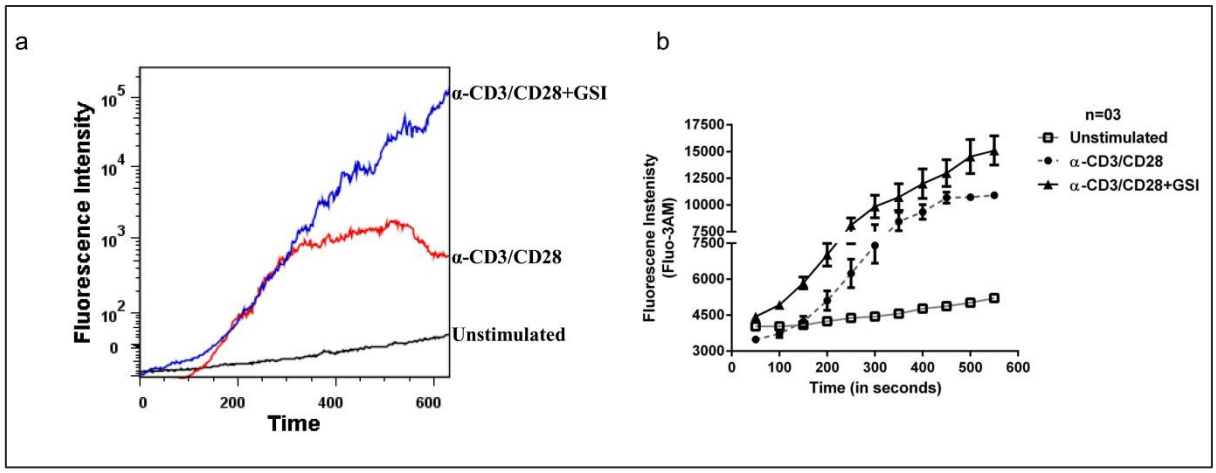

Figure 6.6: Inhibition of Notch signaling results in altered calcium flux in T cells. **a)** The αβ T cells were loaded with the 5 μM of FLuo-3AM in presence or absence of GSI-X at 37 °C for 30 mins. Cells were washed with calcium buffer and acquired on FACSAria for 10 mins. Initially, a 30-second baseline was collected the α-CD3/CD28 was added either alone or in the presence of GSI-X. The graph shows kinetic flux of the intracellular calcium in the T cells stimulated with α-CD3/CD28 mAb in the presence of absence of GSI-X. **b)** Each point on the graph represents the average fluorescence intensity over a range of 50 seconds each. The data represented is the mean of three independent experiments,

6.2.6 Notch is required for distal TCR signaling events in the human $CD3^+$ αβ T cells

Downregulation of CD3-ζ chain has the ability to affect the distal signaling events associated with T cell activation. GSI treatment significantly decreased distal TCR signaling events such as the expression of activation markers CD25, CD69, **(Fig. 6.7a-b)** CD71, and phospho-tyrosine **(Fig. 6.8a-b)**. The effect of GSI was best observed at 48 hr after stimulation which is

in accordance with data published earlier [170] and can be associated with the lag period of 24–30 hrs before the activated T cells begin to divide and execute effector functions [464]. Collectively, this data suggests that inhibition of Notch signaling has the ability to affect the proximal TCR signaling by altering CD3-ζ chain expression which has the capability to alter the distal signaling events as well.

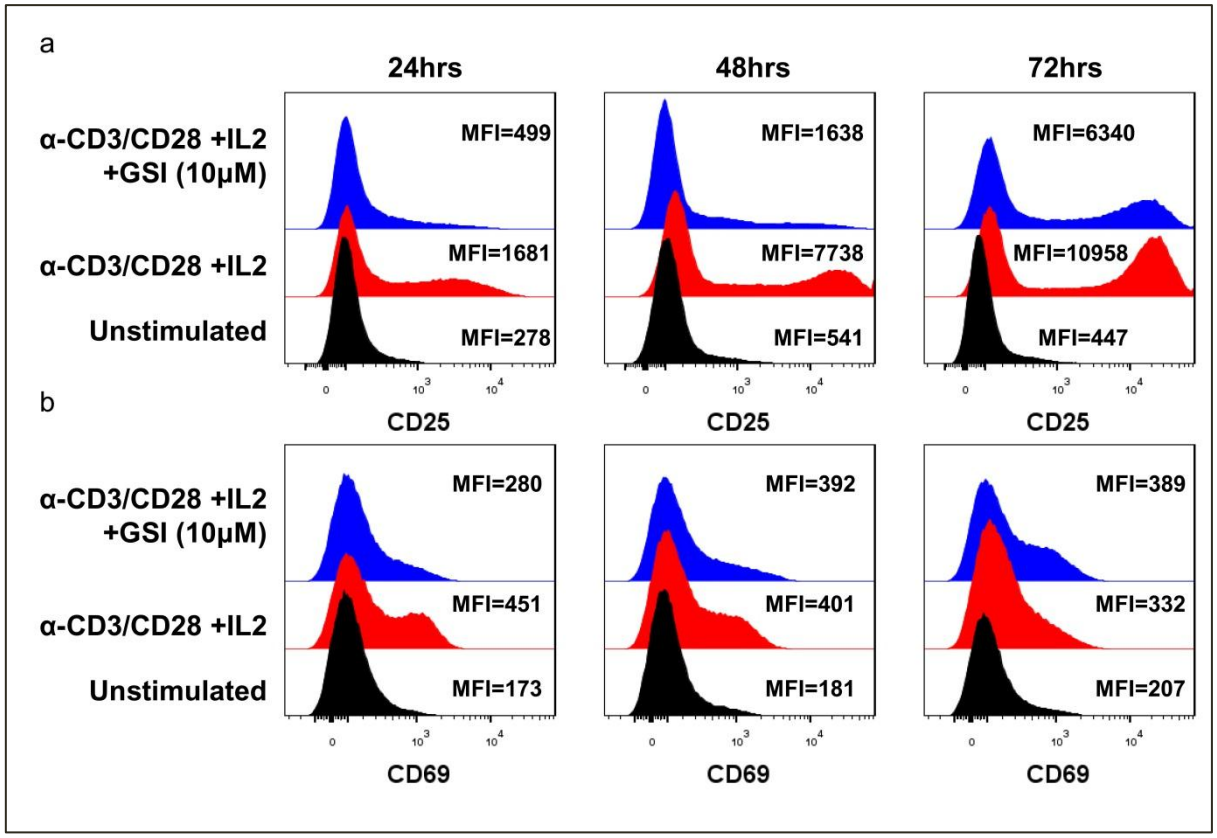

Figure 6.7: Notch is required for distal TCR signaling events in the human CD3$^+$ αβ T cells. Sorted αβ T cells from healthy individuals were stimulated with plate-bound α-CD3ε and α-CD28 for the indicated times either in presence or absence of GSI. Cells were harvested and analyzed by flow cytometry after gating on CD3+ T cells. Median fluorescent intensity (MFI) values are shown in half offset histogram for **(a)** CD25, **(b)** CD69. Results show that effect of Notch inhibition using GSI-X on αβ T cell activation is more prominent after 48 hr post-stimulation. Data is representative of three independent experiments.

6.2.7 Notch signaling is involved in proliferation of human CD3$^+$ αβ T cells

The defect in the TCR CD3-ζ chain has been correlated with the impaired lymphocyte proliferation and cytokine production in cancer patients [29, 465]. GSI-X caused downregulation of CD3-ζ chain and whether it could be associated with decreased

proliferation of αβ T cells in vitro, T cell proliferation in the presence of GSI-X was performed was assessed by ^{3}H-TdR incorporation assay. Purified αβ T cells were incubated with α-CD3/CD28 mAb in the presence or absence of different concentration of GSI-X. The αβ T cells showed robust proliferative responses to α-CD3/CD28 mAb (mean 58203±770cpm) compared to unstimulated αβ T cells (mean 341±11cpm). However, in the presence of various concentrations of GSI-X [10 µM (445.7±147.3), 5 µM (13526±7630), 2.5 µM (37409±5116) and 1.25 µM (44598±772.5)] the proliferative responses of αβ T cells were significantly reduced in a concentration-dependent manner **(Fig. 6.9)**, with the maximum decrease in proliferation of αβ T cells observed at 10 µM concentration of GSI.

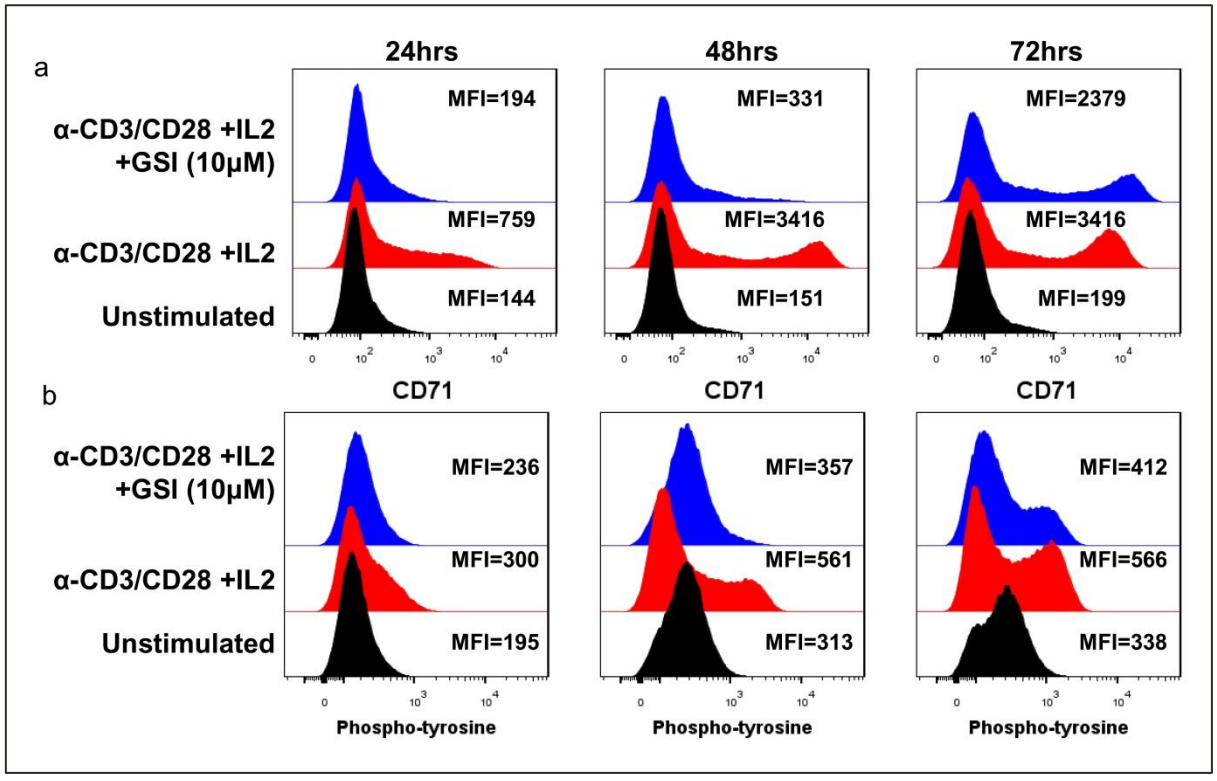

Figure 6.8: Notch regulates activation of human CD3^{+} αβ T cells by regulating CD71 and phopho-Tyrosine: Sorted αβ T cells from healthy individuals were stimulated with plate-bound α-CD3ε and α-CD28 for the indicated times either in presence or absence of GSI. Cells were harvested and analyzed by flow cytometry after gating on CD3+ T cells. Median fluorescent intensity (MFI) values are shown in half offset histogram for **(a)** CD71 and, **(b)** phospho-Tyrosine (pY). Results show that effect of Notch inhibition using GSI-X on αβ T cell activation was more prominent after 48 hr post-stimulation. Data is representative of three independent experiments.

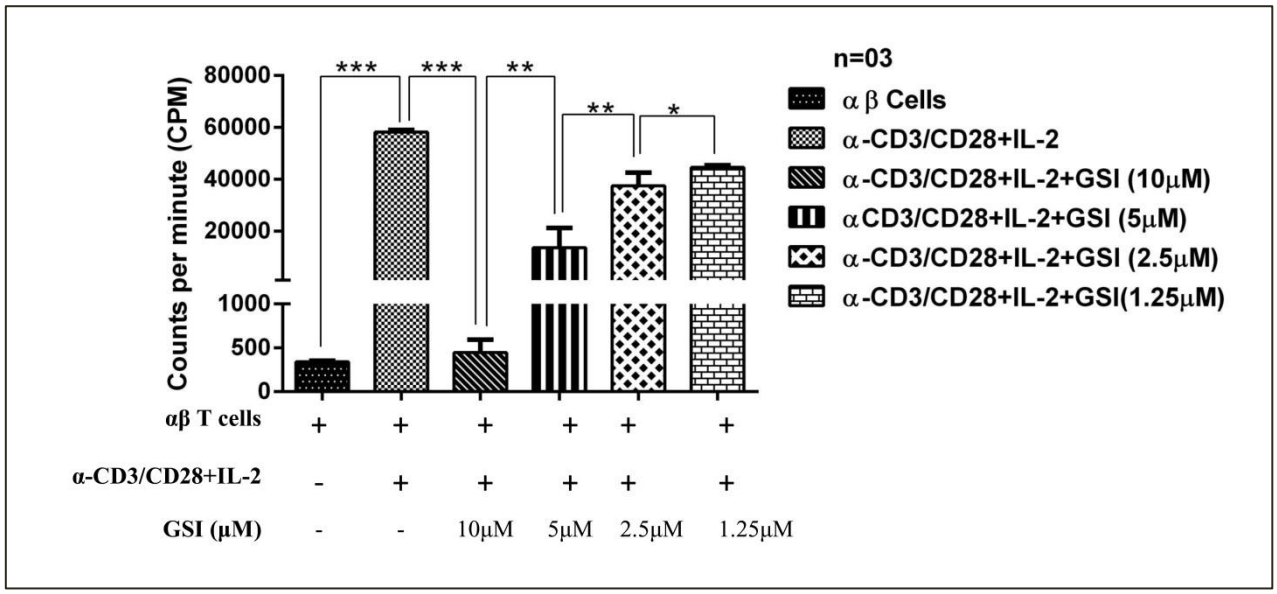

Figure 6.9: Inhibition of α-CD3/CD28 mAb proliferation of αβ T cells by GSI. Human αβ T cells were stimulated with α-CD3/CD28 mAb plus rIL2 in the presence and absence of different concentrations of GSI-X. The data shown are mean of three experiments. (*** represents $p<0.0005$; ** represents $p<0.005$,* represents $p<0.05$).

6.2.8 Notch signaling is involved in cell cycle progression of human $CD3^+$ αβ T cells

We evaluated the effect of Notch signaling pathway in cell cycle progression of αβ T cells. Purified peripheral blood αβ T cells were left untreated or treated with α-CD3/CD28 mAb and IL-2 in the presence and absence of GSI-X. The cells were analyzed for DNA content using propidium iodide (PI). In the absence of strong TCR stimulus, T cells failed to proliferate **(Fig. 6.10a, d).** However in the presence of α-CD3/CD28 mAb and IL2 stimulation for 72 hrs a significant increase number of cells in S phase (31.4%) and G2/M phase (6.5%) was observed **(Fig. 6.10b, d)**. The addition of GSI-X (10 μM) prevented the α-CD3/CD28 mAb and IL2 driven proliferation of αβ T cells and arrested them at G0/G1 phase **(Fig. 6.10c, d)**. This was also reflected in the increased expression of p53 in αβ T cells in GSI-X treated cells suggesting that inhibition of Notch signaling prevents G0/G1- S phase transition in a p53 dependent manner **(Fig. 6.10e).**

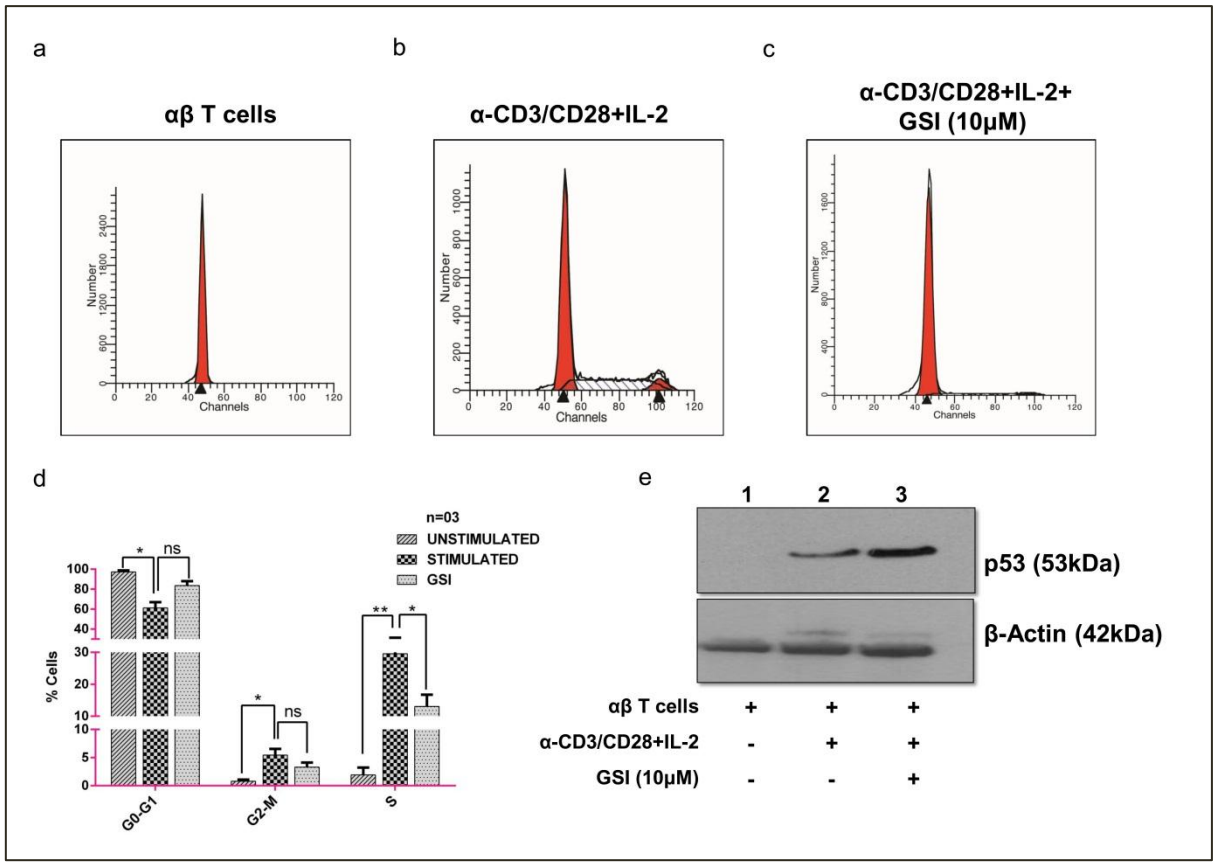

Figure 6.10: Notch and cell cycle progression. (a-c) Cell-cycle analysis was performed on unstimulated αβ T cells **(a)** and **α** -CD3/CD28 mAb plus rIL2 stimulated αβ T cells without GSI-X **(b)** or with GSI-X **(c).** These cells were stained after culturing for 72 hrs and analyzed by flow cytometry. Blocking of Notch signaling by GSI-X prevents S phase progression of cell cycle. FACS plots are representative of three independent experiments. **d)** The graph is the representative of three independent experiments showing percentage of cells in different stages of cell cycle. (** represents $p<0.005$,* represents $p<0.05$ and ns represents non-significant data $p>0.05$). **(e)** In a similar experiment, after 72 hrs total protein lysate was extracted from these cells and was used to detect the levels of p53 and β-Actin by Western blotting. The protein extracts (40-60 μg) prepared from αβ T cells was loaded into the SDS-PAGE and then transferred to the nitrocellulose membrane. The results showed that inhibition of Notch signaling using GSI-X induced higher levels of p53 levels thereby preventing cell cycle progression even after TCR-driven stimulation. β-actin was used as loading control.

6.2.9 Notch signaling regulates activation and cytokine production in activated $CD3^+$ αβ T cells

Given that T cells with low TCR-CD3 ζ chain exhibit a clear proliferative defect, we focused on pathways known to be required for the proliferation of multiple cell types. The c-Myc proto-oncogene confers proliferative and survival signals in numerous neoplastic tissues. Expression of c-Myc is upregulated following TCR-CD3 ligation and inhibition of Notch

signaling prevented c-myc induction in human αβ T cells **(Fig. 6.11a)**. As the expression of c-Myc is regulated by Notch-mediated signaling and another gene- NF-κB that acts as an indicator of Notch activation, were also analyzed. TCR-driven stimulation induced NF-κB induction in αβ T cells which was inhibited in the presence of GSI-X (**Fig. 6.11a)**. NF-κB transcription factor is linked to post-differentiation IFN-γ production and regulator of effector functions [466].

To address whether Notch signal has any role in regulating the effector functions of activated αβ T cells, we examined the effect of GSI on cytokine production. The consequence of GSI-X treatment on the concentration of different Th1 (IL2 and IFN-γ), Th2 (IL4 and IL10) cytokine production was analyzed using CBA kit. The marked increase in production of Th1 cytokines (IFN-γ) compared to Th2 cytokines (IL4 and IL10) were observed after stimulation with α-CD3/CD28 mAb over unstimulated αβ T cells **(Fig. 6.11b)**. It was found that inhibition of Notch signaling by GSI-X leads to a marked reduction in IFN-γ release which was proportional to the concentration of GSI-X. IL-2 levels were analyzed to rule out any difference in the IL-2 levels which might affect the function of T cells.

There was no significant increase observed in IL10 production after α-CD3/CD28 mAb stimulation compared to unstimulated αβ T cells. However, treatment with GSI-X was able to inhibit the IL10 production in a concentration-dependent manner. There were no changes observed in IL-4 production and the levels were found to be very low **(Fig. 6.11c).** Thus, a role of Notch in regulating IFN-γ production in activated αβ T cells is evident which suggests that Notch plays a role in effector functions of T cells.

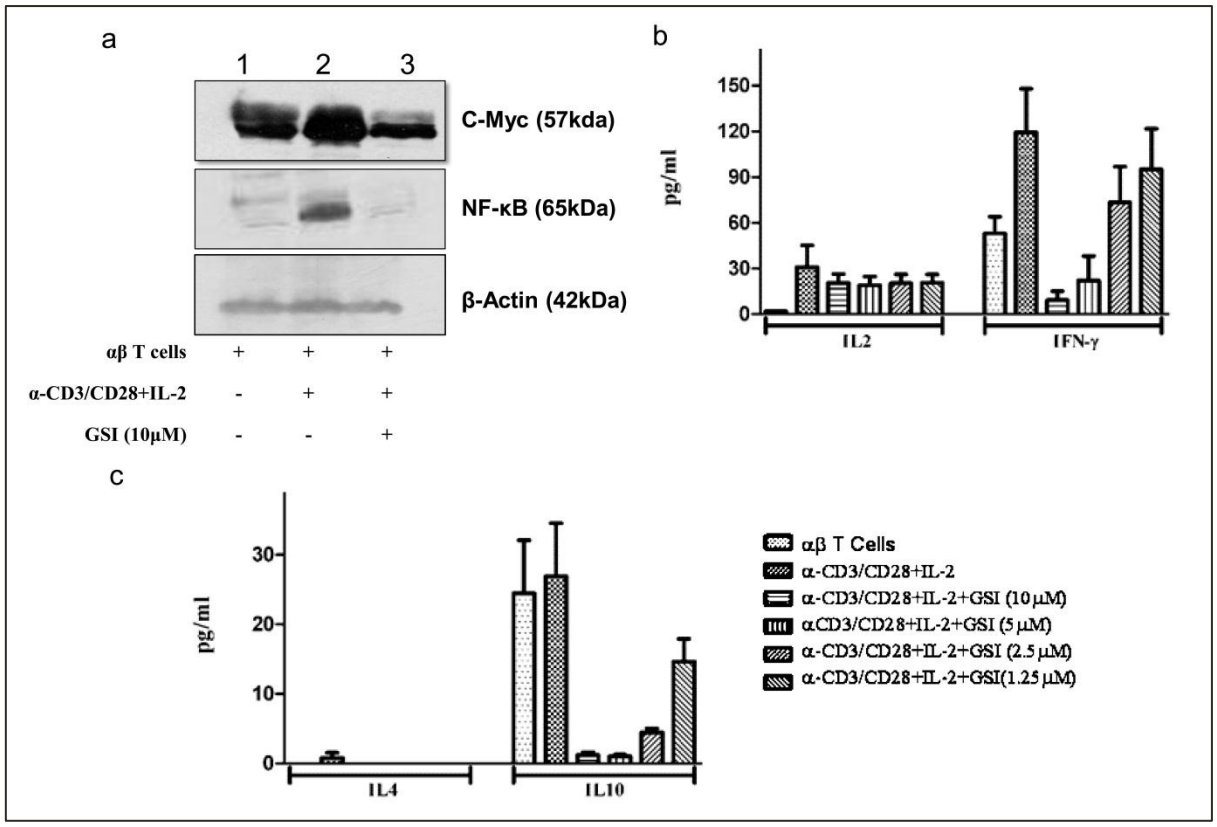

Figure 6.11: **Notch signaling regulates effector functions of αβ T cells. a)** Human αβ T cells were treated with stimulated with α-CD3/CD28 in the presence of absence of GSI-X (10μM) for 24 hr. After 24 hr total protein extract from these cells was used to detect the levels of c-myc, NF-κB, and β-Actin by Western blotting. The protein extracts (40-60 μg) prepared from αβ T cells was loaded oto the SDS-PAGE and then transferred to the nitrocellulose membrane. The results showed that inhibition of Notch signaling using GSI-X prevented TCR-driven upregulation of c-myc and NF-κB. β-actin was used as internal control. **b and c)** αβ T cells were cultured in the absence or presence of different concentration GSI for 24 hr in 96 well plates. Supernatants were collected and level of Th1 (IL2 and IFN-γ) and Th2 (IL4 and IL10) cytokine production were measured by cytometric bead array. In a concentration-dependent manner, GSI-X treatment decreases both Th1 and Th2 production by α-CD3/CD28 mAb plus rIL2 stimulated αβ T cells. The data shown is mean of three independent experiments.

6.3 Discussion

T-cell receptor (TCR) engagement results in the initiation of intracellular signaling cascades that lead to production of different cytokines and chemokines, the induction of anergy, differentiation of T-cell subsets (i.e. Th1/Th2/Tregs) and the generation of cytotoxic T cells (CTLs) [467]. However to accomplish these functions T cell receptor (TCR) cooperates with additional activating receptors (notably NKG2D, NOTCH, and TLR) [468]. Notch signaling can function as positive regulator of T-cell function and has implications in harnessing T cells for cancer immunotherapy. Hence, this study was intended to address the dynamic crosstalk between these two receptors in executing the effector function of human αβ T cells.

Notch signaling pathway plays a prominent role in the regulation of the immune responses. It is known that Notch is essential for the functioning of murine CD4 and CD8 T cells [36, 37]. We have also shown that Notch regulates TCR driven effector function of γδ T cells [168]. However, the role of Notch signaling in regulating TCR-driven effector functions and the point at which these pathways interact in human T cells is not well understood. In the present study, our data showed that peripheral human αβ T cells express Notch receptors and ligands. Their expression increases after α-CD3/CD28 mAb co-ligation suggesting that TCR-driven signaling has the ability to alter the expression of components associated with the Notch signaling. On the other hand, inhibition of Notch signaling in the presence of TCR stimulation prevented TCR-driven induction of Notch receptors and ligands suggesting that there is a dynamic interplay between Notch signaling and TCR-driven signaling in human αβ T cells. It has been reported that Notch signaling involves both canonical and non-canonical pathways in murine T cells and these interactions will influence cell fate decisions and functions [166, 469]. Notch ligand binding is necessary to initiate Notch-mediated induction of target genes. In our study, the expression analysis of Notch target genes confirmed that human αβ T cells expressed the Notch target genes HES1, NF-κB, Deltex, and NRARP.

Despite not using ligands, Notch target genes were upregulated similar to Notch receptors and ligands. It therefore appears that Notch signaling is regulated by TCR signaling and that Notch activation may be *cis or trans* ligand-dependent and confirms the significance of TCR-driven upregulation of Notch ligands on human αβ T cells.

Notch is known to regulate signal strength in thymocytes since constitutive expression of N1ICD in DP thymocytes prevented their maturation into single positive $CD4^+$ and $CD8^+$ T cells by interfering with TCR signal strength [470]. Our data showed higher expression of Notch-1 and Notch-2 compared to Notch 3 and Notch 4 in human αβ T cells which could be outcome of differentiation. The differential expression of Notch receptors observed in human αβ T cells can be attributed to the fact that high Notch activation promotes γδ T cell development, whereas lower levels promote αβ-lineage differentiation [471]. Notch-3, which is a stronger Notch activator and only supports γδ T cell development, was absent in human αβ T cells, whereas Notch-1 is a weaker activator supporting both TCR-αβ and -γδ development was present in αβ T cells [471]. It is argued that the lower levels of Notch-3 in human αβ T cells might be a consequence of T cell differentiation to regulate Notch signal strength. We observed the expression of Dll1, Dll4, Jag1 and Jag2 in the human αβ T cells. Dll4 ligand supports both TCR-αβ and -γδ development while as Jag1 induces mainly αβ-lineage differentiation. Jag1 is known to regulate the T cell signal strength by inducing an inhibitory signal in T cells. Thus, Jag1 expression might be tightly controlled by any signal driving T cell activation and could be reason why TCR stimulation with α-CD3/CD28 mAb or its inhibition failed to alter its expression. Jag1 might be regulated by different pathway which has ability to dampen the TCR driven signaling. Jag1 is also known to regulate the GRAIL expression in T cells which has the ability to decrease T cell signal strength [472]. Jag2 expression is induced after high TCR signal strength which supports the fact that Jag2-mediated Notch activation primarily results in γδ T cell development requiring high TCR

signal strength. Accordingly in peripheral $CD4^+$ T cells, it has been shown that Notch can influence the strength of TCR signaling and thereby influence the threshold of signaling via the TCR [170]. So, inhibiting Notch signaling will induce T cell anergy by increasing the threshold for T cell stimulation. This might result in the up-regulation of the E3 ubiquitin ligases like c-Cbl, Cbl-b, and GRAIL which are associated with anergy in lymphocytes [177]. Similarly, we observed that expression of the E3 ubiquitin ligase GRAIL was induced in human αβ T cells as well as Jurkat T cells on inhibition of Notch signaling. GRAIL also regulates T cell tolerance and regulatory T cell function by mediating T cell receptor-CD3 degradation [462]. Consequently, we observed that inhibition of Notch signaling induced GRAIL also led to the downregulation of CD3-ζ chain without affecting other T cell signaling molecules associated with proximal signaling. TCR stimulation also led to the reduction in the expression of TCR CD3-ζ chain which is known to play an important role in extinguishing the signaling process and reducing T cell responsiveness to antigenic stimulation [158]. This is because effector T cells are susceptible to apoptosis triggered by prolonged TCR stimulation and TCR/CD3ζ down-regulation may also serve to protect differentiating effector T cells from apoptosis. It is also reported that in $GRAIL^{-/-}$ naive T cells, there is no significant difference in total and phosphorylated levels of ZAP70, phospholipase Cγ1, and MAP kinases p38 and JNK but elevated baseline levels of MAP kinase ERK1/2 [473]. T cells marked with decreased CD3ζ protein expression have up-regulation of the related ITAM-containing signaling subunit FcRγ typically associated with the high-affinity IgE FcR [474-476]. In effector cells, the FcRγ subunit forms a new TCR/CD3ε/FcRγ and this complex might have the ability to activate ZAP70. However, how E3 ubiquitin ligases differentially regulate the different proteins associated with T cell signaling needs additional validation.

Jagged-1-induced Notch signaling (using immobilized Jagged-1 fusion protein) during stimulation of purified human $CD4^+$ and $CD8^+$ T cells has been shown to potently inhibit the T cell proliferation and effector function, including both Th1- and Th2-associated cytokines [472]. Jagged-1-mediated signaling also up-regulates expression of GRAIL in human $CD4^+$ T cells. GRAIL is induced in anergic T cells, limiting the transcription of both IL-2 and IL-4 genes [174]. Similarly, our data also confirmed that inhibition of Notch signaling is inducing Jagged-1 expression; although the increase is marginal, it might be having functional implication in inducing the anergy in αβ T cells. The anergic state was further established by the higher intracellular calcium flux. Following anergic stimuli, calcium flux increases which is known to induce dimerization of NFAT1 leading to the expression of two anergy-inducing genes, *GRAIL* and *Caspase-3* [463]. This T cell anergy could be the outcome of decreased Notch signaling which increases the threshold for TCR stimulation. This mechanism can also be correlated with the scenario observed in cancer patients who have decreased CD3-ζ expression and thus could be associated with T cell anergy [29]. Manipulating Notch signaling has been also been shown to rescue the antigen-specific $CD8^+$ T Cells and to overcome the tumor-induced T-cell suppression [181]. This could be used as a strategy to enhance the T cell-based immunotherapy in cancer

Recent reports suggested that NF-κB is a target gene of non-canonical Notch signaling pathway in murine T cells [36, 164]. Our data shows that the expression of mRNA for non-canonical Notch target gene (NF-κB) was higher in human αβ T cells. Activation of Notch signaling has been shown to be involved in the induction of c-Myc and NF-κB in immune cells which in turn are involved in the promotion of cellular proliferation [34, 37, 455]. Previously, we have shown that stimulation of γδ T cells with phosphoantigen was necessary for induction of cell-cycle regulator, c-Myc as well as upregulation of non-canonical Notch target gene, NF-κB [168]. Activation of αβ T cells with α-CD3/CD28 mAb lead to the

induction of the Notch signaling pathway as observed by increased N1ICD expression. Reduction in the expression of NICD by GSI in α-CD3/CD28 mAb-activated αβ T cells showed that Notch signaling is involved in regulating activation of αβ T cells. Besides, our studies demonstrate that inhibiting the Notch signal in activated T cells leads to their decreased proliferative response to α-CD3/CD28 mAb stimulation. We have shown that blocking of Notch signaling pathway by GSI-X leads to decreased proliferation of αβ T cells to α-CD3/CD28 mAb stimulation in healthy individuals in a concentration-dependent manner. Notch signaling is known to support G1-S phase progression of the cell cycle by regulating cyclin D3 promoter activity in CD4+ T cells in spleens of C57BL/6 mice [477]. We observed that α-CD3/CD28 mAb stimulated αβ T cells progress to S-phase of the cell cycle, and their progression was blocked in the presence of GSI-X. Our data indicate that Notch has a role in fine-tuning αβ T cell proliferation in response to α-CD3/CD28 mAb. Notch inhibition diminishes production of IL-2 and the expression of the high-affinity IL-2 receptor (CD25) [167]. Notch signaling operates through augmenting the positive feedback loop involving IL-2 and its high-affinity receptor thereby making T cells more sensitive to both T cell receptor (TCR) stimulation and antigen non-specific signals (IL2). Recently, we have also shown that inhibition of Notch signaling interferes with antigen nonspecific signals of IL2 by decreasing CD25 expression which limits proliferation of γδ T cells [168]. We believe that Notch signaling may be regulating the cytokine-driven bystander proliferation of memory T cells preventing their unnecessary proliferation and exhaustion in the absence of an antigenic signal in a p53 dependent manner.

The involvement of Notch signal in Th1 cytokine production has been previously reported. It has been reported that Notch-1 can regulate IFN-γ production in activated murine T cells [37]. Notch has been reported to regulate IL10 production in murine Th1 cells that converts pro-inflammatory Th1 cells into T cells with regulatory activity [478]. Our data demonstrates

the importance of Notch signal in effector function of human αβ T cells. We observed that GSI treatment of α-CD3/CD28 mAb-activated αβ T cells results in marked reduction of IFN-γ production in activated αβ T cells. Induction of GRAIL in T cells has the capability to inhibit the Th2 effector cytokines [479] and inhibition of Notch signaling in T cells causes overexpression of GRAIL which could be responsible for the decreased cytokines (IL4 and IL10). The expression of Notch in mature T cells and its ability to alter cytokine secretion suggests that Notch signaling has the capability to alter the T cell function as well as Th1/Th2/Treg differentiation because the polarization of helper T cells represents a form of cell fate determination.

The study provided insight into mechanisms that integrate TCR and cytokine signals to determine the outcome of T cell response and identified a central role for Notch signaling in this process. Altogether, the results suggest the key role of Notch in the effector function of αβ T cells and suggest a therapeutic potential of Notch signaling in antigen-specific T cells to reverse T-cell suppression by stabilizing CD3-ζ chain and increase the efficacy of T cell-based immunotherapies in cancer.

Chapter 7
Role of myeloid derived suppressor cells, regulatory T cells and TH17 cells in the observed immune dysfunction of OSCC patients

7.1 Introduction

The pathogenesis of oral squamous cell carcinoma (OSCC) is associated with both inflammation and T cell tolerance [29, 44, 465, 480]. Chronic inflammation is characterized by the overproduction of a range of immunosuppressive/ inflammatory cells like MDSCs, Tregs, TAMs and TH17 cells which have implications for therapeutics in cancers [218, 311, 481]. The immune suppressive cells not only inhibit the host's antitumor immune response but also thwart attempts to augment anticancer immunity through the use of cancer vaccines; or through suppression of T cell activation to impair the efficacy of cancer immunotherapy. This study was intended to unravel the biology of these inflammatory cells in OSCC patients that could be exploited to develop future immunotherapeutic protocols and will provide information whether the existing immunotherapeutic protocols can be employed for the treatment of OSCC patients.

(MDSCs are a heterogeneous population of immature myeloid cells and their frequency is increased in the peripheral blood as well as in the tumor sites of animal tumor models [253, 482] and in human cancers [222, 250, 483]. MDSCs are shunted into the peripheral blood by virtue of emergency myelopoiesis, a demand driven hematopoietic state. MDSCs share characteristics with TAMs in maintaining cancer growth. TAMs exhibit many protumoral features partly by interfering with the function and proliferation of immune effectors cells [484]. MDSCs can suppress T cell activation and are reported to convert naive $CD4^+$ T cells into $FoxP3^+$ expressing regulatory T cells (Tregs) *ex vivo* [485, 486]. Tregs represent a small population (>10%) of $CD4^+$ T cells constitutively expressing the alpha (α) chain (CD25) of the IL-2 receptor complex and FoxP3 transcription factor [487, 488]. However, in few studies it has been shown that MDSCs can promote Th17 differentaion in diverse pathlogical conditions[489, 490] and impair the TGF-β-induced differentiation of $CD4^+CD25^+FoxP3^+$ Tregs from $CD4^+CD25^-FoxP3^-$ T cells in tumor bearing mice [491]. Increased Th17 cell

density, within the tumors in patients with hepatocellular carcinoma, correlated with microvessel density and poor prognosis [492]. IL-17 has been found to increase the suppressive activity of MDSCs through the up-regulation of Arg-1, IDO, and cyclooxygenase (COX)-2[492]. It appears that the interaction of MDSC with different populations of $CD4^+$ T cells is not a one-way traffic and goes beyond the simple direct immune suppressive activity of MDSC on T cells. It would be interesting to know how MDSCs interact with T cells in the setting of inflammation and tolerance. These findings will have important implications for understanding the mechanisms of protective immunity versus tolerance development in OSCC.

In the present chapter, we aimed to address the following questions:

1) Are there increased myeloid derived suppressor cells, regulatory T cells and tumor associated macrophages in the oral cancer patients?
2) Which are the putative factors which are involved in the accumulation of MDSCs in oral cancer patients?
3) How do MDSCs regulate T cell function in oral cancer patients?
4) Is there any cross talk between MDSCs, T regulatory cell and TH17 cells in down regulating cell- mediated anti-tumor immunity in oral cancer patients?

7.2 Results

7.2.1 Expansion of MDSCs during the progression of oral squamous cell carcinoma in humans

Different phenotypic subsets of MDSCs have been reported in different types of cancers and these are characterized either as granulocytic ($HLADR^-CD14^-CD11b^+CD15^+$) or monocytic ($HLDR^-CD14^+CD11b^+CD15^-$). To determine which MDSC subset is predominant in OSCC patients, PBMCs from OSCC patients and age-matched HIs were stained with a combination of antibodies specific for HLADR, CD11b, CD33, CD14, CD15, CD16, CD124, CD66b, CD34, CD38, CD80 and analyzed by flow cytometry. To be more specific with regard to the phenotype of MDSCs, PBMCs of both HIs and OSCC were analyzed for SSC^{high} subpopulation; the OSCC patients have dominant population of SSC^{high} cells suggesting that OSCC patients do have activated myeloid cells which might represent MDSCs **(Fig. 7.1a-b)**. Further, to be more specific with analysing the phenotype of MDSCs, cells were gated on the expression of HLADR and the population of $HLADR^-$ cells were categorized for the expression of myeloid cell markers. The cells with the phenotype like $HLADR^-CD14^+$, $HLA\text{-}DR^-CD33^+$, $HLADR^-CD33^+CD11b^+$ or $HLADR^-CD11b^+CD15^+$ were increased in peripheral blood of OSCC patients compared to the HIs **(Fig. 7.1c)**. However, the weak antigen presenting cells, $HLADR^-$ cells, expressing dual myeloid markers CD33/CD11b (i.e.; $HLADR^-CD33^+CD11b^+$) and CD15/CD11b (i.e.; $HLADR^-CD11b^+CD15^+$) were significantly elevated in OSCC patients. While the subsets $HLADR^-CD14^+$ and $HLA\text{-}DR^-CD33^+$ did not show any significant differences in OSCC patients compared to HIs in the preliminary cases of OSCC patients used to analyze different subsets of MDSCs. The percentage of cells with phenotype $\mathbf{HLADR^-CD33^+CD11b^+}$ were considerably higher than that of $\mathbf{HLADR^-CD11b^+CD15^+}$. Due to the prevalence of $\mathbf{HLADR^-CD33^+CD11b^+}$ in majority of malignancies [493] and higher accumulation in OSCC patients compared to other subsets,

this subset was used for further characterization. The gating strategy used to determine both the subsets (HLADR$^-$CD33$^+$CD11b$^+$ and HLADR$^-$CD11b$^+$CD15$^+$) by flow cytometry in the peripheral blood is shown below **(Fig. 7.2 and Fig. 7.3).** Hereafter, the MDSCs subset with phenotype **HLADR$^-$CD33$^+$CD11b$^+$** will be taken into consideration for further evaluation in OSCC patients.

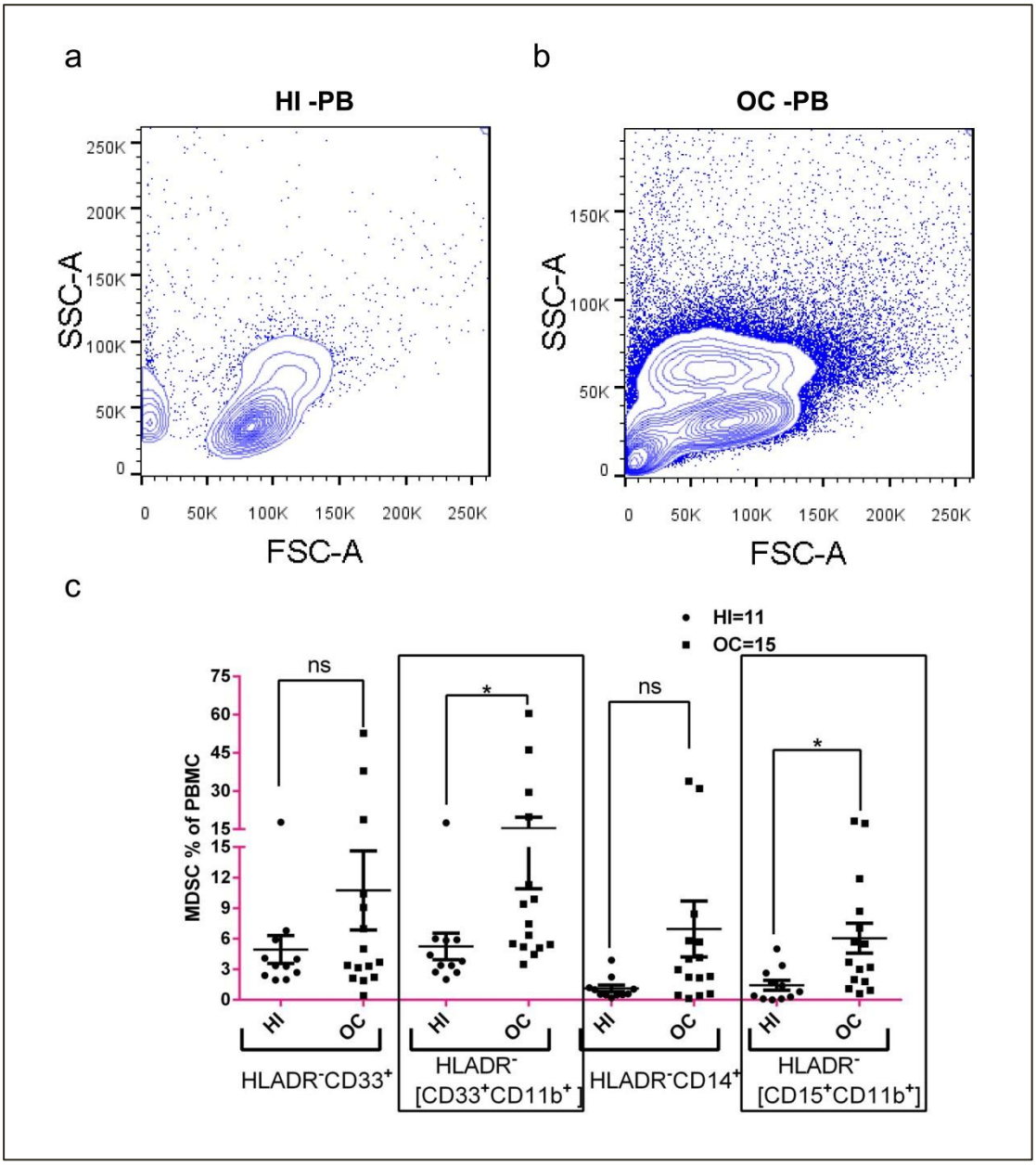

Figure 7.1: Identification and characterization of a highly granular population of cells found in freshly isolated PBMCs of OSCC patients. **a and b)** Using flow cytometry, a distinct SSC^{high} cell population was observed in PBMCs from OSCC patients (right panel) but not in PBMCs from HIs (left panel). **c)** The percentages of different subsets of MDSCs were higher in PBMCs of OSCC patients compared to HIs. The percentage of HLADR$^-$CD33$^+$CD11b$^+$ and HLADR$^-$CD11b$^+$CD15$^+$ subset was significantly higher in OSCC patients

compared to the HIs. The data shown is from the intial 15 OSCC patients and 11 HIs (* represents $p<0.05$, 'ns' represents non-significant data with $p>0.05$)

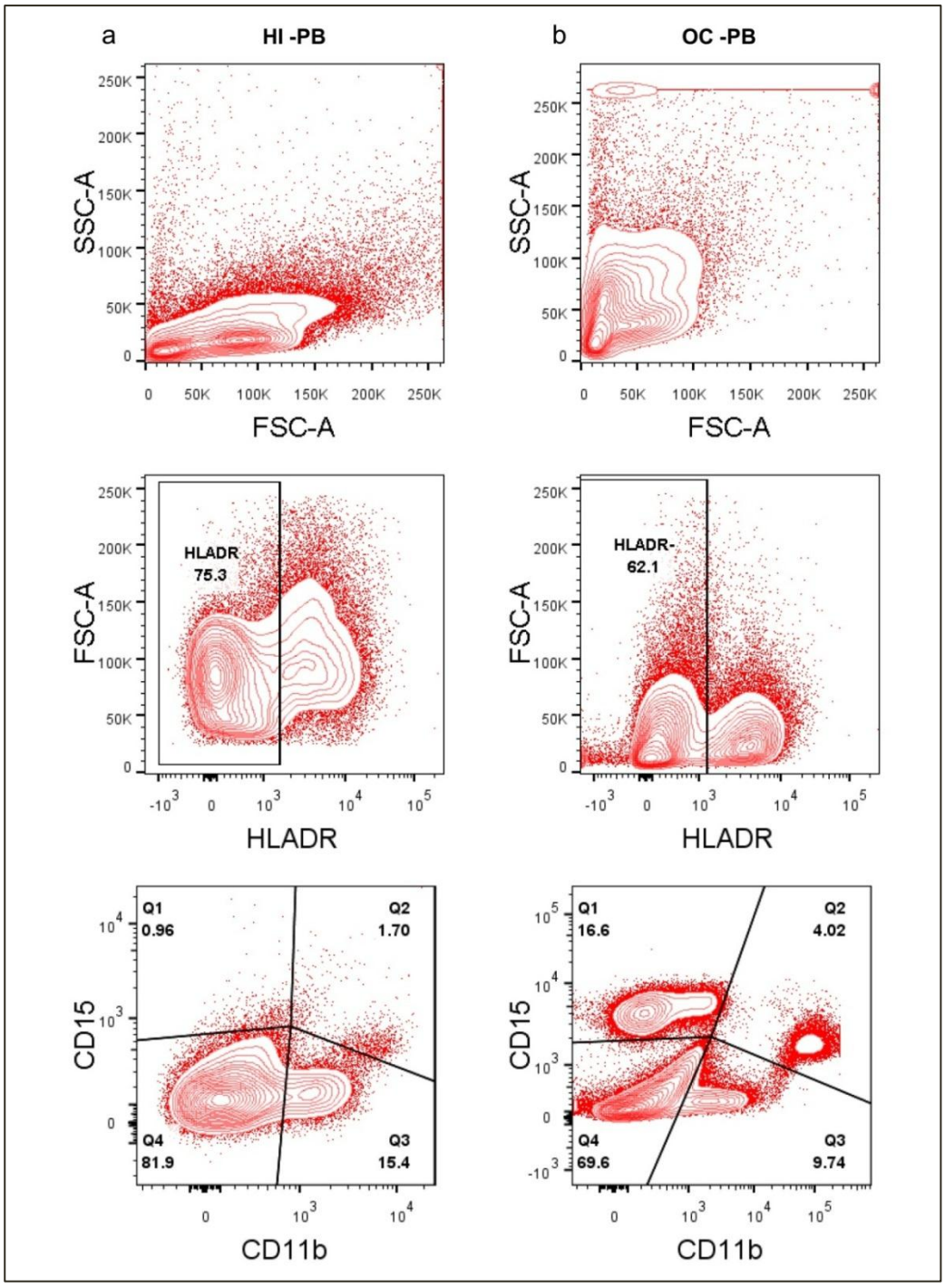

Figure 7.2: Gating strategy to analyze the MDSCs subset ($HLADR^{-}CD11b^{+}CD15^{+}$) prevalent in the peripheral blood of OSCC patients. PBMCs were gated on the basis of forward scatter and side scatter, the gated population was analyzed for the HLADR expression. The $HLADR^{-}$ population depicting weak antigen presenting cells were gated and further assessed for the expression of myeloid cell markers CD15 and CD11b in PBMCs of OSCC patients and healthy donors. **a)** Shows the percentage of $HLADR^{-}CD11b^{+}CD15^{+}$ cells (1.70% of gated $HLADR^{-}$ cells) in the peripheral blood of HIs while **b)** Shows the percentage of $HLADR^{-}CD11b^{+}CD15^{+}$ cells (4.20% of gated $HLADR^{-}$ cells) in the peripheral blood of OSCC patients**.**

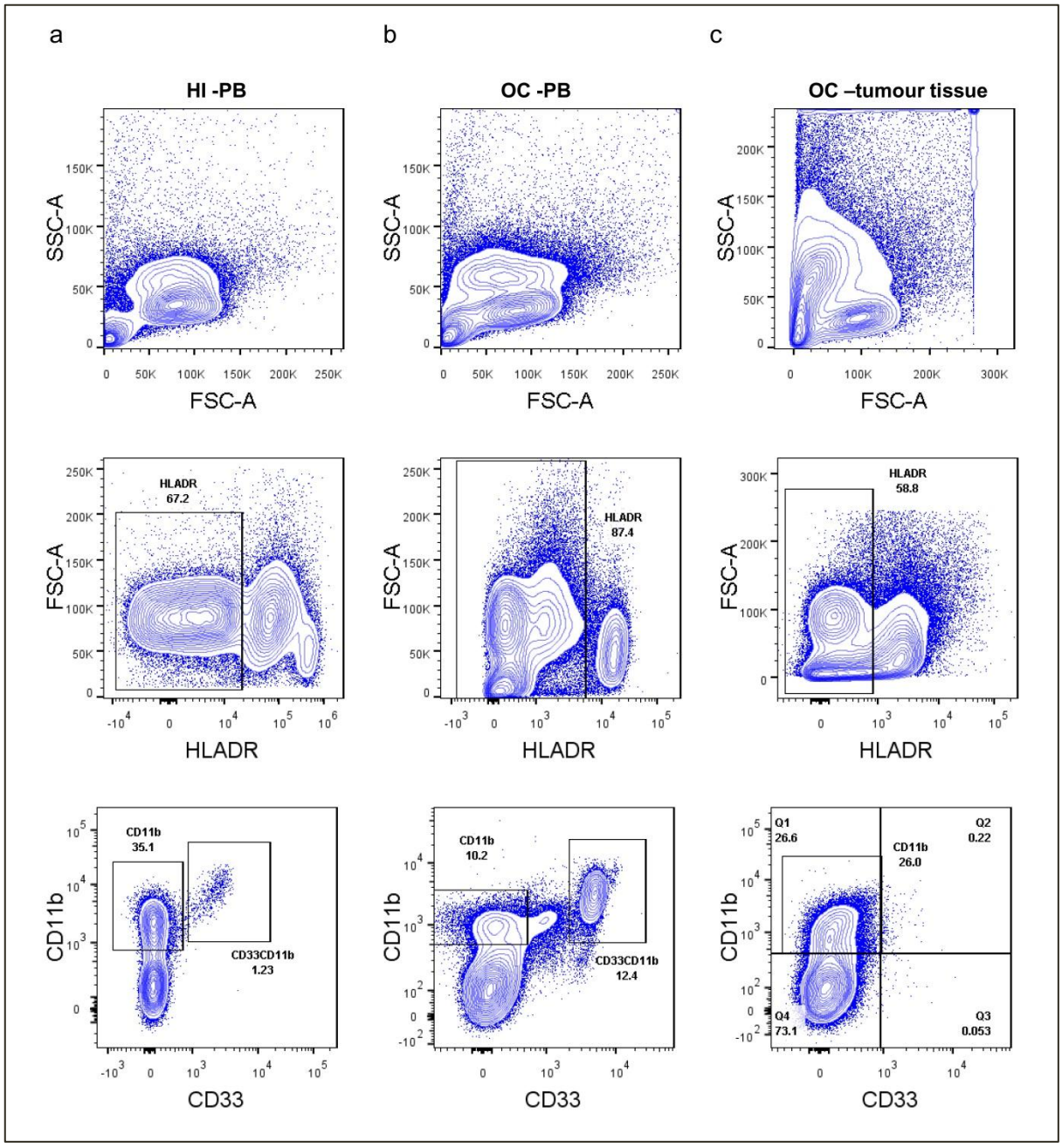

Figure 7.3: Gating strategy to analyze the MDSCs subset (HLADR$^-$CD33$^+$CD11b$^+$) prevalent in the peripheral blood and tumor tissue of OSCC patients. a-b) PBMCs were gated on the basis of forward scatter and side scatter, the gated population was analyzed for the HLADR expression. The HLADR$^-$ population depicting weak antigen presenting cells were gated and further evaluated for the expression of myeloid cell markers CD33 and CD11b in PBMCs of OSCC patients and healthy donors. **c)** Single cell suspension from tumor of OSCC patients was also analyzed for the expression of CD33 and CD11b on the weak antigen presenting cells (HLADR$^-$ cells). In peripheral blood two populations of **HLADR$^-$CD33$^+$CD11b$^+$** and **HLADR$^-$CD33$^-$CD11b$^+$** were observed **(a/b lower panel)** while the tumor tissue in majority of patients was dominated by the HLADR$^-$CD33$^-$CD11b$^+$ MDSC subset (**c lower panel**).

Additionally, the results indicate that the CD11b positive cells encompass two subpopulations within HLADR$^-$ cells i.e; **HLADR$^-$CD33$^+$CD11b$^+$** and **HLADR$^-$CD33$^-$CD11b$^+$** in the peripheral blood of HIs and OSCC patients **(Fig. 7.3a-b**). Interestingly, cells with phenotype **HLADR$^-$CD33$^-$CD11b$^+$** were marginally decreased in the peripheral blood of OSCC patients compared to the HIs. Whereas, the **HLADR$^-$CD33$^+$CD11b$^+$** double positive cells were significantly higher in OSCC patients **(Fig. 7.4a)**.

To further investigate the differences in circulating levels of MDSCs (**HLADR$^-$ CD33$^+$CD11b$^+$)** with respect to clinical stages in OSCC patients, patients were categorized into stages I-IV as per American cancer society TNM classification (stage I n=07, II n=14, III n=09 and IV n=45). The percentage of circulating HLA-DR$^{-/low}$CD33$^+$CD11b$^+$ cells correlated with stage of patients with advanced stage (stage III and IV) OSCC patients having higher percentage of cells (20.35 ± 3.39%) compared to early stage (stage I and II) patients (11.43 ± 2.36 %) **(Fig. 7.4b).** The **HLADR$^-$CD33$^+$CD11b$^+$** subset was further evaluated in the different subsites of oral cavity (buccal mucosa, tongue, mandible, lip, floor of mouth, alveolus, and retromolar). The percentage of these cells did not show any site specific variation in OSCC patients **(Fig. 7.4c)**. The buccal mucosa subsite, which is the most prevalent in Indian patients, also did not show any variation when compared to tongue subsite, which is most common in Western population.

The tumor tissue obtained from OSCC patients were tested for the tumor infiltrating immune cells. Single cell suspension of OSCC tumor was prepared from fresh surgically excised tumor tissues and stained with fluorochrome-conjugated antibodies against markers of myeloid cells. The different subsets of MDSCs especially **HLADR$^-$CD33$^+$CD11b$^+$ and HLADR$^-$CD33$^-$CD11b$^+$** present in peripheral blood were also evaluated in the tumor compartment. However, results demonstrate that OSCC tumors were infiltrated with CD11b expressing weak antigen presenting cells lacking CD33 i.e.; **HLADR$^-$CD33$^-$CD11b$^+$ (Fig.**

7.3c). This subset of myeloid cells was marginally altered in the peripheral blood of OSCC patients but dominated the tumor compartment. On the other hand, the subset of myeloid cells, **HLADR^{-}CD33^{+}CD11b^{+}** which was elevated in the peripheral blood did not show up in the tumor tissue suggesting that two different subsets of myeloid cells are present in the peripheral blood and the tumor compartment of OSCC patients **(Fig. 7.4a).** These MDSCs subsets were assessed for their functional and phenotypic properties. The characterization of tumor-infiltrating MDSCs was ambiguous in OSCC patients as most of the times the cells derived from tumors were contaminated with the microbiota in oral cavity. So, wherever possible, tumor infiltrating MDSCs **HLADR^{-}CD33^{-}CD11b^{+}** were characterized and compared with the **HLADR^{-}CD33^{+}CD11b^{+}** subset of MDSCs present in the peripheral blood of OSCC patients.

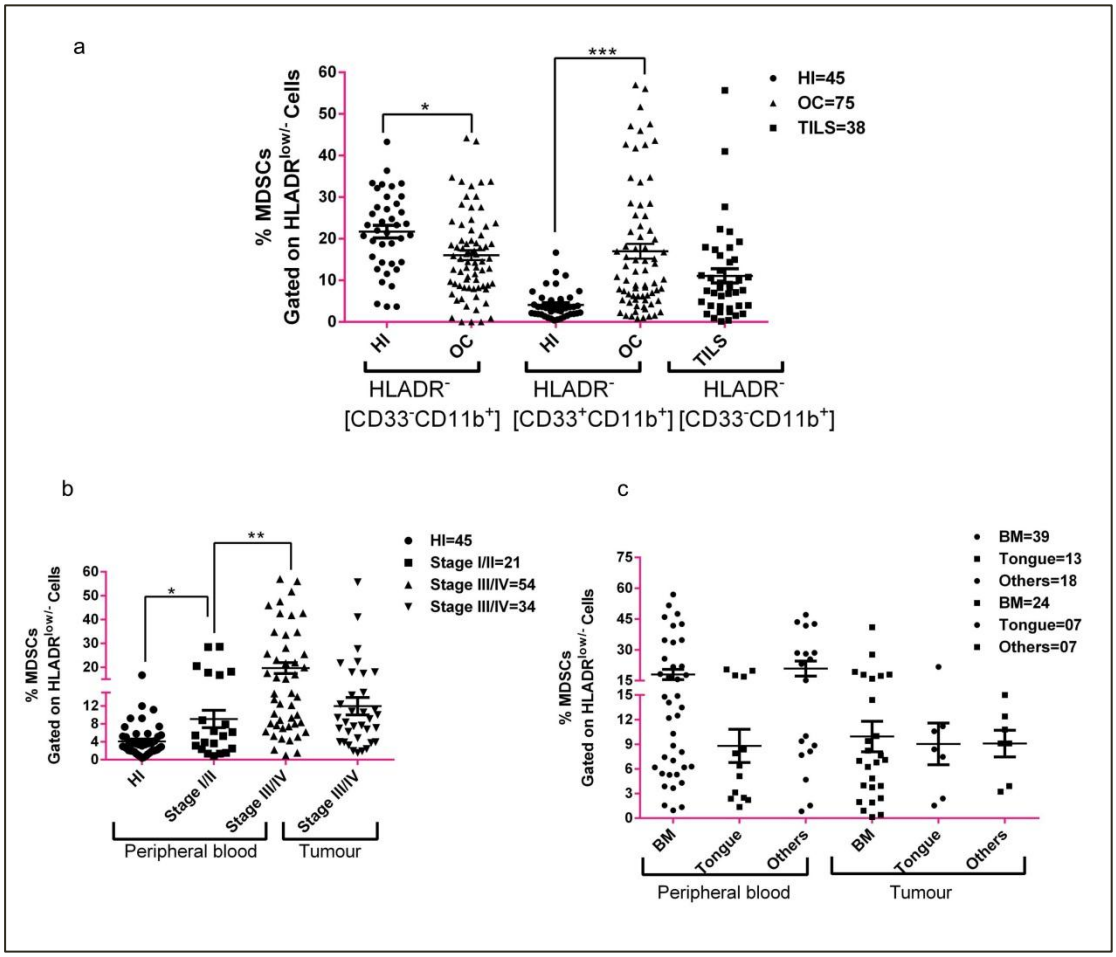

Figure 7.4: Elevated levels of MDSCs in OSCC patients. **a)** The frequency of HLADR^{-} CD33^{+}CD11b^{+} MDSCs was increased in PBMCs of OSCC patients (n = 75) compared with HIs (n = 45). The tumors of OSCC patients (n=38) showed infiltration of HLADR^{-}CD33^{-}

$CD11b^+$ MDSCs. **b)** Increased levels of $HLADR^-CD33^+CD11b^+$ MDSCs were seen in patients with late-stage disease (stage III or IV) compared with those with early-stage disease (stage I or II). **c)** Percentage of $HLADR^-CD33^+CD11b^+$ MDSCs showed no clearly correlation with different subsites of OSCC patients. (* represent $p<0.05$, ** represents $p<0.005$. and *** represents $p<0.0005$)

7.2.2 Characterization of MDSCs prevalent in the peripheral blood and tumor compartment of OSCC patients

To further characterize the circulating and tumor infiltrating MDSCs in OSCC patients, the expression of specific markers on **$HLADR^-CD33^+CD11b^+$** and **$HLADR^-CD33^-CD11b^+$** MDSCs subsets was measured. Circulating $HLADR^-CD33^+CD11b^+$ MDSCs from OSCC patients express CD14, CD15, CD80 and CD124 (IL-4Ra) **(Fig. 7.5)**. A small population, however, expressed CD16 suggesting that the population is heterogeneous with regard to the expression of markers. The expression of CD14 was higher compared to CD15 and CD16. These cells were negative for CD66b, CD38 and CD34. The absence of CD34 and CD38 suggests that they do not possess the potential for haematopoiesis. The subset of MDSCs predominant in periphery of OSCC exhibited dual nature as they express markers which are used to differentiate the monocytic and granulocytic MDSC but being $CD14^{high}$ and $CD66b^-$ can be classified as monocytic MDSC (Mo-MDSCs) **(Fig. 7.5)**. However, the MDSCs subset (**$HLADR^-CD33^-CD11b^+$**) expressed markers like CD15, CD16 and CD66b and lacked expression of CD14 which are characteristic of granulocytic type in both periphery and tumor tissue of OSCC patients **(Fig. 7.6)**. The tumor infiltrating MDSCs **($HLAR^-CD33^-CD11b^+$)** were also heterogeneous for the expression of CD15 and CD16, with a portion of cells (from $HLADR^-CD33^-CD11b^+$) expressing both the markers while other portion of cells predominantly expressing CD16. This CD16 expressing subset might represent the tumor infiltrating neutrophils (TANs). Thus our data confirms that monocytic MDSCs or Mo-MDSCs (**$HLADR^-CD33^+CD11b^+$**) dominate periphery and granulocytic-MDSCs or G-MDSCs (**$HLADR^-CD33^-CD11b^+$**) dominate tumor compartment of OSCC patients.

Hereafter, the terms Mo-MDSCs or HLADR$^-$CD33$^+$CD11b$^+$ and G-MDSCs or HLADR$^-$ CD33$^-$CD11b$^+$ will be used interchangeably.

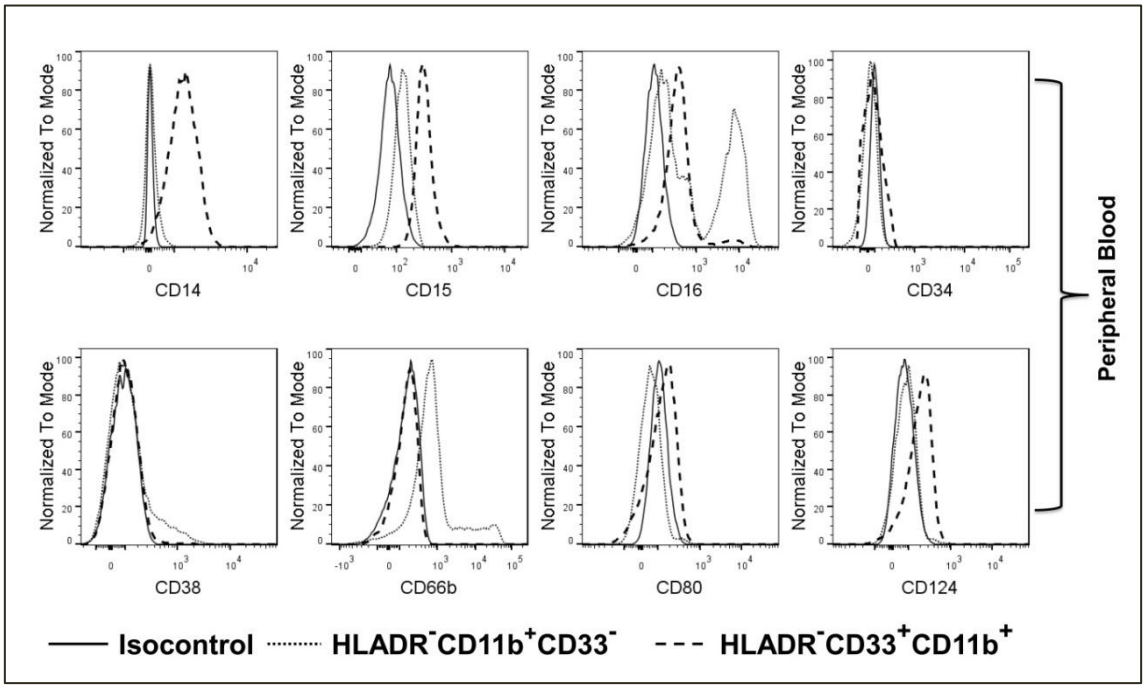

Figure 7.5: Characterization and phenotypic analysis of circulating MDSCs of OSCC patients. The figure is the representative of MDSC markers on HLADR$^-$CD33$^+$CD11b$^+$ cells from peripheral blood (PB) of OSCC patients in comparison to the HLADR$^-$CD33$^-$CD11b$^+$ cells present in PB. The dashed line represents HLADR$^-$CD33$^+$CD11b$^+$ cells, the dotted line represents the HLADR$^-$CD33$^-$CD11b$^+$ while the filled line represents the isotype controls. The presence of CD14, CD15, and CD124 was prominent on the HLADR$^-$CD33$^+$CD11b$^+$ subset while CD16 and CD66b was prominent on the HLADR$^-$CD33$^-$CD11b$^+$ subset in the PB.

7.2.3 Accumulation of monocytic origin tumor associated macrophages in OSCC patients

The infiltrating monocytic origin myeloid cells may be differentiating into macrophages at the tumor site. This differentiation could be enhanced in a spatial manner because of the microbiota and high levels of heat shock proteins in oral tumors. The microbiota and heat shock proteins (HSPs) present in the OSCC will induce the differentiation into monocytes through TLR signaling. To address this, expression of HSPs 60, 70 and 90 were analyzed in three different cells derived from oral cancer AW13516, SSC29B and SSC40. All the three

cell lines expressed these proteins at a higher degree suggesting that they have a capability of inducing TLR signaling in myeloid cells present in the tumor microenvironment and in the peripheral blood **(Fig. 7.7a)**.

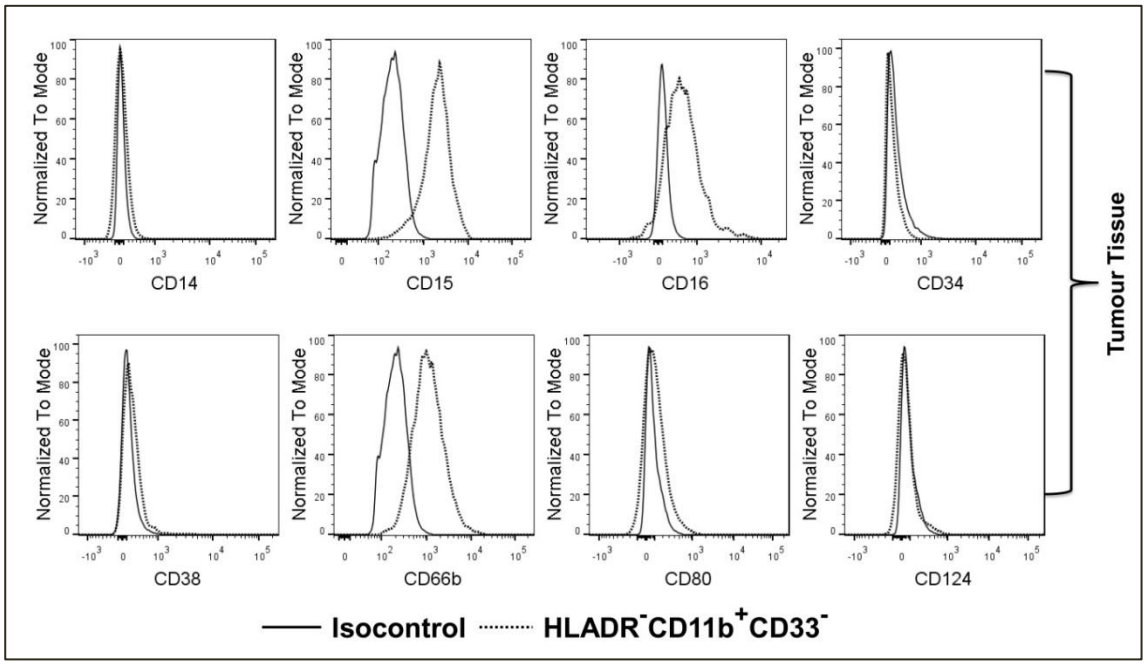

Figure 7.6: Characterization and phenotypic analysis of tumor infiltrating MDSCs of OSCC patients. The figure shows the representative histograms depicting the presence of different MDSC markers on HLADR$^-$CD33$^-$CD11b$^+$ cells present in the tumor tissue. The dotted line represents the HLADR$^-$CD33$^-$CD11b$^+$ while the filled line represents the isotype controls. The presence of CD15, CD16 and CD66b was prominent on the HLADR$^-$CD33$^-$ CD11b$^+$ subset while CD14 CD34, CD38, CD80 and CD124 were absent on the MDSCs subset HLADR$^-$CD33$^-$CD11b$^+$ present in tumor tissue.

The Mo-MDSCs (**HLADR$^-$CD33$^+$CD11b$^+$**) were MACS sorted and the yield of Mo-MDSCs ranged from 1-3x10^6 cells from a starting population of 70±5x10^6 PBMCs. The sorted Mo-MDSCs (**HLADR$^-$CD33$^+$CD11b$^+$**) were stained with Wright stain which confirmed their polymorphonuclear morphology **(shown in material and methods, section 3.14.5.1, Fig. 3.6)**. The purity of Mo-MDSCs (**HLADR$^-$CD33$^+$CD11b$^+$**) confirmed by flow cytometry showed that the sorted cells were 95± 5% pure **(Section 3.14.5, Fig. 3.5)** that was further confirmed by Image stream Flow cytometry **(shown in material and methods, 3.14.5.2, Fig. 3.7)**. These Mo-MDSCs ((**HLADR$^-$CD33$^+$CD11b$^+$**) present in the peripheral blood were analyzed for TLR receptors using real-time PCR and data showed that these MDSCs express

conspicuous amount of TLR receptor transcripts. The myeloid subset expressed higher levels of TLR2 and TRL4 compared to TLR3 and TLR9 **(Fig. 7.7b).** These results suggest that there is an active TLR signaling which might initiate the differentiation of monocytic origin cell into macrophages.

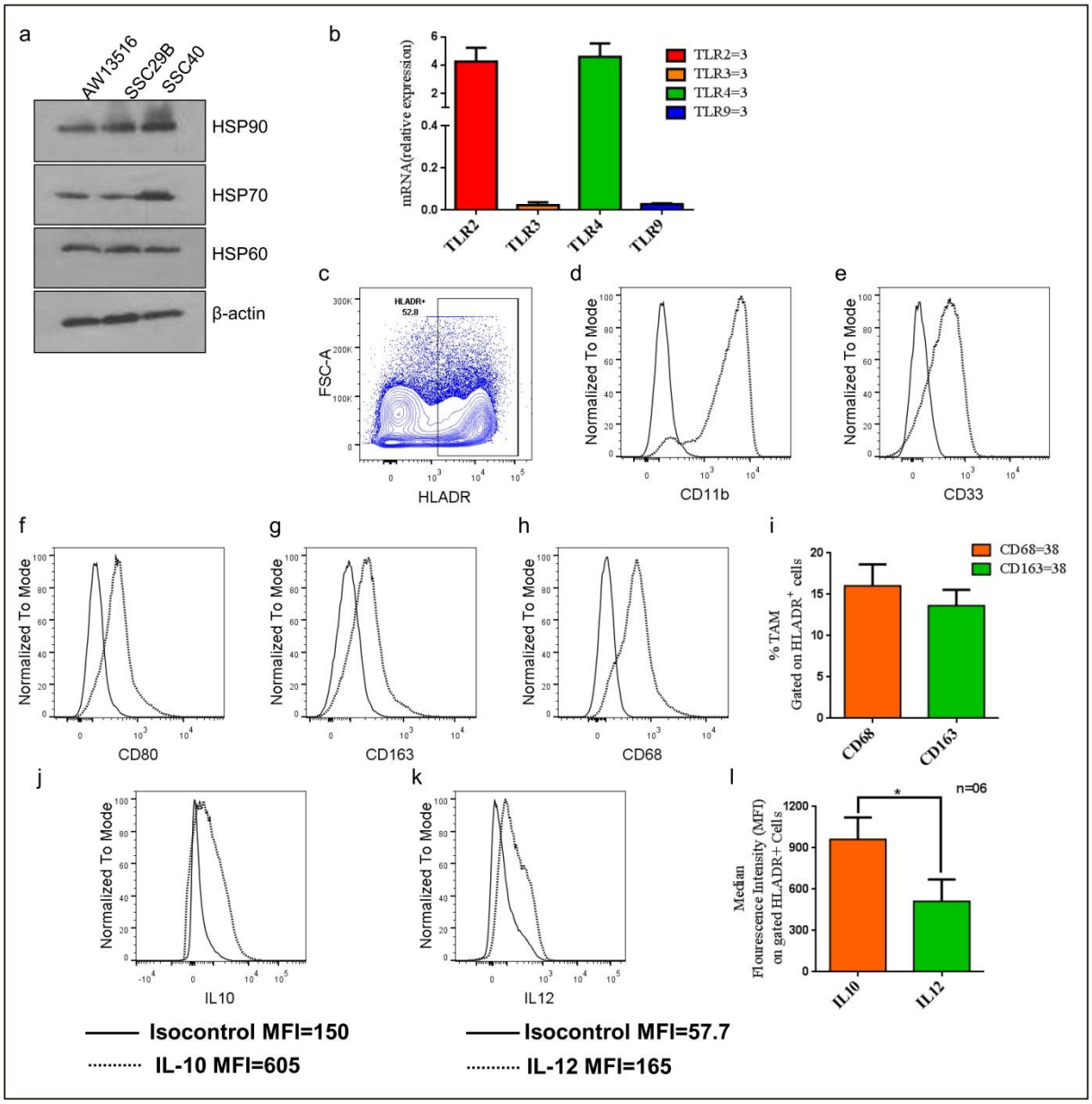

Figure 7.7: Infiltration of monocytic origin alternatively activated macrophages in the tumor tissue of OSCC patients. a) Cell lines of oral tumor origin (AW13516, SCC40, SCC29B) were analyzed for the expression of heat shock proteins (HSP60,70 and 90) to predict the prevalence of molecules inducing TLR signaling. **b)** The sorted Mo-MDSCs present in the peripheral blood expressed the toll like receptors (TLR 2, 3, 4 and 9) with dominant expression of TLR2 and TLR4. **c)** The monocytic origin cells were categorised as $HLADR^{+}$ cells and were analyzed for the expression of myeloid and macrophage associated markers CD11b **(d)**, CD33 **(e),** CD80 **(f),** CD163 **(g)** and CD68 **(h)** and results established

their presence on these cells. **(i)** The presence of CD68 and CD163 positive cells are observed to same degree in tumor tissue of OSCC patients. **(j and k)** The histograms show the expression of IL-10 **(j)** and IL-12 **(k)** on the gated HLADR$^+$ cell**s** in the tumor tissue of OSCC patients**. (l)** The HLADR$^+$ cells appear to be more of M2 phenotype with higher expression of IL-10 compared to IL-12. (* represents $p<0.05$)

This was further confirmed by the observation that tumor-infiltrating myeloid cells of monocytic origin were identified by co-expression of CD33, CD11b, CD80 and HLADR **(Fig. 7.7c-f)**. More detailed cytofluorimetric analysis led us to conclusion that this myeloid cell subset is tumor-associated macrophages (TAM) cells expressing CD68 and CD163 **(Fig. 7.7g-i)**. Further, these cells expressed higher levels of IL10 compared to IL12 **(Fig. 7.7j-l).** These IL10^{high} TAMs represent a phenotype of TAM2 cells which have a pro-tumor role.

7.2.4 Myeloid derived suppressor cells have short life span and undergo high turnover

To monitor whether accumulation of MDSCs in cancer patients could be associated with resistance to apoptosis or not, Mo-MDSCs (**HLADR-CD11b$^+$CD33$^+$**) subset present in patients was isolated from the mononuclear fraction of peripheral blood of OSCC patients and healthy individuals by immuno-magnetic sorting. Mature counterpart represented as **HLADR$^+$** cells were also purified from peripheral blood of the same patients and healthy donors. HLADR$^+$ cells survived 24 hr in culture without cytokines significantly better than Mo-MDSCs (**HLADR$^-$CD33$^+$CD11b$^+$**), as depicted by Annexin V assay **(Fig. 7.8a-b).** The data represented is the combined data from a HI and OSCC patient **(Fig. 7.8c)**. The protein lysates of Mo-MDSCs (**HLADR$^-$CD33$^+$CD11b$^+$**) and their mature counterparts **(HLADR$^+$)** were also analysed for different apoptotic genes using Apoptosis multi-target sandwich ELISA kit. Mo-MDSCs expressed higher levels of phospo-p53 and phospho-BAD compared to the mature counterparts while the expression of cleaved caspase 3 and PARP showed no significant change **(HLADR$^+$)** (**Fig. 7.8d)**. Further analysis suggested Mo-MDSCs produce higher levels of ROS which might aid MDSCs in inducing inflammatory state and immune suppression **(Fig. 7.8e).** Death receptor 5 (DR5) was reported to regulate the apoptosis in

PMN-MDSCs [494] and like PMN-MDSCs, Mo-MDSCs also express DR5 and its regulators p53 and NF-κB **(Fig. 7.8f)**. Endoplasmic (ER) stress was associated with apoptosis in PMN-MDSC but with correlative evidences linking stress, accumulation of oxidative cellular damage and ageing [494, 495]. The connection of p66Shc with increased oxidative stress and decrease life span was investigated. Mo-MDSCs expressed p66Shc which can also act as a downstream target of the tumor suppressor p53 to induce the expression of intracellular oxidants, cytochrome *c* release and apoptosis as depicted by the levels of cleaved caspase 3 **(Fig. 7.8f)**. Therefore, adaptor protein p66Shc could be a mediator of mitochondrial dysfunction and excessive ROS in Mo-MDSCs which triggers the inflammatory response. Manipulating p66Shc might offer a possibility of reducing ROS and thereby reducing inflammation.

7.2.5 HLADR$^-$CD33$^+$CD11b$^+$ myeloid cells are immunosuppressive and affect T cell function

MDSCs were isolated from the peripheral blood of both HIs and OSCC patients as described above. Previous studies have shown that immature myeloid cells are involved in the inhibition of T cell immune responses. The **HLADR$^-$CD33$^+$CD11b$^+$** MDSCs are markedly increased in OSCC patients and were examined whether they could inhibit T cell responses *in vitro*. Sorted Mo-MDSCs were cocultured with anti-CD3/CD28 stimulated autologous T cells at different ratios (0:1; 1:8; 1:4; and 1:2). Mo-MDSCs from all HIs and OSCC patients efficiently suppressed the proliferation of T cells **(Fig. 7.9a-c)**. Mo-MDSCs isolated from the OSCC patients showed higher suppressive potential at 1:2 ratio than the Mo-MDSCs isolated from PB of HIs. This suppressive activity of Mo-MDSCs isolated from both HIs and OSCC patients occurred in a dose dependent fashion. TCR driven activation of T cells is known to induce the production of IFN-γ and in the presence of Mo-MDSCs the TCR driven

production of IFN-γ by T cells was inhibited **(Fig.7.9d).** Therefore, these results confirm that the MDSCs that were identified exhibited immune-suppressive function.

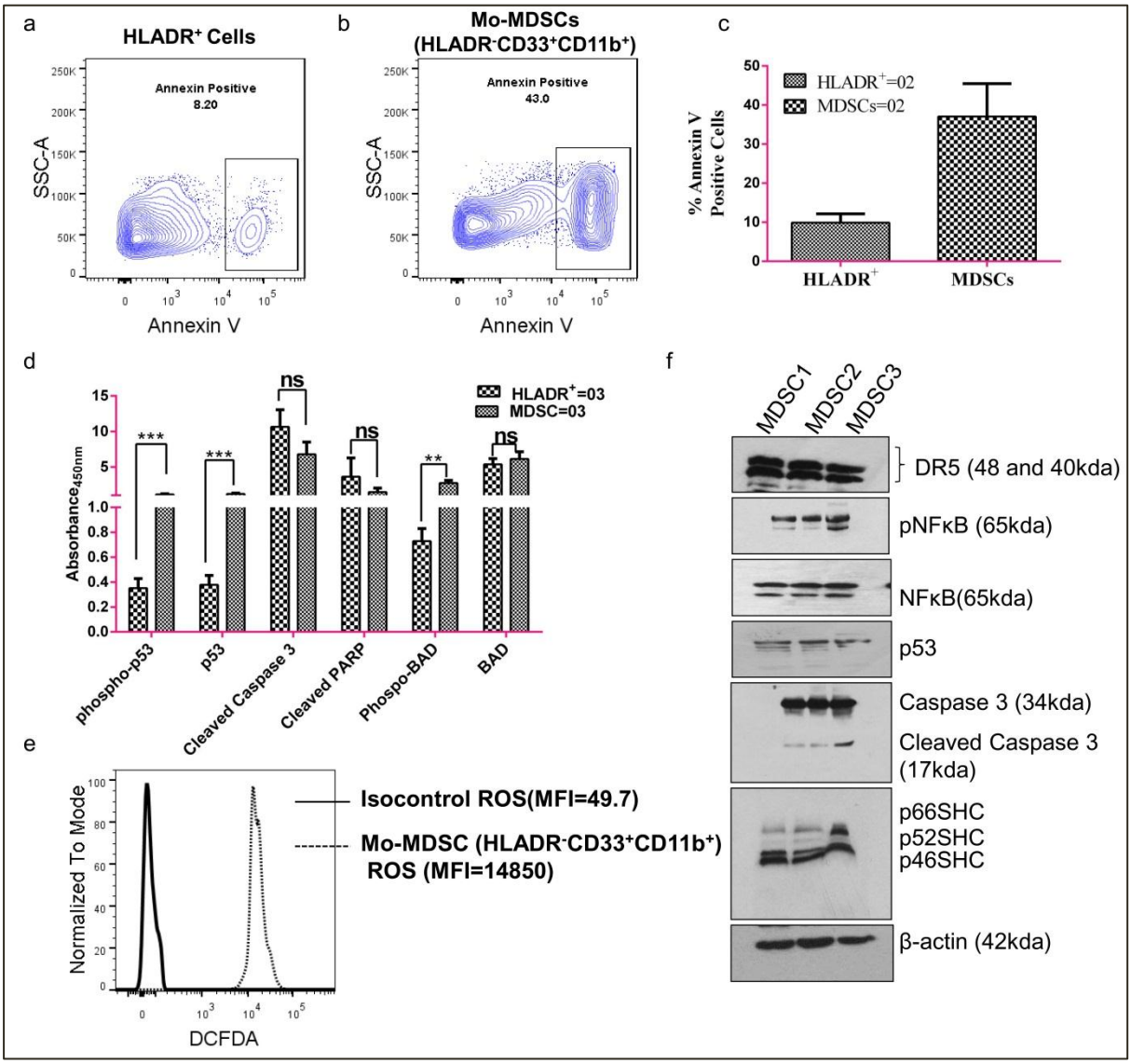

Figure 7.8: MDSCs have shorter survival than mature counterparts. a-b) Apoptosis was analyzed based on percentages of Annexin V^+ cells. Mo-MDSCs (**$HLADR^-CD33^+CD11b^+$**) and $HLADR^+$ cells were cultured in c/RPMI for 24 hr and then evaluated for spontaneous apoptosis using Annexin V assay. Mo-MDSCs have higher propensity to undergo apoptosis than $HLADR^+$ Cells **c)** Shows the average of two independent experiments depicting that MDSCs have 3 fold higher tendencies to undergo spontaneous apoptosis. Data represented is average of two different experiments (one each of HI and OSCC patient). **d)** Amount of p53, phospho-p53, cleaved caspase 3, cleaved PARP, phospho BAD and total BAD was determined in freshly isolated Mo-MDSCs and their mature counterpart ($HLADR^+$ cells) using apoptosis multi-target sandwich ELISA. Experiments were performed on the MDSCs isolated from three different individuals in duplicates. (**e**) The levels of ROS in MDSCs were analyzed using H2DCF-DA dye. The Histogram shows the baseline intensity of ROS produced by the Mo-MDSCs **f)** DR5, NF-κB, phospho-NF-κB, p53, Caspase 3, cleaved caspase 3 , p66shc and its isoforms were analyzed in the Mo-MDSCs isolated from the PB of

different individuals using Western blotting. β-actin was used as loading control (** represents p<0.005 and *** represent p < 0.0005)

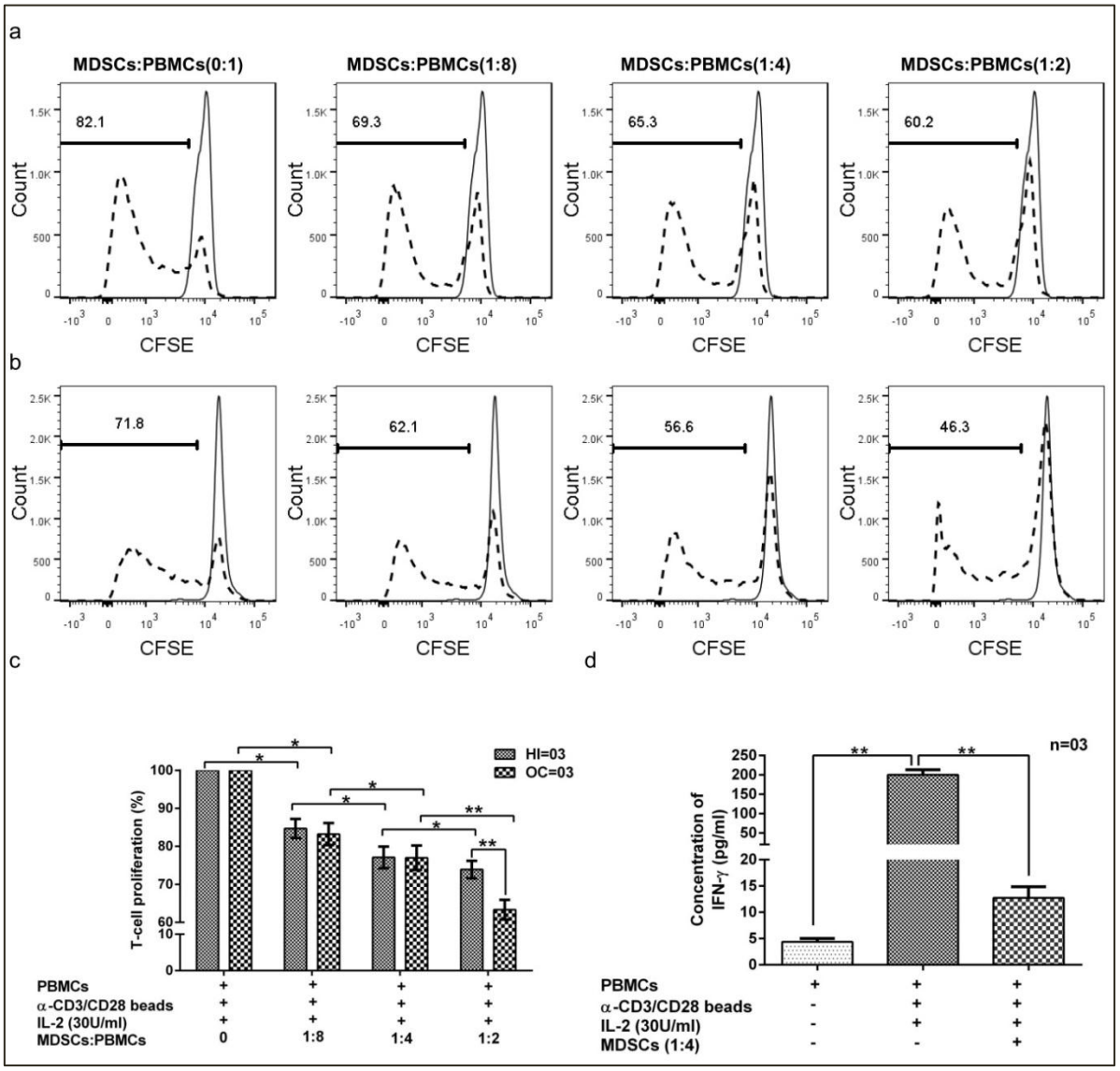

Figure 7.9: Suppression of T cell proliferation and IFN-γ production by HLADR$^-$CD33$^+$CD11b$^+$ MDSCs. Sorted Mo-MDSCs (HLADR$^-$CD33$^+$CD11b$^+$) were cocultured with autologous PBMCs stimulated with anti-CD3/CD28 mAb at different ratios (0:1, 1:8, 1:4, or 1:2) for 5 d. **a-b)** The histograms shows the CFSE fluorescence intensity of gated CD3$^+$ T cells alone or cocultured with MDSCs from HIs **(a)** or OSCC patients **(b).** CFSE fluorescence intensity at the start of culture (undivided cells) is represented by the sold line graphs and that after culture (divided cells) by the dashed line graphs. T cell proliferation was analyzed by the conventional method of gating on the proliferated cells (shifted to the left after stimulation). **c)** T cell proliferation was quantified as the percentage of CFSE dilution (mean increase in percentage of divided cells) of gated CD3$^+$ T cells after stimulation. The proliferation of gated CD3$^+$ T cells in the absence of MDSCs was adjusted to 100% and the proliferation of CD3$^+$ T cells in presence of different ratio of MDSCs was calculated accordingly. **d)** Supernatant from cocultures of autologous T cells with OSCC MDSCs and HIs MDSCs was measured for IFN-γ production by ELISA. The data is represented as the average of three independent experiments carried out in the presence of MDSCs isolated

from OSCC patients (n=2) and HIs (n=1) at 1:4 ratio. (* represents $p<0.05$ and ** represent $p<0.005$)

7.2.6 T cell functionality and correlation of CD3-ζ chain expressions with levels of MDSCs in OSCC patients

CD3-ζ chain, a key T cell signaling molecule, is a marker of a functional activity of T cells. In autologous mixed leukocyte reaction (MLR) experiments, the effect of Mo-MDSCs on the CD3-ζ chain expression was observed. Results demonstrated that the expression of CD3-ζ on $CD8^+$ T cells was significantly downregulated when cocultured with Mo-MDSCs compared to $CD4^+$ T cells. Although CD3-ζ chain was downregulated in both $CD4^+$ and $CD8^+$ T cells, the effect was more prominent on CD8+ T cells **(Fig. 7.10a-b)**. After showing that Mo-MDSCs have capability of altering CD3-ζ chain expression, the levels of CD3-ζ chain were quantitatively assessed in the different T cell subsets in the PBMCs of OSCC patients (n=47) and healthy donors (n = 43) using multicolour flow cytometry. T helper cells ($CD3^+CD4^+$), T cytotoxic cells ($CD3^+CD8^+$) and gamma delta T cells ($CD3^+\gamma\delta^+$) from OSCC patients expressed significantly lower levels of CD3-ζ chain than the T cell subsets from healthy donors **(Fig. 7.11a)**. A substantial variation in the expression of CD3ζ (fluorescence intensity) among different patients was observed, ranging from a marginal decrease to an almost absent expression. However, the levels of CD3-ζ were decreased to the same degree in all the T cell subsets.

The decreased expression of CD3-ζ chain in PBMCs of OSCC patients was also demonstrated by Western blotting experiment. The protein lysate obtained from PBMCs of OSCC patients (n=4) and HIs (n=3) were analyzed for CD3-ζ chain expression and results confirmed that as compared to HIs, the PBMCs of OSCC patients had lower levels of CD3-ζ chain **(Fig. 7.11b)**. This was also reflected at the decreased proliferative response of T cells

obtained from OSCC patients when stimulated with pan mitogen (PHA) or TCR driven stimulation (anti-CD3 mAb) **(Fig. 7.11c)**.

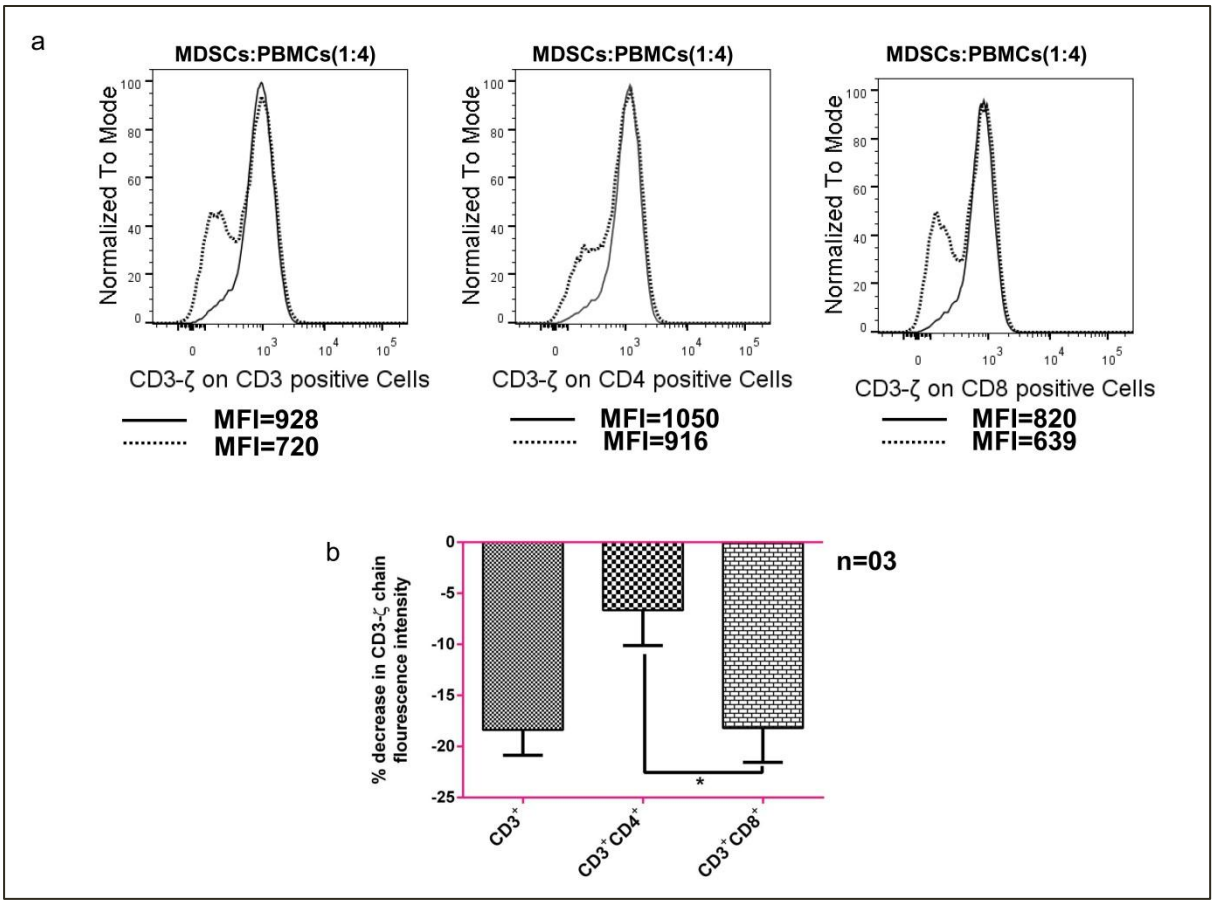

Figure 7.10: Effect of Mo-MDSCs on CD3-ζ chain expression. a) Expression of CD3–ζ chain on $CD4^+$ and $CD8^+$ T cells was analyzed by flow cytometry on autologous T cells after coculture with **$HLADR^-CD33^+CD11b^+$** Mo-MDSCs. Mo-MDSCs were co-cultured with T cells at 1:4 ratio for 5 days and after that cells were stained with CD3, CD4, CD8 and CD3-ζ chain antibodies. The solid line shows the expression of CD3-ζ chain on stimulated T cells subsets ($CD3^+$ left panel; $CD4^+$ middle panel; $CD8^+$ right panel) cultured in absence of Mo-MDSCs while the dotted line shows the expression of CD3-ζ chain on stimulated T cells in presence of Mo-MDSCs. **b)** Data represented is the decrease in CD3-ζ chain expression (median fluorescence intensity) in T cells after being cocultured with MDSCs and is the mean of three independent experiments. (* represents $p<0.05$)

Having shown that Mo-MDSCs have the ability to decrease CD3-ζ chain expression and levels of CD3-ζ chain are decreased in the peripheral blood of OSCC patients, the levels of Mo-MDSCs were correlated with the CD3-ζ chain expression and results confirmed inverse correlation between CD3-ζ chain expression on $CD4^+$ or $CD8^+$ T cells and Mo-MDSCs levels

(Fig. 7.12a-b). However, the correlation was strong in the case of $CD8^+$ T cells **(Fig. 7.12b)**. This result, together with T cell suppression assay, suggests that Mo-MDSCs from OSCC patients have greater ability to induce immunosuppression by affecting T cell function at multiple points.

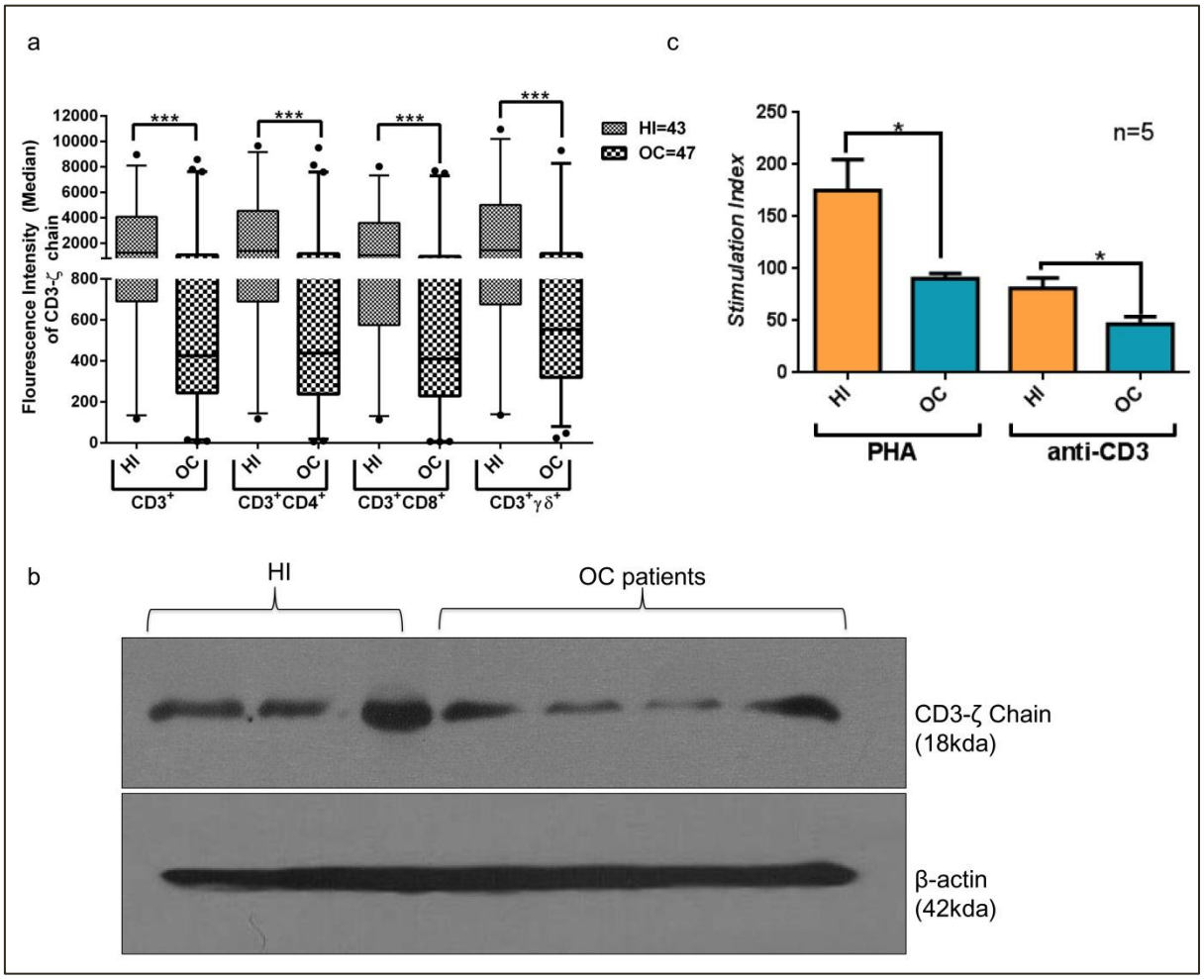

Figure 7.11: Expression of signal transducing molecule CD3-ζ chain in peripheral blood T cell subsets of OSCC patients. a) From FSC/SSC plot, PBMCs were gated and the same gated cells were plotted as SSC on the y-axis versus CD3 fluorescence on the x-axis. CD3 positive population was gated and median fluorescence intensity of CD3-ζ chain was observed on the gated population. Similarly gated cells were plotted as CD3 fluorescence on x-axis and CD4 or CD8 or γδ fluorescence on y- axis respectively. The median fluorescence intensity of CD3-ζ chain on gated $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+$ $\gamma\delta^+$ TCR cells was observed. Data represents the CD3-ζ expression profile of OSCC patients (n=47) compared to the healthy individuals (n=43). **b)** The Western blot shows expression of CD3-ζ expression in PBMCs of OSCC patients and HIs. To ensure equal loading of protein, β-actin was used as loading control. **c)** PBMCs of HIs and OSCC patients were stimulated with PHA and anti-CD3 mAb and their proliferative response was analyzed using tritiated thymidine

incorporation assay. The proliferative response is represented as stimulation index (SI). (* represents p<0.05 and *** represents p<0.0005)

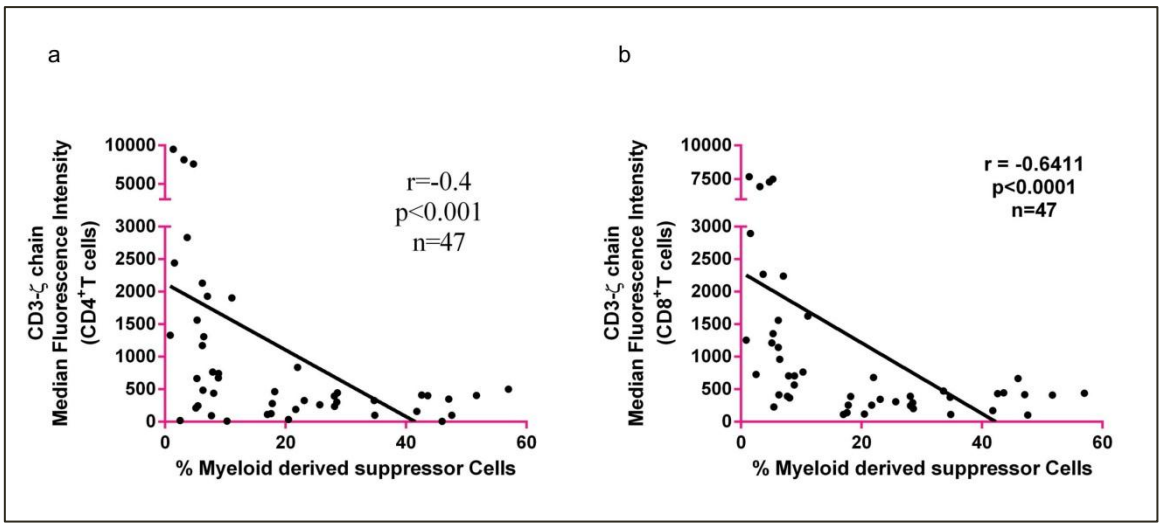

Figure 7.12: Correlation between MDSCs levels and CD3-ζ chain expression. A negative correlation was observed between the CD3-ζ chain expression (Median fluorescence intensity) and the percentage of $HLADR^{-}CD33^{+}CD11b^{+}$ MDSCs in OSCC patients (n = 47) Relationship between two parameters was investigated using the Spearman rank-correlation test. **a)** Shows the correlation between CD3-ζ chain fluorescence intensity of $CD4^{+}$ T cells and % of MDSCs in the peripheral blood of OSCC patients while **b)** shows the correlation between CD3-ζ chain fluorescence intensity of $CD8^{+}$ T cells and % of MDSCs in the peripheral blood of OSCC patients.

7.2.7 Myeloid-derived suppressor cells down-regulate L-Selectin (CD62L) expression on $CD3^{+}$ T cells

To determine whether MDSC is sufficient to affect L-selectin levels, T cells were cultured in the presence or absence of MDSC at 1: 4 ratios. Cells were harvested and stained with CD3 and L-selectin antibodies. The $CD3^{+}$ T cells were gated and then analyzed for L-selectin expression by flow cytometry. Total $CD3^{+}$ T cells cocultured with MDSC displayed an L-$selectin^{low}$ phenotype relative to T cells cultured alone. Although downregulation of L-selectin was observed after stimulation with α-CD3/CD28 mAb, it is a hallmark of newly activated T cells. However, presence of MDSCs further decreased the L-selectin expression (**Fig. 7.13a)**. MDSCs are known to prevent activation of T cells and thus the decreased expression of the L-selectin on $CD3^{+}$ T cells could be attributed to the presence of MDSCs and not due to the activation of T cells. MDSC alters L-selectin expression and MDSCs are

also increased in the peripheral blood of OSCC patients. Hence, T lymphocytes from the OSCC patients and HIs were compared for L-selectin expression. The $CD3^+$ T cells of the OSCC patients have an L-$selectin^{low}$ phenotype (Median 50.4 for $CD3^+$ T cells) while T cells from the HIs have an L-$selectin^{high}$ phenotype (Median 134.6.7 for $CD3^+$ T cells) **(Fig. 7.13b-d)**.Therefore, these results confirm that the MDSCs identified in OSCC patients mediate immunosuppression through multiple mechanisms.

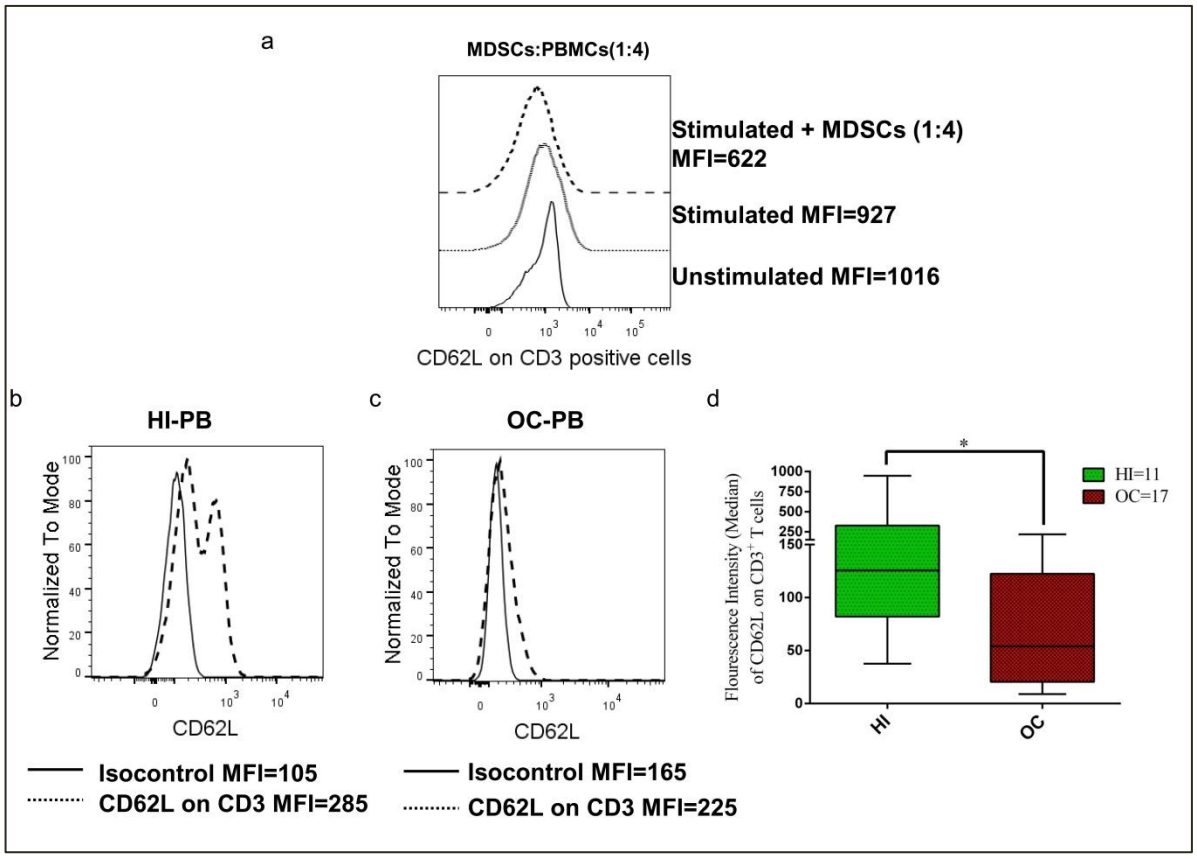

Figure 7.13: T cells cocultured with MDSC have an L-$selectin^{low}$ phenotype. a) PBMCs were harvested and cocultured alone or with MDSC from the peripheral blood in presence of TCR stimulation (α-CD3/CD28 mAb). MDSCs were added at a ratio of 1:4 to the T cells (myeloid cells: T cells). After 5 days of culture, cells were harvested and stained for L-selectin and CD3, and the gated $CD3^+$ T cells were analyzed for L-selectin expression. The activation induced downregulation of L-selectin was observed but presence of MDSCs induced further downregulation. L-selectin expression is represented by MFI (median fluorescence intensity) values. **b-c)** PBMCs were stained for CD3 and L-selectin. $CD3^+$ T cell population was gated and analyzed for L-selectin expression. The expression of L-selectin is represented as median fluorescence intensity. $CD3^+$ T cells of OSCC patients have significantly lower levels of L-selectin than T cells of HIs. **d)** The graph represent the combined data of OSCC patients (n=17) and HIs (n=11). The whisker plot shows the range of L-selectin expression on gated $CD3^+$ T cells. (* represent $p<0.05$)

7.2.8 Diminished production of perforin and granzyme B by T cells in OSCC patients

The presence of an extensive immunosuppressive network weakens the endogenous antitumor immunity by impairing T-cell function. An efficient cellular immune response against cancer requires a robust population of $CD8^+$ T cells with optimal cytotoxic functions. $CD8^+$ T along with $CD4^+$ T cells play a vital role in cell-mediated immune response and secrete cytolytic granules containing proteins, such as perforin and granzyme. To check the functional integrity of $CD4^+$ and $CD8^+$ T cells in OSCC patients, the basal expression of these cytolytic granules was evaluated. The T cell subsets from peripheral blood of HIs and OSCC patients along with tumor tissue were stained for perforin and granzyme B and analyzed by flow cytometry. As anticipated, the $CD8^+$ T cells of HIs expressed higher levels of perforin and granzyme B compared to the $CD8^+$ T cells of present in the peripheral blood and tumour compartment of OSCC patients **(Fig. 7.14a-f)**. The $CD4^+$ T cells with cytotoxic phenotype and function have been identified in humans and the presence of perforin and granzyme B in $CD4^+$ T cells in HIs suggest that they also can contribute in immune surveillance by directly lysing infected or malignant cells. Like $CD8^+$ T cells, $CD4^+$ T cells of OSCC patients in the peripheral blood and tumor tissue expressed lower levels of these molecules compared to $CD4^+$ T cells derived from HIs **(Fig. 7.14a-f)**. This shows that the multiple functions of the immune effector cells are impaired in OSCC patients and these defects could be partly attributed to higher levels of Mo-MDSCs observed in OSCC patients.

7.2.9 Cytokine repertoires of oral cancer patients play pivotal role in the induction and function of myeloid derived suppressor cells

In order to investigate the factors which drive the accumulation of MDSCs in peripheral blood, multiplex cytokine bead array using flow cytometry and enzyme linked Immunosorbent assay (ELISA) was used to monitor relevant cytokines in the serum of OSCC patients.

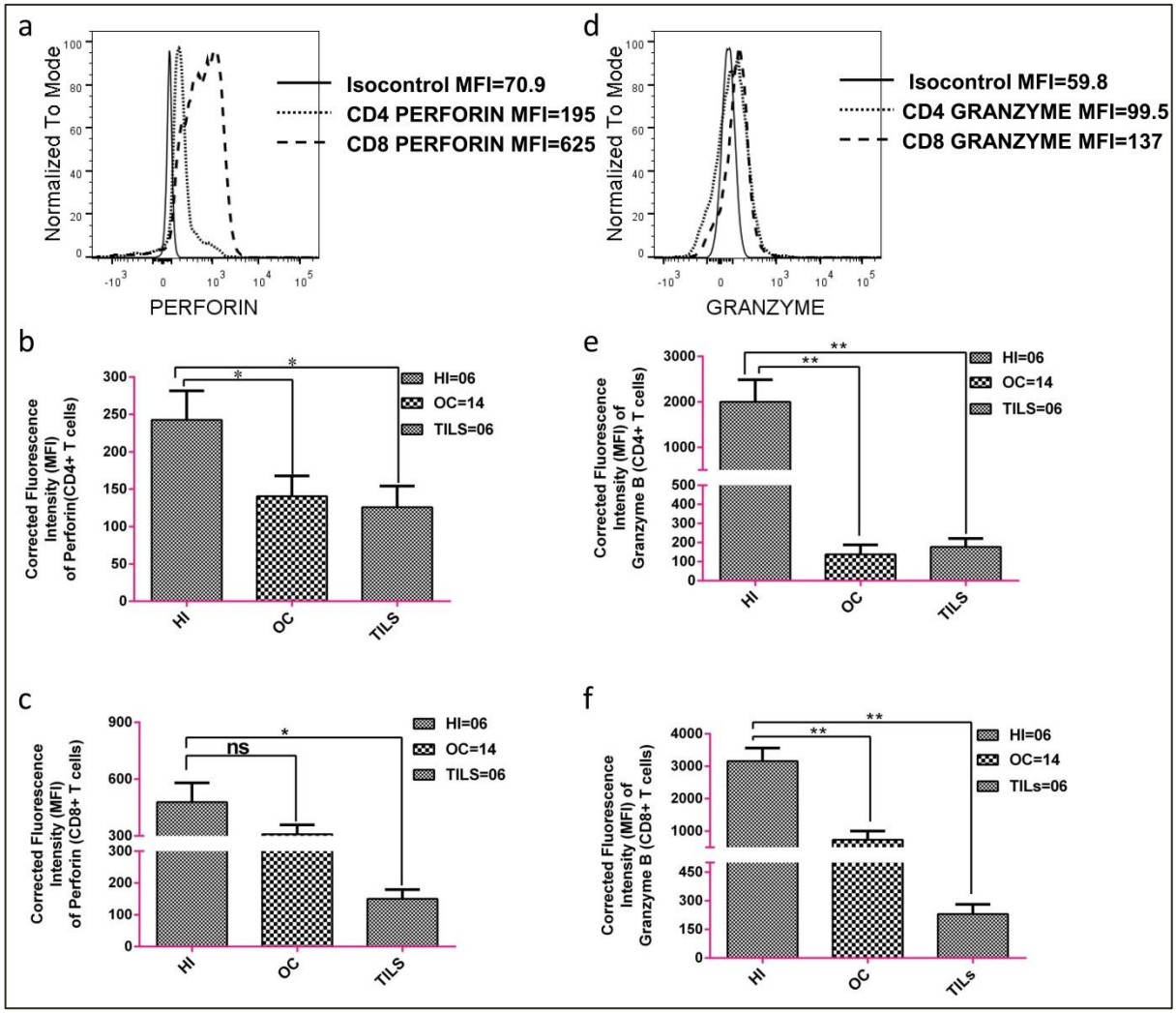

Figure 7.14: Reduced production of perforin and granzyme B by OSCC patient T cells. The PBMCs from OSCC patients and HIs were stained with CD3, CD4, CD8, perforin and granzyme-B antibodies and analyzed by flow cytometry **a)** The Histogram shows the fluorescence intensity of perforin on $CD4^+$ and $CD8^+$ T cells **b)** Shows the comparison of combined data of perforin expression on $CD4^+$ T cells from peripheral blood of HIs with peripheral blood and tumor tissue of OSCC patients while **c)** shows comparison of combined data of perforin expression on $CD8^+$ T. **d)** The Histogram shows the fluorescence intensity of granzyme-B on $CD4^+$ and $CD8^+$ T cells **e)** Shows the comparison of combined data of granzyme-B expression on $CD4^+$ T cells while **f)** shows comparison of combined data of granzyme-B expression on $CD8^+$ T. (* represents $p<0.05$, ** represents $p<0.005$ and ns represent data not significant with $p>0.05$).

The levels of immune modulatory cytokines like IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IFN-γ, IL-17A RANTES, MIG, MCP1, IP10, were analyzed by cytometric bead array while as IL23, PGE_2, VEGF and TGFβ were analysed by ELISA. The data showed that the sera of OSCC patients had significantly higher levels of cytokines like IL-6, IL-10, TNF-α, IL1β, IL8, TGF-β PGE2, and VEGF **(Fig. 7.15a-c)**. Cytokine like IL-6, TNF-

α, IL1β, PGE2, and VEGF are required for the MDSCs accumulation in periphery while IL-10 and TGF-β which are immunosuppressive and have the capability to alter the function and accumulation of Tregs. This suggests that OSCC patients provide favourable environment for the accumulation of immunosuppressive cells which enhance the immunosuppressive network of OSCC patients and promote tumor progression. Apart from this, cytokines like IL-6, IL10, IL1β, and TNF-α have the ability to alter the expression of STAT3 directly or indirectly. STAT3 is known to regulate the effector functions and generation of MDSCs.

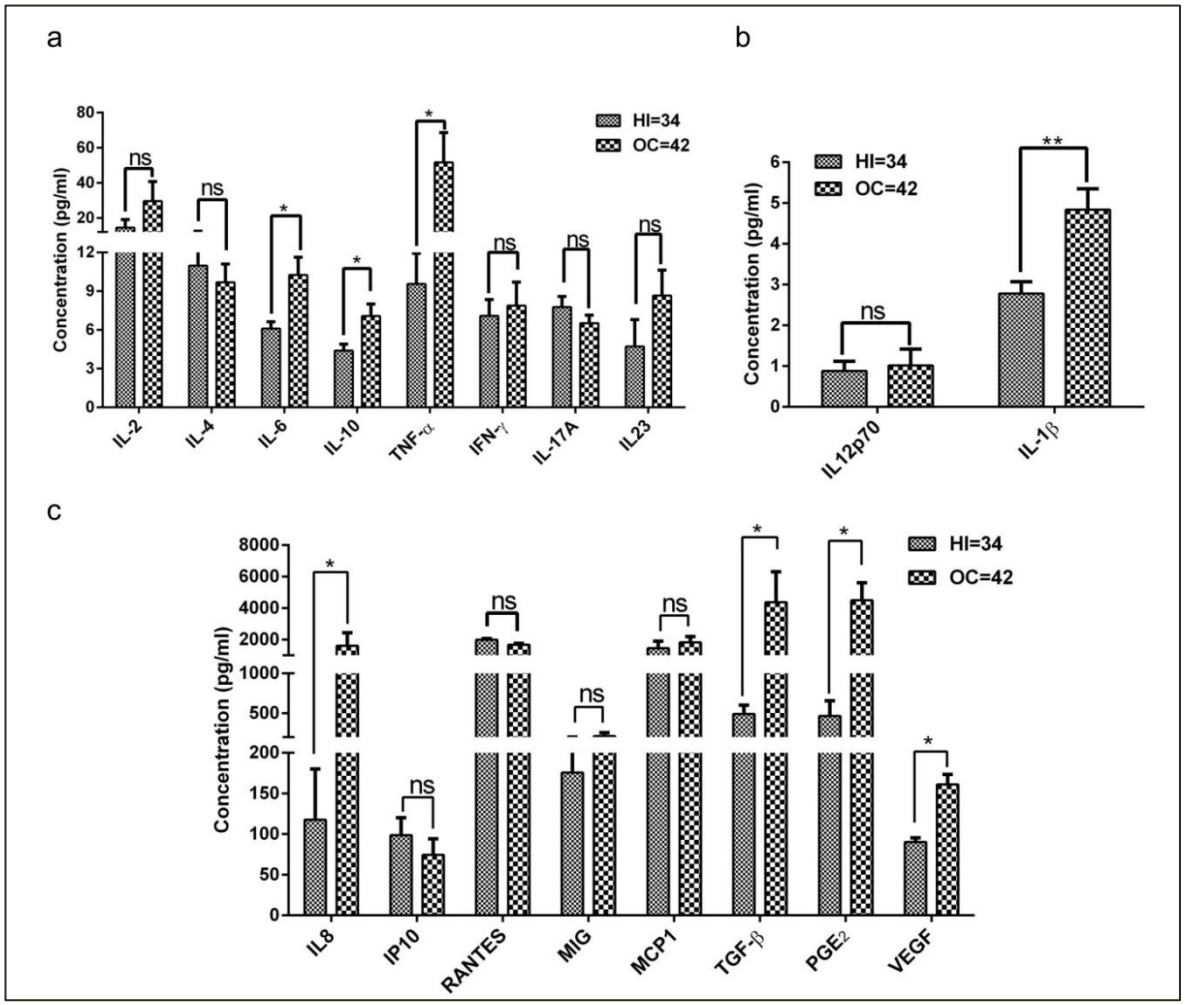

Figure 7.15: Elevated levels of immunosuppressive cytokines in patients with OSCC. **a-c)**, CBA and cytokine-specific sandwich ELISA of serum from OSCC patients (n=42) and healthy individuals (normal subjects, n=34) were used to measure the levels of circulating cytokines and growth factors. (* represent $p<0.05$ and ns represent non-significant data with $p>0.0.5$)

7.2.10 MDSC subsets prevalent in OSCC patients express abundant suppressive markers

MDSCs exert their immunosuppressive activity through multiple mechanisms, which include enzymes that deplete arginine (Arg-I and iNOS), Cox-2 or pSTAT3 or IL-10. As cytokines inducing STAT3 were abundantly present in serum of OSCC patients, the expression of STAT3 was evaluated in the MDSC subsets.

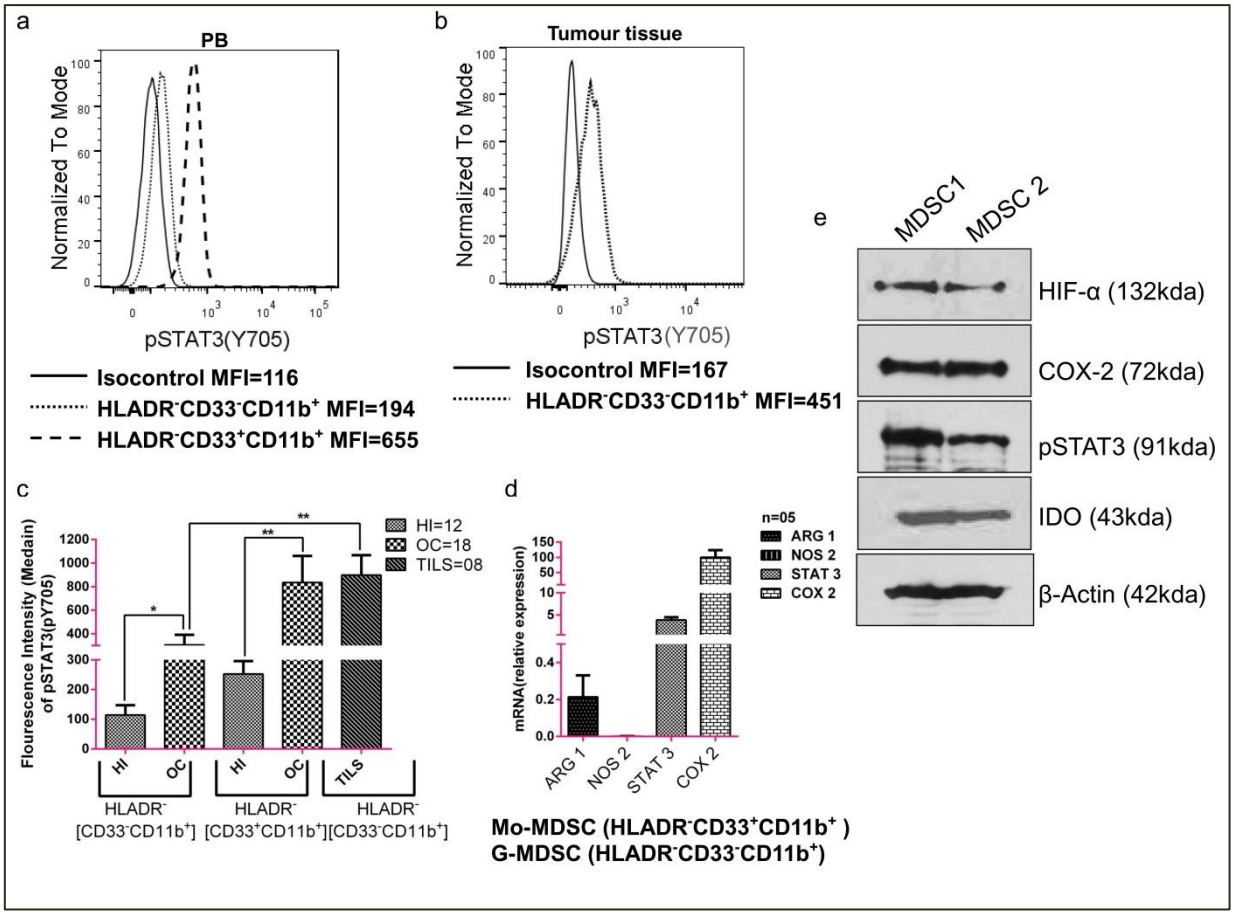

Figure 7.16: MDSCs express abundant suppressive markers. MDSC subsets present in peripheral blood and tumor tissue were analyzed for pSTAT3 expression by flow cytometry. **a)** Histogram show Mo-MDSCs ($HLADR^-CD33^+CD11b^+$) subset elevated in OSCC patients expressed higher levels of pSTAT3 than its other counterparts (G-MDSCs: $HLADR^-CD33^-CD11b^+$) in the peripheral blood of HIs or OSCC patients. **b)** Histogram shows that the G-MDSCs subset present in tumor tissue also expressed pSTAT3. **c)** The combined data showed that the Mo-MDSCs subset ($HLADR^-CD33^+CD11b^+$) dominating peripheral blood and G-MDSCs subset ($HLADR^-CD33^-CD11b^+$) present in tumor tissue expressed comparable levels of pSTAT3 which is higher than the pSTAT3 levels expressed by the MDSCs subsets of HIs. The pSTAT3 expression of MDSCs from peripheral blood of HIs (n=12) and OSCC patients (n=18) and OSCC tumor tissues (n=08) are represented. **d)** Sorted MDSCs expressed Arg-I, Cox-2 and, pSTAT3 mRNA while iNOS was absent or negligible. Data is representative of

five biological replicates **e)** Western blot analysis of Mo-MDSCs protein lysate confirmed that MDSCs express effector molecules like Cox-2, HIF-α, pSTAT3, and IDO. Data represented is from two different individuals from which Mo-MDSCs were sorted immuno-magnetically. (* represents $p<0.05$, ** represents $p<0.005$).

The Mo/G-MDSCs subsets present in OSCC patients expressed higher levels of pSTAT3 compared to the Mo/G-MDSCs subsets present in the peripheral blood of HIs. The pSTAT3 expression in Mo-MDSCs **(HLADR$^-$CD33$^+$CD11b$^+$)** is more prominent than in G-MDSCs (**HLADR$^-$CD33$^-$CD11b$^+$**) subpopulations in the peripheral blood of OSCC patients **(Fig. 7.16a-c)**. The G-MDSCs (**HLADR$^-$CD33$^-$CD11b$^+$**) subset present in the tumor microenvironment has comparable levels of pSTAT3 as observed in the Mo-MDSCs **(HLADR$^-$CD33$^+$CD11b$^+$)** present in the periphery**.**

Further, the Mo- MDSCs were sorted and expression of Arg-I, iNOS, STAT3 and Cox-2 was observed using real-time PCR. The real-time PCR data confirmed that sorted Mo-MDSCs **(HLADR$^-$CD33$^+$CD11b$^+$)** expressed higher levels of STAT3, Arg1 and Cox-2 while the expression of iNOS was negligible or absent **(Fig. 7.16d-e)**. pSTAT3, IDO and other relevant proteins like Cox-2 and HIFα were analyzed in the protein lysate of sorted Mo-MDSCs using western blotting technique. The protein lysate of Mo-MDSCs showed the presence of Cox-2, pSTAT3, HIFα and IDO **(Fig. 7.16d-e)**. This suggested that STAT3 driven signaling is active in the MDSCs subset prevalent in OSCC patients.

7.2.11 STAT3 induced regulators of MDSC function and accumulation

Previous studies have established a critical role for STAT3 in MDSC expansion and in angiogenesis [229, 230] [496]. Several pathways downstream of STAT3 might be involved in the regulation of MDSC expansion and function. To prove this, sorted Mo-MDSCs (**HLADR$^-$CD33$^+$CD11b$^+$**) were stimulated with different concentrations of recombinant human IL-6 to induce pSTAT3 for 24 hr. The IL-6 stimulation led to the induction of pSTAT3 in the sorted MDSCs with concomitant alteration in the expression of C/EBPβ,

C/EBPα, IL-10 and PDL1 **(Fig.7.17a)**. The C/EBPα expression which is required for the steady state haematopoiesis was decreased while the C/EBPβ required for emergency myelopoiesis was enhanced. This enhanced C/EBPβ expression in MDSCs suggests that STAT3 has ability to differentially regulate the myelopoiesis which might lead to the generation of immature myeloid cells. Cox-2 was unaffected on IL-6 stimulation and its induction could be inhibited by IL-6 induced IL-10 **(Fig.7.17a)**. Further STAT3 also regulated PDL1 which is known as a rheostat for T cell function. The pSTAT3 induced expression of IL-10 and PDL1 was observed using flow cytometry and data suggest that MDSCs stimulated with IL-6 led to prominent increase in the expression of IL-10 and PDL1 **(Fig.7.17b-c)**.

Aberrant Notch signaling is associated with the pathogenesis of various myeloid malignancies. Hes1 which plays an important role in Notch signaling pathway is known to inhibit the expression of C/EBPα in lymphoid compartment [497] and STAT3 is known to interact with Hes1 [498]. The differential regulation of C/EBPβ and C/EBPα by pSTAT3 could be attributed to the interaction of pSTAT3 with transcription factor Hes1 (Hairy and enhancer of split-1). IL-6 induced pSTAT3 in Mo-MDSCs **($HLADR^{-}CD33^{+}CD11b^{+}$)** might be enhancing the interaction of transcriptional repressor Hes1 at the C/EBPα promoter. This can be proven as MDSCs had active Notch signaling pathway of which Hes1 is one of the target genes. The sorted Mo-MDSCs expressed Hes1, NF-κB (Notch target genes), Notch-1 to Notch-4 receptors and ligands especially Dll1 and Jag1 **(Fig. 7.17d-f)**. The predominant expression of Jag1on Mo-MDSCs was observed compared to other ligands (Dll1,Dll4 and Jag2) and could be another mechanism used by MDSCs to inhibit T cell function as Jag1 can dampen Notch signaling and thereby T cell function.

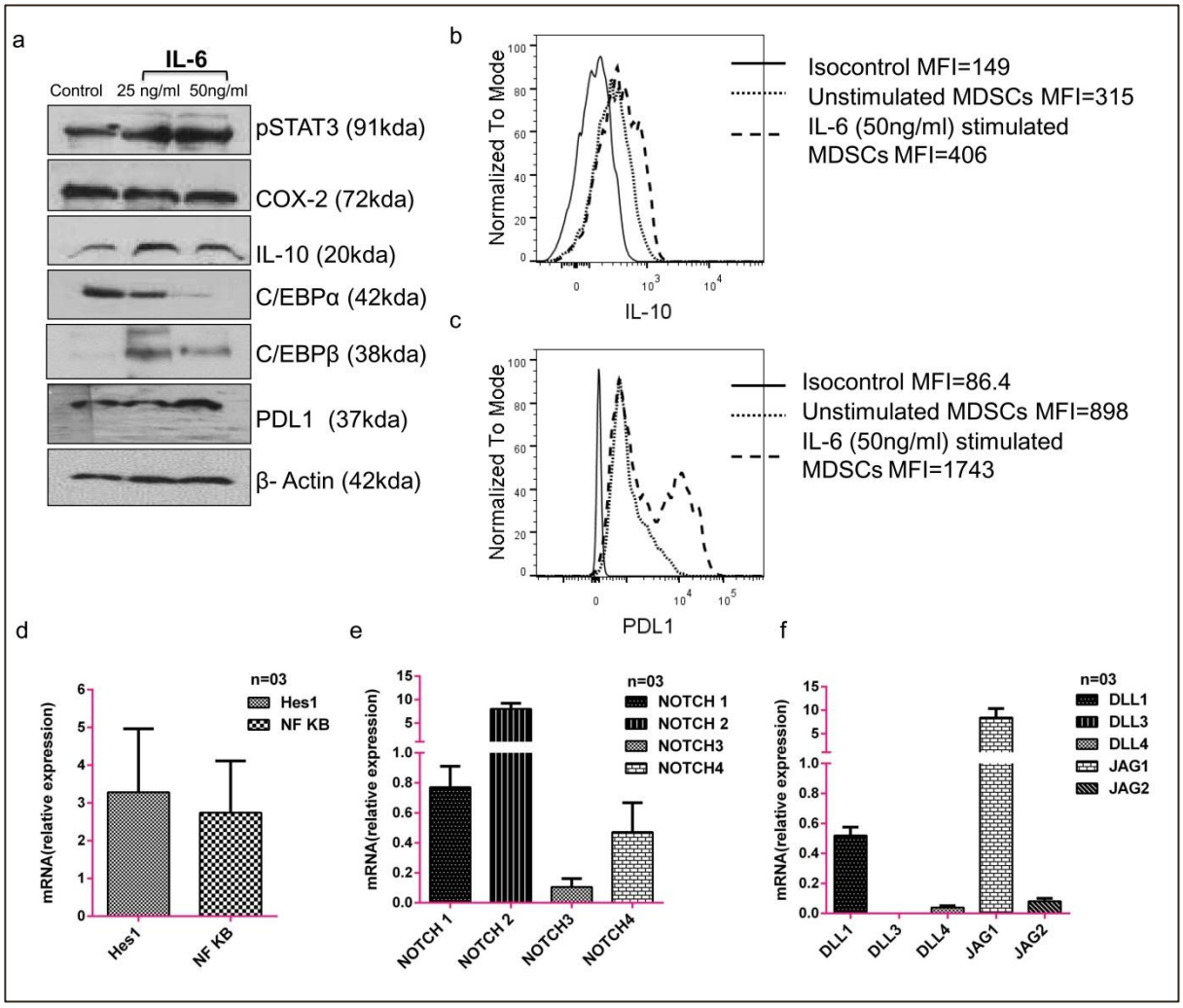

Figure 7.17: Induction of STAT3 increases activity and suppressive potential of MDSCs. MDSCs were sorted from the peripheral blood of HIs. The sorted MDSCs were stimulated with IL-6 (25 ng/ml and 50 ng/ml) for 24 hr to induce pSTAT3 expression and were then monitored for different effector molecules. **a)** IL-6 efficiently induced pSTAT3 expression which also led to the increase in the expression of IL-10, PDL1, C/EBPβ. Cox-2 remained unaffected while C/EBPα was downregulated. **b-c)** The induction of IL-10 and PDL1 by IL-6 was also studied by flow cytometry. Data confirmed that IL-6 induced pSTAT3 might be regulating PDL1 and IL-10 expression in MDSCs. **d-f)** the expression of Hes1 and its regulators (Notch signaling components) were analyzed in the sorted Mo- MDSCs using real-time PCR. The Mo-MDSCs were sorted from three different individuals and the data represented is the mean of three independent experiments run in duplicates.

7.2.12 Differential expression of IL-10, PD-L1 and PDL2 on peripheral blood and tumor-infiltrating MDSCs

IL-10, PDL1 and PDL2 are important players in the MDSC-mediated immune suppression. These were also induced in the MDSCs via IL-6/STAT3 dependent pathway. Therefore, the levels of IL-10, PD-L1 and PD-L2 were analyzed in the MDSCs subsets of HIs and OSCC

patients. The expression of IL-10 in the Mo-MDSC **(HLADR$^-$CD33$^+$CD11b$^+$)** and G-MDSCs **(HLADR$^-$CD33$^-$CD11b$^+$)** in the peripheral blood was analyzed.

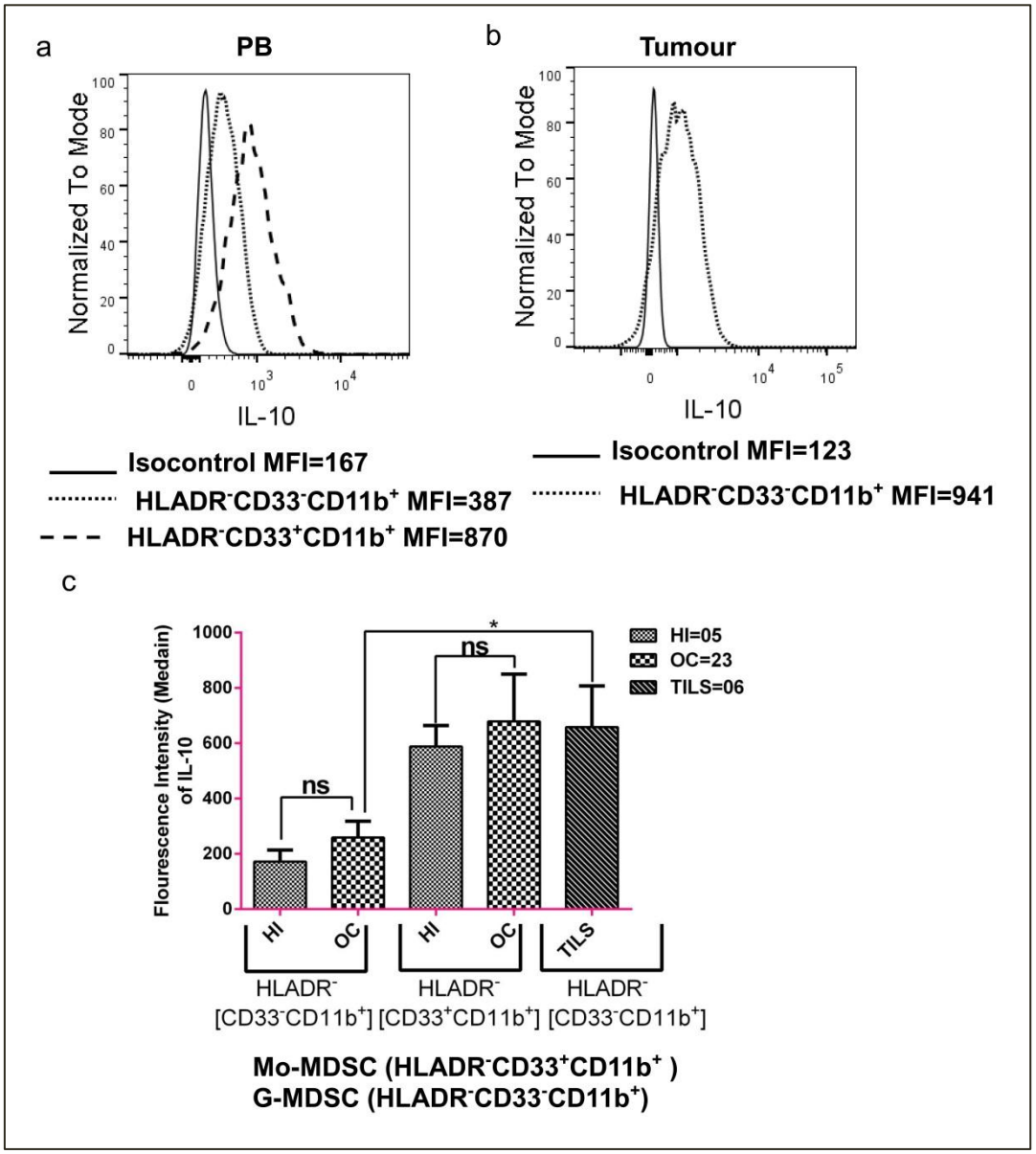

Figure 7.18: IL-10 production by the MDSCs subsets in peripheral blood and tumor tissue of OSCC patients. The PBMCs isolated from the peripheral blood of HIs and OSCC patients were stained with HLADR, CD33, CD11b and IL-10 antibodies. The MDSCs subsets were gated and analyzed for IL-10 represented as Median fluorescence intensity (MFI). **a)** The histogram shows the expression of IL-10 with dashed line representing the IL-10 expression on the Mo-MDSCs (HLADR$^-$CD33$^+$CD11b$^+$) subset while dotted line represents expression on G-MDSCs (HLADR$^-$CD33$^-$CD11b$^+$) **b)** The histogram shows expression of IL-10 on G-MDSCs subset present in tumor compartment (HLADR$^-$CD33$^-$CD11b$^+$). The solid lines represent Isocontrol. **c**) The data represented is the mean of fluorescence intensity of IL-10 expression on the MDSCs subsets from peripheral blood of HIs (n=05), OSCC patients (n=23) and tumor tissues of OSCC patients (n=06). (*represents $p<0.05$ and ns not significant $p>0.05$)

In the peripheral blood, Mo-MDSCs expressed higher levels of IL-10 (MFI 870) than G-MDSCs (MFI 387) **(Fig. 7.18a).** The tumor resident MDSCs (G-MDSCs) also expressed IL-10 (MFI 941) **(Fig. 7.18b).** The data showed that Mo-MDSCs expressed higher levels of IL-10 compared to the G-MDSCs in peripheral blood of OSCC patients and HIs with marginal increase in IL-10 expression in OSCC Mo-MDSCs compared to HI Mo-MDSCs **(Fig. 7.18c)**. The tumor infiltrating G-MDSCs expressed comparable levels of IL-10 produced by the Mo-MDSCs in the peripheral blood of OSCC patients. This shows that MDSCs have an inherent property to produce abundant IL-10 which might facilitate its interaction with other cell types.

Similar to IL-10, the expression of PDL1/2 was analyzed in the MDSCs subset present in the peripheral blood and tumor compartment of OSCC patients. In case of PDL1, it was found that the median fluorescence intensity of PDL1 on both G-MDSCs and Mo-MDSCs of OSCC patients was significantly higher than that seen in HIs. However the Mo-MDSCs of OSCC patients expressed higher levels of PDL1 than G-MDSCs present in the peripheral blood and tumor tissue of OSCC patients **(Fig. 7.19 a-c)**. Like PDL1, PDL2 expression in Mo-MDSC and G-MDSCs showed that Mo-MDSCs express higher levels of these ligands than G-MDSCs subset present in the peripheral blood **(Fig. 7.19d-f)**. The expression of PDL2 on the G-MDSCs subset present in peripheral blood of OSCC patients was higher than G-MDSCs present in the peripheral blood of HIs but remained lower to the Mo-MDSCs present in the peripheral blood of OSCC patients **(Fig. 7.19d-f)**. The higher levels of PDL1/PDL2 molecules on the MDSCs of OSCC patient's might aid in maintaining T cell tolerance. Further, IL-10 secreted by these MDSCs has the ability to potentiate the differentiation of Tregs which might enhance the T cell tolerance and aid in immune evasion of tumors.

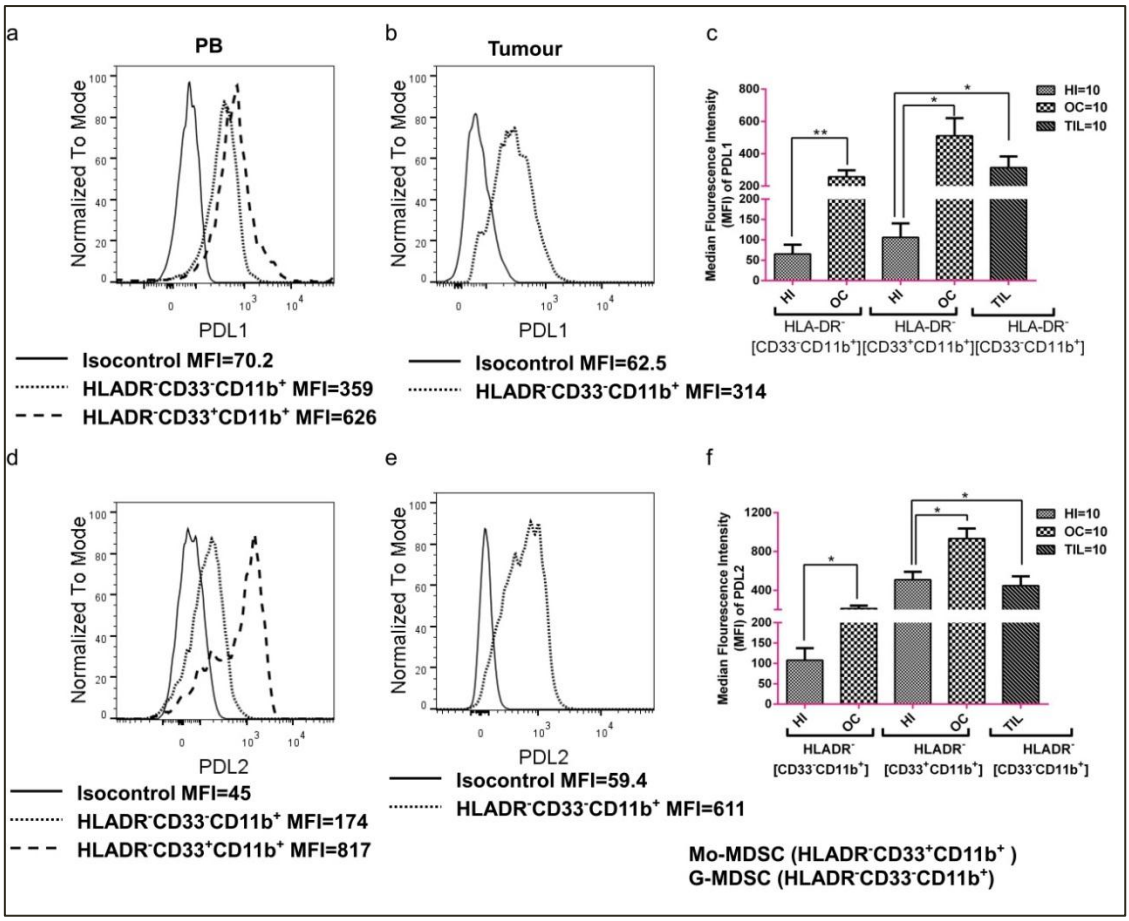

Fig 7.19: Expression of PDL1/PDL2 on the MDSCs subsets present in peripheral blood and tumor tissue of OSCC patients. The PBMCS isolated from the peripheral blood of HIs and OSCC patients were stained with HLADR, CD33, CD11b PDL1, and PDL2 antibodies. The MDSC subsets were gated and analyzed for the expression of PDL1/PDL2 which was represented as Median fluorescence intensity (MFI). **a)** The histogram shows the expression of PDL1 with dashed line representing expression on the $HLADR^{-}CD33^{+}CD11b^{+}$ subset while dotted line represents expression on $HLADR^{-}CD33^{-}CD11b^{+}$ **b)** The histogram shows expression of PDL1 on MDSCs subset present in tumor compartment ($HLADR^{-}CD33^{-}CD11b^{+}$). The solid line represents Isocontrol. **c**) The data represented is the mean of fluorescence intensity of PDL1 on the MDSCs subsets from peripheral blood of HIs (n=10), OSCC patients (n=10) and tumor tissues of OSCC patients (n=10). **d)** The histogram shows the expression of PDL2 with dashed line representing expression on the $HLADR^{-}CD33^{+}CD11b^{+}$ subset while dotted line represents expression on $HLADR^{-}CD33^{-}CD11b^{+}$ **e)** The histogram shows expression of PDL2 on MDSCs subset present in tumor compartment ($HLADR^{-}CD33^{-}CD11b^{+}$). The solid lines represent Isocontrol. **f**) The data represented is the mean of fluorescence intensity of PDL2 on the MDSCs subsets from peripheral blood of HIs (n=10), OSCC patients (n=10) and tumor tissues of OSCC patients (n=10). (*represents $p<0.05$ and ** represents $p<0.005$)

7.2.13 Elevated levels of regulatory T cells (Tregs) in peripheral blood of oral cancer patients correlate with IL-10 levels produced by MDSCs

IL-10 has potential to induce to the differentiation of Tregs. This was validated by analysing the levels of Tregs which are characterized as $CD4^{+}CD25^{+}$ $CD127^{low}$ with FoxP3 positivity

(90%± 5%) in OSCC patients. The gating strategy used to analyze the percentage of Tregs in the peripheral blood and tumor tissue of oral cancer patients is shown below **(Fig. 7.20a-c)**. Briefly, the mononuclear cell were gated on the basis of forward and side scatter, the gated cells were extrapolated for $CD4^+$ fluorescence and the gated $CD4^+$ T cells were further analyzed for the CD25 and CD127 expression. The cell population with phenotype $CD4^+CD25^+CD127^{low}$ was then analyzed for FoxP3 expression. Circulating levels of Tregs in peripheral blood of OSCC patients and healthy individuals were identified by immunostaining for coexpression of CD4 and CD25 cell surface receptors. It was observed that OSCC patients (n=75) exhibited significantly **($p<0.001$, Fig. 7.21a)** high percentages of Tregs (mean=9.17%, range=2-24%) compared to healthy individuals (n=45, mean=4.52%, range= 0.9-12%). The tumor tissue (n=38) of OSCC patients showed even higher accumulation of Tregs compared to the peripheral blood of OSCC patients and HIs.

These patients, when categorized into stages I-IV as per American cancer society TNM classification (stage I n=07, II n=14, III n=09 and IV n=45) demonstrated that Stage III/IV (mean 8.6%± 3.2) had significantly higher percentages of circulating Tregs compared to stage I/II patients (7.1%± 1.7) (**$p<0.001$, Fig. 7.21b)**. The stage I/II OSCC patients (7.1%± 1.7) have higher percentages of Tregs compared to HIs (4.5%± 2.2). The Tregs also infiltrated tumor and their percentage was higher in tumor compartment compared to the peripheral blood. Tumor tissues had higher levels of Tregs compared to the peripheral blood levels of patients with advanced stage (III/IV or (I/II) tumors.

Tregs were further assessed in the different subsites of oral cavity (buccal mucosa, tongue, mandible, lip, floor of mouth, alveolus, and retromolar). However, levels of Tregs did not show any site specific accumulation in OSCC patients (**Fig. 7.21c)**.

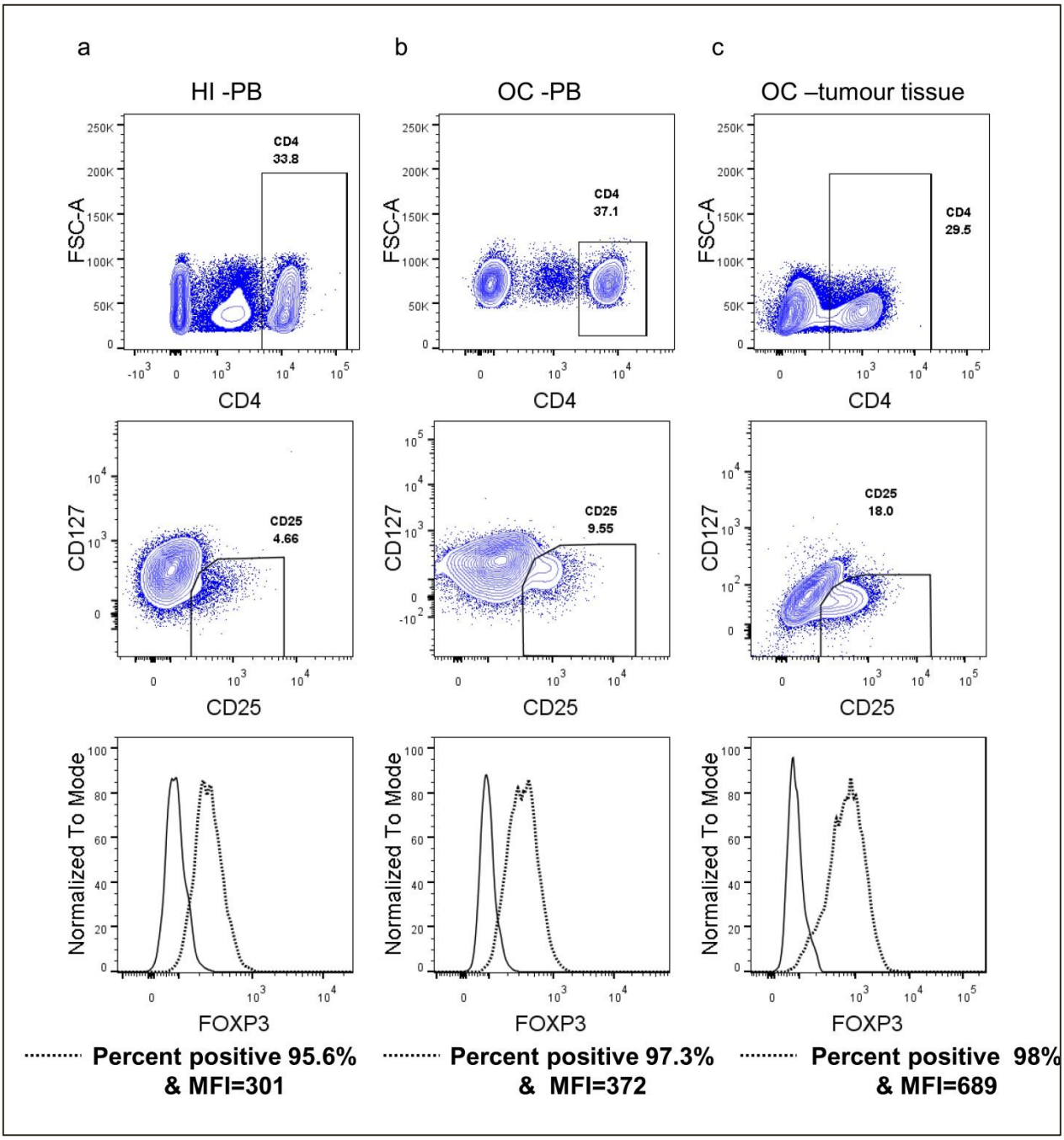

Figure 7.20: Gating strategy to analyze the Regulatory T cells in the peripheral blood and tumor tissue of OSCC patients. The peripheral blood mononuclear cells (PBMCs) were identified using FSC/SSC plot. PBMCs were gated and analyzed for the CD4 expression. The $CD4^{+}$ cells were gated and plotted for CD25 fluorescence on x-axis and CD127 fluorescence on y-axis. The $CD4^{+}CD25^{+}CD127^{low}$ cells expressing FoxP3 (% positive 90±5) were classified as Tregs. a) Shows the gating strategy of Tregs in peripheral blood of HIs, b) shows in the peripheral blood of OSCC patients and **c)** shows gating strategy in the tumor tissue of OSCC patients. Lower panel histogram represents FoxP3 positivity on $CD4^{+}$ $CD25^{high}CD127^{low}$ Tregs.

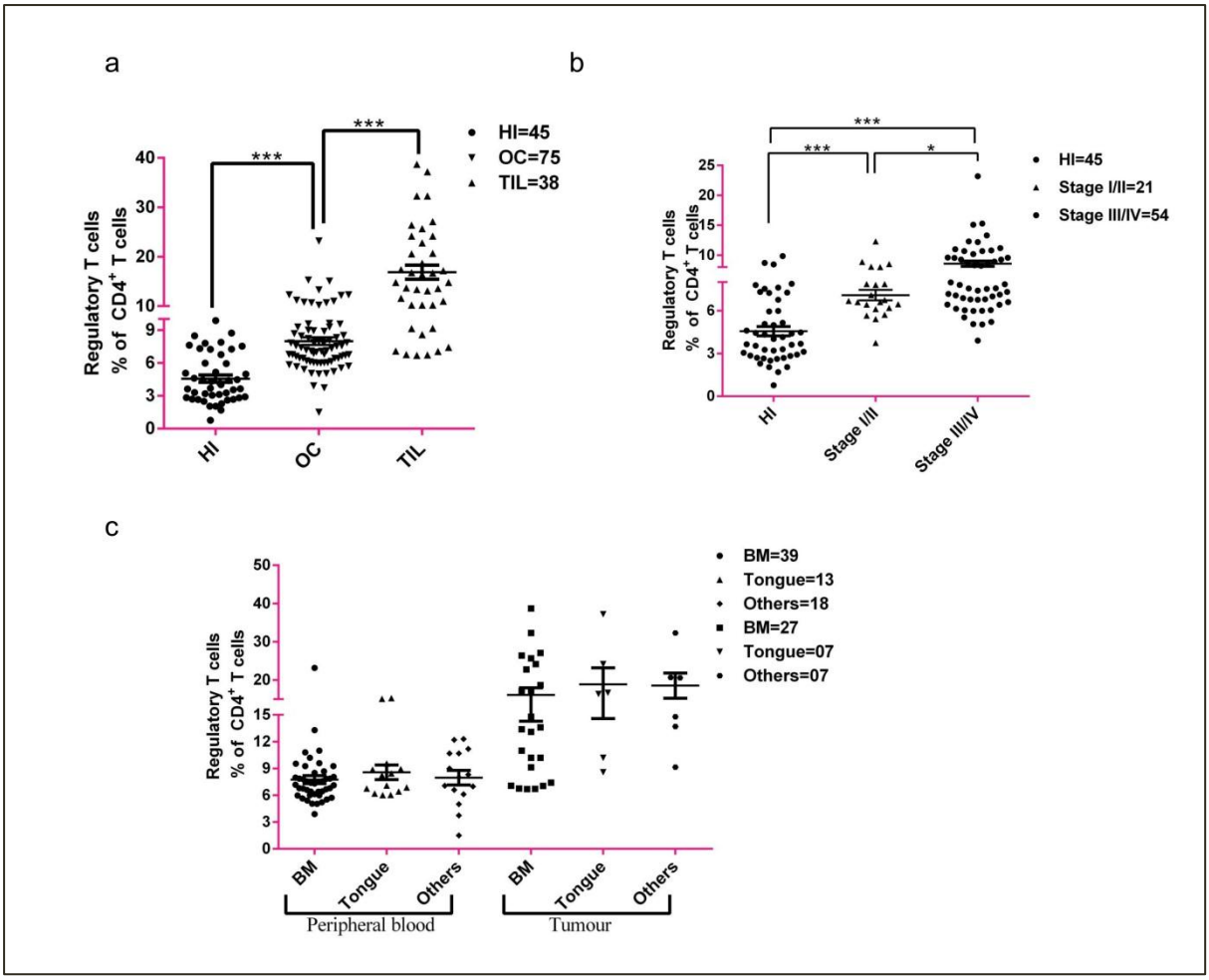

Figure 7.21: Increased levels of Tregs in OSCC patients. **a)** The frequency of $CD4^{+}CD25^{+}CD127^{low}$ Tregs is increased in PBMCs of OSCC patients (n = 75) compared with HIs (n = 45) (upper left panel). The tumors of OSCC patients (n=38) showed higher accumulation of $CD4^{+}CD25^{+}CD127^{low}$ Tregs than those observed in peripheral blood. **b)** Increased levels of Tregs were seen in patients with late-stage disease (stage III or IV) compared with those with early-stage disease (stage I or II). **c)** Abundance pattern of Tregs showed no clearly correlation with different subsites of OSCC patients. (* represent $p<0.05$ and *** represents $p<0.0005$)

The mean fluorescence intensity (MFI) of FoxP3 (master regulator of Tregs) present in Tregs derived from peripheral blood of OSCC patients and HIs was comparable. However, a significant elevation in MFI of FoxP3 was observed in the Tregs of tumor compartment **(Fig 7.22a)**. As it was postulated that IL-10 secreted by MDSCs might be altering the accumulation of Tregs in OSCC patients, the levels of IL-10 (MFI) secreted by the MDSCs and Tregs were correlated. Results showed a positive correlation ($p<0.001$, $r=0.622$) between the levels of Tregs and IL-10 secreted by MDSCs in the peripheral blood of OSCC patients

(Fig 7.22b). This suggests a possible crosstalk between MDSCs and Tregs that may be occurring in the OSCC patients.

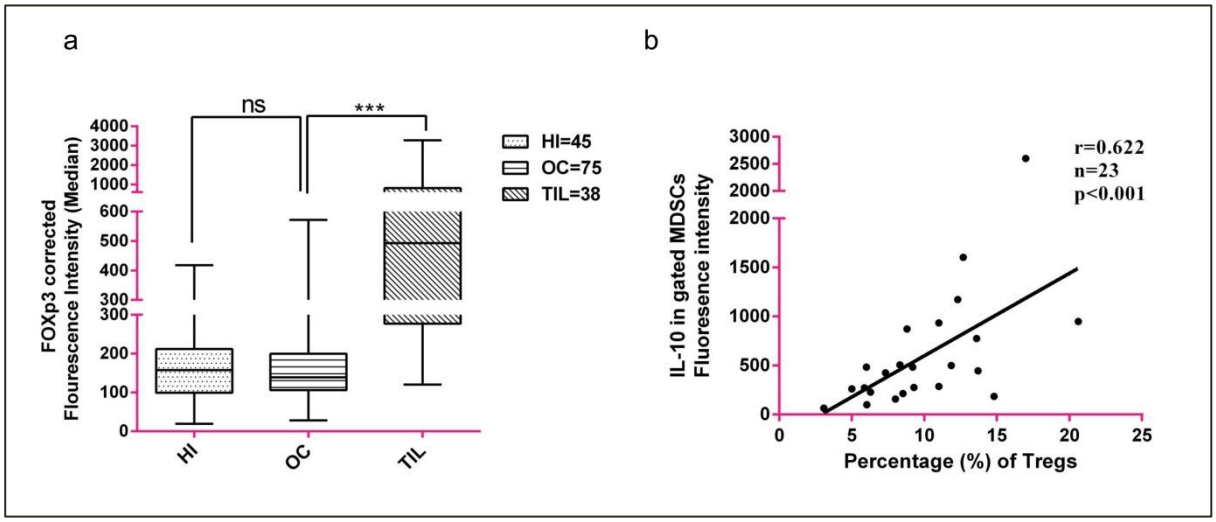

Figure 7.22: FoxP3 expression on regulatory T cells. The gated ($CD4^{+}CD25^{+}CD127^{low}$) Tregs were analyzed for the expression of FoxP3 which is represented as median fluorescence intensity. **a)** The fluorescence intensity of FoxP3 of Tregs from HIs (n=45) was comparable to that expressed by Tregs from the peripheral blood of OSCC patient (n=75). However Tregs from tumor compartment (n=38) expressed significantly higher levels of FoxP3 **b)** A positive correlation was observed between the IL-10 expression (Median fluorescence intensity) and the percentage of Tregs in OSCC patients (n = 23). Relationship between two parameters was investigated using the Spearman rank-correlation test. (*** represents $p<0.0005$ and ns represents not significant data $p>0.05$)

7.2.14 Accumulation of $CCR6^{high}$ and $CXCR4^{high}$ Tregs in the OSCC patients

The percentage of Tregs at tumor site in stage III/IV patients was significantly higher than that in the peripheral blood of stage III/IV patients **(Fig. 7.23a)**. We investigated the possible mechanism regulating Treg accumulation in the tumor compartment. It has been reported that chemokines play a role in the migration of Tregs to the tumor site. The expression of chemokine receptors CCR4, CCR6, CCR7, and CXCR4 was analyzed on the Tregs present in the peripheral blood of OSCC patients. Mononuclear cells were isolated from oral cancer patients and $CD4^{+}CD25^{high}CD127^{low}$ Tregs were analyzed for the expression of CCR4, CCR6, CCR7, and CXCR4. The median fluorescence intensity (MFI) of CCR4, CCR6, CCR7, and CXCR4 on Tregs showed that the Tregs from oral cancer patients expressed higher levels of CCR6 and CXCR4 than CCR4 and CCR7 **(Fig. 7.23b-e)**. Although,

difference observed in the expression of chemokine receptors on Tregs from HIs, peripheral blood and tumor tissue of OSCC patients was not significant **(Fig. 7.23f)**; in OSCC patients, Tregs expressed higher CCR6 and CXCR4 levels. This suggests that chemokines CCL20 and CXCL12 play an important role in recruitment of Tregs expressing CCR6 and CXCR4 receptors to the tumor site in OSCC patients.

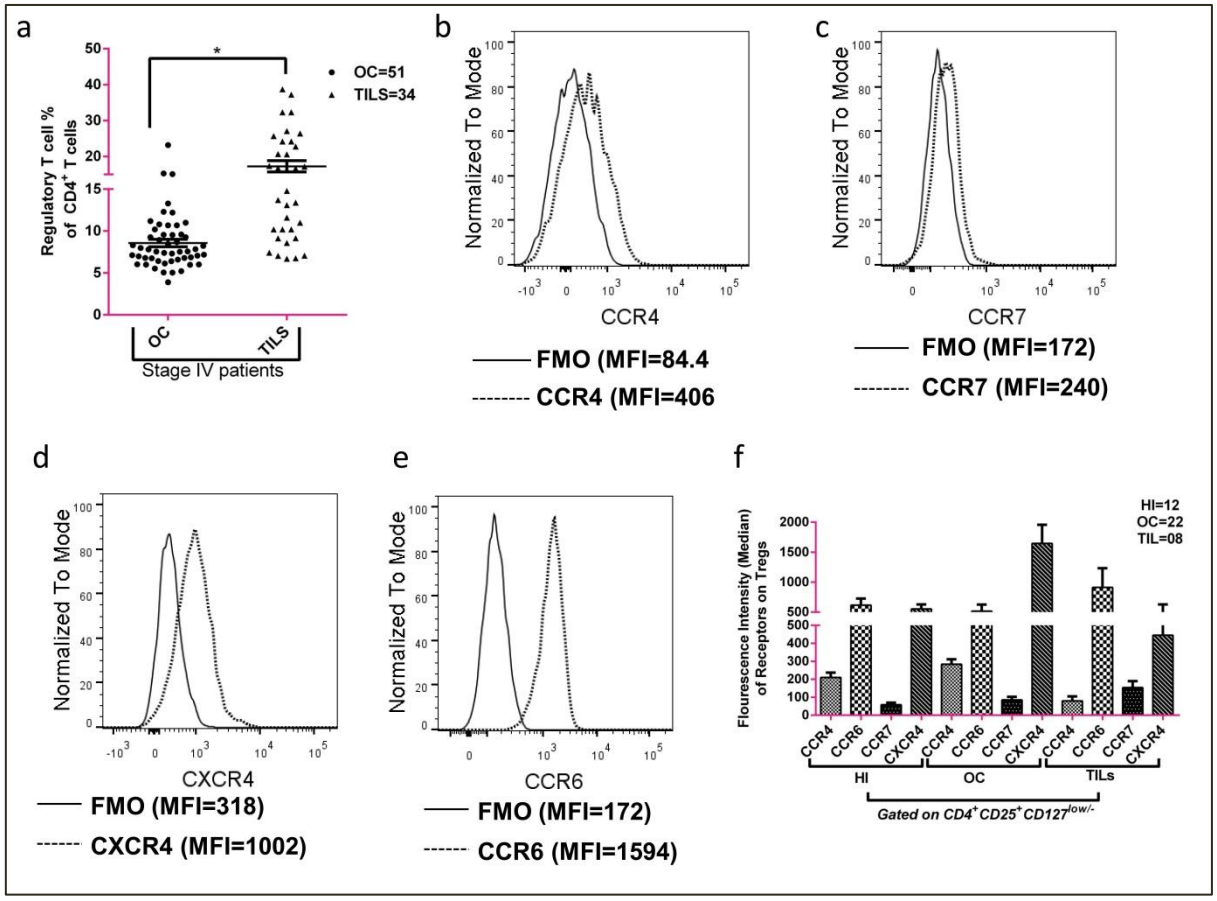

Figure 7.23: Accumulation of CCR6^{high} and CXCR4^{high} Tregs in the tumor tissue of OSCC patients. a) The peripheral blood and tumor tissue of stage IV patients were analyzed for Treg percentage. The data confirmed that tumor tissue of stage IV patients (n=34) had significantly higher levels of Tregs than peripheral blood of stage IV patients (n=51).**b-e)** the histograms show the median fluorescence intensity (MFI) of CCR4, CCR6, CCR7, and CXCR4 on Tregs with dotted line represent fluorescence intensity on Tregs and solid line on the FMO (fluorescence minus one) (f). Data shows that the Tregs from peripheral blood of HIs (n=12), peripheral blood of OSCC patients (n=22) and tumor tissue of OSCC patients (n=08) express CCR6, CXCR4, CCR4 and CCR7 to varying degree. However Tregs from tumor tissue are enriched for CCR6 and CXCR4 expression. (* represents $p<0.05$)

7.2.15 Tregs from OSCC patients have high propensity to migrate in MIP-3α-CCR6/ SDF-1α-CXCR4 axis

CC-chemokine receptor 6 (CCR6) is the only known receptor for macrophage inflammatory protein (MIP)-3alpha (MIP-3α/ CCL20), a CC chemokine chemotactic for lymphocytes and dendritic cells while, CXCR4 is the receptor for the stromal-derived factor (CXCL12/SDF-1α). Oral tumor derived cells (AW13516 and SSC29B) were stained with SDF1α and MIP-3α antibodies and analyzed by confocal microscopy.

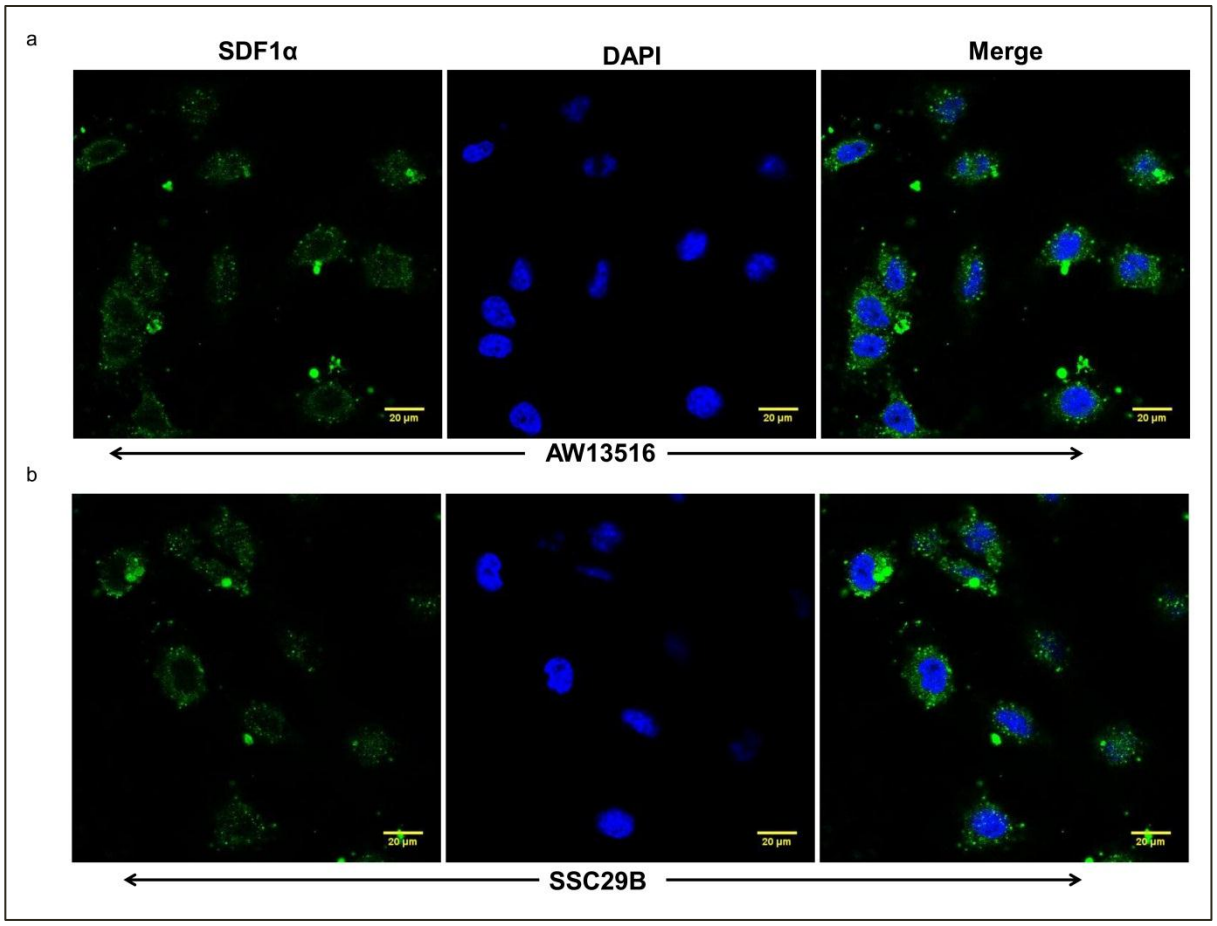

Figure 7.24: SDF-1α expression on oral tumor derived cell line by confocal microscopy. a) Shows the Immunofluorescence staining of AW13516 and **b)** shows the expression of SDF-1α in SSC29B. Green fluorescence (FITC) shows pattern of SDF-1α and nuclei are shown in blue (DAPI). Scale bar of 20 µm is shown at lower right of each image.

Immunofluorescence data confirmed that oral tumor derived cells (AW13516 and SSC29B) expressed variable amounts of the chemokines SDF1α (**Fig. 7.24)** and MIP-3α (**Fig. 7.25)**. Both these ligands showed a predominantly cytoplasmic expression. The presence of these

chemokines in oral tumor derived cell lines further confirms that the MIP-3α-CCR6/ SDF-1α-CXCR4 axis aids in the recruitment of Tregs to the inflammatory site like OSCC tumor.

To determine whether this migratory phenomenon in response to these ligands leads to the accumulation of Tregs in tumor compartment chemotaxis assay was performed. It is reported that TGF-β is known to rapidly and strongly induce CCR6 mRNA expression in T cells as well as CXCR4. Tregs were stimulated with α-CD3/CD28 mAb in presence of TGF-β. The stimulation of freshly isolated Tregs with α-CD3/CD28 + IL-2 and TGF-β induced both CXCR4 and CCR6 in the Tregs **(Fig. 7.26a-b).**

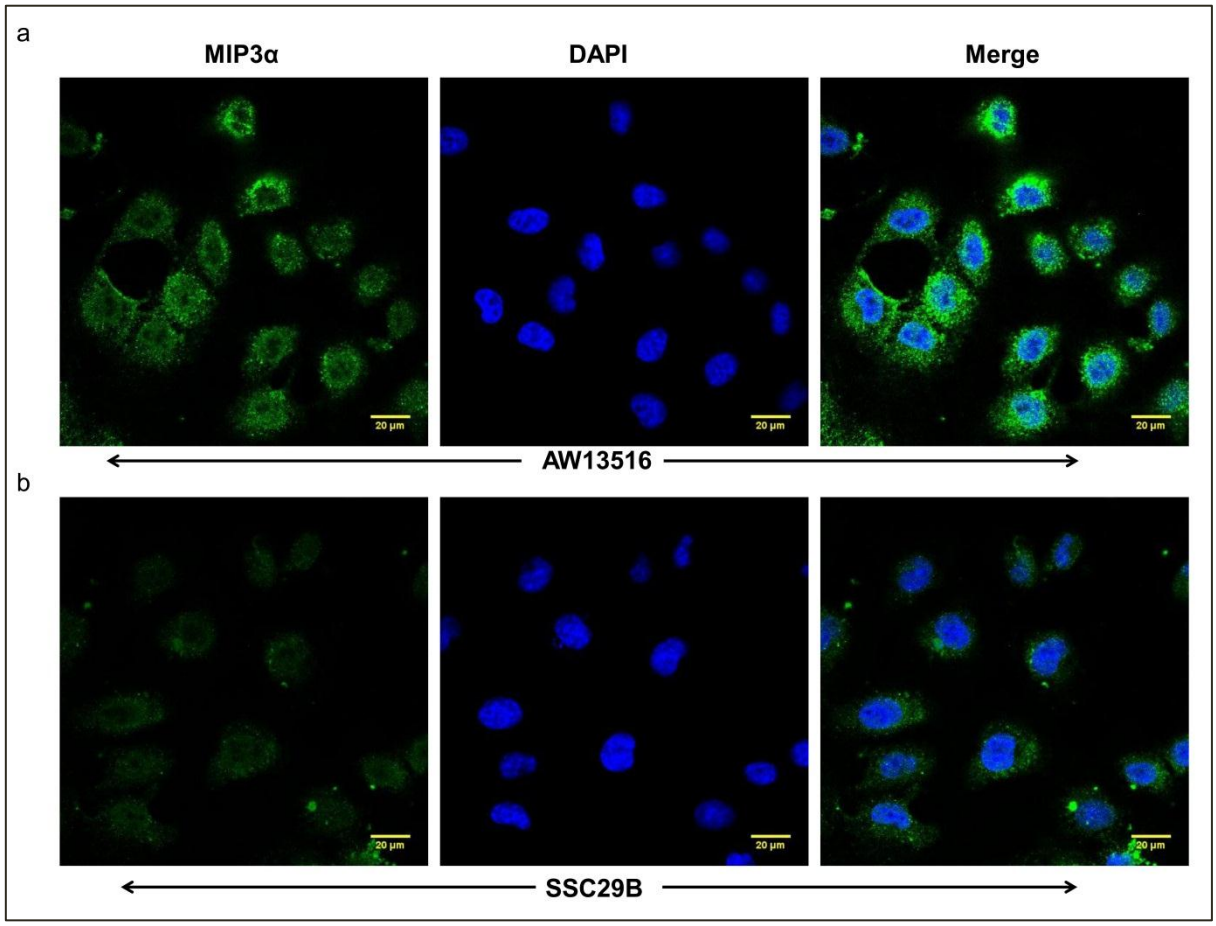

Figure 7.25: MIP-3α expression on oral tumor derived cell line by confocal microscopy. a) Shows the Immunofluorescence staining of AW13516 and **b)** shows the expression of MIP-3α in SSC29B. Green fluorescence (FITC) shows pattern of MIP-3α and nuclei are shown in blue (DAPI). Scale bar of 20 µm is shown at lower right of each image

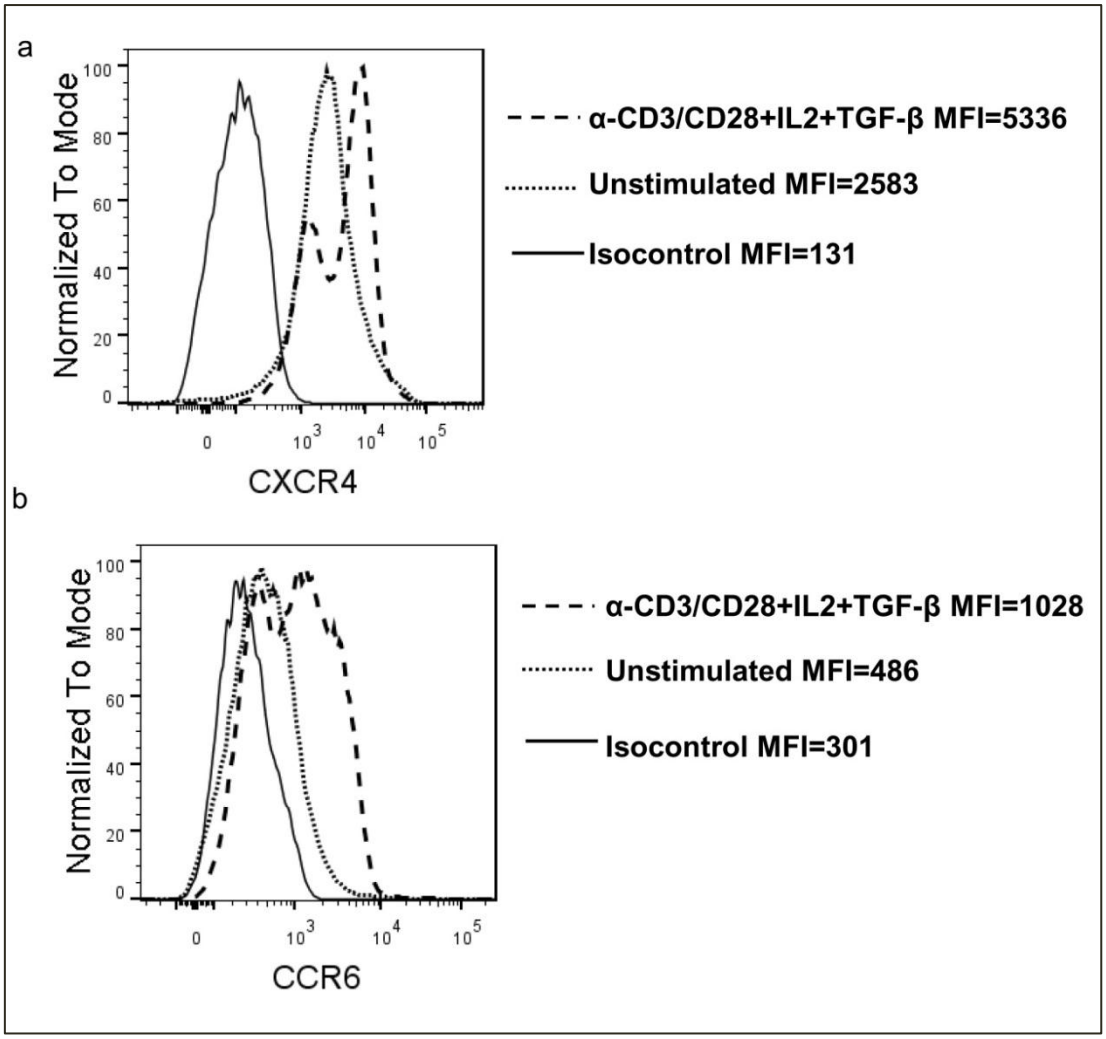

Figure 7.26: Induction of CXCR4 and CCR6 on Tregs. The freshly isolated Tregs were stimulated with α-CD3/CD28 (5 μg/ml) IL-2 (100 U/ml) and TGF-β (20 ng/ml) for 24 hr. After 24 hr cells were stained for CD4, CD25, CD127, CXCR4 and CCR6. a) Histogram shows the induction of CXCR4 on Tregs after stimulation and b) shows induction of CCR6 on Tregs compared to the unstimulated Tregs. The dashed line represents stimulated Tregs; dotted line represents unstimulated Tregs while solid line represents Isocontrol.

TGF-β induced $CCR6^{high}$ and $CXCR4^{high}$ Tregs were analyzed with regard to their migratory capacity toward the CCR6 ligand CCL20 (MIP-3α) and CXCR4 ligand SDF-1α using Trans-well chemotaxis assay. Tregs were efficiently mobilized by SDF-1α and MIP-3α, producing an index of almost 2 and 3, respectively **(Fig. 7.27a-d).** Neutralization of both MIP-3α and SDF-1α resulted in a significant decrease of the chemotactic index; confirming that Tregs cells were in fact migrating under the influence of the chemokines. Thus, chemotaxis assay confirmed that Tregs migrate to the tumor site via CCL20-CCR6, SDF-1α-CXCR4 axis thereby create an immunosuppressive environment.

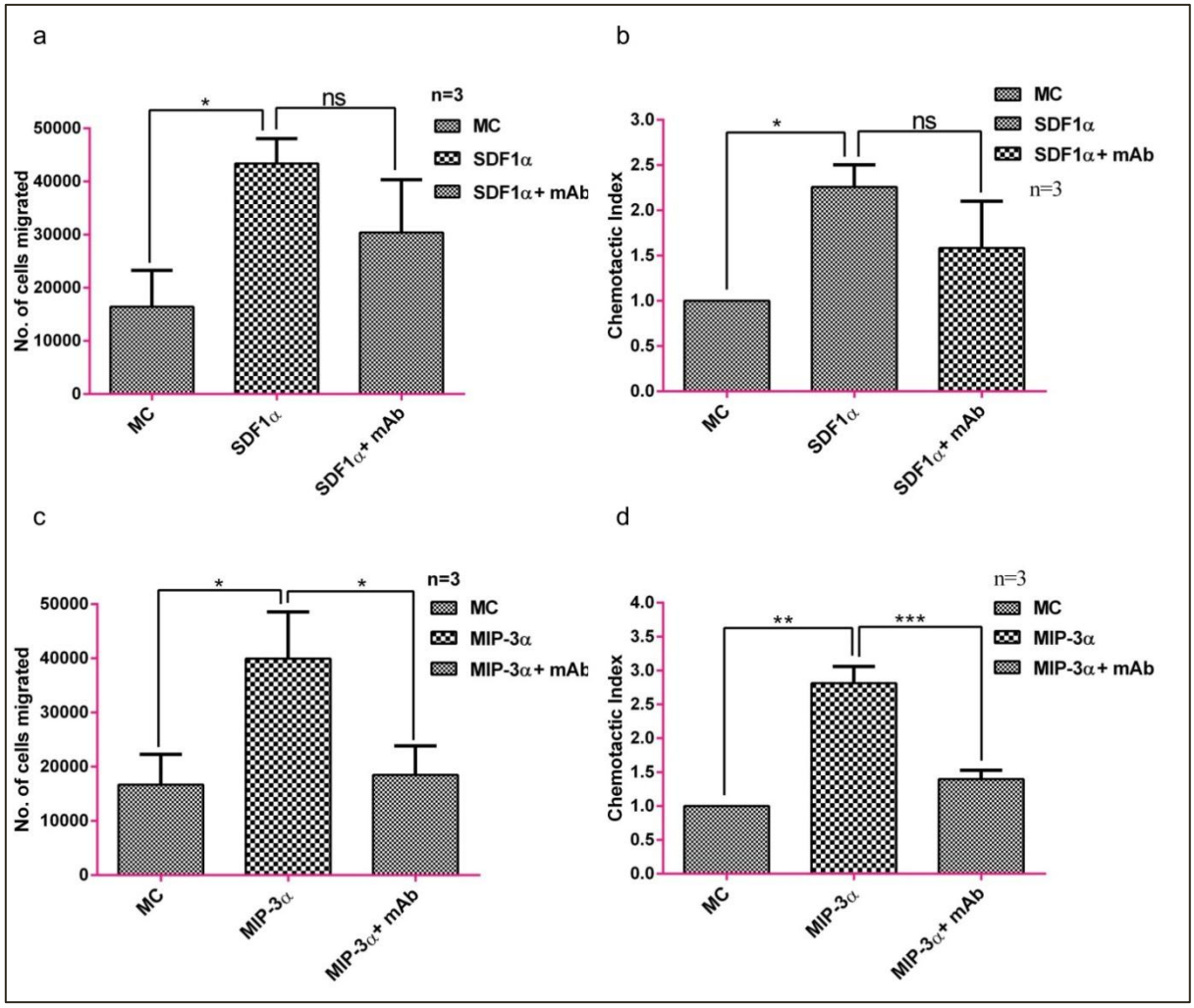

Figure 7.27: Migration of Treg cells is mediated by CCR6-MIP-3α and CXCR4-SDF-1 α interaction. Transwell chemotaxis assay of Tregs in the presence of MIP-3α and SDF-1α was carried out. The migrated cells were analyzed at 7 h of culture. **a)** The number of Tregs migrated in response to the SDF-1α was counted. **b)** Migration is expressed as chemotactic index and was calculated by the ratio of cells migrated in the presence of chemokine to the number of cells that spontaneously migrated with medium alone (medium control, MC). **c)** The number of Tregs migrated in response to the MIP-3α was counted **d)** Migration of Tregs expressed as chemotactic index was calculated by the ratio of cells migrated in the presence of chemokine to the number of cells that spontaneously migrated with medium alone. The result shown is representative of three independent experiments. All data are expressed as the mean±SD. (* represent $p<0.05$, ** represents $p<0.005$, *** represents $p<0.0005$ and ns represent non-significant data with $p>0.05$)

7.2.16 MDSCs inhibit iTreg differentiation under in vitro conditions

MDSCs were shown to secrete IL-10 which is known to potentiate the differentiation of regulatory T cells. Both MDSCs and Tregs are elevated in the peripheral blood of OSCC

patient suggesting that there might be a dynamic crosstalk between these two cell types influencing each other's function and accumulation in OSCC patients.

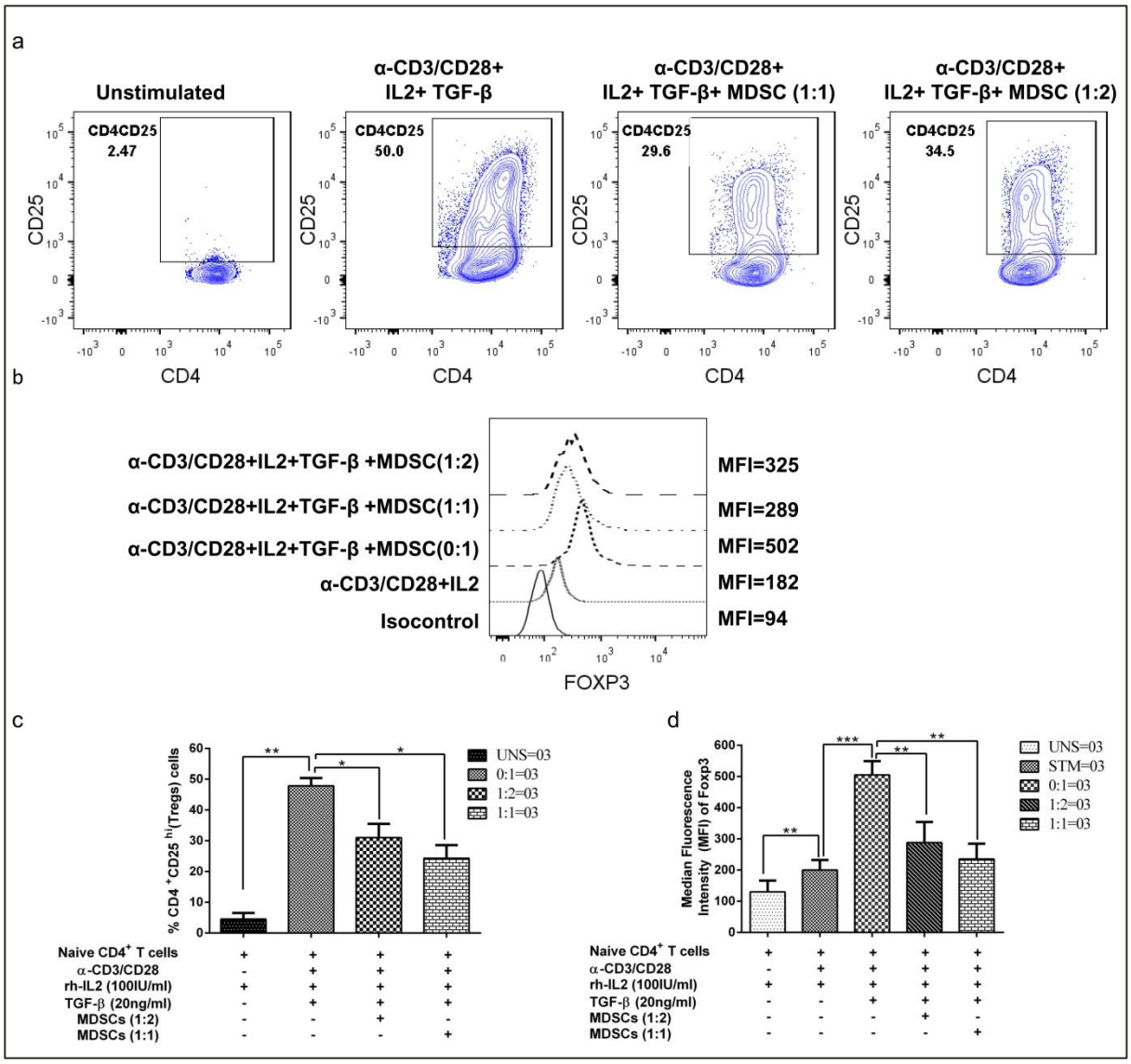

Figure 7.28: Mo-MDSCs suppress TGF-β 1-induced generation of FoxP3$^+$ iTreg. Naïve CD4+ T cells were isolated and cultured for 4 days with activation beads and with or without TGF-β1 (20ng/mL) in the presence or absence of Mo-MDSCs isolated from same individuals at 1:2 or 1:1 ratio (MDSC: T cell). The frequency of CD4$^+$CD25$^+$ T cells with FoxP3$^+$ expression cells was determined after 4th day. **a)** Representative dot plots depict the influence of Mo-MDSCs on generation of CD4$^+$ CD25$^+$ T cells while **b)** shows the effect of Mo-MDSCs on the FoxP3 expression. **c)** Mo-MDSCs prevent the generation of CD4$^+$CD25$^+$ T cells **d)** Mo-MDSCs suppress TGF-β1-induced FoxP3$^+$ expression on CD4$^+$CD25$^+$ T cells in a dose-dependent manner. Data shown is the mean of three independent experiments. (* represent $p<0.05$, ** represents $p<0.005$, and *** represents $p<0.0005$.

Naïve $CD4^+$ T cells were isolated and differentiated in Treg polarizing condition in presence or absence of MDSCs for 4 days. After 4 days, cells were stained and analyzed for Treg differentiation. Surprisingly, our results indicate that MDSCs are not able to promote iTreg differentiation, and instead hamper TGF-β1-induced generation of these cells in a dose-dependent manner **(Fig. 7.28a&c).** Treg differentiation was inhibited in the presence of MDSC as reflected at decreased expression of CD25 on $CD4^+$ T cells. Further, MDSCs also inhibited the TGF-β induced expression of FoxP3 in a dose-dependent manner which is known to regulate the Treg function **(Fig. 7.28b&d).** The data confirmed that the MDSCs might not be responsible for the accumulation of Tregs in the peripheral blood of OSCC patients**.** The positive signals like TGF-β and IL-10 in OSCC patients might be fostering Treg accumulation which might be overriding the negative effects of MDSCs in immunosuppressive tumor microenvironment.

7.2.17 MDSCs promote Th17 cell differentiation under in vitro conditions

To address the MDSCs failure to induce Treg differentiation despite secreting high IL-10, the cytokine repertoires of MDSCs was analyzed. The sorted Mo-MDSCs showed presence of IL-6 and IL-1β cytokines at mRNA level with marginal expression of TGFβ1, RAR-Related Orphan Receptor C (RORC), and IL23A **(Fig 7.29a)**. This was further confirmed by cytometric bead array which showed that the cell free supernatant of Mo-MDSCS have higher levels of IL-6, IL-10, TNF-α, IL1-β, IL8, RANTES, and MCP1 **(Fig. 7.29b)**. More importantly cytokines like IL-6, IL-1β and IL23 have the ability to alter the differentiation of CD4 naïve cells to Th17 cells.

To prove this naïve $CD4^+$ T cells were stimulated with α-CD3/CD28 in the presence or absence of MDSCs. Here IL-2 was not added to the naïve $CD4^+$ T cells. Naïve $CD4^+$ T cells were cocultured with MDSCs for 7 days. Then cells were harvested and stained for CD4, IL17A and IFN-γ. Data showed that MDSCs promote differentiation of IL17A producing

CD4 cells but inhibited IFN-γ production in a dose dependent manner **(Fig 7.29c-d)**. This suggests that MDSCs are capable of inducing TH17 differentiation and inhibit the TH1 response in a dose-dependent manner. It was further proved that in presence of IL1β, IL23 and IL-6, naïve T cells efficiently differentiated into TH17 cells but these cytokines did not affect IFN-γ production by these T cells **(as shown in material and methods, Fig. 3.10)**. Thus, this data confirms that cytokine repertoires along with suppressive markers like IDO might be playing a pivotal role in the induction of IL-17 production by T cells by interfering with the Th1 and Treg differentiation.

7.2.18 IL17 producing T cells are elevated in OSCC patients

In order to study the dominance of circulating IL17 producing cells in OSCC patients, PBMCs were isolated from OSCC patients and HIs. Cells were stimulated with PMA and Ionomycin in the presence of Brefeldin A for 4 h. After 4 h, cells were harvested and stained for Th17 and Tc17. The gating strategy to define and determine Th17 and Tc17 cells in the peripheral blood and tumor tissue is shown in **Figure 7.30 and 7.31**. The Th17 and Tc17 cells were significantly increased in PBMCs of OSCC patients compared to HI. The single cell suspension of tumor tissue (n=38) was prepared by enzyme digestion and analyzed for these cells. Like peripheral blood, tumor tissue of OSCC patients also showed dominant levels of Th17 and Tc17 and comparably higher than that in the peripheral blood of OSCC patients **(Fig. 7.32a-b).** While the IFN-γ producing CD4 and CD8 T cells were found to be low in the peripheral blood and tumor tissue of OSCC patients, compared to the peripheral blood of HIs. **(Fig. 7.32c-d)**

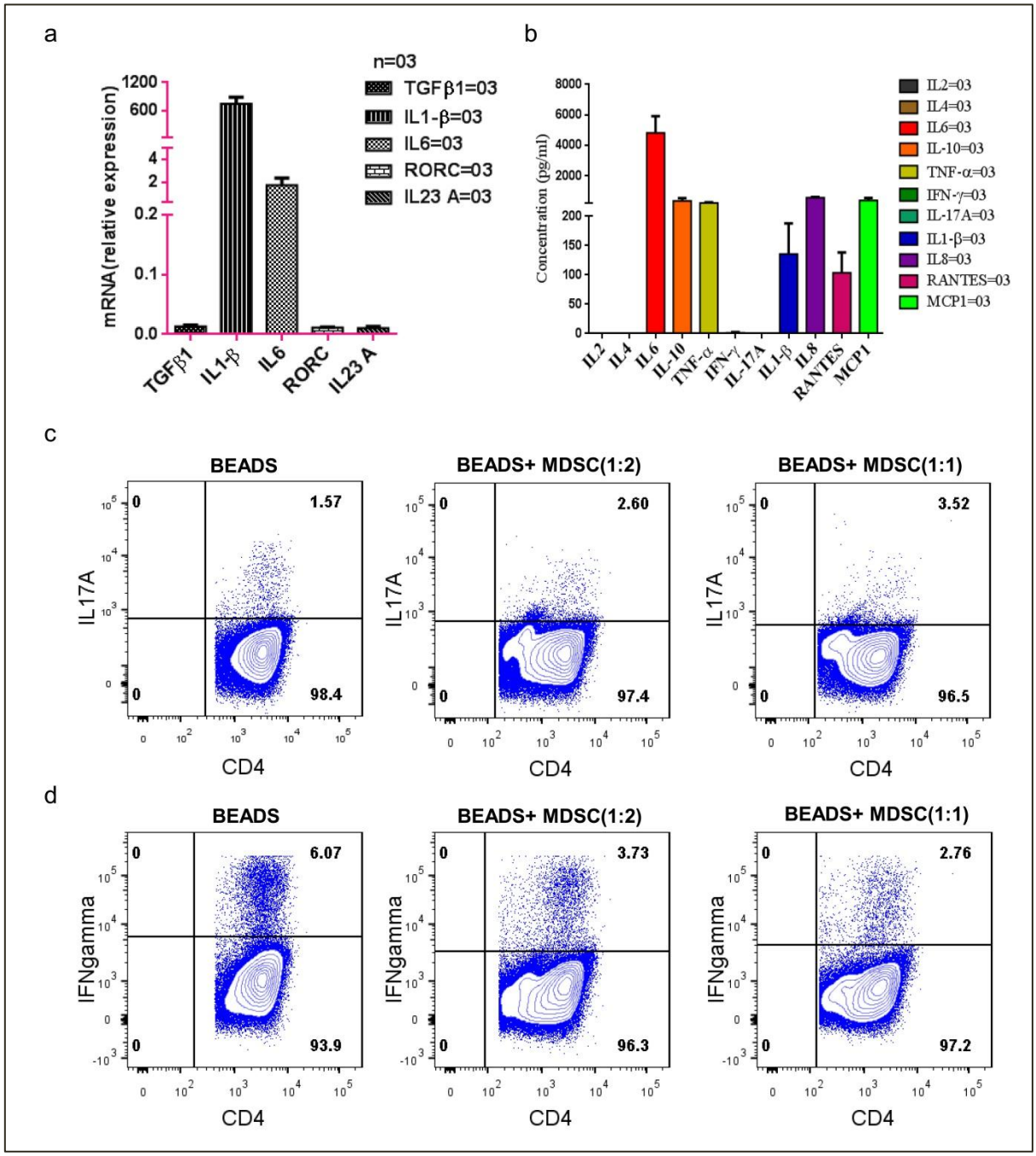

Figure 7.29: Cytokine repertoire of Mo-MDSCs promotes Th17 differentiation. a) Sorted Mo-MDSCs were probed for different cytokines at mRNA level. RNA was extracted from Mo-MDSCs and real time quantitative PCR showed mRNA expression of TGFβ1, IL1β, IL-6, RORC, and IL23A on Mo-MDSCS. Data represented is the mean of three independent experiments. **b)** Sorted Mo-MDSCs were cultured for 24 hr and cell free supernatant was collected. Concentrations of different cytokines were determined by Cytometric Bead Array. Apart from IL-10, Mo-MDSCs also secreted IL-6, TNF-α, IL-1β. Data represented is the mean of three independent experiments. **c)** Naïve CD4+ T cells were isolated and cultured for 7 days with α-CD3/CD28 microbeads in the presence or absence of Mo-MDSCs isolated from same individuals at 1:2 or 1:1 ratio (MDSC: T cells). The frequency of $CD4^{+}IL17A^{+}$ T cells was determined after 7^{th} day. Representative contour plots depict the influence of Mo-MDSCs on generation of $CD4^{+}IL17A^{+}$ T cells while **d)** shows the effect of Mo-MDSCs on the generation of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells. Mo-MDSCs promote the

generation of $CD4^+IL17A^+$ T cells but inhibit generation of $CD4^+IFN\text{-}\gamma^+$ T cells in a dose-dependent manner.

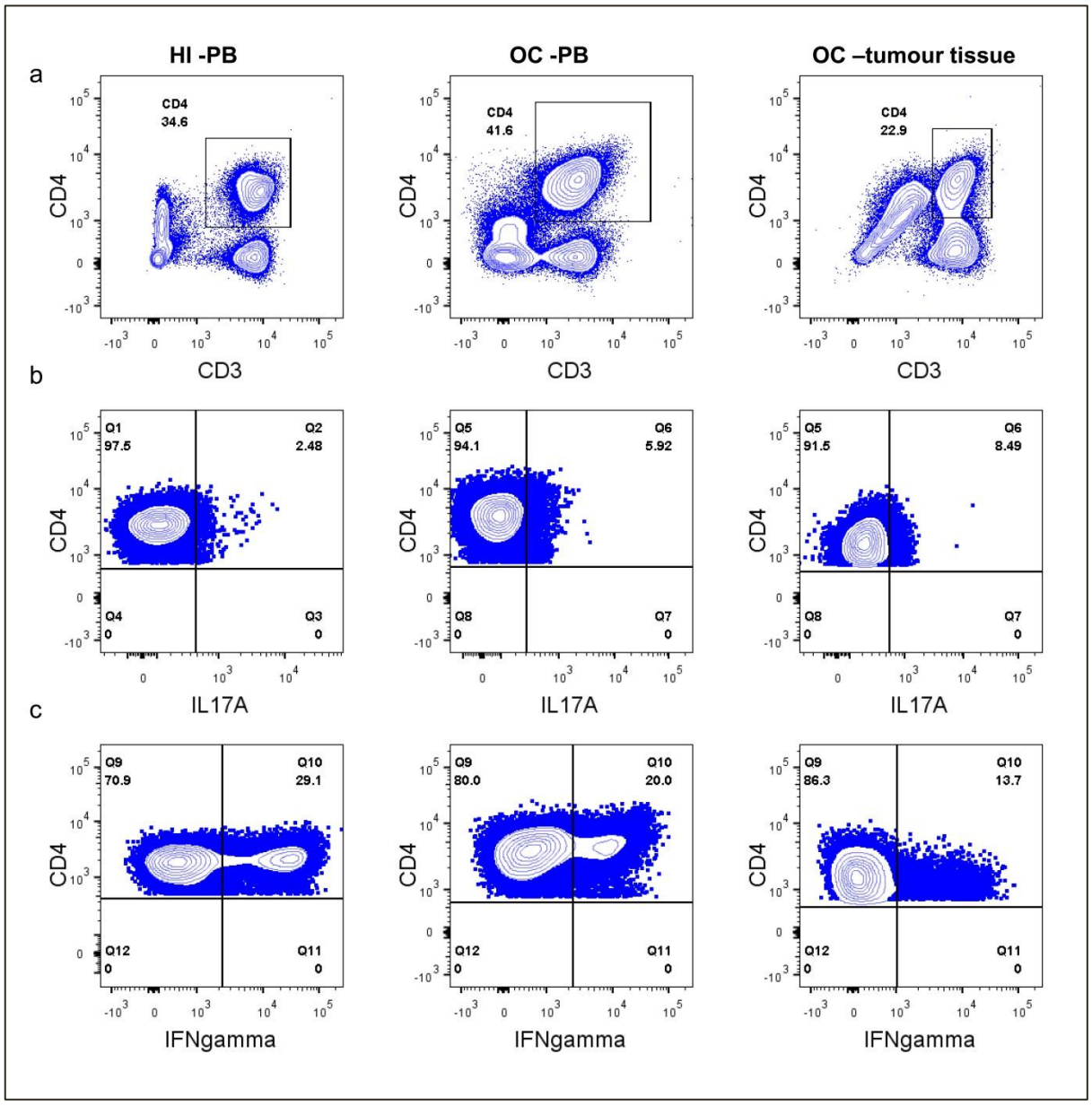

Figure 7.30: Presence of Th17, and Th1 in HIs and OSCC patients. Gating strategy to define the Th17 and Th1 cells in the peripheral blood and tumor tissue of OSCC patients, $CD3^+CD4^+$ cells were gated from which $IL17^+$ and $IFN\gamma^+$ producing cells were analyzed **(a)** Representative contour plot showing the percentage of $CD3^+CD4^+$ cells, **b)** Contour plot showing percentage of Th17 ($CD4^+IL17^+$) and **c)** showing percentage of Th1 cells ($CD4^+IFN\text{-}\gamma^+$) in the peripheral blood of HIs (left), peripheral blood of OSCC patients (middle) and tumor tissue (right).

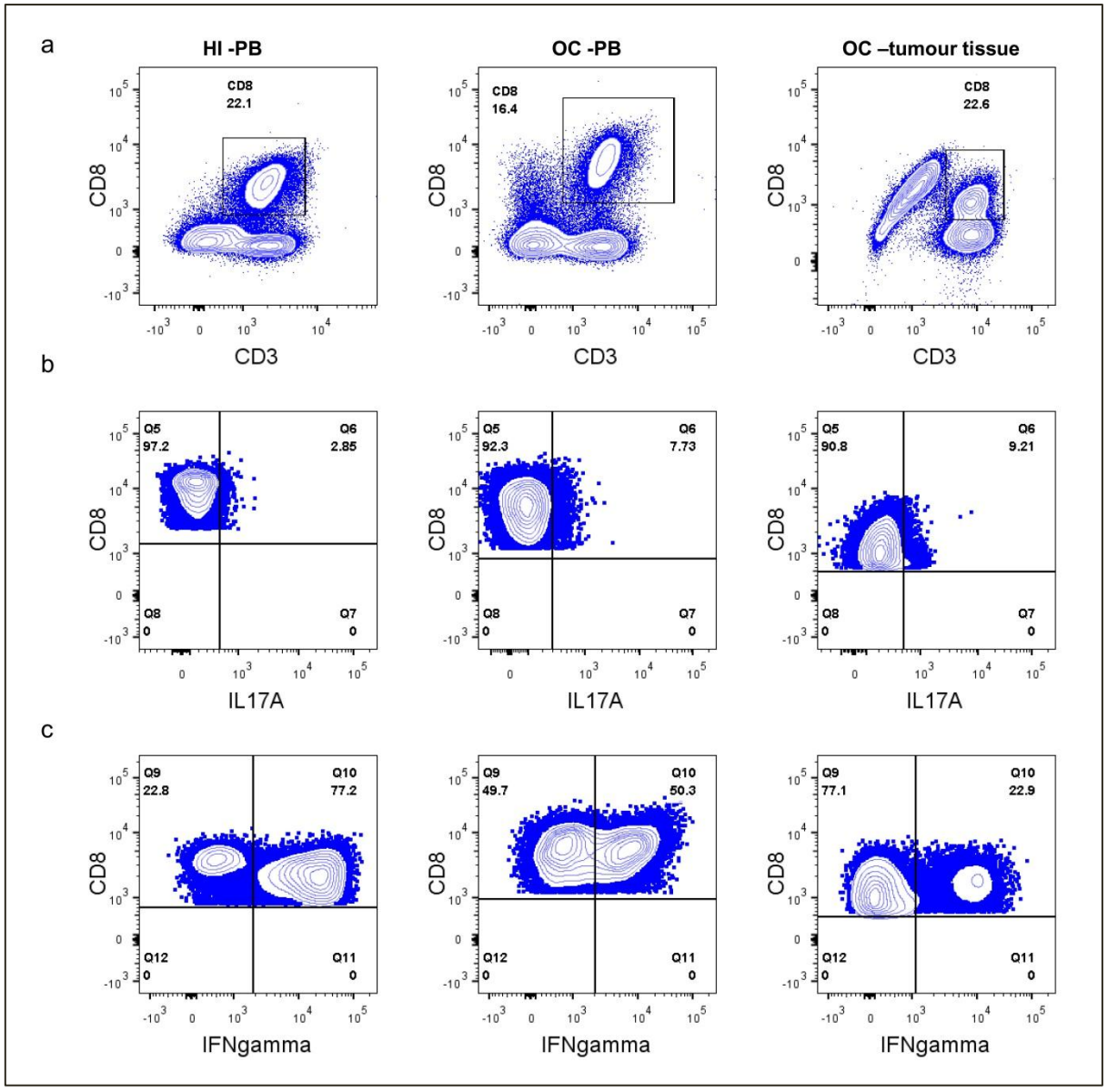

Figure 7.31: Frequency of Tc17, and Tc1 in HIs and OSCC patients. Gating strategy to define the Tc17 and Tc1 cells in the peripheral blood and tumor tissue of OSCC patients, $CD3^{+}CD8^{+}$ cells were gated from which $IL17^{+}$ and $IFN\gamma^{+}$ producing cells were analyzed **(a)** Representative contour plot showing the percentage of $CD3^{+}CD8^{+}$ cells, b) Contour plot showing percentage of Tc17 (CD8+IL17+ and c) showing percentage of Tc1 cells ($CD8^{+}IFN\text{-}\gamma^{+}$) in the peripheral blood of HIs (left), peripheral blood of OSCC patients (middle) and tumor tissue (right).

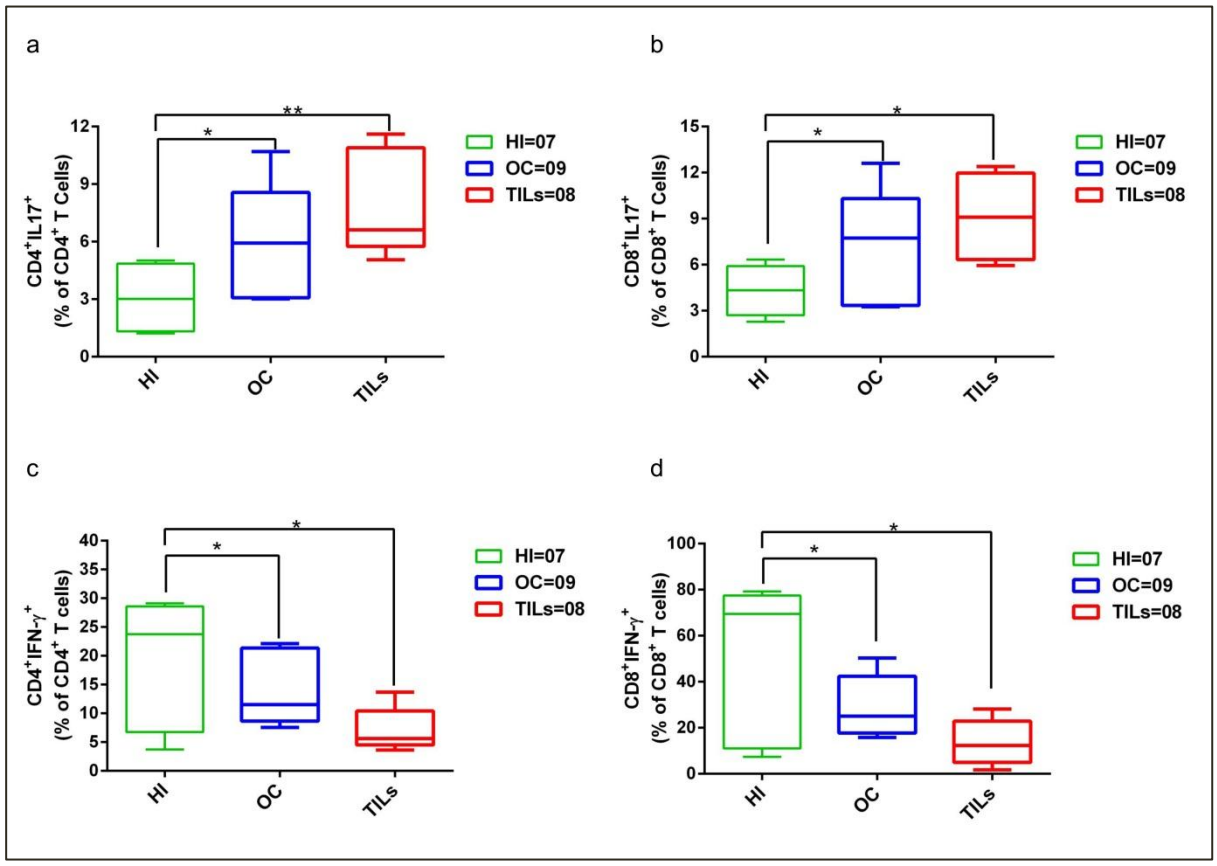

Figure 7.32: Frequency of IL17 and IFN-γ producing cells in the HIs and OSCC patients. (a) A summarized data shows percentages of Th17: **b)** Tc17 in PBMCs and TILs of OSCC patients compared to HIs. **(c)** Frequency of $CD4^{+}IFN\gamma^{+}$ d) $CD8^{+}IFN\gamma^{+}$ cells in PBMCs and TILs of OSCC patients compared to HIs. Data shown from PBMCs (n=09) and tumor tissue (n=08) of OSCC patients and HIs (n=07). Numbers in the plot indicate percent positive cells. Data is analyzed by Mann-Whitney test. (* represents $p < 0.05$; ** represents $p < 0.005$)

7.2.19 T cells derived from the peripheral blood and tumor tissue of OSCC patients are terminally differentiated representing exhausted phenotype

Single cell suspension from oral tumors and mononuclear cells from heparinized venous peripheral blood from healthy individuals and oral cancer patients were analyzed by multiparametric flow cytometry. The cells were then stained with cocktail of labelled antibodies and were acquired on FACSAria. The gating strategy to evaluate different subsets of T cells is shown in **Figure 7.33**. The results confirmed the decreased percentage of $CD3^{+}$ T cells in the peripheral blood of oral cancer patients (n=75) compared to the healthy

individuals (n=45) **(Fig.7.34a).** CD3 is co-receptor of TCR which distinguishes T lymphocytes from other lymphocytes i.e., B cells and NK cells and is vital for propagating the signal from the TCR to cell. The percentage of helper T cells ($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$) were also significantly decreased in peripheral blood of oral cancer patients compared to the healthy individuals **(Fig.7.34b-c)**. The frequency of γδ T cells was also decreased in the peripheral blood of OSCC patients suggesting that both αβ as well as γδ T cells levels were altered in oral cancer patients **(Fig.7.34d)**. Immune profiling of oral tumors depicts infiltration of all the T cell subsets to a higher extent. Infiltration of T cells in oral tumors could be important in rejuvenating the antitumor immunity.

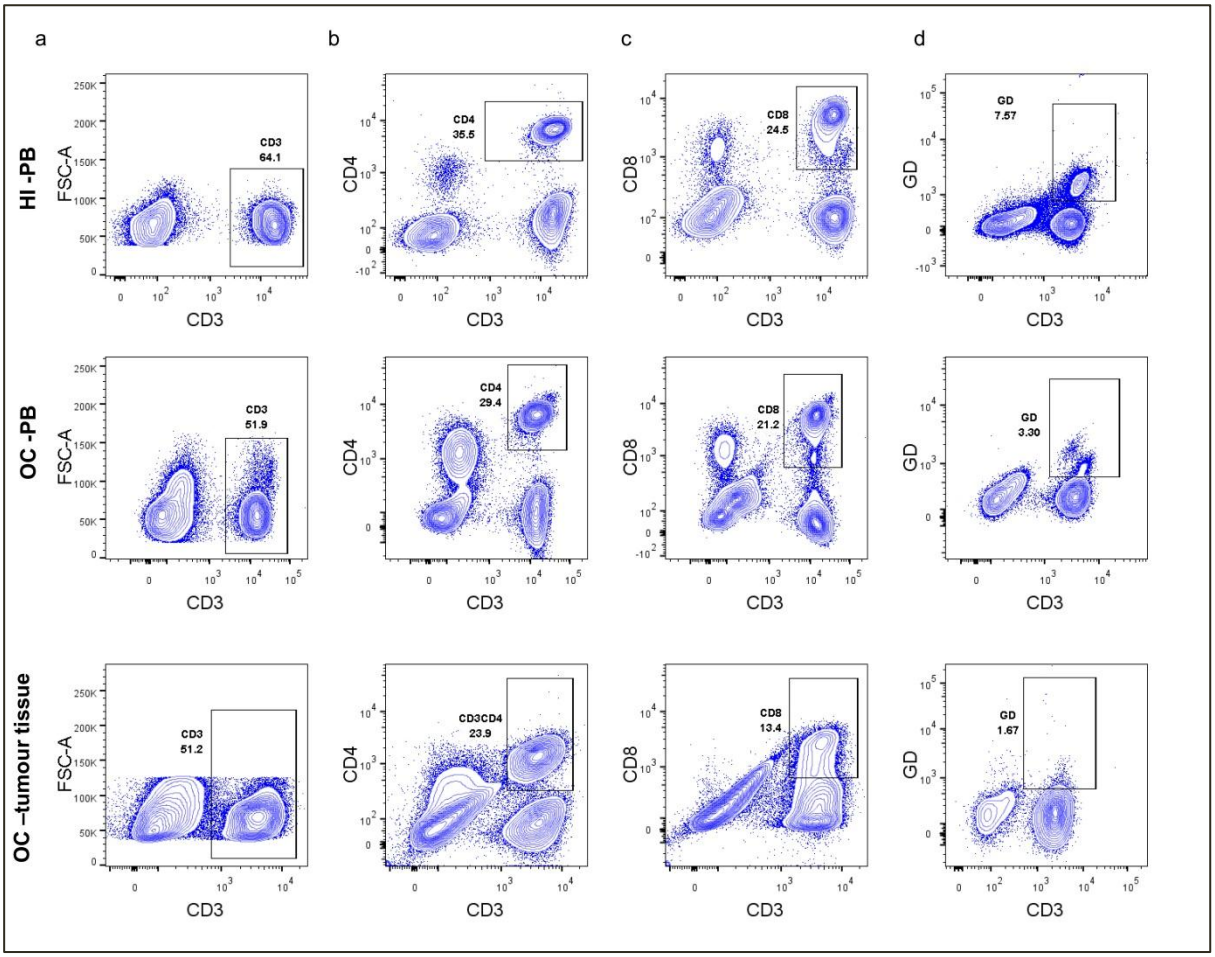

Fig 7.33: Gating strategy to analyze the percentage of effector T cells in the peripheral blood and tumor tissue of OSCC patients. The peripheral blood mononuclear cells (PBMCs) were identified using FSC/SSC plot. The PBMCs were gated and plotted as **a)** CD3 fluorescence on x-axis and FSC on y-axis **b)** CD4 fluorescence on the y-axis versus CD3 fluorescence on the x-axis. **c)** CD8 fluorescence on the y-axis versus CD3 fluorescence on the

x-axis. **d)** γδ fluorescence on the y-axis versus CD3 fluorescence on the x-axis. The upper panel represents peripheral blood of HIs; middle panel of OSCC patients and lower panel represents tumor tissue of OSCC patients.

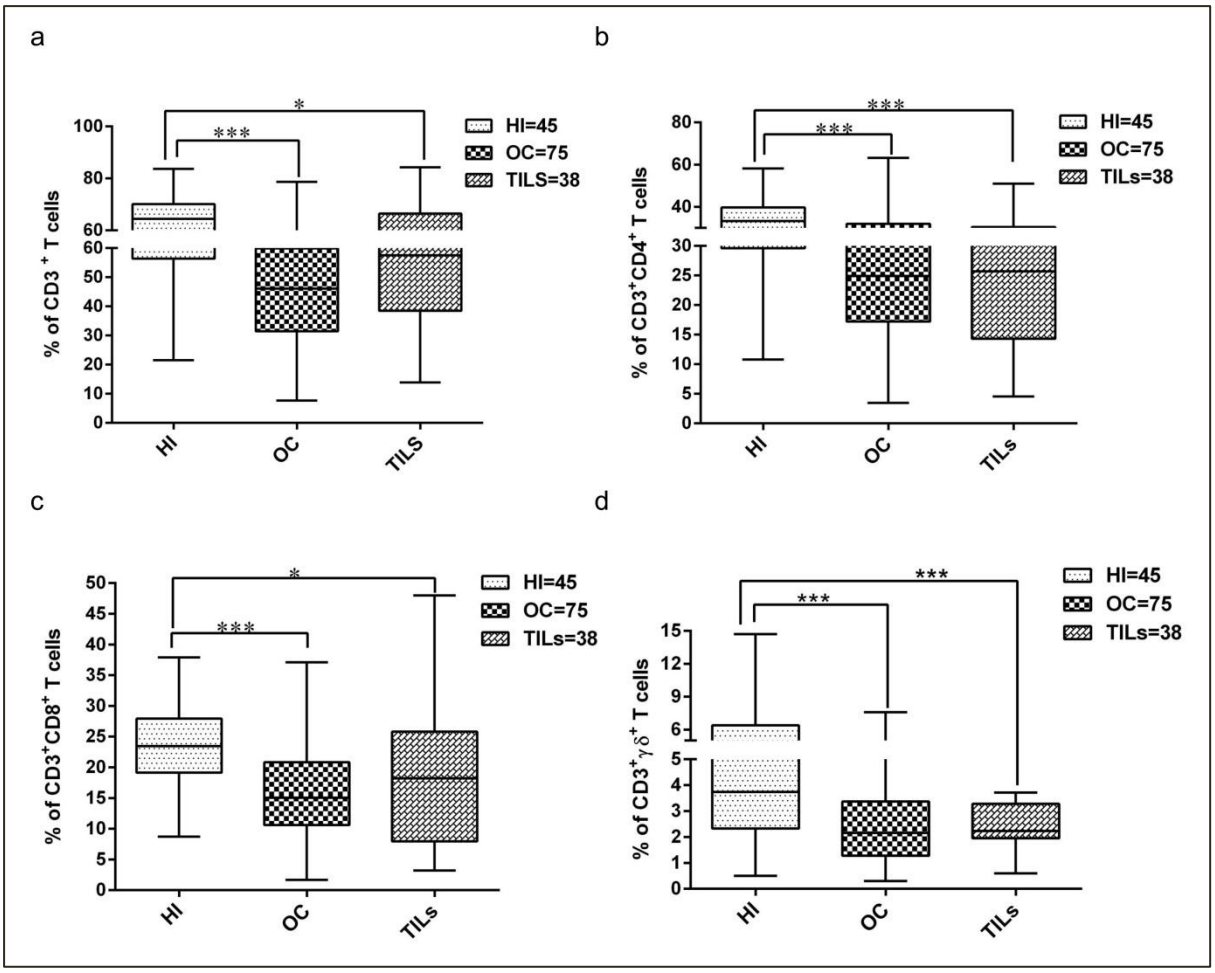

Figure 7.34: Frequency of effector cells in the peripheral blood and tumor tissue of OSCC patients. The whisker plots show the percentage of Total $CD3^+$ **(a**), $CD3^+CD4^+$ **(b)** , $CD3^+CD8^+$ **(c),** and $CD3^+$ $\gamma\delta^+$ **(d)** in healthy individuals (HI=45), peripheral blood of OSCC patients (n=72) and OSCC tumor tissue (n=38). (* represents $p<0.05$ and *** represents $p<0.0005$).

The memory T cell pool functions as a dynamic repository of antigen-experienced T lymphocytes that accumulate over the lifetime of the individual. Memory T lymphocytes contain distinct populations of central memory (TCM) and effector memory (TEM) cells characterized by distinct homing capacity and effector function. To investigate the memory status of lymphocytes, T cells were stained with CD3, CD4, CD8, CD27 and CD45RA antibodies and analyzed by flow cytometry. the cells were classified as naïve ($CD27^+CD45RA^+$), central memory (CM, $CD27^+CD45RA^-$), effector memory (EM, $CD27^-$

CD45RA$^-$) and terminally differentiated or effector memory RA (TEMRA, CD27$^-$CD45RA$^+$) Results showed that in OSCC patients, T cells are majorly of effector phenotype (EM+TEMRA) with cells in tumor compartment mainly of TEMRA phenotype (CD27$^-$ CD45RA$^+$) suggesting that the chronic exposure to tumor antigens is resulting in the exhaustion of T cells **(Fig. 7.35a-d).**

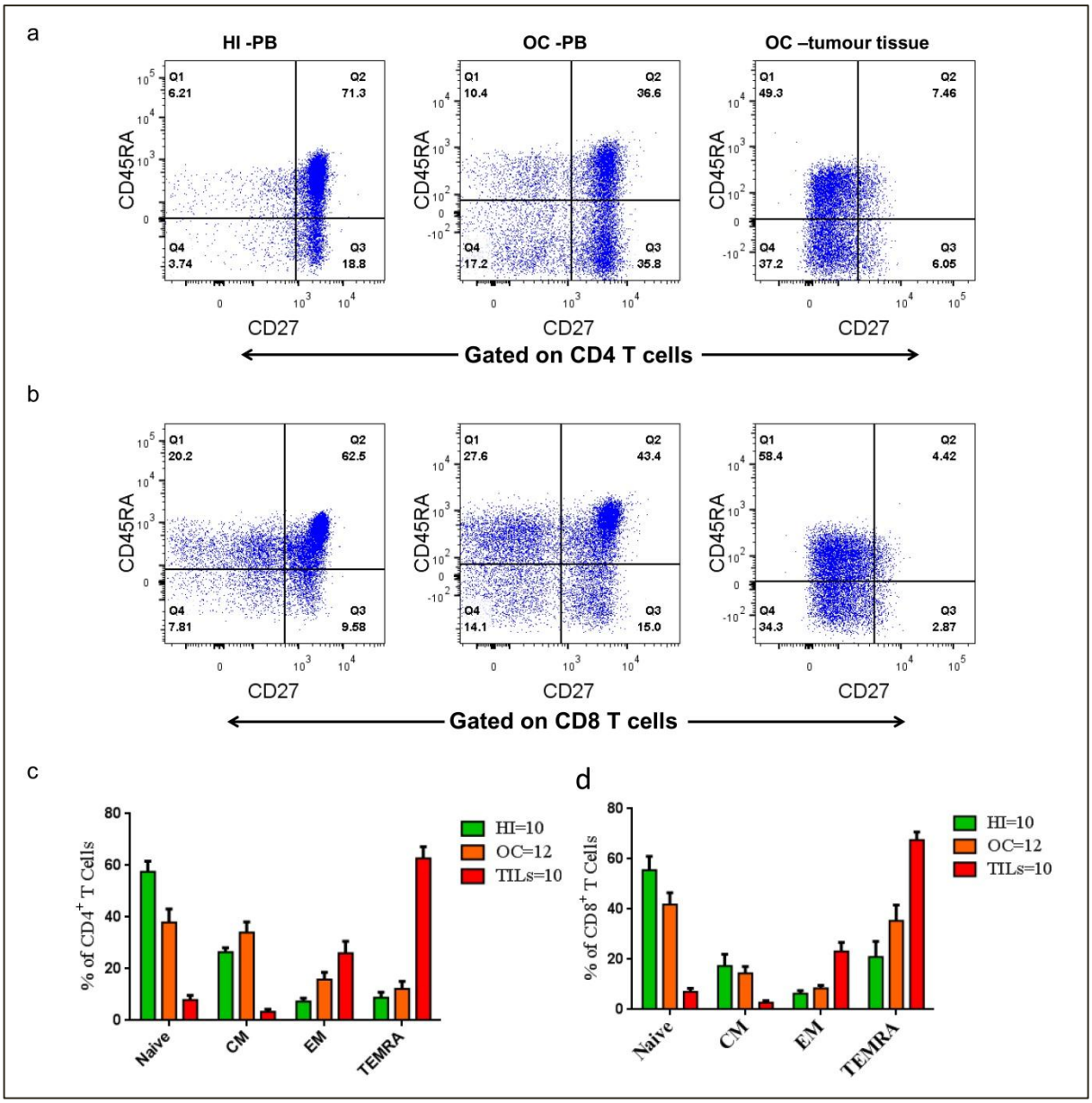

Figure 7.35: Memory status of T cell subsets in the peripheral blood and tumor tissue of OSCC patients. The peripheral blood mononuclear cells (PBMCs) were identified using FSC/SSC plot. **a)** The PBMCs were gated and plotted as CD3 fluorescence on x-axis and CD4 fluorescence on the y-axis. The gated CD3$^+$CD4$^+$ T cells were further extrapolated for CD27 and CD45RA fluorescence. The dot plots show the percentage of naïve, CM, EM, and TEMRA population within the gated CD3$^+$CD4$^+$ T cells. b) Shows the percentage of naïve

CM, EM and TEMRA within the gated $CD3^+CD8^+$ T cells of HIs (left), peripheral blood of OSCC patients (middle) and tumor tissue of OSCC patients (right). **c)** the graph shows the summarized data shows percentages naïve, CM, EM, and TEMRA population within gated $CD3^+CD4^+$ T while **d)** shows on the gated $CD3^+CD8^+$ T cells in PBMCs and TILs of OSCC patients compared to HIs.

7.2.20 The tumor compartment of OSCC patients are infiltrated by DN Tregs

Tumor infiltrates (n=38) were analyzed for different T cell subsets and it was found that the $CD3^+CD4^-$ $CD8^-$ DN T cells were present in tumor tissue **(Fig.7.36a, c).** Although DN T cells were also found in the peripheral blood, the majority of them (>85%±5) expressed γδ TCR^+ **(Fig. 7.36b)**. Accumulating evidence has demonstrated that $CD3^+CD4^-CD8^-$ DN T cells have regulatory phenotype and play an important role in the maintenance of immunologic self-tolerance and in down-regulating various immune responses. DN T cells expressed comparable levels of CD3-ζ chain as was observed in the $CD3^+$ T cells of healthy individuals (**(Fig. 7.36d)**. DN T cells were also analyzed for the markers involved in immune suppression (FasL, CTLA4 and FoxP3) **(Fig. 7.36e).** DN T cells expressed these markers but with varying degree suggesting that prevalence of DN T cells in tumor might be promoting tumor growth by inhibiting the anti-tumor immune response. Altered frequency of double negative T cells could be attributed to presence of defect in events controlling T-cell lineage choice and selection in the thymus or it could be due to extra-thymic generation of these cells.

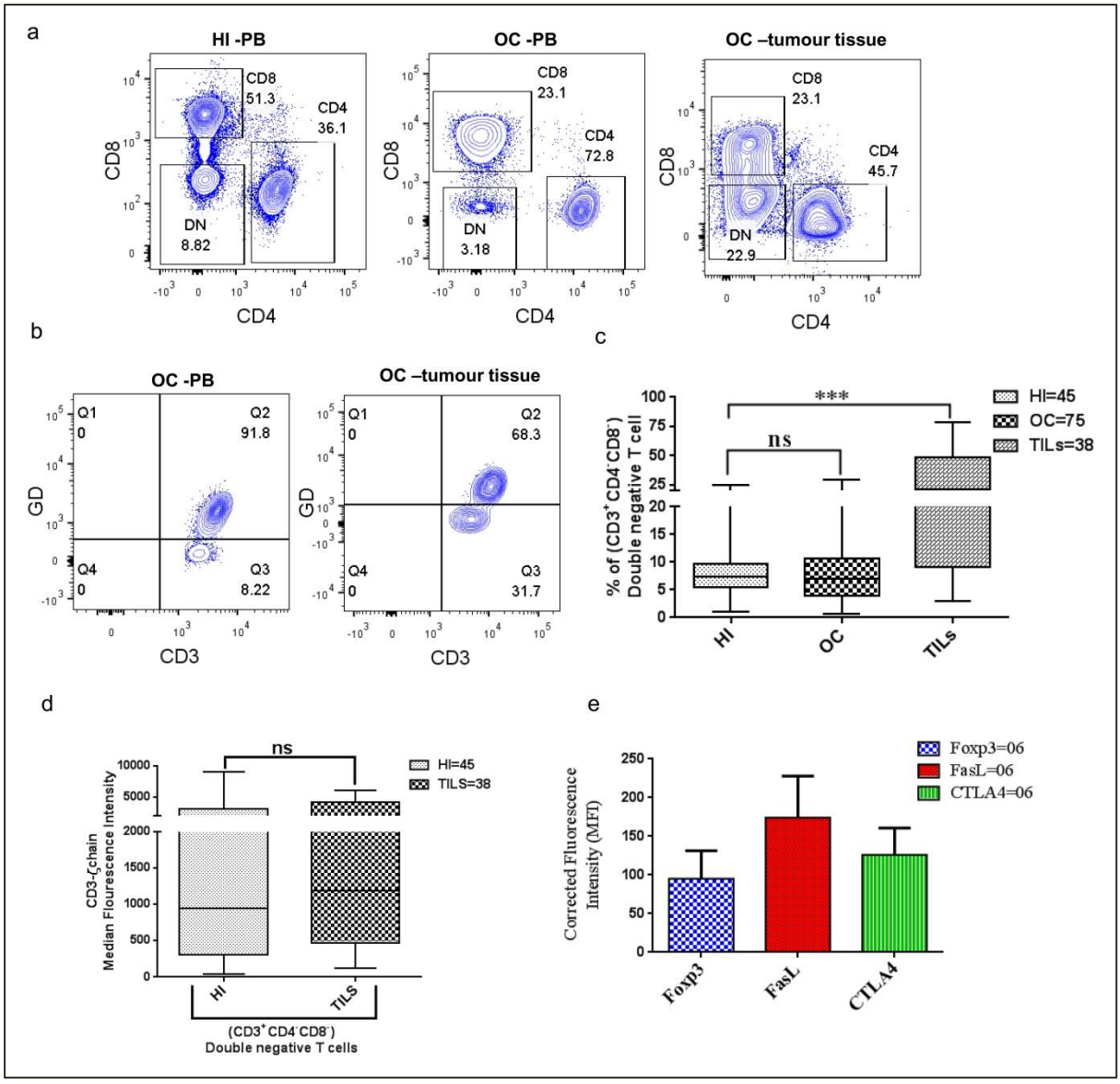

Figure 7.36: Frequency of double negative T cells in the peripheral blood and tumor tissue of OSCC patients. **a**) The peripheral blood mononuclear cells (PBMCs) were identified using FSC/SSC plot. The PBMCs were gated and plotted as CD3 fluorescence on x-axis and FSC on y-axis. The gated $CD3^+$ T cells were plotted as CD8 fluorescence on the y-axis and CD4 fluorescence on the x-axis. The $CD4^-CD8^-$ T cells (DN T cells) were analyzed and the dot plot shows the percentage of DN T cells in the peripheral blood of HI (left), OSCC (middle) and tumor tissue of OSCC patients (right).**b)** Shows the percentage of $CD3^+$ γδ TCR^+ cells (GD) in the gated DN T cell population in the peripheral blood (left panel) and tumor tissue of OSCC patients (right panel). **c)** The whisker plot shows the percentage of $CD3^+CD4^-CD8^-$ (DN) T cells in HIs (45=25), peripheral blood of oral cancer patients (n=75) and tumor tissue of OSCC patients (n=38) in order from left to right. **d-e)** Shows the presence of different effector molecules associated with the T cell function. (*** represents $p<0.0005$ and ns indicates non-significant data with $p>0.05$).

7.3 Discussion

T lymphocytes isolated from tumor tissues display a profound deficiency in their ability to respond to mitogenic stimulation and their effector functions are severely compromised [499]. One possible explanation for this is the presence of the immunosuppressive cells (like MDSCs and Tregs) in the tumor microenvironment [500]. MDSCs are immune-modulatory cells that exhibit suppressive activities on adaptive immunity and aid tumor progression and metastasis [218]. These cells represent an important barrier that most likely limit the full potential of immune-based cancer therapies or endogenous host responses to developing tumors. The present study was carried out to demonstrate how MDSCs influence T cell function in OSCC patients and how these cells interact with other immunosuppressive cells in these patients to promote immune evasion of tumors. In this study, MDSCs were identified and characterized in OSCC patients. Different markers have been used to analyze human MDSC subtypes in various clinical settings. Most studies concur with the observation that MDSCs express CD11b and CD33 but lack the expression of markers of mature myeloid cells, such as CD80, CD40, and HLADR [501]. In OSCC patients, a significantly higher percentage of MDSCs (mean = 13.75%) was observed and is likely due to the use of fresh rather than frozen PBMCs in our study. This also concurs with the data reported by Wang and colleagues in gastric cancer patients [502]. The accumulation of immature myeloid cells lin(CD3/CD19/CD56/CD14)$^-$HLADR$^-$CD33$^+$ in the blood correlated with the tumor burden, as well as with the stage of the disease in two independent studies that included diverse types of cancer [222, 272]. In addition, CD14$^+$HLADR$^-$CD11b$^+$, as well as CD14$^+$CD15$^+$HLADR$^-$ CD11b$^+$, MDSCs have been described in patients with melanoma or renal cell carcinoma (RCC) [250, 483]. In our study of OSCC patients, it was consistently observed that cells lacking mature lymphoid and myeloid marker (HLADR) and expressing dual myeloid markers CD33 and CD11b were higher. This phenotype is also reported to be increased in

melanoma/ colorectal patients and correlated with poor responsiveness and disease severity [503-505]. Our results are consistent with others showing that levels of MDSCs correlate linearly with tumor burden. Compared to the patients of stage I/II, patients of III/IV stage had significantly elevated number of circulating MDSCs. However, there was no difference in the percentage of circulating MDSCs by tumor subsites in OSCC patients which is in accordance with the data reported earlier in subgroups of hypopharynx/larynx compared to oral cavity in HNSCC patients. Close correlation between expansion of MDSCs and tumor burden indicates MDSCs may play a role in tumor invasion and metastasis in OSCC patients and consequently influence the clinical outcomes of OSCC patients. The OSCC patients have high cargo of bacteria which might be inducing myeloproliferation in these patients leading to differentiation arrest of myeloid-committed haematopoietic progenitors [506]. The mutations of Notch-1 and 2 in HNSCC have appeared to be loss-of-function mutations [507]. Inactivation of Notch signaling in mouse hematopoietic stem cells (HSC) had resulted in an aberrant accumulation of granulocyte/monocyte progenitors (GMP) [508]. This could be another reason behind the abnormal generation and accumulation of immature myeloid cells (MDSCs) in OSCC patients.

In addition, another subpopulation of cells lacking the expression of HLADR but expressing only CD11b was observed in the peripheral blood of OSCC patients. This population was marginally decreased in the peripheral blood of OSCC patients compared to healthy individuals. However, in tumor compartment, this subset of $HLADR^{-}CD33^{-}CD11b^{+}$ phenotype was present predominantly. Surprisingly, the subset dominating the peripheral blood was rarely present in the tumor compartment. Exhaustive analysis of subsets suggest that MDSCs present in peripheral blood express CD14, CD15, CD80, and CD124 but lack CD66b, CD16, CD34 and CD38 while that present in tumor compartment express CD16,CD15, CD66b lack expression of CD80, CD124, CD34 and CD38. Results confirm

that monocytic MDSCs (Mo-MDSCs) are dominant in the periphery whereas granulocytic MDSCs (G-MDSCs) are present in the tumor compartment. Further, our data reported the overlap of CD14 and CD15 expression in MDSCs subset present is the peripheral blood of OSCC patients and is consistent with data reported in subgroup of HNSCC, colon cancer and melanoma patients [226, 509]. In other words, these data along with ours suggest that CD14 and CD15 may not clearly define the morphologic subtypes of MDSC in the human system which is currently used by the majority in the field. This discrepancy of accumulation of different subsets of MDSCs in OSCC patients could be attributed to the nature of OSCC tumors which are ruled by hypoxia, heat shock proteins (HSPs) and microbiota. These factors have the ability to regulate function and differentiation of MDSCs into macrophages in tumor microenvironment [218, 510]. Accordingly, the presence of monocyte–macrophage myeloid cells in OSCC tumor tissue was established and could be identified as HLA-DR^{+} cell expressing markers like CD33, CD11b, CD80, CD68, and CD163. These cells were IL-10^{high} and IL-12^{low} which is a trait of M2 macrophages promoting tumor growth. This is also in accordance to the earlier data reported in bladder cancer showing accumulation of both G-MDSCs and monocytic origin macrophages in tumor tissue [511]. There are studies which have shown accumulation of G-MDSCs in the peripheral blood of cancer patients which in our case were marginally decreased compared to HIs [512, 513]. Although the G-MDSCs were present in the peripheral blood, we did not observe increased levels of G-MDSCs in the peripheral blood of OSCC patients compared to HIs. The lack of G-MDSCs accumulation in our case could be attributed to the fact that PBMCs separated by ficoll density gradient were used to characterize MDSCs which might have ignored G-MDSCs.

The peripheral blood of OSCC patients had elevated numbers of Mo-MDSCs suggest that they might have mechanism preventing their apoptosis. However, Mo-MDSC goes through spontaneous apoptosis compared to their mature counterparts (HLADR^{+} cells) in the

peripheral blood. This is similar to what is observed by Condamine and colleagues showing that MDSCs have less lifespan than monocytes and polymorphonuclear cells (PMNs) [494]. The higher levels of apoptotic genes also reflected the tendency of MDSCs to undergo spontaneous turnover. The expression of NF-κB and p53 might be regulating the expression of DR-5 which is reported to regulate the shorter survival of MDSCs [494]. The presence of higher levels of ROS and its regulator p66Shc observed in Mo-MDSCs suggest that mitochondrial dysfunction could be one of the mechanisms leading to the spontaneous turnover of Mo-MDSCs in OSCC patients. The presence of ROS creates inflammatory BM microenvironment which in turn promotes oxygen consumption and HIF-1α expression throughout the BM and can induce emergency myelopoiesis [216, 514]. This in turn can promote progenitor cell expansion and mobilization from BM to peripheral blood [515]. Manipulating p66Shc might offer a possibility of reducing ROS and thereby decreasing inflammation induced emergency myelopoiesis which is a possible source of MDSCs in tumor bearing host. All trans-retinoic acid (ATRA) through activation of ERKI/2 can cause upregulation of ROS scavenger Glutathione (GSH) and decrease ROS which in turn can successfully induce differentiation of MDSCs in both mice and humans [223, 264]. Similar to ATRA, catalase (ROS scavenger) can also lead to differentiation of MDSCs obtained from tumor bearing mice [265]. This confirms that targeting ROS or its upstream regulator will have potential to disrupt the differentiation halt in MDSCs.

The Mo-MDSCs subset, $HLADR^{-}CD33^{+}CD11b^{+}$, identified in OSCC patients was capable of suppressing T cell proliferation in a dose dependent manner. MDSCs from myeloproliferative disease like chronic myeloid leukemia [516] or in chronic lymphocytic leukemia patients [517] were also reported to be monocytic and were suppressive. Our data showed that Mo-MDSCs could suppress IFN-γ production and expression level of CD3-ζ chain on T cells in coculture assays. The effect of MDSCs on the expression of CD3-ζ chain was more

prominent on $CD8^+$ T cells and marginal on CD4+ T cells. Inhibition of CD3-ζ chain by MDSCs appears to be MHC restricted and thus affected majorly $CD8^+$ T cells. This corroborates earlier report showing inhibition by MDSCs is dependent on MHC class I expression on the MDSCs [518]. However, in case of OSCC patients the expression level of CD3-ζ chain was decreased to the same level across all the subsets of T cells. This suggests that other factors apart from MDSCs may be contributing to the downregulation of CD3-ζ chain in OSCC patients. MDSCs also produce soluble factors which can affect T cell function non-specifically. Diminished granzyme B and perforin production by T cells of OSCC patients may render them dysfunctional. The T cells infiltrating the tumor of OSCC patients are functionally exhausted as portrayed by their phenotype ($CD27^-CD45RA^+$) and expression of cytolytic effector molecules (perforin and granzyme B). Increased prevalence of MDSCs in patients with OSCC may be compromising the cytolytic effector function of T cells at least in part by inhibiting their activation and expression of perforin and granzyme. This may further compromise the T cell mediated tumor cell killing in OSCC patients and thereby evade immune recognition. Our data showed that MDSCs down-regulate L-selectin levels on T cells in coculture assay. It was interesting to note that reduced L-selectin expression on T cells was observed in OSCC patients which could be correlated with the increased levels of MDSCs in these patients. This will also decrease the ability of T cells to home to sites where they would be activated and could be another mechanism by which MDSCs inhibit antitumor immunity. These findings, together with the increased number of MDSCs in the peripheral blood and tumor tissue, establish the immunosuppressive functions of MDSCs in OSCC patients.

The expansion and activation of MDSCs is a two-signal model, one signal is primarily responsible for MDSC expansion and the second one for driving MDSC activation [227]. The various cytokines and growth factors regulating these two steps include GM-CSF, M-

CSF, G-CSF, IL-6, VEGF, IL-1β, IL-6, cyclooxygenase 2 (COX2)–generated PGE_2, IL-10, TGF-β, IDO, fms-related tyrosine kinase 3 ligand (FLT3L), stem cell factor (SCF), IFN-γ,IL-13, TLR ligands etc. [227, 519]. Our data showed higher levels of IL-6, IL-1β, TNF-α, VEGF, PGE_2 in the sera of OSCC patients. We also observed the expression of TLR2, 3, 4 and 9 on Mo-MDSCs. Further, the presence of HSPs in oral tumor cells may be activating MDSCs via TLR4 signaling. This data indicates that signals required for MDSC expansion and activation are present in OSCC patients. Cytokines like IL-6, TNF-α, IL1-β and IL-10 can directly activate STAT3 signaling which prevents differentiation of mature myeloid cells and promotes proliferation of immature myeloid cells.

The combination of GM-CSF with IL-6 has also been shown to cause sufficient expansion of MDSCs under *in vitro* conditions [519]. The MDSCs from OSCC patients expressed higher levels of pSTAT3 and the presence of STAT3 regulated Arginase 1 and IDO in MDSCs indicates the mechanism used by MDSCs to suppress T cell proliferation and decrease CD3-ζ chain expression in T cells. Further, the STAT3 dependent differential regulation of C/EBPα and C/EBPβ in MDSCs predicts that this pathway has capability to alter myelopoiesis. STAT3 dependent upregulation of C/EBPβ and downregulation of C/EBPα can shift the myelopoiesis from steady state to emergency state leading to generation of MDSCs [520, 521]. Notch signaling playing a crucial role in inducing the emergency myelopoiesis as Hes1 (Notch target gene) is known to inhibit the C/EBP-α hence shifting myelopoiesis to emergency state [497]. Hes1 is also known to interact with STAT3 [498] and the presence of the components of Notch signaling pathway depicts the relevance of this interaction in MDSCs. It appears that the coordinated action of these two signaling pathways regulate myelopoiesis and hence MDSC generation. This data signifies that the STAT3 driven signaling would be responsible for MDSC expansion and function in OSCC patients. The inhibition of STAT3's in MDSC function is clinically important. Epidermal growth factor

receptor (EGFR)/signal transducer and activator of transcription 3 (STAT3) and PI3 kinase/PTEN/Akt pathways are activated in HNSCC including the subgroup of OSCC patients [522, 523]. EGFR overexpression correlates with aggressive tumor behaviour and poor clinical outcome [524]. EGFR signals through STAT3 and anti-EGFR therapy (Cetuximab) is used to treat HNSCC patients [525]. Inhibition of STAT3 signaling in OSCC patients will not only inhibit the EGFR mediated tumor progression, but will also help in rejuvenating the antitumor immunity by inhibiting the generation and function of MDSCs.

The presence of immune inhibitor molecules like IL-10 and PDL1/PDL2 observed by us on MDSCs are suggestive of the diverse mechanisms that are utilized by MDSCs to promote immune suppressive network in OSCC patients. Although, it is well known that IL-10 is an important mediator of Treg suppression, IL-10 can also potentiate the differentiation of iTregs from naïve CD4 T cells [526, 527]. This confirms that the dynamic crosstalk between MDSCs and Tregs is on in OSCC patients. Accordingly, the levels of Tregs observed were higher in OSCC patients and correlated with the stage of OSCC patients. Like MDSCs, Tregs did not show any subsite specific variation of numbers in OSCC patients. The Tregs from the tumor tissue of OSCC patients were $FOXP3^{high}$ compared to those present in the peripheral blood indicating the presence of highly immunosuppressive microenvironment. This property of Tregs might be induced by the higher levels of TGF-β and IL-10 present in the OSCC tumor microenvironment [528, 529]. The higher accumulation of Tregs at tumor site could be through the migration of Tregs via CCR6-MIP3α/CXCR4-SDF-1α axis where these cells are stabilized by TGF-β and IL-10 presence. The induction of memory phenotype in Tregs ($CCR6^{high}$) could be due to TCR ligation along with cytokines. $CCR6^{+}$ Treg cells can be considered as the regulatory equivalent to $CD4^{+}$ effector-memory T cells. CCR6 appears associated directly with effector-memory function and mediates migration into peripheral sites via CCL20 and β-defensin [530, 531]. CCL20 is expressed mostly in inflamed tissues

and mucosal surfaces and β-defensin is secreted by macrophages and DCs on encounter with microbial pathogens. Both MIP3α/CCL20 and microbial pathogens are part of OSCC tumors. Thus, $CCR6^+$ effector T cells which act to promote anti-tumor immune responses in inflamed tissues will be counterbalanced by $CCR6^+$ Treg cells, thereby dampening the antitumor immunity.

The IL-10 secreted by the MDSCs showed a positive correlation with the levels of Tregs in OSCC patients. However, in vitro assay confirmed that MDSCs inhibited the TGF-β driven generation of iTregs from naïve $CD4^+$ T cells. This contradicted many earlier reports showing that MDSCs have capability to induce Treg generation [485, 486, 527]. IL-2 signaling is necessary for the induction of Tregs and MDSCs have ability to downmodulate the IL-2 signaling by preventing activation induced expression of α-chain of IL-2 receptor (CD25). Thus, MDSCs induced CD25 downregulation which prevent activation of T cells interferes with the TGF-β induced generation of Tregs. The MDSCs in coculture were able to inhibit the TCR driven upregulation of CD25 in T cells. The engagement of PD-1 expressed on T cells with PD-L1 expressed by MDSCs can downregulate the phosphorylation of STAT-5 by inhibiting IL-2 signaling. This is also in accordance with what is reported by Centuori and colleagues showing that MDSCs inhibit Treg generation in IDO dependent manner [491]. Our data was further validated by the fact that apart from IL-10, MDSCs also secreted IL-1β, IL-6 and expressed IL23A mRNA. IL-1β interferes with Treg generation by either inducing phosphorylation of STAT3 or alternative splicing of FoxP3 and promotes TH17 differentiation [532, 533]. MDSCs derived interleukin-1β (IL-1β) has been shown to curtail the anticancer immunity and also decrease the anticancer efficacy of the chemotherapy by inducing secretion of IL-17 by $CD4^+$ T cells [534]. Tregs were higher in OSCC patients suggesting that differentiating Tregs may be under the pressure of both negative signals and positive signals. However the positive signals favouring Treg expansion and function are

dominating in OSCC patients which cause their accumulation. The production of IL-β, IL-6 and IL23 by MDSCs advocates that they may alter differentiation of naive $CD4^+$ T cells into TH17 cells and our *in vitro* experiments confirmed this hypothesis. The ability of MDSCs to induce Th17 differentiation is also reported by different studies[490, 535]. The levels of MDSCs have been correlated with the levels of Th17 cells in esophageal cancer [536] and likewise levels of Th17 were also elevated in OSCC patients. The presence of MDSCs, Tregs and Th17 cells in OSCC patients will induce both chronic inflammation and T cell tolerance thereby promoting OSCC progression.

The immunotherapy is likely to succeed if tumor mediated immunosuppressive networks are blocked. To accomplish this, the extent of immunosuppression should be measured accurately. This is the first study in OSCC patients which has systematically evaluated the immune suppressive network. These results add to our understanding of the immunobiology of OSCC patients. This may also be informative for future immunotherapeutic trials. There are excellent evidences suggesting that MDSCs, Tregs and Th17 are detrimental for antitumor immunity and that removal of these cells or by blocking their function or by using checkpoint inhibitor antibodies will greatly improve antitumor responses in OSCC patients.

Chapter 8
Summary and Conclusion

Summary and Conclusion

In recent years no significant progress in the management of oral cancer has been noted. For early stage oral cancer, simple surgical excision or radiotherapy is usually adequate but, in the late stage of oral cancer, a combination therapy such as, surgical excision with post-operative radiotherapy or induction chemotherapy with surgical excision, has been suggested. In spite of preoperative or adjuvant chemotherapies, the prognosis of patients with advanced OSCC still remains poor. This may be due to a systemic immunosuppressive effect that cancer exerts in these patients. Therefore, identification and characterization of the immune players in OSCC patients may aid in their treatment

Immune dysfunction is a hallmark of patients with oral cancer. The effect of a progressively growing tumor on the immune response presents a significant challenge to the success of immunotherapy and cancer vaccines. A better understanding of the factors that cause immune dysfunction in OSCC patients might be relevant for the development of new therapeutic or prophylactic approaches. The aim of this study was to understand the role of immuno-suppressive factors/cells which are utilized by the OSCC tumors to evade immune destruction mainly by affecting T cell functioning. This will provide a rationale for designing immunotherapies which would be aimed at changing the tumor microenvironment from tumor-promoting to tumor-rejecting.

Lymphocytes especially T cells are important players in the antitumor immune response but T cells from cancer patients are reported to be dysfunctional with decreased CD3-ζ chain expression, a key T cell signaling molecule. Several distinct mechanisms may be responsible for decreased/absent expression of CD3-ζ chain in T cells of patients with cancer. CD3-ζ chain has appeared as a prognostic marker which can predict a favourable response to biological therapies and could be helpful in a selection of patients for immunotherapy trials [117]. Absent or low expression of CD3-ζ chain in TILs of patients with stage III or IV

HNSCC predicts a poor survival compared to patients expressing a normal CD3-ζ chain [138]. Our study in OSCC patients confirmed their T cells are severely impaired with respect to the decreased ability to proliferate, decreased CD3-ζ chain expression (key T cell signaling molecule), decreased expression of perforin, granzyme B, and CD62L and presence of terminally differentiated cells ($CD27^{-}CD45RA^{+}$). The decreased CD3-ζ chain expression associated with T cells of OSCC patients' might be contributed by the factors secreted by tumors. Studies from our laboratory have shown a detailed mechanism responsible of CD3-ζ chain downregulation with transcriptional defect in the tumor compartment and translational defect in the peripheral blood. In the present thesis, the role of tumor derived factors in downregulation of CD3-ζ chain was investigated. Different approaches were exploited to look at the possible candidates (proteins) which have the ability to alter CD3-ζ chain expression in T cells derived from OSCC patients. In one of the approach (pragmatic), the cell free supernatants from fresh OSCC tumors and cell lines were used to demonstrate their effect on CD3-ζ chain expression. The OSCC tumor cell free supernatants led to the progressive decrease in the CD3-ζ chain expression with marginal effect on the other key T cell signaling molecules (CD3-ε, ZAP70, p56Lck) while the cell free supernatant collected from established oral tumor cell lines failed to do so. These supernatants were subjected to proteomic analysis using LC-MS/MS to identify possible factor(s) that mediate downregulation of CD3-ζ chain expression. The proteins present exclusively in OSCC tumors were selected and Oligoadenylate synthetase (OAS2) was identified as a potential candidate leading to CD3-ζ chain expression. The presence of OAS2 in OSCC tumors was further confirmed at both mRNA and protein level. Results confirmed that the secreted OAS2 initiated downregulation of CD3-ζ chain expression in a caspase-3 dependent manner in T cells. For the first time, a potentially important function of tumor-derived Oligoadenylate synthetase (OAS2) as a paracrine negative regulator of T-cell functions was identified.

In another approach (idealistic), Tumor necrosis factor-alpha (TNF-α) which is pro-inflammatory cytokine and is reported to attenuate the T cell response was studied to analyze its effect on CD3-ζ chain downregulation. The mechanism employed by TNF-α, an important negative regulator of T cell function, was addressed. The effect of TNF-α on T cell function appears to be spatiotemporal. The T cells in OSCC patients are reprogrammed to overexpress TNFR-I receptor rendering them susceptible to TNF-α induced downregulation of CD3-ζ chain. Blocking TNFR-I prevented TNF-α induced CD3-ζ chain downregulation while blocking of TNFR-II enhanced the TNF-α induced CD3-ζ chain downregulation. The TNF-α stimulus effectively induced ROS and capase-3 levels in T cells which are known to be involved in the downregulation of CD3-ζ chain. The antagonist ligands aimed at a single TNF receptor family has on one hand potential to selectively turn off immunosuppressive microenvironment [537, 538]. However, at the same time, it might lead to TNF-α induced TCR signaling defect in antigen encountered T cells via TNFR-I. Therefore, to design future therapies using TNF inhibitors in different disease conditions these aspects should be taken into account.

Apart from negative regulators, this study has also unravelled the positive regulator of T cell signaling which have the competency to increase the antitumor immune response in oral cancer patients. The study provided new insights into mechanisms that integrate TCR and cytokine signals to determine the outcome of T cell response with a central role of Notch signaling. The Notch signaling was found to be one of the positive regulators of T cell signaling and inhibiting this signaling affected both proximal as well as distal TCR signaling molecules. The CD3-ζ chain was a proximal signaling molecule which was affected while ZAP70 and p56Lck remained unaffected. Inhibition of Notch signaling also induced T cell anergy reflected by increased calcium flux and induction of GRAIL. Thus manipulation of Notch signaling has ability to reverse the tumor induced anergy in T cells.

Both the molecules present in the tumor microenvironment, OAS2 and TNF-α, shared the ability of inducing caspase-3 levels in T cells. This insinuates that a common mechanism might be employed which involves caspase-3 activation as a functional readout. The most relevant phenomenon which appears to be ensuing is the induction of T cell anergy which is associated with the activation of caspase-3. The T cell anergy is a common feature in cancer patients due to high co-inhibitory and poor co-stimulatory molecules. The dampened Notch signaling observed in cancer patients might also be the reason behind the anergic T cells. In summary, tumor induced T cell anergy may be one of the immune evasion mechanisms in patients with OSCC. TNF-α, OAS2, and reduced Notch signaling may be the potential factors controlling T cell anergy. However, preclinical and clinical studies suggest that T cell dysfunction is functionally reversible. This paves the way for targeting T cell dysfunctional mechanisms and introducing/promoting T cell stemness to treat patients with cancer. Active specific immunotherapy in the form of prostate specific antigen (PSA) based vaccines results in the restoration of impaired CD3-ζ chain expression and generation of PSA-reactive circulating T cells [539]. Thus, immunotherapy targeting restoration of CD3-ζ chain expression might hold clinical importance for enhancing the efficacy of T cell based therapies. Chimeric antigen receptors (CARs) are recombinant receptors for antigen which in a single molecule redirect the specificity and function of T lymphocytes and other immune cells [540]. CD3-ζ chain is one of the fusion proteins of the receptor and modification of receptor domains of CAR receptors like CD3-ζ chain will provide T cells with array of signals required for their optimal clonal expansion and targeted immune responses.

The adoptive transfer of T cells, expanded ex vivo, into cancer patients is the most promising strategy. Unfortunately, the clinical investigation of immune-based therapeutic strategies, especially T cell transfer, has yielded disappointing results. This may be due in part to the

fact that these T cells are grown in the absence of immunosuppressive networks and when re-infused into patients have to expand in the presence of tumor-mediated immune suppression.

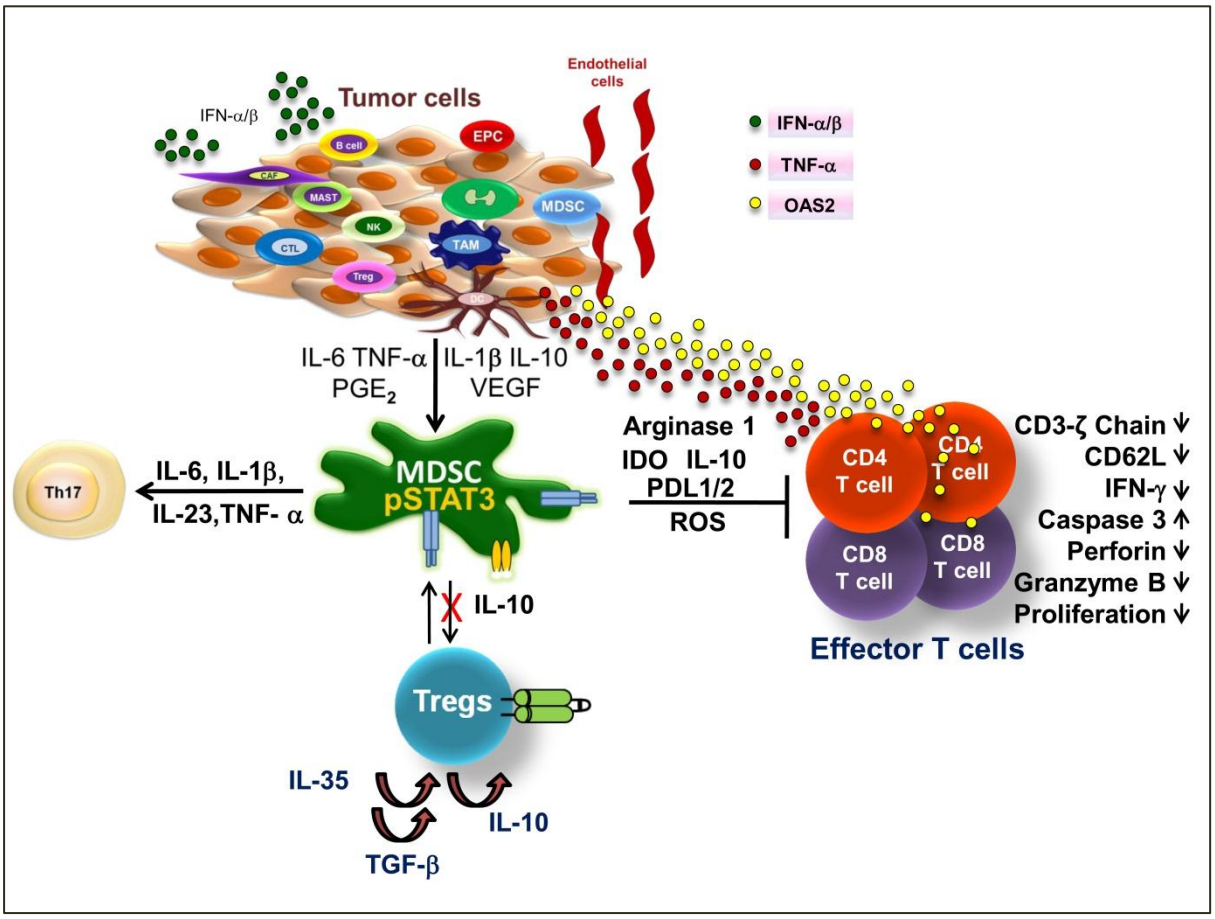

Figure 8.1: Immunosuppressive network in OSCC patients. The different players involved in the immune dysfunction of OSCC patients. The oral tumor micro-environment is enriched by immunosuppressive/inflammatory factors and cells contributing to the dampened immune response. The oral tumor micro-environment secretes cytokines or growth factors which either directly affect the T cells or indirectly by inducing the suppressive potential of myeloid derived suppressor cells by activating STAT3. MDSCs express the immunosuppressive molecules like arginase 1, IDO, IL-10, PDL1/2 and ROS. The factors like TNF-α and OAS2 present in oral tumor microenvironment along with MDSCs collectively execute immune suppression by downregulating CD3-ζ chain, CD62L, decreasing IFN-γ, perforin and granzyme production of T cells, impair TCR driven proliferation of T cells and increase caspase 3 levels. IL-6, IL-1β, IL-23 secreted by MDSCs induces TH17 differentiation in OSCC patients. Although, the Tregs were increased in OSCC patients MDSCs failed to induce iTreg differentiation under Treg polarizing condition.

MDSC and Treg cells are major components of the immune suppressive tumor microenvironment (TME). Both cell types expand systematically in preclinical tumor models and promote T-cell dysfunction that in turn favours tumor progression **(Fig. 8.1).** MDSCs play a crucial role in the cellular network that regulates immune responses in cancer,

inflammation and in other pathological conditions. One of the most important hallmarks of MDSCs is the morphological, phenotypic and functional heterogeneity. Therefore, the role of specific MDSC subsets in mediating T cell suppression and the molecular mechanisms for their accumulation was elucidated. In this study, different subsets of MDSCs were found to amass in the peripheral blood and tumor compartments. The monocytic-MDSCs were dominant in the peripheral blood while granulocytic-MDSCs were prevalent in the tumor compartment. STAT3 played an important role in MDSC activation and accumulation in OSCC patients. The Mo-MDSCs secreted high levels of IL-10, IL-1β, and IL-6 which have differential capability to induce T cell differentiation. Although Tregs were also higher in OSCC patients, it was not mediated by MDSCs, as they inhibited the in vitro generation of Tregs, but promoted TH17 differentiation. This suggests that MDSCs contribute in enhancing the inflammation as well as inducing T cell tolerance in oral cancer patients.

The reversibility of the immune dysfunction suggests that it is possible to overcome the deleterious effects of the tumors on the immune response, either pharmacologically or through more-effective strategies of active specific immunotherapy. STAT3 plays a central role in many molecular events governing tumor cell proliferation, survival and invasion. At the same time, STAT3 is involved in inhibition of antitumor immune responses. In myeloid cells, STAT3 regulates numerous functions as shown by us and others**.** STAT3 is crucial for MDSCs accumulation and its immunosuppressive function. The inhibition of STAT3 is clinically significant as STAT3 is activated in the majority of OSCC cell lines and human tumors. Levels of pYSTAT3 (activated) STAT3 are associated with decreased survival in HNSCC patients [541]. Epidermal growth factor receptor (EGFR)/signal transducer and activator of transcription 3 (STAT3) are activated in HNSCC including the subgroup of OSCC patients [522, 523]. Like STAT3, EGFR overexpression correlates with aggressive tumor behaviour and poor clinical outcome [524]. EGFR signals through STAT3 and anti-

EGFR therapy (Cetuximab) is used to treat HNSCC patients [525]. Inhibition of STAT3 signaling in OSCC patients will not only inhibit the EGFR mediated tumor progression but will also help in rejuvenating the antitumor immunity by inhibiting the generation and function of MDSCs.

Further, several receptors have been identified that are expressed on exhausted, dysfunctional lymphocytes, including CTLA-4, lymphocyte-activation gene 3 (LAG-3; CD223), T cell immunoglobulin mucin protein-3 (TIM-3), and PD-1. The ligand for PD-1, PD-L1 (B7-H1, CD274) [542], is upregulated in multiple tumor cell lines, including OSCC, and induces a loss of function of cytotoxic T lymphocytes (CTLs) [543]. The Mo-MDSCs present in OSCC patients also expressed PDL1 and is regulated by STAT3. More recently, the anti–PD-1 (Pembrolizumab, Nivolumab, Lambrolizumab) or PD-L1 (Durvalumab) Abs have demonstrated clinical efficacy in various malignancies including HNSCC [542, 544-546]. Investigation of the PD-L1: PD-1 pathway in OSCC patients provides a robust support for its blockade to ease the MDSCs and tumor induced immune suppression in these patients.

Bibliography

1. Torre, L.A., et al., *Global cancer statistics, 2012.* CA Cancer J Clin, 2015. **65**(2): p. 87-108.
2. Rajaraman, P., et al., *Recommendations for screening and early detection of common cancers in India.* The Lancet Oncology, 2015. **16**(7): p. e352-e361.
3. Danaei, G., et al., *Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors.* Lancet, 2005. **366**(9499): p. 1784-93.
4. Hashibe, M., et al., *Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium.* Cancer Epidemiol Biomarkers Prev, 2009. **18**(2): p. 541-50.
5. Simard, E.P., L.A. Torre, and A. Jemal, *International trends in head and neck cancer incidence rates: differences by country, sex and anatomic site.* Oral Oncol, 2014. **50**(5): p. 387-403.
6. Wen, C.P., et al., *Cancer risks from betel quid chewing beyond oral cancer: a multiple-site carcinogen when acting with smoking.* Cancer Causes Control, 2010. **21**(9): p. 1427-35.
7. Pignon, J.P., et al., *Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data.* The Lancet. **355**(9208): p. 949-955.
8. Pan, Q., M.A. Gorin, and T.N. Teknos, *Pharmacotherapy of head and neck squamous cell carcinoma.* Expert Opin Pharmacother, 2009. **10**(14): p. 2291-302.
9. Burchhardt, D.M. and A. Sukari, *Chemotherapy in Head and Neck Squamous Cell Cancer*, in *Targeting Oral Cancer*, A. M. Fribley, Editor 2016, Springer International Publishing: Cham. p. 53-68.
10. Seiwert, T.Y. and E.E. Cohen, *State-of-the-art management of locally advanced head and neck cancer.* Br J Cancer, 2005. **92**(8): p. 1341-8.
11. Marur, S. and A.A. Forastiere, *Head and neck cancer: changing epidemiology, diagnosis, and treatment.* Mayo Clin Proc, 2008. **83**(4): p. 489-501.
12. Sacco, A.G. and E.E. Cohen, *Current Treatment Options for Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma.* J Clin Oncol, 2015. **33**(29): p. 3305-13.
13. Begley, J. and A. Ribas, *Targeted therapies to improve tumor immunotherapy.* Clin Cancer Res, 2008. **14**(14): p. 4385-91.
14. Balermpas, P., et al., *Tumour-infiltrating lymphocytes predict response to definitive chemoradiotherapy in head and neck cancer.* Br J Cancer, 2014. **110**(2): p. 501-9.
15. Ward, M.J., et al., *Tumour-infiltrating lymphocytes predict for outcome in HPV-positive oropharyngeal cancer.* Br J Cancer, 2014. **110**(2): p. 489-500.
16. Seiwert, T.Y., et al. *Antitumor activity and safety of pembrolizumab in patients (pts) with advanced squamous cell carcinoma of the head and neck (SCCHN): preliminary results from KEYNOTE-012 expansion cohort*. in *ASCO Annual Meeting Proceedings*. 2015.
17. Motz, G.T. and G. Coukos, *Deciphering and reversing tumor immune suppression.* Immunity, 2013. **39**(1): p. 61-73.
18. Chen, D.S. and I. Mellman, *Oncology meets immunology: the cancer-immunity cycle.* Immunity, 2013. **39**(1): p. 1-10.
19. Ansell, S.M. and R.H. Vonderheide, *Cellular composition of the tumor microenvironment.* Am Soc Clin Oncol Educ Book, 2013.
20. Predina, J., et al., *Changes in the local tumor microenvironment in recurrent cancers may explain the failure of vaccines after surgery.* Proc Natl Acad Sci U S A, 2013. **110**(5): p. E415-24.
21. Wang, L., et al., *Immune evasion of mantle cell lymphoma: expression of B7-H1 leads to inhibited T-cell response to and killing of tumor cells.* Haematologica, 2013. **98**(9): p. 1458-66.
22. Reichert, T.E., et al., *Signaling abnormalities, apoptosis, and reduced proliferation of circulating and tumor-infiltrating lymphocytes in patients with oral carcinoma.* Clin Cancer Res, 2002. **8**(10): p. 3137-45.

23. Zea, A.H., et al., *Alterations in T cell receptor and signal transduction molecules in melanoma patients.* Clin Cancer Res, 1995. **1**(11): p. 1327-35.
24. Lai, P., et al., *Alterations in expression and function of signal-transducing proteins in tumor-associated T and natural killer cells in patients with ovarian carcinoma.* Clin Cancer Res, 1996. **2**(1): p. 161-73.
25. Lockhart, D.C., et al., *Loss of T-cell receptor-CD3zeta and T-cell function in tumor-infiltrating lymphocytes but not in tumor-associated lymphocytes in ovarian carcinoma.* Surgery, 2001. **129**(6): p. 749-56.
26. Choi, S.H., et al., *Alteration of signal-transducing molecules in tumor-infiltrating lymphocytes and peripheral blood T lymphocytes from human colorectal carcinoma patients.* Cancer Immunol Immunother, 1998. **45**(6): p. 299-305.
27. Kono, K., et al., *Decreased expression of signal-transducing zeta chain in peripheral T cells and natural killer cells in patients with cervical cancer.* Clin Cancer Res, 1996. **2**(11): p. 1825-8.
28. Kuss, I., et al., *Clinical significance of decreased zeta chain expression in peripheral blood lymphocytes of patients with head and neck cancer.* Clin Cancer Res, 1999. **5**(2): p. 329-34.
29. Kulkarni, D.P., et al., *Mechanisms involved in the down-regulation of TCR zeta chain in tumor versus peripheral blood of oral cancer patients.* Int J Cancer, 2009. **124**(7): p. 1605-13.
30. Mahoney, K.M., P.D. Rennert, and G.J. Freeman, *Combination cancer immunotherapy and new immunomodulatory targets.* Nat Rev Drug Discov, 2015. **14**(8): p. 561-84.
31. Schwabe, R.F. and C. Jobin, *The microbiome and cancer.* Nat Rev Cancer, 2013. **13**(11): p. 800-12.
32. Thomas, R.M. and C. Jobin, *The Microbiome and Cancer: Is the 'Oncobiome' Mirage Real?* Trends Cancer, 2015. **1**(1): p. 24-35.
33. Birnbaum, M.E., et al., *Molecular architecture of the alphabeta T cell receptor-CD3 complex.* Proc Natl Acad Sci U S A, 2014. **111**(49): p. 17576-81.
34. Guy, C.S., et al., *Distinct TCR signaling pathways drive proliferation and cytokine production in T cells.* Nat Immunol, 2013. **14**(3): p. 262-70.
35. Ishigami, S., et al., *CD3-zetachain expression of intratumoral lymphocytes is closely related to survival in gastric carcinoma patients.* Cancer, 2002. **94**(5): p. 1437-42.
36. Cho, O.H., et al., *Notch regulates cytolytic effector function in CD8+ T cells.* J Immunol, 2009. **182**(6): p. 3380-9.
37. Palaga, T., et al., *TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells.* J Immunol, 2003. **171**(6): p. 3019-24.
38. Ostrand-Rosenberg, S., *Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity.* Cancer Immunol Immunother, 2010. **59**(10): p. 1593-600.
39. Bronte, V., et al., *L-arginine metabolism in myeloid cells controls T-lymphocyte functions.* Trends Immunol, 2003. **24**(6): p. 302-6.
40. Nagaraj, S., et al., *Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer.* Nat Med, 2007. **13**(7): p. 828-35.
41. Serafini, P., et al., *Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells.* Cancer Res, 2008. **68**(13): p. 5439-49.
42. Sakaguchi, S., *Regulatory T cells: key controllers of immunologic self-tolerance.* Cell, 2000. **101**(5): p. 455-8.
43. Onizuka, S., et al., *Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody.* Cancer Res, 1999. **59**(13): p. 3128-33.
44. Feller, L., M. Altini, and J. Lemmer, *Inflammation in the context of oral cancer.* Oral Oncol, 2013. **49**(9): p. 887-92.
45. Tosolini, M., et al., *Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer.* Cancer Res, 2011. **71**(4): p. 1263-71.

46. Grivennikov, S.I., et al., *Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth.* Nature, 2012. **491**(7423): p. 254-8.
47. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012.* Int J Cancer, 2015. **136**(5): p. E359-86.
48. Ahluwalia, K.P., *Assessing the oral cancer risk of South-Asian immigrants in New York City.* Cancer, 2005. **104**(12 Suppl): p. 2959-61.
49. Dikshit, R., et al., *Cancer mortality in India: a nationally representative survey.* Lancet, 2012. **379**(9828): p. 1807-16.
50. Tsantoulis, P.K., et al., *Advances in the biology of oral cancer.* Oral Oncol, 2007. **43**(6): p. 523-34.
51. Pathak, K.A., et al., *Advanced squamous cell carcinoma of lower gingivobuccal complex: patterns of spread and failure.* Head Neck, 2005. **27**(7): p. 597-602.
52. da Silva, S.D., et al., *Advances and applications of oral cancer basic research.* Oral Oncol, 2011. **47**(9): p. 783-91.
53. Graham, S., et al., *Dentition, diet, tobacco, and alcohol in the epidemiology of oral cancer.* J Natl Cancer Inst, 1977. **59**(6): p. 1611-8.
54. Jussawalla, D.J. and V.A. Deshpande, *Evaluation of cancer risk in tobacco chewers and smokers: an epidemiologic assessment.* Cancer, 1971. **28**(1): p. 244-52.
55. Gupta, P.C. and C.S. Ray, *Smokeless tobacco and health in India and South Asia.* Respirology, 2003. **8**(4): p. 419-31.
56. Nair, U., H. Bartsch, and J. Nair, *Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: a review of agents and causative mechanisms.* Mutagenesis, 2004. **19**(4): p. 251-62.
57. Vincent, R.G. and F. Marchetta, *The Relationship of the Use of Tobacco and Alcohol to Cancer of the Oral Cavity, Pharynx or Larynx.* Am J Surg, 1963. **106**: p. 501-5.
58. Kaminagakura, E., et al., *High-risk human papillomavirus in oral squamous cell carcinoma of young patients.* Int J Cancer, 2012. **130**(8): p. 1726-32.
59. Gillison, M.L., et al., *Epidemiology of Human Papillomavirus-Positive Head and Neck Squamous Cell Carcinoma.* J Clin Oncol, 2015. **33**(29): p. 3235-42.
60. Gillison, M.L., *Human papillomavirus-related diseases: oropharynx cancers and potential implications for adolescent HPV vaccination.* J Adolesc Health, 2008. **43**(4 Suppl): p. S52-60.
61. Li, X., et al., *Human papillomavirus infection and laryngeal cancer risk: a systematic review and meta-analysis.* J Infect Dis, 2013. **207**(3): p. 479-88.
62. Ha, P.K., et al., *Molecular techniques and genetic alterations in head and neck cancer.* Oral Oncol, 2009. **45**(4-5): p. 335-9.
63. Chen, H.C., et al., *MicroRNA deregulation and pathway alterations in nasopharyngeal carcinoma.* Br J Cancer, 2009. **100**(6): p. 1002-11.
64. *Comprehensive genomic characterization of head and neck squamous cell carcinomas.* Nature, 2015. **517**(7536): p. 576-82.
65. Riaz, N., et al., *Unraveling the molecular genetics of head and neck cancer through genome-wide approaches.* Genes Dis, 2014. **1**(1): p. 75-86.
66. Cerami, E., et al., *The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data.* Cancer Discov, 2012. **2**(5): p. 401-4.
67. Kalyankrishna, S. and J.R. Grandis, *Epidermal growth factor receptor biology in head and neck cancer.* J Clin Oncol, 2006. **24**(17): p. 2666-72.
68. Argiris, A., et al., *Head and neck cancer.* Lancet, 2008. **371**(9625): p. 1695-709.
69. Gregoire, V., J.A. Langendijk, and S. Nuyts, *Advances in Radiotherapy for Head and Neck Cancer.* J Clin Oncol, 2015. **33**(29): p. 3277-84.
70. Prabhu, R.S., et al., *Lymph node ratio influence on risk of head and neck cancer locoregional recurrence after initial surgical resection: implications for adjuvant therapy.* Head Neck, 2015. **37**(6): p. 777-82.

71. Vanderveken, O.M., et al., *Gemcitabine-Based Chemoradiation in the Treatment of Locally Advanced Head and Neck Cancer: Systematic Review of Literature and Meta-Analysis.* Oncologist, 2016. **21**(1): p. 59-71.
72. Muro, K., et al., *Pembrolizumab for patients with PD-L1-positive advanced gastric cancer (KEYNOTE-012): a multicentre, open-label, phase 1b trial.* Lancet Oncol, 2016.
73. Ferris, R.L., *Immunology and Immunotherapy of Head and Neck Cancer.* J Clin Oncol, 2015. **33**(29): p. 3293-304.
74. Schoenfeld, J.D., *Immunity in head and neck cancer.* Cancer Immunol Res, 2015. **3**(1): p. 12-7.
75. Ehrlich, P., *Ueber den Jetzigen stand der Karzinomforschung. .* Ned. Tijdschr.Geneeskd, 1909. **5**: p. 273.290.
76. Burnet, M., *Cancer; a biological approach. I. The processes of control.* Br Med J, 1957. **1**(5022): p. 779-86.
77. Smyth, M.J., D.I. Godfrey, and J.A. Trapani, *A fresh look at tumor immunosurveillance and immunotherapy.* Nat Immunol, 2001. **2**(4): p. 293-9.
78. Senovilla, L., et al., *An immunosurveillance mechanism controls cancer cell ploidy.* Science, 2012. **337**(6102): p. 1678-84.
79. Fuchs, E.J. and P. Matzinger, *Is cancer dangerous to the immune system?* Semin Immunol, 1996. **8**(5): p. 271-80.
80. Willimsky, G. and T. Blankenstein, *Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance.* Nature, 2005. **437**(7055): p. 141-6.
81. Stutman, O., *Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice.* Science, 1974. **183**(4124): p. 534-6.
82. Stutman, O., *Chemical carcinogenesis in nude mice: comparison between nude mice from homozygous matings and heterozygous matings and effect of age and carcinogen dose.* J Natl Cancer Inst, 1979. **62**(2): p. 353-8.
83. Smyth, M.J., et al., *Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma.* J Exp Med, 2000. **192**(5): p. 755-60.
84. Dunn, G.P., et al., *Cancer immunoediting: from immunosurveillance to tumor escape.* Nat Immunol, 2002. **3**(11): p. 991-8.
85. Dunn, G.P., L.J. Old, and R.D. Schreiber, *The three Es of cancer immunoediting.* Annu Rev Immunol, 2004. **22**: p. 329-60.
86. Balkwill, F. and A. Mantovani, *Inflammation and cancer: back to Virchow?* Lancet, 2001. **357**(9255): p. 539-45.
87. Shankaran, V., et al., *IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity.* Nature, 2001. **410**(6832): p. 1107-11.
88. Dunn, G.P., C.M. Koebel, and R.D. Schreiber, *Interferons, immunity and cancer immunoediting.* Nat Rev Immunol, 2006. **6**(11): p. 836-48.
89. Schreiber, R.D., L.J. Old, and M.J. Smyth, *Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion.* Science, 2011. **331**(6024): p. 1565-70.
90. Mittal, D., et al., *New insights into cancer immunoediting and its three component phases — elimination, equilibrium and escape.* Current Opinion in Immunology, 2014. **27**(0): p. 16-25.
91. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-99.
92. Dighe, A.S., et al., *Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors.* Immunity, 1994. **1**(6): p. 447-56.
93. Kaplan, D.H., et al., *Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice.* Proc Natl Acad Sci U S A, 1998. **95**(13): p. 7556-61.
94. Girardi, M., et al., *Regulation of cutaneous malignancy by gammadelta T cells.* Science, 2001. **294**(5542): p. 605-9.

95. Girardi, M., et al., *The distinct contributions of murine T cell receptor (TCR)gammadelta+ and TCRalphabeta+ T cells to different stages of chemically induced skin cancer.* J Exp Med, 2003. **198**(5): p. 747-55.
96. Swann, J.B., et al., *Type I natural killer T cells suppress tumors caused by p53 loss in mice.* Blood, 2009. **113**(25): p. 6382-5.
97. Smyth, M.J., et al., *Differential tumor surveillance by natural killer (NK) and NKT cells.* J Exp Med, 2000. **191**(4): p. 661-8.
98. Smyth, M.J., N.Y. Crowe, and D.I. Godfrey, *NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma.* Int Immunol, 2001. **13**(4): p. 459-63.
99. Simson, L., et al., *Regulation of carcinogenesis by IL-5 and CCL11: a potential role for eosinophils in tumor immune surveillance.* J Immunol, 2007. **178**(7): p. 4222-9.
100. Obeid, M., et al., *Calreticulin exposure dictates the immunogenicity of cancer cell death.* Nat Med, 2007. **13**(1): p. 54-61.
101. Kagi, D., et al., *Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice.* Nature, 1994. **369**(6475): p. 31-7.
102. Bolitho, P., et al., *Perforin-mediated suppression of B-cell lymphoma.* Proc Natl Acad Sci U S A, 2009. **106**(8): p. 2723-8.
103. Vesely, M.D., et al., *Natural innate and adaptive immunity to cancer.* Annu Rev Immunol, 2011. **29**: p. 235-71.
104. Koebel, C.M., et al., *Adaptive immunity maintains occult cancer in an equilibrium state.* Nature, 2007. **450**(7171): p. 903-907.
105. Teng, M.W., et al., *Opposing roles for IL-23 and IL-12 in maintaining occult cancer in an equilibrium state.* Cancer Res, 2012. **72**(16): p. 3987-96.
106. Loeser, S., et al., *Spontaneous tumor rejection by cbl-b-deficient CD8+ T cells.* J Exp Med, 2007. **204**(4): p. 879-91.
107. Casanovas, O., et al., *Incomplete inhibition of the Rb tumor suppressor pathway in the context of inactivated p53 is sufficient for pancreatic islet tumorigenesis.* Oncogene, 2005. **24**(44): p. 6597-604.
108. Bergers, G., et al., *Effects of angiogenesis inhibitors on multistage carcinogenesis in mice.* Science, 1999. **284**(5415): p. 808-12.
109. Casanovas, O., et al., *Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors.* Cancer Cell, 2005. **8**(4): p. 299-309.
110. Forster, I., et al., *Limited capacity for tolerization of CD4+ T cells specific for a pancreatic beta cell neo-antigen.* Immunity, 1995. **2**(6): p. 573-85.
111. Muller-Hermelink, N., et al., *TNFR1 signaling and IFN-gamma signaling determine whether T cells induce tumor dormancy or promote multistage carcinogenesis.* Cancer Cell, 2008. **13**(6): p. 507-18.
112. Braumuller, H., et al., *T-helper-1-cell cytokines drive cancer into senescence.* Nature, 2013. **494**(7437): p. 361-5.
113. Wu, X., et al., *Immune microenvironment profiles of tumor immune equilibrium and immune escape states of mouse sarcoma.* Cancer Lett, 2013. **340**(1): p. 124-33.
114. Grandis, J.R., et al., *Human leukocyte antigen class I allelic and haplotype loss in squamous cell carcinoma of the head and neck: clinical and immunogenetic consequences.* Clin Cancer Res, 2000. **6**(7): p. 2794-802.
115. Kim, J.W., et al., *Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes.* Clin Cancer Res, 2005. **11**(3): p. 1010-20.
116. Dong, H., et al., *Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion.* Nat Med, 2002. **8**(8): p. 793-800.

117. Reichert, T.E., et al., *The number of intratumoral dendritic cells and zeta-chain expression in T cells as prognostic and survival biomarkers in patients with oral carcinoma.* Cancer, 2001. **91**(11): p. 2136-47.
118. Vinay, D.S., et al., *Immune evasion in cancer: Mechanistic basis and therapeutic strategies.* Semin Cancer Biol, 2015. **35 Suppl**: p. S185-98.
119. Kayagaki, N., et al., *Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: A novel mechanism for the antitumor effects of type I IFNs.* J Exp Med, 1999. **189**(9): p. 1451-60.
120. Cullen, S.P., M. Brunet, and S.J. Martin, *Granzymes in cancer and immunity.* Cell Death Differ, 2010. **17**(4): p. 616-23.
121. Lin, J. and A. Weiss, *T cell receptor signalling.* J Cell Sci, 2001. **114**(Pt 2): p. 243-4.
122. Okkenhaug, K., et al., *Phosphoinositide 3-kinase in T cell activation and survival.* Biochem Soc Trans, 2004. **32**(Pt 2): p. 332-5.
123. Adams, E.J., Y.H. Chien, and K.C. Garcia, *Structure of a gammadelta T cell receptor in complex with the nonclassical MHC T22.* Science, 2005. **308**(5719): p. 227-31.
124. Brenner, M.B., I.S. Trowbridge, and J.L. Strominger, *Cross-linking of human T cell receptor proteins: association between the T cell idiotype beta subunit and the T3 glycoprotein heavy subunit.* Cell, 1985. **40**(1): p. 183-90.
125. Samelson, L.E., J.B. Harford, and R.D. Klausner, *Identification of the components of the murine T cell antigen receptor complex.* Cell, 1985. **43**(1): p. 223-31.
126. Sussman, J.J., et al., *Failure to synthesize the T cell CD3-zeta chain: structure and function of a partial T cell receptor complex.* Cell, 1988. **52**(1): p. 85-95.
127. Wegener, A.M., et al., *Distinct domains of the CD3-gamma chain are involved in surface expression and function of the T cell antigen receptor.* J Biol Chem, 1995. **270**(9): p. 4675-80.
128. Cantrell, D.A., *T-cell antigen receptor signal transduction.* Immunology, 2002. **105**(4): p. 369-74.
129. van Leeuwen, J.E. and L.E. Samelson, *T cell antigen-receptor signal transduction.* Curr Opin Immunol, 1999. **11**(3): p. 242-8.
130. Zhang, W. and L.E. Samelson, *The role of membrane-associated adaptors in T cell receptor signalling.* Semin Immunol, 2000. **12**(1): p. 35-41.
131. Saoudi, A., et al., *Rho-GTPases as key regulators of T lymphocyte biology.* Small GTPases, 2014. **5**.
132. Baniyash, M., *TCR zeta-chain downregulation: curtailing an excessive inflammatory immune response.* Nat Rev Immunol, 2004. **4**(9): p. 675-87.
133. Manolios, N., et al., *Pairwise, cooperative and inhibitory interactions describe the assembly and probable structure of the T-cell antigen receptor.* EMBO J, 1991. **10**(7): p. 1643-51.
134. Minami, Y., et al., *Building a multichain receptor: synthesis, degradation, and assembly of the T-cell antigen receptor.* Proc Natl Acad Sci U S A, 1987. **84**(9): p. 2688-92.
135. Finke, J.H., et al., *Loss of T-cell receptor zeta chain and p56lck in T cells infiltrating human renal cell carcinoma.* Cancer Res, 1993. **53**(23): p. 5613-6.
136. Whiteside, T.L. and G. Parmiani, *Tumor-infiltrating lymphocytes: their phenotype, functions and clinical use.* Cancer Immunol Immunother, 1994. **39**(1): p. 15-21.
137. Frey, A.B. and N. Monu, *Signaling defects in anti-tumor T cells.* Immunol Rev, 2008. **222**: p. 192-205.
138. Whiteside, T.L., *Down-regulation of zeta-chain expression in T cells: a biomarker of prognosis in cancer?* Cancer Immunol Immunother, 2004. **53**(10): p. 865-78.
139. Maurice, M.M., et al., *Defective TCR-mediated signaling in synovial T cells in rheumatoid arthritis.* J Immunol, 1997. **159**(6): p. 2973-8.
140. Liossis, S.N., et al., *Altered pattern of TCR/CD3-mediated protein-tyrosyl phosphorylation in T cells from patients with systemic lupus erythematosus. Deficient expression of the T cell receptor zeta chain.* J Clin Invest, 1998. **101**(7): p. 1448-57.

141. Zea, A.H., et al., *Changes in expression of signal transduction proteins in T lymphocytes of patients with leprosy.* Infect Immun, 1998. **66**(2): p. 499-504.
142. Stefanova, I., et al., *HIV infection--induced posttranslational modification of T cell signaling molecules associated with disease progression.* J Clin Invest, 1996. **98**(6): p. 1290-7.
143. Solomou, E.E., et al., *Decreased TCR zeta-chain expression in T cells from patients with acquired aplastic anaemia.* Br J Haematol, 2007. **138**(1): p. 72-6.
144. Mizoguchi, H., et al., *Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice.* Science, 1992. **258**(5089): p. 1795-8.
145. Whiteside, T.L., *Down-regulation of ζ-chain expression in T cells: a biomarker of prognosis in cancer?* Cancer Immunology, Immunotherapy, 2004. **53**(10): p. 865-878.
146. Taylor, D.D., et al., *Modulation of TcR/CD3-zeta chain expression by a circulating factor derived from ovarian cancer patients.* Br J Cancer, 2001. **84**(12): p. 1624-9.
147. Otsuji, M., et al., *Oxidative stress by tumor-derived macrophages suppresses the expression of CD3 zeta chain of T-cell receptor complex and antigen-specific T-cell responses.* Proc Natl Acad Sci U S A, 1996. **93**(23): p. 13119-24.
148. Cemerski, S., et al., *Reactive oxygen species differentially affect T cell receptor-signaling pathways.* J Biol Chem, 2002. **277**(22): p. 19585-93.
149. Nambiar, M.P., et al., *Heat stress downregulates TCR zeta chain expression in human T lymphocytes.* J Cell Biochem, 2000. **79**(3): p. 416-26.
150. Nambiar, M.P., et al., *Oxidative stress is involved in the heat stress-induced downregulation of TCR zeta chain expression and TCR/CD3-mediated [Ca(2+)](i) response in human T-lymphocytes.* Cell Immunol, 2002. **215**(2): p. 151-61.
151. Nambiar, M.P., et al., *Dexamethasone modulates TCR zeta chain expression and antigen receptor-mediated early signaling events in human T lymphocytes.* Cell Immunol, 2001. **208**(1): p. 62-71.
152. Nambiar, M.P., et al., *Effect of trichostatin A on human T cells resembles signaling abnormalities in T cells of patients with systemic lupus erythematosus: a new mechanism for TCR zeta chain deficiency and abnormal signaling.* J Cell Biochem, 2002. **85**(3): p. 459-69.
153. Rodriguez, P.C., et al., *L-arginine consumption by macrophages modulates the expression of CD3 zeta chain in T lymphocytes.* J Immunol, 2003. **171**(3): p. 1232-9.
154. Bronte, V. and P. Zanovello, *Regulation of immune responses by L-arginine metabolism.* Nat Rev Immunol, 2005. **5**(8): p. 641-54.
155. Gastman, B.R., et al., *Caspase-mediated degradation of T-cell receptor zeta-chain.* Cancer Res, 1999. **59**(7): p. 1422-7.
156. Wieckowski, E., et al., *Granzyme B-mediated degradation of T-cell receptor zeta chain.* Cancer Res, 2002. **62**(17): p. 4884-9.
157. Hanaoka, N., et al., *NKG2D initiates caspase-mediated CD3zeta degradation and lymphocyte receptor impairments associated with human cancer and autoimmune disease.* J Immunol, 2010. **185**(10): p. 5732-42.
158. Valitutti, S., et al., *Degradation of T cell receptor (TCR)-CD3-zeta complexes after antigenic stimulation.* J Exp Med, 1997. **185**(10): p. 1859-64.
159. Friend, S.F., et al., *Regulation of T cell receptor complex-mediated signaling by ubiquitin and ubiquitin-like modifications.* Am J Clin Exp Immunol, 2014. **3**(3): p. 107-23.
160. Myers, M.D., et al., *Src-like adaptor protein regulates TCR expression on thymocytes by linking the ubiquitin ligase c-Cbl to the TCR complex.* Nat Immunol, 2006. **7**(1): p. 57-66.
161. Ersek, B., et al., *CD3zeta-chain expression of human T lymphocytes is regulated by TNF via Src-like adaptor protein-dependent proteasomal degradation.* J Immunol, 2012. **189**(4): p. 1602-10.
162. Artavanis-Tsakonas, S., M.D. Rand, and R.J. Lake, *Notch signaling: cell fate control and signal integration in development.* Science, 1999. **284**(5415): p. 770-6.

163. Maillard, I., T. Fang, and W.S. Pear, *Regulation of lymphoid development, differentiation, and function by the Notch pathway.* Annu Rev Immunol, 2005. **23**: p. 945-74.
164. Shin, H.M., et al., *Notch1 augments NF-kappaB activity by facilitating its nuclear retention.* EMBO J, 2006. **25**(1): p. 129-38.
165. Minter, L.M., et al., *Inhibitors of gamma-secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21.* Nat Immunol, 2005. **6**(7): p. 680-8.
166. Minter, L.M. and B.A. Osborne, *Canonical and non-canonical Notch signaling in CD4(+) T cells.* Curr Top Microbiol Immunol, 2012. **360**: p. 99-114.
167. Adler, S.H., et al., *Notch signaling augments T cell responsiveness by enhancing CD25 expression.* J Immunol, 2003. **171**(6): p. 2896-903.
168. Gogoi, D., A.A. Dar, and S.V. Chiplunkar, *Involvement of Notch in activation and effector functions of gammadelta T cells.* J Immunol, 2014. **192**(5): p. 2054-62.
169. Sugimoto, K., et al., *Notch2 signaling is required for potent antitumor immunity in vivo.* J Immunol, 2010. **184**(9): p. 4673-8.
170. Dongre, A., et al., *Non-Canonical Notch Signaling Drives Activation and Differentiation of Peripheral CD4(+) T Cells.* Front Immunol, 2014. **5**: p. 54.
171. Jain, J., et al., *The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun.* Nature, 1993. **365**(6444): p. 352-5.
172. Macian, F., C. Lopez-Rodriguez, and A. Rao, *Partners in transcription: NFAT and AP-1.* Oncogene, 2001. **20**(19): p. 2476-89.
173. Macian, F., et al., *Transcriptional mechanisms underlying lymphocyte tolerance.* Cell, 2002. **109**(6): p. 719-31.
174. Anandasabapathy, N., et al., *GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells.* Immunity, 2003. **18**(4): p. 535-47.
175. Heissmeyer, V., et al., *Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins.* Nat Immunol, 2004. **5**(3): p. 255-65.
176. Jeon, M.S., et al., *Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction.* Immunity, 2004. **21**(2): p. 167-77.
177. Mueller, D.L., *E3 ubiquitin ligases as T cell anergy factors.* Nat Immunol, 2004. **5**(9): p. 883-90.
178. Rathmell, J.C. and C.B. Thompson, *The central effectors of cell death in the immune system.* Annu Rev Immunol, 1999. **17**: p. 781-828.
179. Puga, I., A. Rao, and F. Macian, *Targeted cleavage of signaling proteins by caspase 3 inhibits T cell receptor signaling in anergic T cells.* Immunity, 2008. **29**(2): p. 193-204.
180. Huang, Y., et al., *Resuscitating cancer immunosurveillance: selective stimulation of DLL1-Notch signaling in T cells rescues T-cell function and inhibits tumor growth.* Cancer Res, 2011. **71**(19): p. 6122-31.
181. Sierra, R.A., et al., *Rescue of Notch-1 signaling in antigen-specific CD8+ T cells overcomes tumor-induced T-cell suppression and enhances immunotherapy in cancer.* Cancer Immunol Res, 2014. **2**(8): p. 800-11.
182. Kusmartsev, S. and D.I. Gabrilovich, *Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer.* Cancer Metastasis Rev, 2006. **25**(3): p. 323-31.
183. Groh, V., et al., *Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation.* Nature, 2002. **419**(6908): p. 734-8.
184. Zheng, X., et al., *Reinstalling antitumor immunity by inhibiting tumor-derived immunosuppressive molecule IDO through RNA interference.* J Immunol, 2006. **177**(8): p. 5639-46.
185. Gabrilovich, D.I., et al., *Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells.* Nat Med, 1996. **2**(10): p. 1096-103.

186. Bronte, V., et al., *Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits CD8+ T cell responses by dysregulating antigen-presenting cell maturation.* J Immunol, 1999. **162**(10): p. 5728-37.
187. Seo, N., et al., *Interleukin-10 expressed at early tumour sites induces subsequent generation of CD4(+) T-regulatory cells and systemic collapse of antitumour immunity.* Immunology, 2001. **103**(4): p. 449-57.
188. Mullins, D.W., et al., *Tumor cell-derived TGF-beta and IL-10 dysregulate paclitaxel-induced macrophage activation.* J Leukoc Biol, 2001. **69**(1): p. 129-37.
189. Akdis, C.A., et al., *A molecular basis for T cell suppression by IL-10: CD28-associated IL-10 receptor inhibits CD28 tyrosine phosphorylation and phosphatidylinositol 3-kinase binding.* FASEB J, 2000. **14**(12): p. 1666-8.
190. Menetrier-Caux, C., et al., *Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor.* Blood, 1998. **92**(12): p. 4778-91.
191. Birkle, S., et al., *Role of tumor-associated gangliosides in cancer progression.* Biochimie, 2003. **85**(3-4): p. 455-63.
192. de Caestecker, M.P., E. Piek, and A.B. Roberts, *Role of transforming growth factor-beta signaling in cancer.* J Natl Cancer Inst, 2000. **92**(17): p. 1388-402.
193. Wrzesinski, S.H., Y.Y. Wan, and R.A. Flavell, *Transforming growth factor-beta and the immune response: implications for anticancer therapy.* Clin Cancer Res, 2007. **13**(18 Pt 1): p. 5262-70.
194. Thomas, D.A. and J. Massague, *TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance.* Cancer Cell, 2005. **8**(5): p. 369-80.
195. Salih, H.R., H.G. Rammensee, and A. Steinle, *Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding.* J Immunol, 2002. **169**(8): p. 4098-102.
196. Spear, P., et al., *NKG2D ligands as therapeutic targets.* Cancer Immun, 2013. **13**: p. 8.
197. Soliman, H., M. Mediavilla-Varela, and S. Antonia, *Indoleamine 2,3-dioxygenase: is it an immune suppressor?* Cancer J, 2010. **16**(4): p. 354-9.
198. Di Giovine, F.S., G. Nuki, and G.W. Duff, *Tumour necrosis factor in synovial exudates.* Ann Rheum Dis, 1988. **47**(9): p. 768-72.
199. Saxne, T., et al., *Detection of tumor necrosis factor alpha but not tumor necrosis factor beta in rheumatoid arthritis synovial fluid and serum.* Arthritis Rheum, 1988. **31**(8): p. 1041-5.
200. Szlosarek, P.W. and F.R. Balkwill, *Tumour necrosis factor alpha: a potential target for the therapy of solid tumours.* Lancet Oncol, 2003. **4**(9): p. 565-73.
201. Wong, G.H., et al., *Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor.* Cell, 1989. **58**(5): p. 923-31.
202. Cope, A.P., et al., *Chronic exposure to tumor necrosis factor (TNF) in vitro impairs the activation of T cells through the T cell receptor/CD3 complex; reversal in vivo by anti-TNF antibodies in patients with rheumatoid arthritis.* J Clin Invest, 1994. **94**(2): p. 749-60.
203. Cope, A.P., et al., *Chronic tumor necrosis factor alters T cell responses by attenuating T cell receptor signaling.* J Exp Med, 1997. **185**(9): p. 1573-84.
204. Isomaki, P., et al., *Prolonged exposure of T cells to TNF down-regulates TCR zeta and expression of the TCR/CD3 complex at the cell surface.* J Immunol, 2001. **166**(9): p. 5495-507.
205. Wang, X. and Y. Lin, *Tumor necrosis factor and cancer, buddies or foes?* Acta Pharmacol Sin, 2008. **29**(11): p. 1275-88.
206. Sade-Feldman, M., et al., *Tumor necrosis factor-alpha blocks differentiation and enhances suppressive activity of immature myeloid cells during chronic inflammation.* Immunity, 2013. **38**(3): p. 541-54.
207. Lindau, D., et al., *The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells.* Immunology, 2013. **138**(2): p. 105-15.

208. Weed, D.T., et al., *Tadalafil reduces myeloid-derived suppressor cells and regulatory T cells and promotes tumor immunity in patients with head and neck squamous cell carcinoma.* Clin Cancer Res, 2015. **21**(1): p. 39-48.
209. Weide, B., et al., *Myeloid-derived suppressor cells predict survival of patients with advanced melanoma: comparison with regulatory T cells and NY-ESO-1- or melan-A-specific T cells.* Clin Cancer Res, 2014. **20**(6): p. 1601-9.
210. Iwasaki, H. and K. Akashi, *Myeloid lineage commitment from the hematopoietic stem cell.* Immunity, 2007. **26**(6): p. 726-40.
211. Manz, M.G., et al., *Dendritic cell potentials of early lymphoid and myeloid progenitors.* Blood, 2001. **97**(11): p. 3333-41.
212. Traver, D., et al., *Development of CD8alpha-positive dendritic cells from a common myeloid progenitor.* Science, 2000. **290**(5499): p. 2152-4.
213. Saleem, S.J. and D.H. Conrad, *Hematopoietic cytokine-induced transcriptional regulation and Notch signaling as modulators of MDSC expansion.* Int Immunopharmacol, 2011. **11**(7): p. 808-15.
214. Young, M.R., M. Newby, and H.T. Wepsic, *Hematopoiesis and suppressor bone marrow cells in mice bearing large metastatic Lewis lung carcinoma tumors.* Cancer Res, 1987. **47**(1): p. 100-5.
215. Robinson, W.A., *Granulocytosis in neoplasia.* Ann N Y Acad Sci, 1974. **230**: p. 212-8.
216. Manz, M.G. and S. Boettcher, *Emergency granulopoiesis.* Nat Rev Immunol, 2014. **14**(5): p. 302-14.
217. Bennett, J.A., V.S. Rao, and M.S. Mitchell, *Systemic bacillus Calmette-Guerin (BCG) activates natural suppressor cells.* Proc Natl Acad Sci U S A, 1978. **75**(10): p. 5142-4.
218. Gabrilovich, D.I. and S. Nagaraj, *Myeloid-derived suppressor cells as regulators of the immune system.* Nat Rev Immunol, 2009. **9**(3): p. 162-74.
219. Talmadge, J.E. and D.I. Gabrilovich, *History of myeloid-derived suppressor cells.* Nat Rev Cancer, 2013. **13**(10): p. 739-52.
220. Peranzoni, E., et al., *Myeloid-derived suppressor cell heterogeneity and subset definition.* Curr Opin Immunol, 2010. **22**(2): p. 238-44.
221. Ochoa, A.C., et al., *Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma.* Clin Cancer Res, 2007. **13**(2 Pt 2): p. 721s-726s.
222. Almand, B., et al., *Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer.* J Immunol, 2001. **166**(1): p. 678-89.
223. Mirza, N., et al., *All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients.* Cancer Res, 2006. **66**(18): p. 9299-307.
224. Condamine, T., et al., *Regulation of tumor metastasis by myeloid-derived suppressor cells.* Annu Rev Med, 2015. **66**: p. 97-110.
225. Damuzzo, V., et al., *Complexity and challenges in defining myeloid-derived suppressor cells.* Cytometry B Clin Cytom, 2015. **88**(2): p. 77-91.
226. Vasquez-Dunddel, D., et al., *STAT3 regulates arginase-I in myeloid-derived suppressor cells from cancer patients.* J Clin Invest, 2013. **123**(4): p. 1580-9.
227. Condamine, T. and D.I. Gabrilovich, *Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function.* Trends Immunol, 2011. **32**(1): p. 19-25.
228. Yu, H., D. Pardoll, and R. Jove, *STATs in cancer inflammation and immunity: a leading role for STAT3.* Nat Rev Cancer, 2009. **9**(11): p. 798-809.
229. Nefedova, Y., et al., *Hyperactivation of STAT3 is involved in abnormal differentiation of dendritic cells in cancer.* J Immunol, 2004. **172**(1): p. 464-74.
230. Nefedova, Y., et al., *Regulation of dendritic cell differentiation and antitumor immune response in cancer by pharmacologic-selective inhibition of the janus-activated kinase 2/signal transducers and activators of transcription 3 pathway.* Cancer Res, 2005. **65**(20): p. 9525-35.

231. Ostrand-Rosenberg, S. and P. Sinha, *Myeloid-derived suppressor cells: linking inflammation and cancer.* J Immunol, 2009. **182**(8): p. 4499-506.
232. Markiewski, M.M., et al., *Modulation of the antitumor immune response by complement.* Nat Immunol, 2008. **9**(11): p. 1225-35.
233. Sinha, P., et al., *Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells.* Cancer Res, 2007. **67**(9): p. 4507-13.
234. Turovskaya, O., et al., *RAGE, carboxylated glycans and S100A8/A9 play essential roles in colitis-associated carcinogenesis.* Carcinogenesis, 2008. **29**(10): p. 2035-43.
235. El Gazzar, M. and C.E. McCall, *MicroRNAs regulatory networks in myeloid lineage development and differentiation: regulators of the regulators.* Immunol Cell Biol, 2012. **90**(6): p. 587-93.
236. Boldin, M.P., et al., *miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice.* J Exp Med, 2011. **208**(6): p. 1189-201.
237. Liu, Q., et al., *miR-223 suppresses differentiation of tumor-induced CD11b(+) Gr1(+) myeloid-derived suppressor cells from bone marrow cells.* Int J Cancer, 2011. **129**(11): p. 2662-73.
238. Liu, Y., et al., *MicroRNA-494 is required for the accumulation and functions of tumor-expanded myeloid-derived suppressor cells via targeting of PTEN.* J Immunol, 2012. **188**(11): p. 5500-10.
239. Li, L., et al., *MicroRNA-155 and MicroRNA-21 promote the expansion of functional myeloid-derived suppressor cells.* J Immunol, 2014. **192**(3): p. 1034-43.
240. Zhang, M., et al., *Both miR-17-5p and miR-20a alleviate suppressive potential of myeloid-derived suppressor cells by modulating STAT3 expression.* J Immunol, 2011. **186**(8): p. 4716-24.
241. Marvel, D. and D.I. Gabrilovich, *Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected.* J Clin Invest, 2015. **125**(9): p. 3356-64.
242. Movahedi, K., et al., *Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity.* Blood, 2008. **111**(8): p. 4233-44.
243. Dunsky, I.L., *Snellen acuity, refractive error, and the Scheerer phenomenon.* Optom Vis Sci, 1990. **67**(12): p. 898-902.
244. Huang, B., et al., *Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host.* Cancer Res, 2006. **66**(2): p. 1123-31.
245. Srivastava, M.K., et al., *Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine.* Cancer Res, 2010. **70**(1): p. 68-77.
246. Hanson, E.M., et al., *Myeloid-derived suppressor cells down-regulate L-selectin expression on CD4+ and CD8+ T cells.* J Immunol, 2009. **183**(2): p. 937-44.
247. Corzo, C.A., et al., *Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells.* J Immunol, 2009. **182**(9): p. 5693-701.
248. Kusmartsev, S., et al., *Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species.* J Immunol, 2004. **172**(2): p. 989-99.
249. Rodriguez, P.C., et al., *Regulation of T cell receptor CD3zeta chain expression by L-arginine.* J Biol Chem, 2002. **277**(24): p. 21123-9.
250. Zea, A.H., et al., *Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion.* Cancer Res, 2005. **65**(8): p. 3044-8.
251. Bronte, V., et al., *IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice.* J Immunol, 2003. **170**(1): p. 270-8.
252. Serafini, P., et al., *Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function.* J Exp Med, 2006. **203**(12): p. 2691-702.

253. Kusmartsev, S.A., Y. Li, and S.H. Chen, *Gr-1+ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation.* J Immunol, 2000. **165**(2): p. 779-85.
254. Bingisser, R.M., et al., *Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway.* J Immunol, 1998. **160**(12): p. 5729-34.
255. Fischer, T.A., et al., *Activation of cGMP-dependent protein kinase Ibeta inhibits interleukin 2 release and proliferation of T cell receptor-stimulated human peripheral T cells.* J Biol Chem, 2001. **276**(8): p. 5967-74.
256. Duhe, R.J., et al., *Nitric oxide and thiol redox regulation of Janus kinase activity.* Proc Natl Acad Sci U S A, 1998. **95**(1): p. 126-31.
257. Gallatin, W.M., I.L. Weissman, and E.C. Butcher, *A cell-surface molecule involved in organ-specific homing of lymphocytes.* Nature, 1983. **304**(5921): p. 30-4.
258. Veltman, J.D., et al., *COX-2 inhibition improves immunotherapy and is associated with decreased numbers of myeloid-derived suppressor cells in mesothelioma. Celecoxib influences MDSC function.* BMC Cancer, 2010. **10**: p. 464.
259. Fujita, M., et al., *COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells.* Cancer Res, 2011. **71**(7): p. 2664-74.
260. Wesolowski, R., J. Markowitz, and W.E. Carson, 3rd, *Myeloid derived suppressor cells - a new therapeutic target in the treatment of cancer.* J Immunother Cancer, 2013. **1**: p. 10.
261. Rodriguez, P.C., et al., *Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma.* J Exp Med, 2005. **202**(7): p. 931-9.
262. Chen, E.P. and E.M. Smyth, *COX-2 and PGE2-dependent immunomodulation in breast cancer.* Prostaglandins Other Lipid Mediat, 2011. **96**(1-4): p. 14-20.
263. Hengesbach, L.M. and K.A. Hoag, *Physiological concentrations of retinoic acid favor myeloid dendritic cell development over granulocyte development in cultures of bone marrow cells from mice.* J Nutr, 2004. **134**(10): p. 2653-9.
264. Nefedova, Y., et al., *Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells.* Cancer Res, 2007. **67**(22): p. 11021-8.
265. Kusmartsev, S., et al., *All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination.* Cancer Res, 2003. **63**(15): p. 4441-9.
266. Meyer, C., et al., *Chronic inflammation promotes myeloid-derived suppressor cell activation blocking antitumor immunity in transgenic mouse melanoma model.* Proc Natl Acad Sci U S A, 2011. **108**(41): p. 17111-6.
267. Suzuki, E., et al., *Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity.* Clin Cancer Res, 2005. **11**(18): p. 6713-21.
268. Le, H.K., et al., *Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice.* Int Immunopharmacol, 2009. **9**(7-8): p. 900-9.
269. Vincent, J., et al., *5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity.* Cancer Res, 2010. **70**(8): p. 3052-61.
270. Ozao-Choy, J., et al., *The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies.* Cancer Res, 2009. **69**(6): p. 2514-22.
271. Xin, H., et al., *Sunitinib inhibition of Stat3 induces renal cell carcinoma tumor cell apoptosis and reduces immunosuppressive cells.* Cancer Res, 2009. **69**(6): p. 2506-13.
272. Diaz-Montero, C.M., et al., *Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy.* Cancer Immunol Immunother, 2009. **58**(1): p. 49-59.

273. Albeituni, S.H., C. Ding, and J. Yan, *Hampering immune suppressors: therapeutic targeting of myeloid-derived suppressor cells in cancer.* Cancer J, 2013. **19**(6): p. 490-501.
274. Verschoor, C.P., A. Puchta, and D.M. Bowdish, *The macrophage.* Methods Mol Biol, 2012. **844**: p. 139-56.
275. Gordon, S., *Biology of the macrophage.* J Cell Sci Suppl, 1986. **4**: p. 267-86.
276. Burke, B., et al., *Macrophages in gene therapy: cellular delivery vehicles and in vivo targets.* J Leukoc Biol, 2002. **72**(3): p. 417-28.
277. Epelman, S., K.J. Lavine, and G.J. Randolph, *Origin and functions of tissue macrophages.* Immunity, 2014. **41**(1): p. 21-35.
278. Epelman, S., et al., *Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation.* Immunity, 2014. **40**(1): p. 91-104.
279. Schulz, C., et al., *A lineage of myeloid cells independent of Myb and hematopoietic stem cells.* Science, 2012. **336**(6077): p. 86-90.
280. Yona, S., et al., *Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis.* Immunity, 2013. **38**(1): p. 79-91.
281. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment.* F1000Prime Rep, 2014. **6**: p. 13.
282. Biswas, S.K., P. Allavena, and A. Mantovani, *Tumor-associated macrophages: functional diversity, clinical significance, and open questions.* Semin Immunopathol, 2013. **35**(5): p. 585-600.
283. Allavena, P., et al., *The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance.* Immunol Rev, 2008. **222**: p. 155-61.
284. Biswas, S.K. and A. Mantovani, *Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm.* Nat Immunol, 2010. **11**(10): p. 889-96.
285. Weigert, A., D. Sekar, and B. Brune, *Tumor-associated macrophages as targets for tumor immunotherapy.* Immunotherapy, 2009. **1**(1): p. 83-95.
286. Celada, A., et al., *Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity.* J Exp Med, 1984. **160**(1): p. 55-74.
287. Devaud, C., et al., *Immune modulation of the tumor microenvironment for enhancing cancer immunotherapy.* Oncoimmunology, 2013. **2**(8): p. e25961.
288. Joyce, J.A. and J.W. Pollard, *Microenvironmental regulation of metastasis.* Nat Rev Cancer, 2009. **9**(4): p. 239-52.
289. Keller, R., et al., *Mechanisms of macrophage-mediated tumor cell killing: a comparative analysis of the roles of reactive nitrogen intermediates and tumor necrosis factor.* Int J Cancer, 1990. **46**(4): p. 682-6.
290. Noy, R. and J.W. Pollard, *Tumor-associated macrophages: from mechanisms to therapy.* Immunity, 2014. **41**(1): p. 49-61.
291. Franklin, R.A., et al., *The cellular and molecular origin of tumor-associated macrophages.* Science, 2014. **344**(6186): p. 921-5.
292. Gershon, R.K. and K. Kondo, *Cell interactions in the induction of tolerance: the role of thymic lymphocytes.* Immunology, 1970. **18**(5): p. 723-37.
293. Gershon, R.K. and K. Kondo, *Infectious immunological tolerance.* Immunology, 1971. **21**(6): p. 903-14.
294. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.
295. Sakaguchi, S., *Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses.* Annu Rev Immunol, 2004. **22**: p. 531-62.
296. Shevach, E.M., *Mechanisms of foxp3+ T regulatory cell-mediated suppression.* Immunity, 2009. **30**(5): p. 636-45.

297. Ziegler, S.F., *FOXP3: of mice and men.* Annu Rev Immunol, 2006. **24**: p. 209-26.
298. Huehn, J., J.K. Polansky, and A. Hamann, *Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage?* Nat Rev Immunol, 2009. **9**(2): p. 83-9.
299. Liu, W., et al., *CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells.* J Exp Med, 2006. **203**(7): p. 1701-11.
300. Seddiki, N., et al., *Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells.* J Exp Med, 2006. **203**(7): p. 1693-700.
301. Malek, T.R., *The main function of IL-2 is to promote the development of T regulatory cells.* J Leukoc Biol, 2003. **74**(6): p. 961-5.
302. Gavin, M.A., et al., *Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo.* Nat Immunol, 2002. **3**(1): p. 33-41.
303. Wang, H.Y. and R.F. Wang, *Regulatory T cells and cancer.* Curr Opin Immunol, 2007. **19**(2): p. 217-23.
304. Ha, T.Y., *The role of regulatory T cells in cancer.* Immune Netw, 2009. **9**(6): p. 209-35.
305. Robinson, D.S., M. Larche, and S.R. Durham, *Tregs and allergic disease.* J Clin Invest, 2004. **114**(10): p. 1389-97.
306. Mougiakakos, D., et al., *Regulatory T cells in cancer.* Adv Cancer Res, 2010. **107**: p. 57-117.
307. Liyanage, U.K., et al., *Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma.* J Immunol, 2002. **169**(5): p. 2756-61.
308. Kawaida, H., et al., *Distribution of CD4+CD25high regulatory T cells in tumor-draining lymph nodes in patients with gastric cancer.* J Surg Res, 2005. **124**(1): p. 151-7.
309. Boucek, J., et al., *Regulatory T cells and their prognostic value for patients with squamous cell carcinoma of the head and neck.* J Cell Mol Med, 2010. **14**(1-2): p. 426-33.
310. Curiel, T.J., et al., *Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival.* Nat Med, 2004. **10**(9): p. 942-9.
311. Zou, W., *Regulatory T cells, tumour immunity and immunotherapy.* Nat Rev Immunol, 2006. **6**(4): p. 295-307.
312. Menetrier-Caux, C., et al., *Targeting regulatory T cells.* Target Oncol, 2012. **7**(1): p. 15-28.
313. Iellem, A., et al., *Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells.* J Exp Med, 2001. **194**(6): p. 847-53.
314. Sutmuller, R.P., et al., *Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses.* J Exp Med, 2001. **194**(6): p. 823-32.
315. Nishikawa, H., et al., *Accelerated chemically induced tumor development mediated by CD4+CD25+ regulatory T cells in wild-type hosts.* Proc Natl Acad Sci U S A, 2005. **102**(26): p. 9253-7.
316. Xu, L., et al., *Enrichment of CCR6+Foxp3+ regulatory T cells in the tumor mass correlates with impaired CD8+ T cell function and poor prognosis of breast cancer.* Clin Immunol, 2010. **135**(3): p. 466-75.
317. Ahmadzadeh, M. and S.A. Rosenberg, *IL-2 administration increases CD4+ CD25(hi) Foxp3+ regulatory T cells in cancer patients.* Blood, 2006. **107**(6): p. 2409-14.
318. Jensen, H.K., et al., *Increased intratumoral FOXP3-positive regulatory immune cells during interleukin-2 treatment in metastatic renal cell carcinoma.* Clin Cancer Res, 2009. **15**(3): p. 1052-8.
319. Ghiringhelli, F., et al., *Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation.* J Exp Med, 2005. **202**(7): p. 919-29.

320. Zhou, G. and H.I. Levitsky, *Natural regulatory T cells and de novo-induced regulatory T cells contribute independently to tumor-specific tolerance.* J Immunol, 2007. **178**(4): p. 2155-62.
321. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.
322. Liu, V.C., et al., *Tumor evasion of the immune system by converting CD4+CD25- T cells into CD4+CD25+ T regulatory cells: role of tumor-derived TGF-beta.* J Immunol, 2007. **178**(5): p. 2883-92.
323. Audia, S., et al., *Increase of CD4+ CD25+ regulatory T cells in the peripheral blood of patients with metastatic carcinoma: a Phase I clinical trial using cyclophosphamide and immunotherapy to eliminate CD4+ CD25+ T lymphocytes.* Clin Exp Immunol, 2007. **150**(3): p. 523-30.
324. Correale, P., et al., *Chemo-immunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients.* J Clin Oncol, 2005. **23**(35): p. 8950-8.
325. Tang, Q. and J.A. Bluestone, *The Foxp3+ regulatory T cell: a jack of all trades, master of regulation.* Nat Immunol, 2008. **9**(3): p. 239-44.
326. Cao, X., et al., *Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance.* Immunity, 2007. **27**(4): p. 635-46.
327. Strauss, L., C. Bergmann, and T.L. Whiteside, *Human circulating CD4+CD25highFoxp3+ regulatory T cells kill autologous CD8+ but not CD4+ responder cells by Fas-mediated apoptosis.* J Immunol, 2009. **182**(3): p. 1469-80.
328. Ren, X., et al., *Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells.* Cell Death Differ, 2007. **14**(12): p. 2076-84.
329. Oderup, C., et al., *Costimulation blockade-induced cardiac allograft tolerance: inhibition of T cell expansion and accumulation of intragraft cD4(+)Foxp3(+) T cells.* Transplantation, 2006. **82**(11): p. 1493-500.
330. Onishi, Y., et al., *Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation.* Proc Natl Acad Sci U S A, 2008. **105**(29): p. 10113-8.
331. Tadokoro, C.E., et al., *Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo.* J Exp Med, 2006. **203**(3): p. 505-11.
332. Fallarino, F., et al., *The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells.* J Immunol, 2006. **176**(11): p. 6752-61.
333. Munn, D.H. and A.L. Mellor, *Indoleamine 2,3-dioxygenase and tumor-induced tolerance.* J Clin Invest, 2007. **117**(5): p. 1147-54.
334. Deaglio, S., et al., *Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression.* J Exp Med, 2007. **204**(6): p. 1257-65.
335. Vignali, D.A., L.W. Collison, and C.J. Workman, *How regulatory T cells work.* Nat Rev Immunol, 2008. **8**(7): p. 523-32.
336. Bopp, T., et al., *Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression.* J Exp Med, 2007. **204**(6): p. 1303-10.
337. Byrne, W.L., et al., *Targeting regulatory T cells in cancer.* Cancer Res, 2011. **71**(22): p. 6915-20.
338. Liu, C., C.J. Workman, and D.A. Vignali, *Targeting Regulatory T Cells in Tumors.* FEBS J, 2016.
339. Pere, H., et al., *Comprehensive analysis of current approaches to inhibit regulatory T cells in cancer.* Oncoimmunology, 2012. **1**(3): p. 326-333.
340. Jacobs, J.F., et al., *Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy?* Lancet Oncol, 2012. **13**(1): p. e32-42.

341. Le, D.T. and E.M. Jaffee, *Regulatory T-cell modulation using cyclophosphamide in vaccine approaches: a current perspective.* Cancer Res, 2012. **72**(14): p. 3439-44.
342. Nair, S., et al., *Vaccination against the forkhead family transcription factor Foxp3 enhances tumor immunity.* Cancer Res, 2007. **67**(1): p. 371-80.
343. Shalev, I., et al., *Making sense of regulatory T cell suppressive function.* Semin Immunol, 2011. **23**(4): p. 282-92.
344. Wing, K., et al., *CTLA-4 control over Foxp3+ regulatory T cell function.* Science, 2008. **322**(5899): p. 271-5.
345. Peggs, K.S., et al., *Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies.* J Exp Med, 2009. **206**(8): p. 1717-25.
346. Ghiringhelli, F., et al., *CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative.* Eur J Immunol, 2004. **34**(2): p. 336-44.
347. Stagg, J. and M.J. Smyth, *Extracellular adenosine triphosphate and adenosine in cancer.* Oncogene, 2010. **29**(39): p. 5346-58.
348. Ohta, A., et al., *A2A adenosine receptor protects tumors from antitumor T cells.* Proc Natl Acad Sci U S A, 2006. **103**(35): p. 13132-7.
349. Jin, D., et al., *CD73 on tumor cells impairs antitumor T-cell responses: a novel mechanism of tumor-induced immune suppression.* Cancer Res, 2010. **70**(6): p. 2245-55.
350. Mandapathil, M., et al., *Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells.* J Biol Chem, 2010. **285**(10): p. 7176-86.
351. Stagg, J., et al., *CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis.* Cancer Res, 2011. **71**(8): p. 2892-900.
352. Stagg, J., et al., *Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis.* Proc Natl Acad Sci U S A, 2010. **107**(4): p. 1547-52.
353. Tatake, R.J., et al., *Establishment and characterization of four new squamous cell carcinoma cell lines derived from oral tumors.* J Cancer Res Clin Oncol, 1990. **116**(2): p. 179-86.
354. Ellis, G.I., et al., *Generation of induced regulatory T cells from primary human naive and memory T cells.* J Vis Exp, 2012(62).
355. de Waal Malefyt, R. and K. Boniface, *_In vitro_ human Th17 development.* 2007.
356. Laurence, A., et al., *Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation.* Immunity, 2007. **26**(3): p. 371-81.
357. Burnet, M., *Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications.* Br Med J, 1957. **1**(5023): p. 841-7.
358. Thomas, L., *Cellular and humoral aspects of the hypersensitive states.* Acta Medica Scandinavica, 1961. **170**(1): p. 128-128.
359. Seliger, B., *Strategies of tumor immune evasion.* BioDrugs, 2005. **19**(6): p. 347-54.
360. Zou, W., *Immunosuppressive networks in the tumour environment and their therapeutic relevance.* Nat Rev Cancer, 2005. **5**(4): p. 263-74.
361. Drake, C.G., E. Jaffee, and D.M. Pardoll, *Mechanisms of Immune Evasion by Tumors*, in *Advances in Immunology*2006, Academic Press. p. 51-81.
362. Whiteside, T.L., *Immune cells in the tumor microenvironment. Mechanisms responsible for functional and signaling defects.* Adv Exp Med Biol, 1998. **451**: p. 167-71.
363. Cheng, J., A. Montecalvo, and L.P. Kane, *Regulation of NF-kappaB induction by TCR/CD28.* Immunol Res, 2011. **50**(2-3): p. 113-7.
364. Burbach, B.J., et al., *T-cell receptor signaling to integrins.* Immunol Rev, 2007. **218**: p. 65-81.
365. Chen, L. and D.B. Flies, *Molecular mechanisms of T cell co-stimulation and co-inhibition.* Nat Rev Immunol, 2013. **13**(4): p. 227-42.

366. Rebouillat, D., et al., *The 100-kDa 2',5'-oligoadenylate synthetase catalyzing preferentially the synthesis of dimeric pppA2'p5'A molecules is composed of three homologous domains.* J Biol Chem, 1999. **274**(3): p. 1557-65.
367. Ghosh, A., et al., *A specific isozyme of 2'-5' oligoadenylate synthetase is a dual function proapoptotic protein of the Bcl-2 family.* J Biol Chem, 2001. **276**(27): p. 25447-55.
368. Hovanessian, A.G., *On the discovery of interferon-inducible, double-stranded RNA activated enzymes: the 2'-5'oligoadenylate synthetases and the protein kinase PKR.* Cytokine Growth Factor Rev, 2007. **18**(5-6): p. 351-61.
369. Thornberry, N.A. and Y. Lazebnik, *Caspases: enemies within.* Science, 1998. **281**(5381): p. 1312-6.
370. Rapidis, A.D. and G.T. Wolf, *Immunotherapy of Head and Neck Cancer: Current and Future Considerations.* Journal of Oncology, 2009. **2009**.
371. Kiessling, R., et al., *Tumor-induced immune dysfunction.* Cancer Immunology, Immunotherapy, 1999. **48**(7): p. 353-362.
372. Frydecka, I., et al., *Expression of signal-transducing zeta chain in peripheral blood T cells and natural killer cells in patients with Hodgkin's disease in different phases of the disease.* Leuk Lymphoma, 1999. **35**(5-6): p. 545-54.
373. Catalano, A., et al., *Semaphorin-3A is expressed by tumor cells and alters T-cell signal transduction and function.* Blood, 2006. **107**(8): p. 3321-9.
374. Benech, P., et al., *Interferon-responsive regulatory elements in the promoter of the human 2',5'-oligo(A) synthetase gene.* Mol Cell Biol, 1987. **7**(12): p. 4498-504.
375. Melchjorsen, J., et al., *Differential regulation of the OASL and OAS1 genes in response to viral infections.* J Interferon Cytokine Res, 2009. **29**(4): p. 199-207.
376. Mihm, U., et al., *Clinical relevance of the 2'-5'-oligoadenylate synthetase/RNase L system for treatment response in chronic hepatitis C.* J Hepatol, 2009. **50**(1): p. 49-58.
377. Kim, K.I., et al., *2'-,5'-Oligoadenylate synthetase response ratio predicting virological response to PEG-interferon-alpha2b plus ribavirin therapy in patients with chronic hepatitis C.* J Clin Pharm Ther, 2006. **31**(5): p. 441-6.
378. Shindo, M., et al., *In vivo interferon system assessed by 2'-5' oligoadenylate synthetase activity in chronic hepatitis C virus patients treated with pegylated interferon and ribavirin.* Hepatol Res, 2008. **38**(12): p. 1213-20.
379. Kristiansen, H., et al., *Extracellular 2'-5' oligoadenylate synthetase stimulates RNase L-independent antiviral activity: a novel mechanism of virus-induced innate immunity.* J Virol, 2010. **84**(22): p. 11898-904.
380. Feng, X., et al., *Low expression of interferon-stimulated genes in active multiple sclerosis is linked to subnormal phosphorylation of STAT1.* J Neuroimmunol, 2002. **129**(1-2): p. 205-15.
381. Sanda, C., et al., *Differential gene induction by type I and type II interferons and their combination.* J Interferon Cytokine Res, 2006. **26**(7): p. 462-72.
382. Hovanessian, A.G. and J. Justesen, *The human 2'-5'oligoadenylate synthetase family: unique interferon-inducible enzymes catalyzing 2'-5' instead of 3'-5' phosphodiester bond formation.* Biochimie, 2007. **89**(6-7): p. 779-88.
383. Chi, L.M., et al., *Enhanced interferon signaling pathway in oral cancer revealed by quantitative proteome analysis of microdissected specimens using 16O/18O labeling and integrated two-dimensional LC-ESI-MALDI tandem MS.* Mol Cell Proteomics, 2009. **8**(7): p. 1453-74.
384. Bower, J.E., et al., *Yoga reduces inflammatory signaling in fatigued breast cancer survivors: a randomized controlled trial.* Psychoneuroendocrinology, 2014. **43**: p. 20-9.
385. Vaarala, M.H., et al., *Differentially expressed genes in two LNCaP prostate cancer cell lines reflecting changes during prostate cancer progression.* Lab Invest, 2000. **80**(8): p. 1259-68.
386. Liu, Y.P., et al., *Induction of antiviral genes by the tumor microenvironment confers resistance to virotherapy.* Sci Rep, 2013. **3**: p. 2375.

387. Wang, B., et al., *IL-17A but not IL-22 suppresses the replication of hepatitis B virus mediated by over-expression of MxA and OAS mRNA in the HepG2.2.15 cell line.* Antiviral Res, 2013. **97**(3): p. 285-92.
388. Lallemand, C., et al., *Constitutive expression of specific interferon isotypes in peripheral blood leukocytes from normal individuals and in promonocytic U937 cells.* J Leukoc Biol, 1996. **60**(1): p. 137-46.
389. Baechler, E.C., et al., *Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2610-5.
390. van Baarsen, L.G., et al., *A subtype of multiple sclerosis defined by an activated immune defense program.* Genes Immun, 2006. **7**(6): p. 522-31.
391. van der Pouw Kraan, T.C., et al., *Rheumatoid arthritis subtypes identified by genomic profiling of peripheral blood cells: assignment of a type I interferon signature in a subpopulation of patients.* Ann Rheum Dis, 2007. **66**(8): p. 1008-14.
392. Tough, D.F., et al., *Stimulation of naive and memory T cells by cytokines.* Immunol Rev, 1999. **170**: p. 39-47.
393. Rebouillat, D., I. Marie, and A.G. Hovanessian, *Molecular cloning and characterization of two related and interferon-induced 56-kDa and 30-kDa proteins highly similar to 2'-5' oligoadenylate synthetase.* Eur J Biochem, 1998. **257**(2): p. 319-30.
394. Mashimo, T., et al., *Structural and functional genomics and evolutionary relationships in the cluster of genes encoding murine 2',5'-oligoadenylate synthetases.* Genomics, 2003. **82**(5): p. 537-52.
395. Hartmann, R., et al., *p59OASL, a 2'-5' oligoadenylate synthetase like protein: a novel human gene related to the 2'-5' oligoadenylate synthetase family.* Nucleic Acids Res, 1998. **26**(18): p. 4121-8.
396. Hovnanian, A., et al., *The human 2',5'-oligoadenylate synthetase-like gene (OASL) encoding the interferon-induced 56-kDa protein maps to chromosome 12q24.2 in the proximity of the 2',5'-OAS locus.* Genomics, 1999. **56**(3): p. 362-3.
397. Lin, R.J., et al., *Distinct antiviral roles for human 2',5'-oligoadenylate synthetase family members against dengue virus infection.* J Immunol, 2009. **183**(12): p. 8035-43.
398. Bonnevie-Nielsen, V., et al., *Variation in antiviral 2',5'-oligoadenylate synthetase (2'5'AS) enzyme activity is controlled by a single-nucleotide polymorphism at a splice-acceptor site in the OAS1 gene.* Am J Hum Genet, 2005. **76**(4): p. 623-33.
399. Soto-Nieves, N., et al., *Transcriptional complexes formed by NFAT dimers regulate the induction of T cell tolerance.* J Exp Med, 2009. **206**(4): p. 867-76.
400. Takahashi, A., et al., *Elevated caspase-3 activity in peripheral blood T cells coexists with increased degree of T-cell apoptosis and down-regulation of TCR zeta molecules in patients with gastric cancer.* Clin Cancer Res, 2001. **7**(1): p. 74-80.
401. Maki, A., et al., *Decreased expression of CD28 coincides with the down-modulation of CD3zeta and augmentation of caspase-3 activity in T cells from hepatocellular carcinoma-bearing patients and hepatitis C virus-infected patients.* J Gastroenterol Hepatol, 2004. **19**(12): p. 1348-56.
402. Woo, M., et al., *Caspase-3 regulates cell cycle in B cells: a consequence of substrate specificity.* Nat Immunol, 2003. **4**(10): p. 1016-22.
403. Alam, A., et al., *Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells.* J Exp Med, 1999. **190**(12): p. 1879-90.
404. Algeciras-Schimnich, A., B.C. Barnhart, and M.E. Peter, *Apoptosis-independent functions of killer caspases.* Curr Opin Cell Biol, 2002. **14**(6): p. 721-6.
405. Denis, F., et al., *The role of caspases in T cell development and the control of immune responses.* Cell Mol Life Sci, 1998. **54**(9): p. 1005-19.

406. Krishnan, S., et al., *Increased caspase-3 expression and activity contribute to reduced CD3zeta expression in systemic lupus erythematosus T cells.* J Immunol, 2005. **175**(5): p. 3417-23.
407. Rusch, L., A. Zhou, and R.H. Silverman, *Caspase-dependent apoptosis by 2',5'-oligoadenylate activation of RNase L is enhanced by IFN-beta.* J Interferon Cytokine Res, 2000. **20**(12): p. 1091-100.
408. Ludwiczek, O., et al., *Activation of caspase-3 by interferon alpha causes interleukin-16 secretion but fails to modulate activation induced cell death.* Eur Cytokine Netw, 2001. **12**(3): p. 478-86.
409. Dugan, J.W., et al., *Nucleotide oligomerization domain-2 interacts with 2'-5'-oligoadenylate synthetase type 2 and enhances RNase-L function in THP-1 cells.* Mol Immunol, 2009. **47**(2-3): p. 560-6.
410. Takeuchi, T., et al., *CD3 zeta defects in systemic lupus erythematosus.* Ann Rheum Dis, 2012. **71 Suppl 2**: p. i78-81.
411. Clavien, P.A., *Interferon: the magic bullet to prevent hepatocellular carcinoma recurrence after resection?* Ann Surg, 2007. **245**(6): p. 843-5.
412. Sun, H.C., et al., *Postoperative interferon alpha treatment postponed recurrence and improved overall survival in patients after curative resection of HBV-related hepatocellular carcinoma: a randomized clinical trial.* J Cancer Res Clin Oncol, 2006. **132**(7): p. 458-65.
413. Urba, S.G., et al., *Intensive recombinant interleukin-2 and alpha-interferon therapy in patients with advanced head and neck squamous carcinoma.* Cancer, 1993. **71**(7): p. 2326-31.
414. Mukherjee, K.K., et al., *IFNalpha2b augments immune responses of cisplatin+5-fluorouracil treated tongue squamous cell carcinoma patients--a preliminary study.* Indian J Med Res, 2012. **136**(1): p. 54-9.
415. Honda, M., et al., *Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C.* Gastroenterology, 2010. **139**(2): p. 499-509.
416. Burkholder, B., et al., *Tumor-induced perturbations of cytokines and immune cell networks.* Biochim Biophys Acta, 2014. **1845**(2): p. 182-201.
417. Bemelmans, M.H., L.J. van Tits, and W.A. Buurman, *Tumor necrosis factor: function, release and clearance.* Crit Rev Immunol, 1996. **16**(1): p. 1-11.
418. Vandenabeele, P., et al., *Two tumour necrosis factor receptors: structure and function.* Trends Cell Biol, 1995. **5**(10): p. 392-9.
419. Tartaglia, L.A. and D.V. Goeddel, *Two TNF receptors.* Immunol Today, 1992. **13**(5): p. 151-3.
420. Aggarwal, B.B., *Signalling pathways of the TNF superfamily: a double-edged sword.* Nat Rev Immunol, 2003. **3**(9): p. 745-56.
421. Ashkenazi, A. and V.M. Dixit, *Death receptors: signaling and modulation.* Science, 1998. **281**(5381): p. 1305-8.
422. Degterev, A., M. Boyce, and J. Yuan, *A decade of caspases.* Oncogene, 2003. **22**(53): p. 8543-67.
423. Bertrand, F., et al., *Blocking Tumor Necrosis Factor alpha Enhances CD8 T-cell-Dependent Immunity in Experimental Melanoma.* Cancer Res, 2015. **75**(13): p. 2619-28.
424. Ding, X., et al., *TNF receptor 1 mediates dendritic cell maturation and CD8 T cell response through two distinct mechanisms.* J Immunol, 2011. **187**(3): p. 1184-91.
425. Zheng, L., et al., *Induction of apoptosis in mature T cells by tumour necrosis factor.* Nature, 1995. **377**(6547): p. 348-51.
426. Ratner, A. and W.R. Clark, *Role of TNF-alpha in CD8+ cytotoxic T lymphocyte-mediated lysis.* J Immunol, 1993. **150**(10): p. 4303-14.
427. Kim, E.Y. and H.S. Teh, *TNF type 2 receptor (p75) lowers the threshold of T cell activation.* J Immunol, 2001. **167**(12): p. 6812-20.

428. Chatzidakis, I., et al., *An essential role for TNF in modulating thresholds for survival, activation, and tolerance of CD8+ T cells.* J Immunol, 2007. **178**(11): p. 6735-45.
429. Chopra, M., et al., *Tumor necrosis factor receptor 2-dependent homeostasis of regulatory T cells as a player in TNF-induced experimental metastasis.* Carcinogenesis, 2013. **34**(6): p. 1296-303.
430. Okubo, Y., et al., *Homogeneous expansion of human T-regulatory cells via tumor necrosis factor receptor 2.* Sci Rep, 2013. **3**: p. 3153.
431. Chen, X., et al., *TNFR2 is critical for the stabilization of the CD4+Foxp3+ regulatory T. cell phenotype in the inflammatory environment.* J Immunol, 2013. **190**(3): p. 1076-84.
432. Zhao, X., et al., *TNF signaling drives myeloid-derived suppressor cell accumulation.* J Clin Invest, 2012. **122**(11): p. 4094-104.
433. Kim, E.Y. and H.S. Teh, *Critical role of TNF receptor type-2 (p75) as a costimulator for IL-2 induction and T cell survival: a functional link to CD28.* J Immunol, 2004. **173**(7): p. 4500-9.
434. Aspalter, R.M., H.M. Wolf, and M.M. Eibl, *Chronic TNF-alpha exposure impairs TCR-signaling via TNF-RII but not TNF-RI.* Cell Immunol, 2005. **237**(1): p. 55-67.
435. Bryl, E., et al., *Down-regulation of CD28 expression by TNF-alpha.* J Immunol, 2001. **167**(6): p. 3231-8.
436. Okkenhaug, K. and R. Rottapel, *Grb2 forms an inducible protein complex with CD28 through a Src homology 3 domain-proline interaction.* J Biol Chem, 1998. **273**(33): p. 21194-202.
437. Cope, A.P., *Regulation of autoimmunity by proinflammatory cytokines.* Current Opinion in Immunology, 1998. **10**(6): p. 669-676.
438. Chen, G. and D.V. Goeddel, *TNF-R1 signaling: a beautiful pathway.* Science, 2002. **296**(5573): p. 1634-5.
439. D'Oro, U., et al., *Regulation of constitutive TCR internalization by the zeta-chain.* J Immunol, 2002. **169**(11): p. 6269-78.
440. Redmond, W.L. and L.A. Sherman, *Peripheral tolerance of CD8 T lymphocytes.* Immunity, 2005. **22**(3): p. 275-84.
441. Marrack, P. and J. Kappler, *Control of T cell viability.* Annu Rev Immunol, 2004. **22**: p. 765-87.
442. Finetti, F., et al., *The proapoptotic and antimitogenic protein p66SHC acts as a negative regulator of lymphocyte activation and autoimmunity.* Blood, 2008. **111**(10): p. 5017-27.
443. Orsini, F., et al., *The life span determinant p66Shc localizes to mitochondria where it associates with mitochondrial heat shock protein 70 and regulates trans-membrane potential.* J Biol Chem, 2004. **279**(24): p. 25689-95.
444. Kamata, H., et al., *Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases.* Cell, 2005. **120**(5): p. 649-61.
445. Hong, D.S., et al., *BRAF(V600) inhibitor GSK2118436 targeted inhibition of mutant BRAF in cancer patients does not impair overall immune competency.* Clin Cancer Res, 2012. **18**(8): p. 2326-35.
446. Wilmott, J.S., et al., *Dynamics of chemokine, cytokine, and growth factor serum levels in BRAF-mutant melanoma patients during BRAF inhibitor treatment.* J Immunol, 2014. **192**(5): p. 2505-13.
447. Menon, D.R., et al., *TGF-beta1 and TNF-alpha differentially regulate Twist1 mediated resistance towards BRAF/MEK inhibition in melanoma.* Pigment Cell Melanoma Res, 2013. **26**(6): p. 912-6.
448. Guruharsha, K.G., M.W. Kankel, and S. Artavanis-Tsakonas, *The Notch signalling system: recent insights into the complexity of a conserved pathway.* Nat Rev Genet, 2012. **13**(9): p. 654-66.
449. Artavanis-Tsakonas, S. and M.A. Muskavitch, *Notch: the past, the present, and the future.* Curr Top Dev Biol, 2010. **92**: p. 1-29.
450. Maillard, I., S.H. Adler, and W.S. Pear, *Notch and the immune system.* Immunity, 2003. **19**(6): p. 781-91.

451. Bray, S.J., *Notch signalling: a simple pathway becomes complex.* Nat Rev Mol Cell Biol, 2006. **7**(9): p. 678-89.
452. Lecourtois, M. and F. Schweisguth, *Indirect evidence for Delta-dependent intracellular processing of Notch in Drosophila embryos.* Curr Biol, 1998. **8**(13): p. 771-4.
453. Schroeter, E.H., J.A. Kisslinger, and R. Kopan, *Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain.* Nature, 1998. **393**(6683): p. 382-6.
454. Fiuza, U.M. and A.M. Arias, *Cell and molecular biology of Notch.* J Endocrinol, 2007. **194**(3): p. 459-74.
455. Radtke, F., N. Fasnacht, and H.R. Macdonald, *Notch signaling in the immune system.* Immunity, 2010. **32**(1): p. 14-27.
456. Yuan, J.S., et al., *Functions of Notch signaling in the immune system: consensus and controversies.* Annu Rev Immunol, 2010. **28**: p. 343-65.
457. Pui, J.C., et al., *Notch1 expression in early lymphopoiesis influences B versus T lineage determination.* Immunity, 1999. **11**(3): p. 299-308.
458. Tanigaki, K., et al., *Regulation of alphabeta/gammadelta T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling.* Immunity, 2004. **20**(5): p. 611-22.
459. Zheng, Y., Y. Zha, and T.F. Gajewski, *Molecular regulation of T-cell anergy.* EMBO Rep, 2008. **9**(1): p. 50-5.
460. Yun, T.J. and M.J. Bevan, *Notch-regulated ankyrin-repeat protein inhibits Notch1 signaling: multiple Notch1 signaling pathways involved in T cell development.* J Immunol, 2003. **170**(12): p. 5834-41.
461. Pollizzi, K.N. and J.D. Powell, *Integrating canonical and metabolic signalling programmes in the regulation of T cell responses.* Nat Rev Immunol, 2014. **14**(7): p. 435-46.
462. Nurieva, R.I., et al., *The E3 ubiquitin ligase GRAIL regulates T cell tolerance and regulatory T cell function by mediating T cell receptor-CD3 degradation.* Immunity, 2010. **32**(5): p. 670-80.
463. Baine, I., B.T. Abe, and F. Macian, *Regulation of T-cell tolerance by calcium/NFAT signaling.* Immunol Rev, 2009. **231**(1): p. 225-40.
464. Jelley-Gibbs, D.M., et al., *Influencing the fates of CD4 T cells on the path to memory: lessons from influenza.* Immunol Cell Biol, 2008. **86**(4): p. 343-52.
465. Dar, A.A., et al., *Extracellular 2'5'-oligoadenylate synthetase 2 mediates T-cell receptor CD3-zeta chain down-regulation via caspase-3 activation in oral cancer.* Immunology, 2016. **147**(2): p. 251-64.
466. Corn, R.A., et al., *T cell-intrinsic requirement for NF-kappa B induction in postdifferentiation IFN-gamma production and clonal expansion in a Th1 response.* J Immunol, 2003. **171**(4): p. 1816-24.
467. Wilkinson, B., H. Wang, and C.E. Rudd, *Positive and negative adaptors in T-cell signalling.* Immunology, 2004. **111**(4): p. 368-74.
468. Dar, A.A., R.S. Patil, and S.V. Chiplunkar, *Insights into the Relationship between Toll Like Receptors and Gamma Delta T Cell Responses.* Front Immunol, 2014. **5**: p. 366.
469. Auderset, F., et al., *Redundant Notch1 and Notch2 signaling is necessary for IFNgamma secretion by T helper 1 cells during infection with Leishmania major.* PLoS Pathog, 2012. **8**(3): p. e1002560.
470. Izon, D.J., et al., *Notch1 regulates maturation of CD4+ and CD8+ thymocytes by modulating TCR signal strength.* Immunity, 2001. **14**(3): p. 253-64.
471. Van de Walle, I., et al., *Specific Notch receptor-ligand interactions control human TCR-alphabeta/gammadelta development by inducing differential Notch signal strength.* J Exp Med, 2013. **210**(4): p. 683-97.
472. Kostianovsky, A.M., et al., *Up-regulation of gene related to anergy in lymphocytes is associated with Notch-mediated human T cell suppression.* J Immunol, 2007. **178**(10): p. 6158-63.

473. Kriegel, M.A., C. Rathinam, and R.A. Flavell, *E3 ubiquitin ligase GRAIL controls primary T cell activation and oral tolerance.* Proc Natl Acad Sci U S A, 2009. **106**(39): p. 16770-5.
474. Krishnan, S., et al., *Generation and biochemical analysis of human effector CD4 T cells: alterations in tyrosine phosphorylation and loss of CD3zeta expression.* Blood, 2001. **97**(12): p. 3851-9.
475. Blank, U., et al., *Complete structure and expression in transfected cells of high affinity IgE receptor.* Nature, 1989. **337**(6203): p. 187-9.
476. Krishnan, S., D.L. Farber, and G.C. Tsokos, *T cell rewiring in differentiation and disease.* J Immunol, 2003. **171**(7): p. 3325-31.
477. Joshi, I., et al., *Notch signaling mediates G1/S cell-cycle progression in T cells via cyclin D3 and its dependent kinases.* Blood, 2009. **113**(8): p. 1689-98.
478. Rutz, S., et al., *Notch regulates IL-10 production by T helper 1 cells.* Proc Natl Acad Sci U S A, 2008. **105**(9): p. 3497-502.
479. Sahoo, A., et al., *Grail controls Th2 cell development by targeting STAT6 for degradation.* Nat Commun, 2014. **5**: p. 4732.
480. Reichert, T.E., et al., *Absent or low expression of the zeta chain in T cells at the tumor site correlates with poor survival in patients with oral carcinoma.* Cancer Res, 1998. **58**(23): p. 5344-7.
481. Woo, E.Y., et al., *Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer.* Cancer Res, 2001. **61**(12): p. 4766-72.
482. Bronte, V., et al., *Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells.* Blood, 2000. **96**(12): p. 3838-46.
483. Filipazzi, P., et al., *Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine.* J Clin Oncol, 2007. **25**(18): p. 2546-53.
484. Ugel, S., et al., *Tumor-induced myeloid deviation: when myeloid-derived suppressor cells meet tumor-associated macrophages.* J Clin Invest, 2015. **125**(9): p. 3365-76.
485. Hoechst, B., et al., *A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+)Foxp3(+) T cells.* Gastroenterology, 2008. **135**(1): p. 234-43.
486. Kalathil, S., et al., *Higher frequencies of GARP(+)CTLA-4(+)Foxp3(+) T regulatory cells and myeloid-derived suppressor cells in hepatocellular carcinoma patients are associated with impaired T-cell functionality.* Cancer Res, 2013. **73**(8): p. 2435-44.
487. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.
488. Shevach, E.M., et al., *The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells.* Immunol Rev, 2006. **212**: p. 60-73.
489. Yi, H., et al., *Mouse CD11b+Gr-1+ myeloid cells can promote Th17 cell differentiation and experimental autoimmune encephalomyelitis.* J Immunol, 2012. **189**(9): p. 4295-304.
490. Chatterjee, S., et al., *Myeloid derived suppressor cells (MDSCs) can induce the generation of Th17 response from naive CD4+ T cells.* Immunobiology, 2013. **218**(5): p. 718-24.
491. Centuori, S.M., et al., *Myeloid-derived suppressor cells from tumor-bearing mice impair TGF-beta-induced differentiation of CD4+CD25+FoxP3+ Tregs from CD4+CD25-FoxP3- T cells.* J Leukoc Biol, 2012. **92**(5): p. 987-97.
492. Zhang, J.P., et al., *Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients.* J Hepatol, 2009. **50**(5): p. 980-9.
493. Greten, T.F., M.P. Manns, and F. Korangy, *Myeloid derived suppressor cells in human diseases.* Int Immunopharmacol, 2011. **11**(7): p. 802-7.
494. Condamine, T., et al., *ER stress regulates myeloid-derived suppressor cell fate through TRAIL-R-mediated apoptosis.* J Clin Invest, 2014. **124**(6): p. 2626-39.

495. Trinei, M., et al., *A p53-p66Shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis.* Oncogene, 2002. **21**(24): p. 3872-8.
496. Kujawski, M., et al., *Stat3 mediates myeloid cell-dependent tumor angiogenesis in mice.* J Clin Invest, 2008. **118**(10): p. 3367-77.
497. De Obaldia, M.E., et al., *T cell development requires constraint of the myeloid regulator C/EBP-alpha by the Notch target and transcriptional repressor Hes1.* Nat Immunol, 2013. **14**(12): p. 1277-84.
498. Kamakura, S., et al., *Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling.* Nat Cell Biol, 2004. **6**(6): p. 547-54.
499. Prins, R.M., M.R. Graf, and R.E. Merchant, *Cytotoxic T cells infiltrating a glioma express an aberrant phenotype that is associated with decreased function and apoptosis.* Cancer Immunol Immunother, 2001. **50**(6): p. 285-92.
500. Rabinovich, G.A., D. Gabrilovich, and E.M. Sotomayor, *Immunosuppressive strategies that are mediated by tumor cells.* Annu Rev Immunol, 2007. **25**: p. 267-96.
501. Filipazzi, P., V. Huber, and L. Rivoltini, *Phenotype, function and clinical implications of myeloid-derived suppressor cells in cancer patients.* Cancer Immunol Immunother, 2012. **61**(2): p. 255-63.
502. Wang, L., et al., *Increased myeloid-derived suppressor cells in gastric cancer correlate with cancer stage and plasma S100A8/A9 proinflammatory proteins.* J Immunol, 2013. **190**(2): p. 794-804.
503. Rudolph, B.M., et al., *Increased frequencies of CD11b(+) CD33(+) CD14(+) HLA-DR(low) myeloid-derived suppressor cells are an early event in melanoma patients.* Exp Dermatol, 2014. **23**(3): p. 202-4.
504. Sade-Feldman, M., et al., *Clinical significance of circulating CD33+CD11b+HLA-DR- myeloid cells in Stage-IV melanoma patients treated with ipilimumab.* Clin Cancer Res, 2016.
505. Zhang, B., et al., *Circulating and tumor-infiltrating myeloid-derived suppressor cells in patients with colorectal carcinoma.* PLoS One, 2013. **8**(2): p. e57114.
506. Hermouet, S., E. Bigot-Corbel, and B. Gardie, *Pathogenesis of Myeloproliferative Neoplasms: Role and Mechanisms of Chronic Inflammation.* Mediators Inflamm, 2015. **2015**: p. 145293.
507. Stransky, N., et al., *The mutational landscape of head and neck squamous cell carcinoma.* Science, 2011. **333**(6046): p. 1157-60.
508. Klinakis, A., et al., *A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia.* Nature, 2011. **473**(7346): p. 230-3.
509. Mandruzzato, S., et al., *IL4Ralpha+ myeloid-derived suppressor cell expansion in cancer patients.* J Immunol, 2009. **182**(10): p. 6562-8.
510. Corzo, C.A., et al., *HIF-1alpha regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment.* J Exp Med, 2010. **207**(11): p. 2439-53.
511. Eruslanov, E., et al., *Circulating and tumor-infiltrating myeloid cell subsets in patients with bladder cancer.* Int J Cancer, 2012. **130**(5): p. 1109-19.
512. Najjar, Y.G. and J.H. Finke, *Clinical perspectives on targeting of myeloid derived suppressor cells in the treatment of cancer.* Front Oncol, 2013. **3**: p. 49.
513. Hossain, D.M., et al., *TLR9-Targeted STAT3 Silencing Abrogates Immunosuppressive Activity of Myeloid-Derived Suppressor Cells from Prostate Cancer Patients.* Clin Cancer Res, 2015. **21**(16): p. 3771-82.
514. Takizawa, H., S. Boettcher, and M.G. Manz, *Demand-adapted regulation of early hematopoiesis in infection and inflammation.* Blood, 2012. **119**(13): p. 2991-3002.
515. Xing, Z., et al., *Increased hematopoietic stem cell mobilization in aged mice.* Blood, 2006. **108**(7): p. 2190-7.

516. Christiansson, L., et al., *Increased level of myeloid-derived suppressor cells, programmed death receptor ligand 1/programmed death receptor 1, and soluble CD25 in Sokal high risk chronic myeloid leukemia.* PLoS One, 2013. **8**(1): p. e55818.
517. Jitschin, R., et al., *CLL-cells induce IDOhi CD14+HLA-DRlo myeloid-derived suppressor cells that inhibit T-cell responses and promote TRegs.* Blood, 2014. **124**(5): p. 750-60.
518. Gabrilovich, D.I., et al., *Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells.* J Immunol, 2001. **166**(9): p. 5398-406.
519. Lechner, M.G., D.J. Liebertz, and A.L. Epstein, *Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells.* J Immunol, 2010. **185**(4): p. 2273-84.
520. Zhang, H., et al., *STAT3 controls myeloid progenitor growth during emergency granulopoiesis.* Blood, 2010. **116**(14): p. 2462-71.
521. Zhang, P., et al., *Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha.* Immunity, 2004. **21**(6): p. 853-63.
522. Grandis, J.R. and D.J. Tweardy, *Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer.* Cancer Res, 1993. **53**(15): p. 3579-84.
523. Grandis, J.R., et al., *Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo.* Proc Natl Acad Sci U S A, 2000. **97**(8): p. 4227-32.
524. Shin, D.M., et al., *Dysregulation of epidermal growth factor receptor expression in premalignant lesions during head and neck tumorigenesis.* Cancer Res, 1994. **54**(12): p. 3153-9.
525. Vermorken, J.B., et al., *Platinum-based chemotherapy plus cetuximab in head and neck cancer.* N Engl J Med, 2008. **359**(11): p. 1116-27.
526. Hsu, P., et al., *IL-10 Potentiates Differentiation of Human Induced Regulatory T Cells via STAT3 and Foxo1.* J Immunol, 2015. **195**(8): p. 3665-74.
527. Fujimura, T., Y. Kambayashi, and S. Aiba, *Crosstalk between regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) during melanoma growth.* Oncoimmunology, 2012. **1**(8): p. 1433-1434.
528. White, R.A., S.P. Malkoski, and X.J. Wang, *TGFbeta signaling in head and neck squamous cell carcinoma.* Oncogene, 2010. **29**(40): p. 5437-46.
529. Bornstein, S., et al., *IL-10 and integrin signaling pathways are associated with head and neck cancer progression.* BMC Genomics, 2016. **17**: p. 38.
530. Cook, D.N., et al., *CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue.* Immunity, 2000. **12**(5): p. 495-503.
531. Yang, D., et al., *Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6.* Science, 1999. **286**(5439): p. 525-8.
532. Basu, R., et al., *IL-1 signaling modulates activation of STAT transcription factors to antagonize retinoic acid signaling and control the TH17 cell-iTreg cell balance.* Nat Immunol, 2015. **16**(3): p. 286-95.
533. Mailer, R.K., et al., *IL-1beta promotes Th17 differentiation by inducing alternative splicing of FOXP3.* Sci Rep, 2015. **5**: p. 14674.
534. Bruchard, M., et al., *Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth.* Nat Med, 2013. **19**(1): p. 57-64.
535. Zhang, H., et al., *Myeloid-derived suppressor cells are proinflammatory and regulate collagen-induced arthritis through manipulating Th17 cell differentiation.* Clin Immunol, 2015. **157**(2): p. 175-86.
536. Jiao, Z.J., et al., *Correlation between circulating myeloid-derived suppressor cells and Th17 cells in esophageal cancer.* World J Gastroenterol, 2012. **18**(38): p. 5454-61.

537. van Horssen, R., T.L. Ten Hagen, and A.M. Eggermont, *TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility.* Oncologist, 2006. **11**(4): p. 397-408.
538. Schaer, D.A., D. Hirschhorn-Cymerman, and J.D. Wolchok, *Targeting tumor-necrosis factor receptor pathways for tumor immunotherapy.* J Immunother Cancer, 2014. **2**: p. 7.
539. Meidenbauer, N., et al., *Recovery of zeta-chain expression and changes in spontaneous IL-10 production after PSA-based vaccines in patients with prostate cancer.* Br J Cancer, 2002. **86**(2): p. 168-78.
540. Sadelain, M., R. Brentjens, and I. Riviere, *The basic principles of chimeric antigen receptor design.* Cancer Discov, 2013. **3**(4): p. 388-98.
541. Geiger, J.L., J.R. Grandis, and J.E. Bauman, *The STAT3 pathway as a therapeutic target in head and neck cancer: Barriers and innovations.* Oral Oncol, 2016. **56**: p. 84-92.
542. Zandberg, D.P. and S.E. Strome, *The role of the PD-L1:PD-1 pathway in squamous cell carcinoma of the head and neck.* Oral Oncol, 2014. **50**(7): p. 627-32.
543. Zou, W. and L. Chen, *Inhibitory B7-family molecules in the tumour microenvironment.* Nat Rev Immunol, 2008. **8**(6): p. 467-77.
544. Topalian, S.L., et al., *Safety, activity, and immune correlates of anti-PD-1 antibody in cancer.* N Engl J Med, 2012. **366**(26): p. 2443-54.
545. Topalian, S.L., C.G. Drake, and D.M. Pardoll, *Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity.* Curr Opin Immunol, 2012. **24**(2): p. 207-12.
546. Badoual, C., et al., *PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer.* Cancer Res, 2013. **73**(1): p. 128-38.

Extracellular 2′5′-oligoadenylate synthetase 2 mediates T-cell receptor CD3-ζ chain down-regulation via caspase-3 activation in oral cancer

Asif A. Dar,[1] Trupti N. Pradhan,[1] Dakshayni P. Kulkarni,[1] Sagar U. Shah,[1] Kanury V. Rao,[2] Devendra A. Chaukar,[3] Anil K. D'Cruz[3] and Shubhada V. Chiplunkar[1]

[1]*Chiplunkar Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai,* [2]*Immunology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, and* [3]*Tata Memorial Hospital, Mumbai, India*

doi:10.1111/imm.12560
Received 6 July 2015; revised 11 November 2015; accepted 13 November 2015.
Correspondence: Prof. Shubhada V. Chiplunkar, Principal investigator, Chiplunkar Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai, Maharashtra 410210, India. Email: schiplunkar@actrec.gov.in
Senior author: Prof. Shubhada V. Chiplunkar

Summary

Decreased expression of CD3-ζ chain, an adaptor protein associated with T-cell signalling, is well documented in patients with oral cancer, but the mechanistic justifications are fragmentary. Previous studies in patients with oral cancer have shown that decreased expression of CD3-ζ chain was associated with decreased responsiveness of T cells. Tumours are known to induce localized as well as systemic immune suppression. This study provides evidence that oral tumour-derived factors promote immune suppression by down-regulating CD3-ζ chain expression. 2′5′-Oligoadenylate synthetase 2 (OAS2) was identified by the proteomic approach and our results established a causative link between CD3-ζ chain down-regulation and OAS2 stimulation. The surrogate situation was established by over-expressing OAS2 in a HEK293 cell line and cell-free supernatant was collected. These supernatants when incubated with T cells resulted in down-regulation of CD3-ζ chain, which shows that the secreted OAS2 is capable of regulating CD3-ζ chain expression. Incubation of T cells with cell-free supernatants of oral tumours or recombinant human OAS2 (rh-OAS2) induced caspase-3 activation, which resulted in CD3-ζ chain down-regulation. Caspase-3 inhibition/down-regulation using pharmacological inhibitor or small interfering RNA restored down-regulated CD3-ζ chain expression in T cells induced by cell-free tumour supernatant or rh-OAS2. Collectively these results show that OAS2 leads to impairment in CD3-ζ chain expression, so offering an explanation that might be applicable to the CD3-ζ chain deficiency observed in cancer and diverse disease conditions.

Keywords: CD3-ζ chain; humans; T cells; tumour immunology; tumour-secreted factors.

Introduction

The cancer immunoediting hypothesis stresses the dual role of the immune system: host protection and tumour shaping. The immune system, apart from eliminating the nascent malignant cells, also shapes the tumour through equilibrium and escape phases.[1] The ability of tumour cells to escape obliteration by immune cells could be because of the plethora of strategies used to evade immune attack. One of these is represented by the production of soluble immunosuppressive factors that may prevent the pro-inflammatory effects and promote T-cell dysfunction in the tumour microenvironment. Immune dysfunction appears to be more frequent and profound in patients with cancer. Immune effector cells obtained from the peripheral blood of cancer patients, including oral cancer have been reported to have a variety of functional abnormalities, which may vary in magnitude from patient

Abbreviations: HIs, healthy individuals; IFN, interferon; MxA, myxovirus resistance gene A; OAS2, 2′5′-oligoadenylate synthetase 2; PBMCs, peripheral blood mononuclear cells; rh-OAS2, recombinant human OAS2; TCR, T-cell receptor

to patient and may be related to the extent of the disease.[2,3] These abnormalities include defects in T-cell signalling via the T-cell receptor (TCR), decreased tyrosine kinase activity following triggering with anti-CD3 monoclonal antibodies, poor lymphocytic proliferative responses, defects in lytic capacity, and decreased ability for cytokine production.[3–6] The immune dysfunction is also associated with the down-regulation of expression of the TCR-ζ chain (CD3-ζ) or of intracellular protein kinases in cancer-bearing hosts.[7]

Decreased expression of the CD3-ζ chain has been reported in several autoimmune, inflammatory and malignant diseases. It has been reported that cancer cells produce several ligands that function to prevent optimal T-cell activation through CD3-ζ chain down-regulation and induces either T-cell anergy or apoptosis.[1,8] Studies from our laboratory have shown that post-translational down-regulation is primarily responsible for decreased CD3-ζ chain expression in the peripheral blood of patients with oral cancer whereas a dominant transcriptional defect is observed in the tumour compartment. The down-regulation of CD3-ζ chain culminates in impaired lymphocyte responses in these patients.[9]

The cytoplasmic domain of CD3-ζ chain has several consensus target sequences for caspases, among which caspase-3 and caspase-7 have been shown to cleave *in vitro* translated CD3-ζ chain.[10] Caspase-3, an effector caspase, is expressed during T-cell anergy induction and recognizes proteins with a common DXXD motif and cleaves after the second aspartic residue.[11,12] Circumstantial evidence for a physiological involvement of active caspase-3 in generating a CD3-ζ-chain-deficient T-cell phenotype has been described in patients with gastric and liver cancers.[13,14] T and B cells from Casp3$^{-/-}$ mice show hyperproliferative responses, which have been attributed to reduced activation-induced cell death and to alterations of cell cycle regulation[15,16] in these cells, respectively. Caspase-3 also regulates many non-apoptotic cellular processes, such as cell proliferation, cell-cycle regulation and cell differentiation.[17,18]

The loss of CD3-ζ chain is a common observation in cancer patients. However, the mechanism responsible for cancer-associated decreased expression of CD3-ζ chain remains controversial. This study reports the identification of a tumour-secreted factor isolated from oral cancer patients that can mediate down-regulation of CD3-ζ chain expression. This study unravels the potential role of tumour-secreted 2′5′-oligoadenylate synthetase 2 (OAS2), identified by the proteomic approach, in down-regulation of CD3-ζ chain. Defining the mechanism, through which this factor modulates CD3-ζ chain levels, might ultimately provide a therapeutic target leading to the generation of effective anti-tumour cellular immune responses in patients with cancer.

Materials and methods

Study group

The study was approved by the institutional ethics committee. After written informed consent, surgically resected tumours ($n = 31$) were obtained from patients with newly diagnosed oral cancer (stage I–IV) before initiation of treatment. Blood specimens were obtained from healthy individuals (HIs). Peripheral blood mononuclear cells (PBMCs) were isolated by differential density gradient centrifugation (Ficoll–Hypaque, Sigma-Aldrich, St Louis, MO) from HIs. The mononuclear cell fraction was washed twice with normal saline, counted and analysed.

Cell culture

The PBMCs isolated by Ficoll–Hypaque gradient were cultured with RPMI-1640 medium supplemented with 10% fetal calf serum. The PBMCs from HIs were seeded in 24-well plates at 1×10^6 cells/ml in each well. Oral tumour supernatants were added to HI PBMCs at a final dilution of 1 : 1 with RPMI-1640 medium supplemented with 10% fetal calf serum. After incubation for different times, PBMCs were harvested and analysed for CD3-ζ chain expression. The HI PBMCs were also stimulated with different concentrations of recombinant human OAS2 (rh-OAS2) (Abnova, Taipei City, Taiwan) or rh-OAS1 (Abnova; 3–96 ng/ml) for 24 hr. PBMCs were harvested and stained for CD3-ζ chain and analysed by flow cytometry.

Tumours were collected in sterile plain RPMI medium (Invitrogen Life-Technology, Carlsbad, CA) supplemented with antibiotics. The necrotic, haemorrhagic and fatty tissues were removed and tumour tissues were thoroughly washed with antibiotic-containing plain RPMI. The tumour tissues were minced finely and incubated in plain RPMI containing an enzyme mixture (0·05% collagenase, 0·02% DNase, and 5 U/ml hyaluronidase) (Sigma-Aldrich), at 37° for 2 hr with intermittent shaking. The tumour tissues were then passed through a 200-gauge wire mesh. The cells were washed with plain RPMI medium and were tested for cell viability. Cells with > 90% viability were used to carry out further experiments.

Oral tumour-derived cell lines (AW13516, AW8507 and AW9803) and HEK293 cells were cultured in 10% fetal calf serum supplemented Iscove's modified Dulbecco's medium and Dulbecco's modified Eagle's medium, respectively. These adherent cell lines were subcultured at 65–75% confluence.

Magnetic activated cell sorting

The PBMCs were isolated by differential density gradient centrifugation using Ficoll Hypaque (Sigma-Aldrich).

$CD3^+$ T cells were purified from PBMCs using MicroBeads (Miltenyi Biotch, Bergisch Gladbach, Germany) by positive selection. The separation procedure was conducted according to the manufacturer's instructions. The purity of separated cells was > 95% as determined by flow cytometry (BD Biosciences, San Jose, CA).

Flow cytometry

Cells were either stained with FITC or phycoerythrin-labelled mouse anti-human CD3-ε and CD3-ζ antibodies (BD Biosciences, San Diego, CA) or with anti-human $p56^{Lck}$ and ZAP70 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min at 4°. Thereafter, cells were washed and incubated with FITC-labelled goat anti-mouse IgG (Sigma-Aldrich) for another 45 min at 4°. Briefly, PBMCs ($0{\cdot}5 \times 10^6$ to 1×10^6) were rinsed in cold PBS and cold-fixed in 1% paraformaldehyde in PBS for 10 min at 4°. The cells were washed and permeabilized for 15 min with 0·1% saponin in PBS. The permeabilized cells were stained with the cocktail of antibodies and 50 000 events/sample were acquired with a FACS Aria flow cytometer (Becton Dickinson, Mountain View, CA). For the above experiments, cells were gated on the basis of their forward and side scatter characteristics and the fluorescence intensity was measured. The analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Cloning and cell transfection

The cDNA encoding OAS2 gene was amplified by PCR using a forward primer containing the *Pme*I restriction site and a reverse primer containing the *Sal*I restriction site. The PCR with 35 cycles at 95° for 1 min, 58° for 2 min, and 72° for 2 min using the forward primer 5′-AGTTTAAACCATGGGAAATGGGGAGTCCCAGCTGTC-3′ and a reverse primer 5′-ACCGTCGACTGATGACTTTTACCGGCACTTTC-3′ was used. The PCR product was gel purified and by TA cloning inserted into pTZ57R/T vector. The pTZ57R/t vector was digested with *Eco*RI and *Sal*I to release the OAS2 cDNA, which was purified and cloned into *Eco*RI- and *Sal*I-digested pEGFP-N2 vector. The vector was sequenced and sequence confirmed the in-frame addition of OAS2 gene with green fluorescent protein (GFP) present in the pEGFP-N2 vector. The vector was transfected into HEK293 cells and supernatant was collected. The collected supernatant was added to the HI PBMCs to monitor CD3-ζ chain down-regulation.

Small interfering RNA

The PBMCs were transfected with small interfering RNA (siRNA) specific for caspase-3 and fluorescent oligonucleotide control siRNA (Cell Signaling Technology, Danvers, MA) at a concentration of 100 nM using X-tremeGENE HP transfection reagent (Roche Diagnostics, Indianapolis, IN). Briefly, cells were transfected with 100 nM control siRNA, or caspase-3-specific siRNA, or were left untransfected. After 40 hr, untransfected cells and caspase-3 knockdown cells were stimulated with rh-OAS2 (24 ng/ml) and at 60 hr post-transfection cells were harvested; and samples were subjected to Western blot analysis for caspase-3, CD3-ζ chain or β-actin. The inhibition of caspase 3 expression was assessed at the 60th hr post transfection.

Western blotting

Cells (PBMCs and oral tumour/cell lines) were washed and lysed using 1% Nonidet P-40 lysis buffer containing 10 mM Tris–HCl, 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 μg aprotinin, 10 μg leupeptin and 1% Nonidet P-40. The cells were sonicated at 4° then centrifuged for 10 min. Protein lysate was harvested and quantified using Bradford reagent (Sigma-Aldrich). Samples were resolved on 12% SDS–PAGE and then transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was probed with the primary antibodies of CD3-ζ chain (1 : 300) (6B10.2; Santa Cruz Biotechnology), OAS2 (1 : 500) (Santa Cruz Biotechnology), caspase-3 (Cell Signaling Technology) and β-actin (1 : 1000) (Sigma-Aldrich) as loading control. Immunostaining was performed using appropriate secondary antibody at a dilution of 1 : 3000 and developed with ECL plus, a western blot detection system (Amersham Pharmacia).

Semi-quantitative PCR

RNA was extracted from oral tumour and cell lines using Trizol reagent (Invitrogen Life Technologies, Grand Island, NY) in accordance with the company's instructions. RNA obtained from cells was reverse transcribed in the presence of 5 mM $MgCl_2$, 1 × PCR Buffer II, 1 mM dNTPs, 25 u MuLV reverse transcriptase, 1 unit RNA guard Ribonuclease inhibitor (Amersham Pharmacia Biotech, Uppsala, Sweden). The OAS2 mRNA levels were measured by semi-quantitative RT-PCR. The forward and reverse primer pair for OAS2 5′-TTAAATGATAATCCCAGCCCC-3′ and 5′-TCAGCGAGGCCAGTAATCTT-3′, respectively.

Caspase-3 detection by fluorometry

The PBMCs were washed in PBS, lysed in ice cold caspase lysis buffer for 30 min and the lysate was collected after centrifugation. The caspase enzymatic reaction was performed according to the manufacturer's instruction (Caspase-3 assay kit, Fluorimetric CASP3F; Sigma-Aldrich).

Mass spectroscopy

The cell-free supernatant (1 ml) from surgically excised oral tumours (HPV-negative), oral cancer cells lines AW8501, AW13516[19] and medium alone (control) were lyophilized. The dried samples were dissolved in 0·5 ml of ammonium bicarbonate buffer (0·1 M, pH 8·5) and the samples were concentrated on a centricon (membrane with a molecular weight cut-off of 3000). Samples were dried in a lyophilizer overnight and the dried samples were dissolved in dissolution buffer supplied with the i-TRAQ kit (Applied Biosystems, Foster City, CA). Samples were digested with trypsin according to the manufacturer's protocol and the samples were labelled with the i-TRAQ reagents 114, 115, 116 and 117. Tryptic peptides of medium control were labelled with 114, AW8501 peptides with 115, AW13516 peptides with 116 and finally tumour supernatants tryptic peptides with 117. Samples were pooled and dried in a speed-vac. The dried samples were fractionated on strong cation exchange chromatography and fractions were collected using salt gradient. Each of these fractions was analysed using liquid chromatography tandem mass spectrometry (LC/MS/MS) on a QSTAR XL mass spectrometer. By differential labelling, peptides labelled with isotope 117 (threefold excess) were selected by subtracting the common peptides found in AW8501 and AW13516 cell lines from cell-free supernatants of oral tumours. Mascot analysis was performed to identify the peptide and the protein from which the peptide was derived.

Annexin–propidium iodide staining

To analyse an effect of rh-OAS2 on cell viability, propidium iodide (PI)/annexin V staining kit (BD PharMingen, San Diego, CA) was used. The PBMCs were stimulated with rh-OAS2 and after 24 hr cells were harvested, suspended in binding buffer (10 mM HEPES pH 7·4, 150 mM NaCl, 0·25 mM $CaCl_2$) and incubated with PI and FITC-conjugated annexin V (BD Biosciences) in the dark for 15 min at room temperature. After incubation, 400 µl of binding buffer was added and cells were analysed immediately on FACSAria (10 000 events/sample).

Statistical analysis

Results are expressed as the mean ± standard error of mean (SEM). Statistical analysis was performed using PRISM software (Prism Software, Lake Forest, CA) and the *P*-value was calculated using Student's *t*-test. Two-sided *P*-values $< 0{\cdot}05$ were considered statistically significant. Densitometry analysis was performed using IMAGEJ software (National Institutes of Health, Bethesda, MD).

Results

Tumour-derived factor from oral tumours down-regulates CD3-ζ chain in peripheral blood lymphocytes of healthy individuals

Our earlier published data demonstrated that compared with other T-cell signalling molecules, ($p56^{Lck}$, Zap70) CD3-ζ chain is markedly decreased in peripheral blood T cells of patients with oral cancer.[9] This study was proposed to investigate the role of an oral tumour-derived factor in regulating CD3-ζ chain expression. Single cell suspensions of oral tumours were prepared by enzyme disintegration. Oral tumour cells were cultured in serum-free media and cell-free supernatant (referred to as tumour supernatant) was collected after 72 hr in culture. The cell-free supernatants of two cell lines derived from oral tumours (AW8501 and AW13516) were also used. The oral tumour supernatant (stage III patient) was added to the PBMCs of HI and incubated for 24–96 hr. As seen in Fig. 1(a) the expression of CD3-ζ chain was monitored in HI PBMCs at defined time intervals after incubation with tumour supernatant. Time kinetics demonstrated that incubation of HI PMBCs with tumour supernatant led to the progressive decrease in CD3-ζ chain expression compared with PBMCs incubated with medium alone (control). However, the supernatants derived from the AW8501 and AW13516 cell lines were not able to decrease the CD3-ζ chain expression (Fig. 1b, c).

The effect of tumour supernatant on other T-cell signalling molecules ($p56^{Lck}$, Zap70 and CD3ε) was also studied after incubating for 72 hr. The supernatants collected from the patients (Stage I–III) led to a marked decrease in CD3-ζ chain expression whereas its effect on CD3-ε chain, ZAP-70 and $p56^{Lck}$ expression was marginal (Fig. 1d–g). However, there was a decrease in expression of all these signalling molecules when incubated with the tumour supernatant collected from stage IV patients (Fig. 1d–g). Interestingly, the effect was best observed on the CD3-ζ chain levels and it inversely correlated with stage of oral cancer in patients, as shown in Fig. 1(d). The broad range effect of supernatant collected from the patients with advanced stage cancer (stage IV) on all the T-cell signalling molecules (CD3-ζ chain, $p56^{Lck}$, ZAP-70) could be attributed to a general state of immunosuppression in these patients contributed by the accumulation of immunosuppressive cells and the secreted immune suppressive factors.

To identify the oral tumour cell-derived factor (s) that degrade CD3-ζ chain, a pragmatic approach was adopted. The cell-free supernatants derived from oral tumours and cell lines (AW8501 and AW13516) were subjected to LC-MS/MS. The identities of proteins observed exclusively in cell-free tumour supernatants were shortlisted (Table 1).

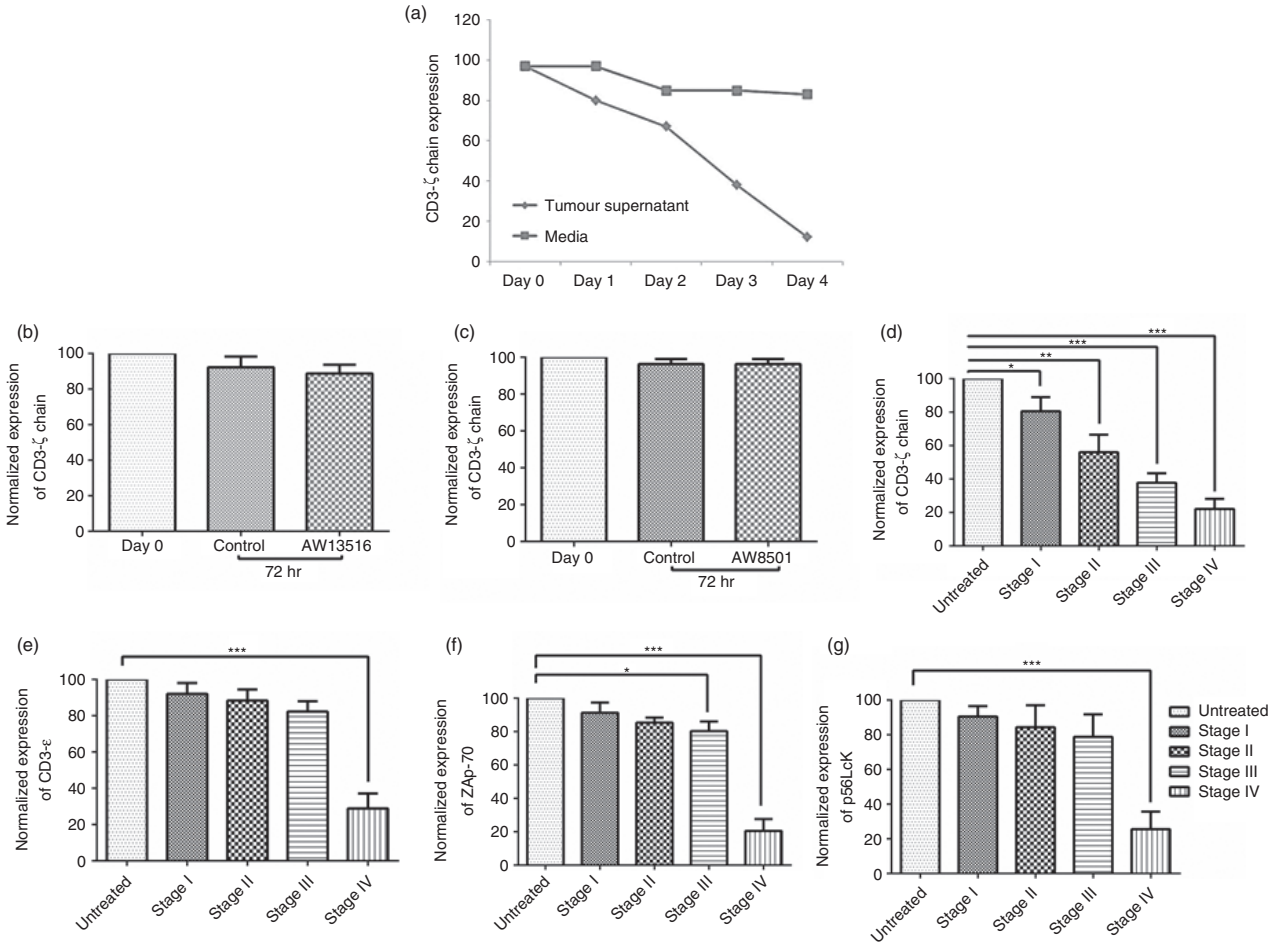

Figure 1. Healthy individual peripheral blood mononuclear cells (PBMCs) were cultured in the presence of tumour supernatant to monitor the expression of T-cell signalling molecules. (a) Representative figure showing the expression of CD3-ζ chain on gated $CD3^+$ T cells in healthy individuals' (HI) PBMCs at different time-points (24, 48, 72 and 96 hr) after incubation with cell-free supernatant (diluted 1 : 1) obtained from patients with oral cancer (Stage III patient) and medium (control). (b, c) Cell-free supernatants collected from oral tumour-derived cell lines (AW13516 and AW8501) did not cause any change in CD3-ζ chain expression after incubating with HI PBMCs for 72 hr. The graph shown is representative of four independent experiments. (d–g) Oral tumour supernatants were collected after 72 hr in plain RPMI from different tumour stages (Stage 1, $n = 4$; stage II, $n = 3$; stage III, $n = 4$; and stage IV, $n = 6$). Oral tumour supernatants were added to the HI PBMCs at a 1 : 1 ratio and after 72 hr the effects of tumour supernatant on CD3-ζ chain and other T-cell signalling molecules (CD3-ε chain ZAP70 and $p56^{Lck}$) were analysed. The graph indicates the expression (normalized median fluorescence intensity) of T-cell signalling molecules on incubation with tumour supernatants obtained from different stage tumours. Fluorescence intensity was adjusted to 100 for control. Accordingly intensity for the test was calculated and represented as normalized fluorescence intensity. ***$P < 0{\cdot}0005$, **$P < 0{\cdot}005$ and *$P < 0{\cdot}05$.

The proteins – Nuclear migration protein nudC, SET-binding protein, Pleckstrin homology-like domain family B member 2, Kinesin-like protein KIF14, OAS2, Protocadherin $\beta 16$ precursors – had a higher score (> 23). OAS2 belongs to the 2′5′-oligoadenylate synthetase family of antiviral proteins consisting of OAS1, OAS2, OAS3 and OASL proteins, and was taken for further analysis.[20] It is known to control cellular apoptosis;[21] which may alter the expression of caspase-3 and thereby regulate CD3-ζ chain expression. OAS2 is also characterized by different subcellular locations and enzymatic parameters, suggesting that this protein might have distinct roles.[22] Hence, based on LC-MS/MS data and previously documented literature OAS2 was shortlisted as the key molecule present in the cell-free supernatants of the oral tumours that may play a role in CD3-ζ chain down-regulation.

Expression of OAS2 in oral tumours and tumour-derived cell lines

To show the presence of OAS2 in the tumour microenvironment, the expression of OAS2 at both protein and mRNA level was analysed in oral tumours. The expression analysis of OAS2 by western blotting showed that oral tumours (Stage II) express OAS2 (69 000 MW) at

Table 1. Screening of proteins present exclusively in tumour supernatant by liquid chromatography tandem mass spectrometry

Description of protein	Peptide sequence	Mass/Score
Nuclear migration protein nudC	K.SMGLPTSDEQKK.Q	885·1/23
Dedicator of cytokinesis protein 7	R.SYTEDWAIVIRK.Y	885/19
SET-binding protein (SEB)	R.SYEGFGTYREK.D	884·9/23
Dual specificity tyrosine-phosphorylation-regulated kinase 4	R.NLKPQPRPQTLRK.S	860·8/18
Toll-like receptor 6 precursor (CD286 antigen)	K.SIVENIINCIEK.S	860·4/15
Microtubule-associated proteins 1A/1B light chain 3C precursor (Microtubule-associated protein 1 light chain 3γ) (MAP1A/MAP1B LC3 C) (MAP1A/1B light chain 3 C) (MAP1 light chain 3-like protein 3) (Autophagy-related protein LC3 C).	K.SLVSMSATMAEIYR.D	860·4/17
U6 snRNA-associated Sm-like protein LSm2	K.SLVGKDVVVELK.N	860·1/19
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1 (EC 3·6·1.-)	K.LNYAIFDEGHMLK.N	855·4/15
Protein GPR89A (Putative MAPK-activating protein PM01)	R.RLLQTMDMIISK.K	590·6/20
Ephexin-1 (Eph-interacting exchange protein)	K.SVNEPLTLNIPWSR.M	590·3/15
Semenogelin-1 precursor	K.EQTSVSGAQK.G	590·3/17
Aryl hydrocarbon receptor nuclear translocator 2 (ARNT protein 2)	R.SGMDFDDEDGEGPSK.F	582·3/22
Pleckstrin homology-like domain family B member 2	R.SGAASMPSSPK.Q	582/23
Kinesin-like protein KIF14	R.SGHLTTKPTQSK.L	534·0/16
Chromatin-modifying protein 4c	K.KHGTQNK.R	478·3/16
PHD finger protein 21B	R.HQNGDLK.K	478·3/16
CD109 antigen precursor (p180)	R.TYTWLK.G	478·3/16
Ankyrin repeat and SOCS box protein 1 (ASB-1)	K.WESLGPESRGR.R	473·3/14
Interleukin-1 family member 8 (IL-1F8)	R.TNIGMPGR.M	502·8/11
Mastermind-like protein 3 (Mam-3)	R.SLQGMPGR.T	502·8/11
Kinesin-like protein KIF14	R.SGHLTTKPTQSK.L	524·9/27
Microtubule-associated proteins 1A/1B light chain 3C precursor	K.SLVSMSATMAEIYR.D	860·4/15
Integrin α-L precursor	R.RGLFPGGR.H	503·8/14
Ras-related protein Rab-6B	M.SAGGDFGNPLRK.F	503·3/14
Kelch domain-containing protein 5	R.SNFKLVAVNSK.L	450·8/11
2′-5′-oligoadenylate synthetase 2 (EC 2·7·7.-)	R.ILNNNSK.R +	473·3/23
Epididymal secretory protein E1 precursor	K.TYSYLNK.L	517·3/19
Pericentrin (Pericentrin B) (Kendrin)	R.SLTEQQGR.L	531·2/22
Ubiquitin-conjugating enzyme E2 J1 (EC 6·3·2·19)	R.GPPDSDFDGGVYHGR.I	573·8/16
Protocadherin β16 precursor (PCDH-β16)	R.VIDINDHSPMFTEKEMILK.	855·1/23

the protein level as shown in Fig. 2(a). For OAS2 mRNA expression, total RNA was extracted from surgically excised oral tumours and adjacent normal tissue. Complementary DNA (cDNA) was prepared from the total RNA using MuLV RT enzyme. Gene-specific PCR was performed for GAPDH and OAS2. The heterogeneous expression of OAS2 mRNA was observed in oral tumours (Stage II and III) (Fig. 2b). The overall expression of mRNA for OAS2 remained high in oral tumours with two out of three tumours showing higher mRNA of OAS2 compared with the histologically adjacent normal tissues (Fig. 2c). In one of the oral tumour samples, mRNA expression of OAS2 was not observed but in its adjacent normal tissue OAS2 expression was noted. However, in oral tumour-derived cell lines (AW13516, AW8507 and AW9803) OAS2 expression was observed only at mRNA level (Fig. 2d) but not at the protein level. This may suggest that the tumour microenvironment contributes to OAS2 expression.

Secreted OAS2 mediates CD3-ζ chain down-regulation in PBMCs of healthy individuals

To analyse the role of extracellular OAS2 in degrading CD3-ζ chain, the key T-cell signalling molecule, a surrogate situation, was created by transfecting vector control (pEGFP-N2) or an OAS2-encoding pEGFP-N2 vector (pEGFP-N2 + OAS2) into HEK293 cells. Total RNA was extracted from transfected cells, and mRNA expression levels were analysed by RT-PCR. As shown in Fig. 3(a; upper panel) OAS2 expression was higher in cells transfected with (pEGFP-N2 + OAS2) compared with the untransfected or vector-control-transfected HEK293 cells. Transfected cells were also analysed by flow cytometry for GFP positivity, which indirectly also confirms over-expression of OAS2 protein as depicted in Fig. 3(a; lower panel).

Supernatants were collected from OAS2 (pEGFP-N2 + OAS2) and vector control (pEGFP-N2) -transfected HEK293 (referred to as control) cells after 48 hr. The

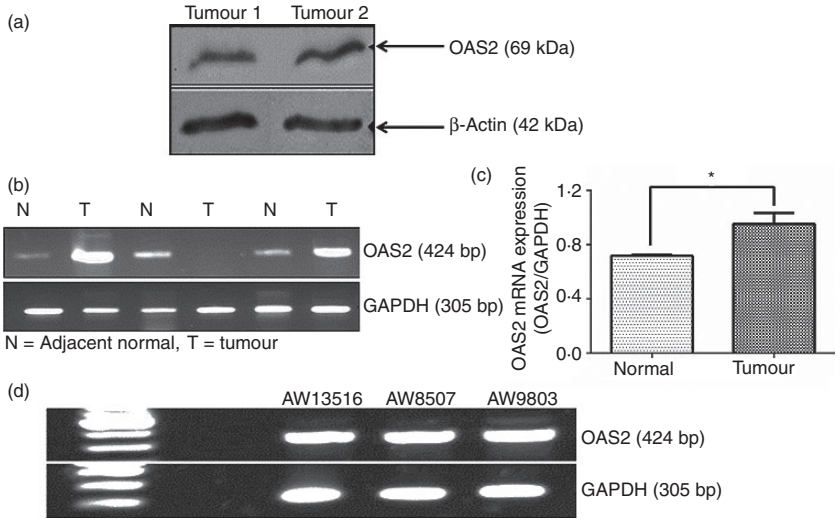

Figure 2. Expression of 2′5′-oligoadenylate synthetase 2 (OAS2) in oral tumour cells. (a) Total protein extract from oral tumours was used to detect the levels of OAS2 and β-actin by Western blotting. The protein extracts (40–60 μg) prepared from oral tumours ($n = 2$) was loaded into the SDS–PAGE and then transferred to the nitrocellulose membrane. The results showed a presence of the OAS2 (p69) in these tumours, β-actin was used as internal control. (b) Total RNA (150–300 ng/μl) was isolated from the oral tumours and cell lines of oral tumour origin. The isolated RNA was converted into cDNA and cDNA was subjected to PCR, as described in the Materials and methods, to detect OAS2 and GAPDH transcripts. The expression of OAS2 transcript in oral tumours (T) and its adjacent normal tissues (N) is shown. (c) Band densitometry illustrating total OAS2 transcript expression is higher in oral tumours compared with the adjacent normal tissue. (d) Expression of OAS2 mRNA in oral tumour-derived cell lines (AW13516, AW8507 and AW9803). *$P < 0.05$

collected supernatants from control- and OAS2-transfected HEK293 cells were added to the HI PBMCs at 1 : 1 [supernatant: medium (RPMI)] dilution for different time intervals. As shown in Fig. 3(b, c), the CD3-ζ chain expression was observed in gated $CD3^+$ T cells after 24 and 72 hr of incubation, respectively. The results confirmed that at both time-points there was a decreased expression of CD3-ζ chain when incubated with supernatant collected from OAS2 over-expressing HEK293 cells compared with control HEK293 cells (vector-control-transfected) as shown in Fig. 3(d). On the other hand, CD3-ε expression at both time intervals 24 and 72 hr remained unaltered on incubation with supernatant collected from OAS2-transfected HEK293 cells as shown in Fig. 3(e, f). This confirms that the effect of secreted OAS2 was specific and altered only CD3-ζ chain expression. The presence of OAS2 in supernatants was confirmed by the immuno-dot blot assay (data not shown).

Recombinant human OAS2 protein decreases CD3-ζ chain expression in PBMCs of healthy individuals

To elucidate the role of OAS family proteins in down-regulating the CD3-ζ chain expression, HI PBMCs were stimulated with different concentrations of rh-OAS2 and rh-OAS1 (3–96 ng/ml) for 24 hr. The PBMCs were harvested and expression of CD3-ζ and CD3-ε chain expression was examined by flow cytometry.

The HI PBMCs stimulated with rh-OAS2 showed decreased expression of CD3-ζ chain on a gated population of $CD3^+$ T cells (Fig. 4a). The treatment of PBMCs with rh-OAS2 at 6, 12 and 24 ng/ml concentrations for 24 hr led to a significant decrease in CD3-ζ chain expression as shown in Fig. 4(a, c), although a dose–response was not evident. However, rh-OAS2 treatment was not able to induce the changes in CD3-ε expression as studied in Fig. 4(b), which is consistent with the earlier findings. The data clearly indicated that as observed with the supernatant collected from OAS2 transfected HEK293 cells, rh-OAS2 was unable to affect the CD3-ε chain expression on $CD3^+$ T cells, again confirming that the effect of rh-OAS2 was specific in modulating only CD3-ζ chain expression.

Upon stimulation with another member of OAS family, i.e. rh-OAS1, PBMCs were unable to decrease the CD3-ζ chain expression on gated population of $CD3^+$ T cells as shown in Fig. 4(d). Data in Fig. 4(f) also show that the treatment of PBMCs with different concentrations of rh-OAS1 did not alter the CD3-ζ chain expression. Similar to rh-OAS2, rh-OAS1 was also incapable of decreasing the CD3-ε chain expression on T cells as shown in Fig. 4(e). These data confirmed that OAS2 but not OAS1 was capable of modulating the CD3-ζ chain expression.

Tumour supernatant and rh-OAS2 decreases CD3-ζ chain expression in sorted T cells

To understand whether oral tumour supernatants or rhOAS2 directly acted on T cells to down-regulate CD3-ζ

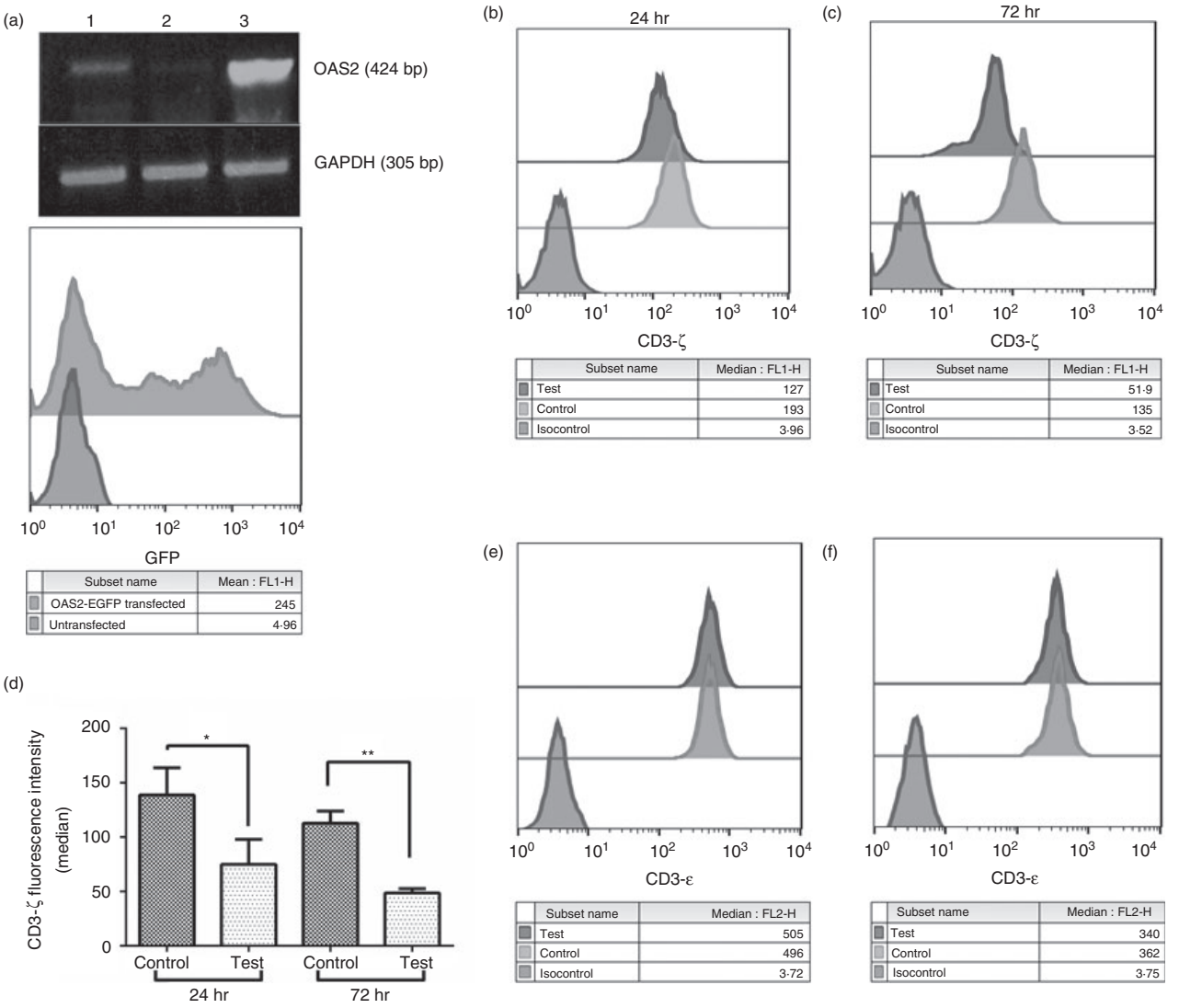

Figure 3. Effect of supernatant collected from 2′5′-oligoadenylate synthetase 2 (OAS2) over-expressing HEK293 cells on T-cell signalling molecules. (a) HEK293 cells were transfected with either vector control (pEGFP-N2) or vector encoding the OAS2 cDNA (pEGFP-N2 + OAS2). Total RNA was extracted from transfected cells, and mRNA expression levels were analysed by RT-PCR (upper panel) using gene-specific primers for OAS2 and GAPDH. Lanes 1, 2 and 3 represent untransfected HEK293, vector control transfected HEK293 and pEGFP-N2 + OAS2 transfected HEK293 cells, respectively. The pEGFP-N2 + OAS2 transfected cells were also monitored for GFP expression by flow cytometry (lower panel). (b, c) Cell-free supernatant collected from pEGFP-OAS2 transfected HEK293 cells led to the decrease in expression of CD3-ζ chain on gated $CD3^+$ T cells in healthy individuals' (HI) peripheral blood mononuclear cells (PBMCs) at different time-points (24 and 72 hr) compared with the supernatant collected from vector control transfected cells. (d) The median fluorescence intensity of CD3-ζ chain staining in HI PBMCs treated with cell-free supernatant of vector control and pEGFP-OAS2 transfected HEK293 cells at 24 and 72 hr. (e, f) Unaffected fluorescence intensity (median) of CD3-ε chain after incubating HI PBMCs with cell-free supernatant collected from vector control and pEGFP-OAS2 transfected HEK293 cells at different time intervals 24 and 72 hr, respectively. The data represented here are the mean of three experiments carried out using supernatants collected from three independent transfections. $^{**}P < 0{\cdot}005$ and $^{*}P < 0{\cdot}05$.

chain expression, experiments were performed on sorted T cells ($CD3^+$ T-cell purity 99·5%, Fig. 5a). Incubation of tumour supernatant (diluted 1 : 1) with purified T cells decreased the CD3-ζ chain expression in T cells. The decrease in CD3-ζ chain expression observed in purified T cells after incubation with tumour supernatant for 24 hr was comparable to that observed in gated $CD3^+$ T cells in PBMCs (Fig. 5b, d). Incubation of T cells with the various concentration of rh-OAS2 (6, 12, 24 ng/ml) also led to the decrease in CD3-ζ chain expression in accordance with the earlier data obtained with PBMCs incubated with rh-OAS2 (Fig. 5c, e). These results demonstrate that tumour supernatant and rh-OAS2 specifically acted on CD3-ζ chain expressed in T cells.

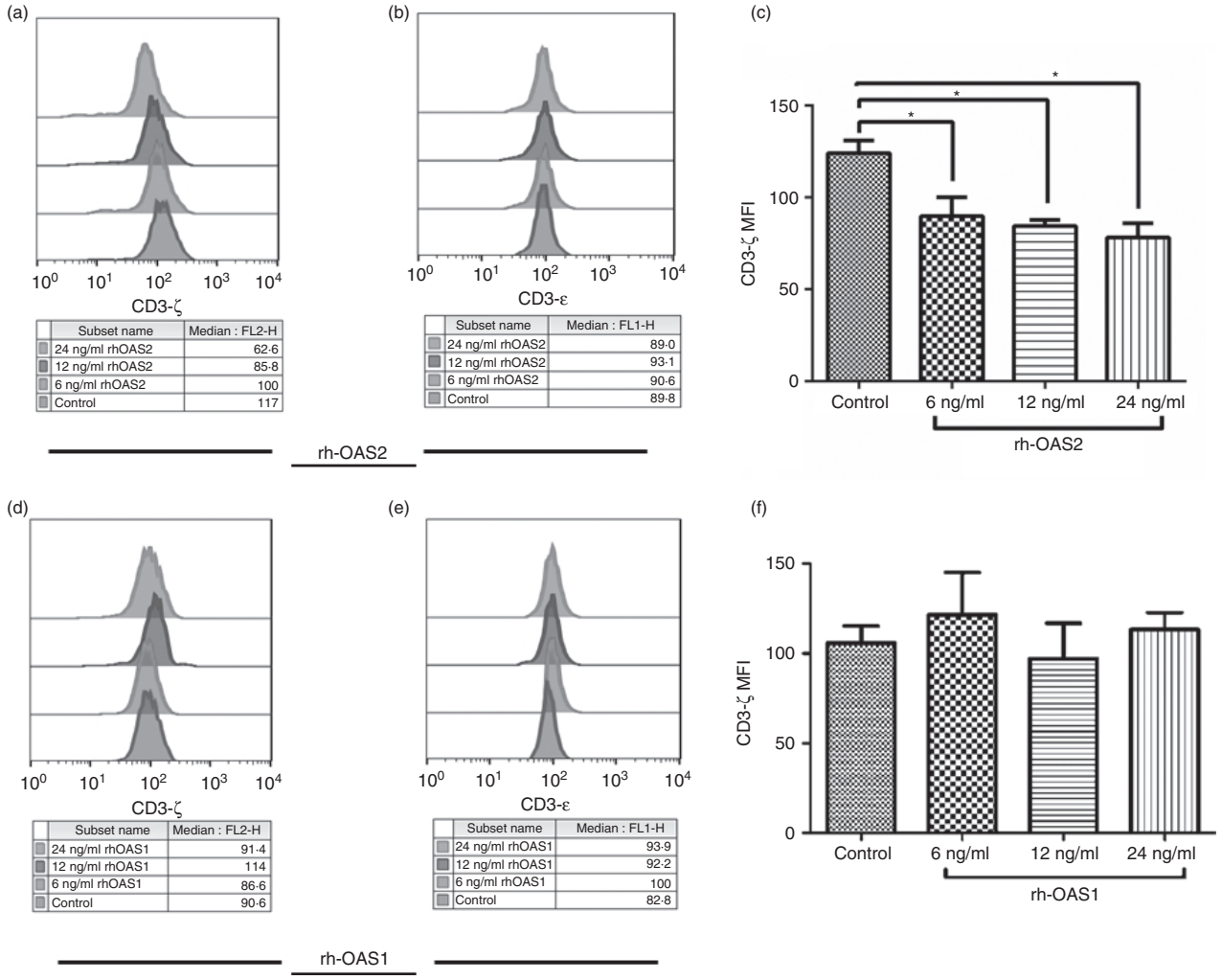

Figure 4. Effect of different concentrations of recombinant human 2′5′-oligoadenylate synthetase 2 (rh-OAS2) and rh-OAS1 on the expression of CD3-ζ and CD3-ε chain on $CD3^+$ T cells of healthy individuals' (HI) peripheral blood mononuclear cells (PBMCs). (a, c) The fluorescence intensity (median) of CD3-ζ chain decreased after incubation of HI PBMCs with rh-OAS2. Significant reduction in the expression of CD3-ζ chain was observed on gated $CD3^+$ T cells when treated with 6, 12 and 24 ng/ml of rh-OAS2. (b) The fluorescence intensity (median) of CD3-ε after incubation of HI PBMCs with rh-OAS2 did not show any change. (d, f) the fluorescence intensity (median) of CD3-ζ chain upon rh-OAS1 stimulation of HI PBMCs remained unaltered on $CD3^+$ T cells compared with the medium control used. (e) CD3-ε expression was not affected on $CD3^+$ T cells after treatment with rh-OAS1 at different concentrations (6, 12 and 24 ng/ml). The data represented here are the mean of four independent experiments (*$P < 0{\cdot}05$).

Tumour-derived supernatants and rh-OAS2 increased caspase-3 levels in T cells of healthy individuals

Single-cell suspension of oral tumours prepared by enzyme digestion was cultured in serum-free medium and cell-free tumour supernatants were collected after 72 hr of culture. The tumour supernatants were added to the HI PBMCs and incubated for 24 hr. Treatment of HI PBMCs with H_2O_2 served as positive control for caspase-3 induction. As shown in Fig. 6(a) a marked increase in caspase-3 levels was observed in HI PBMCs after H_2O_2 treatment (positive control). The addition of the tumour supernatant was also effectively able to induce caspase-3 levels in HI PBMCs, indicating that tumour supernatant may alter CD3-ζ expression by increasing caspase-3 levels.

To validate the role of caspase-3 in decreasing CD3-ζ chain expression, PBMCs of HI were co-incubated with the tumour supernatant and caspase-3 inhibitor (DEVD-CHO). As shown in Fig. 6(b), CD3-ζ chain expression was decreased after incubation with cell-free oral tumour supernatant (lane 3), which was almost restored back to its normal levels after addition of the caspase-3 inhibitor, DEVD-CHO (Lane 2). These data clearly suggest that caspase-3 is involved in the regulation of CD3-ζ chain expression.

To validate further, HI PBMCs were also incubated with rh-OAS2 to analyse caspase-3 activation. As observed

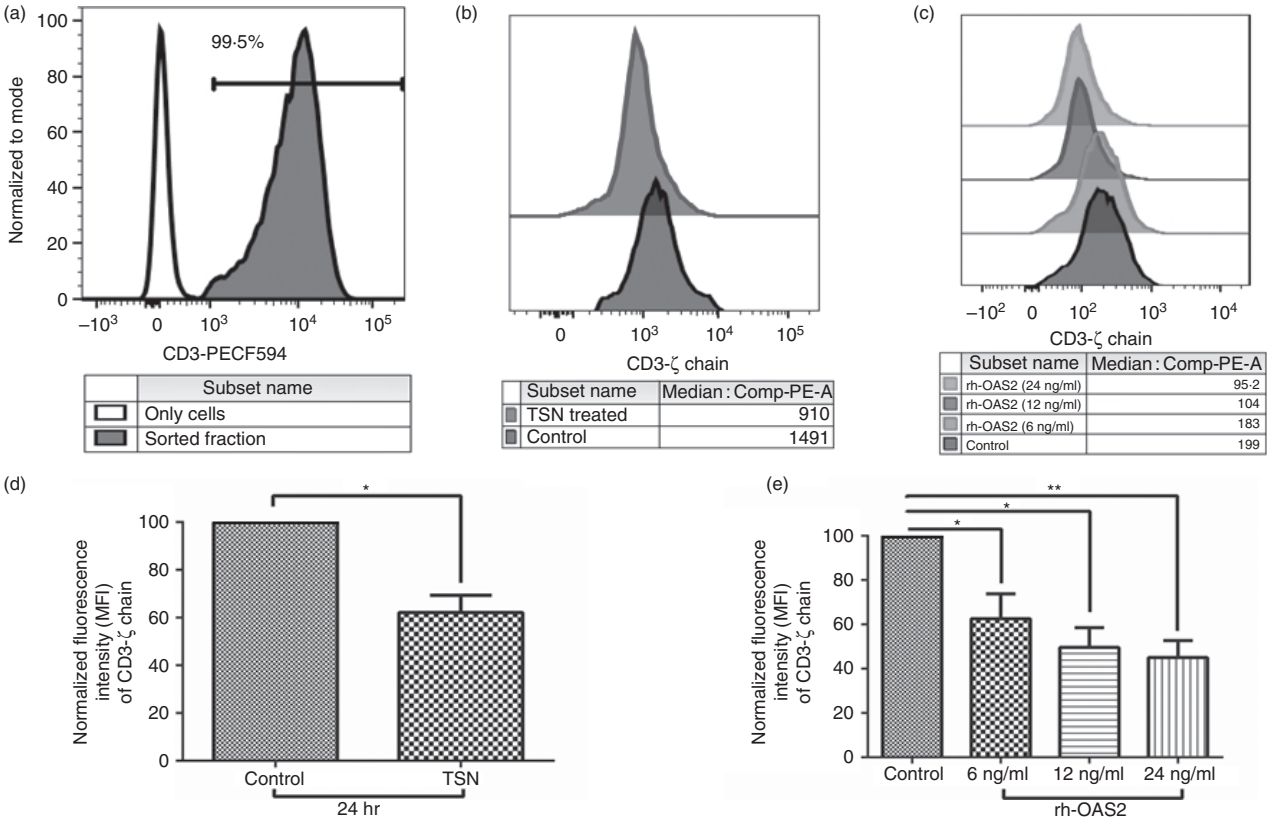

Figure 5. Effect of tumour supernatant and recombinant human 2′5′-oligoadenylate synthetase 2 (rh-OAS2) on the sorted CD3$^+$ T cells. (a) The purity of CD3$^+$ T cells isolated from the peripheral blood mononuclear cells (PBMCs). The shaded histogram is the sorted fraction (99·5% purity) whereas the unfilled histogram represents only cells. (b) Representative figure showing the expression of CD3-ζ chain in CD3$^+$ T cells after incubation with cell-free supernatant (TSN) (diluted 1 : 1) obtained after culturing oral tumours or with medium (control). (c) Representative figure showing that fluorescence intensity (median) of CD3-ζ chain decreased after incubation of CD3$^+$ T cells with different concentrations of rh-OAS2. Significant reduction in the expression of CD3-ζ chain (MFI) was observed on CD3$^+$ T cells when treated with 6, 12 and 24 ng/ml of rh-OAS2. (d, e) Expression (normalized median fluorescence intensity) of CD3-ζ chain on incubation with tumour supernatants obtained from oral tumours (d) or rh-OAS2 (e). Fluorescence intensity was adjusted to 100 for control. Accordingly intensity for the test was calculated and represented as normalized fluorescence intensity. The graph shown is representative of three independent experiments (**$P < 0{\cdot}005$ and *$P < 0{\cdot}05$).

with tumour supernatant, rh-OAS2 also induced caspase-3 activation in the PBMCs of HI compared with the untreated controls (Fig. 6c). The synthetic siRNA sequences targeting the caspase-3 were transfected into the PBMCs (Fig. 6d) and purified CD3$^+$ T cells (Fig. 6e). PBMCs were transfected with 100 nM of control siRNA or caspase-3-specific siRNA duplexes or left untransfected. Forty hours post-transfection, cells were stimulated with rh-OAS2 (24 ng/ml) and at 60 hr cells were harvested and lysates were prepared. Caspase-3 expression was compared in unstimulated control siRNA transfected PBMCs, PBMCs stimulated with rh-OAS2, and in PBMCs transfected with caspase-3 siRNA and stimulated with rh-OAS2 (Fig. 6d). The result showed that compared with control siRNA transfected PBMCs (unstimulated, lane 1) caspase-3 expression after rh-OAS2 stimulation appeared to be marginally increased in these cells (lane 2). Transfection with caspase-3 siRNA led to the reduction in the expression of active caspase-3 (lane 3) compared with untransfected PBMCs (lane 2) (Fig. 6d). Silencing of caspase-3 prevented rh-OAS2 induced down-regulation of CD3-ζ chain expression in PBMCs (lane 3 versus lane 2; Fig. 6d). This clearly demonstrates that expression of CD3-ζ chain was restored and remained comparable to that observed with control siRNA. In purified CD3$^+$ T cells, rh-OAS2 stimulation had a more pronounced effect on caspase-3 activation leading to CD3-ζ chain down-regulation. This down-regulation of CD3-ζ chain in purified T cells was rescued by knockdown of caspase-3 and was comparable to the expression observed in control siRNA transfected cells (Fig. 6e). The results confirm that caspase-3 is the key downstream molecule induced by OAS2 present in the tumour supernatant that is responsible for CD3-ζ chain down-regulation in T cells.

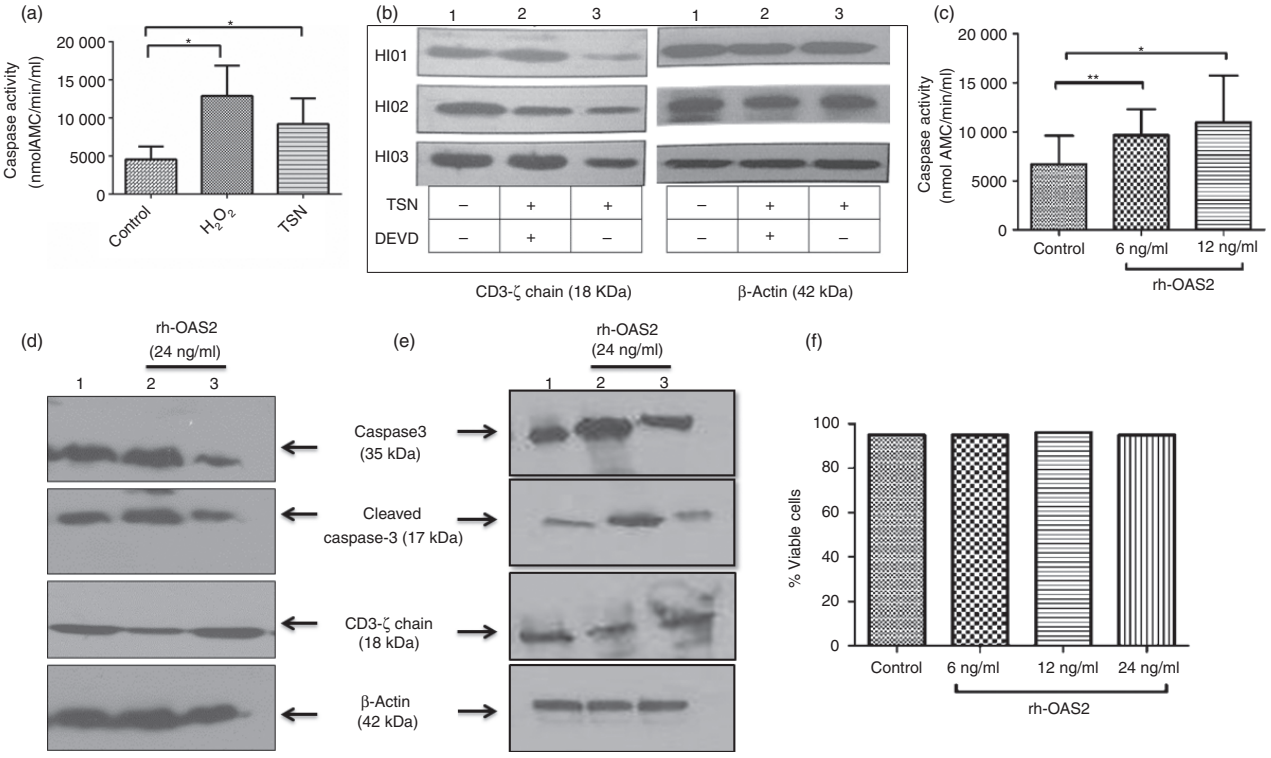

Figure 6. Caspase-3 levels after treatment with tumour supernatant and recombinant human 2′5′-oligoadenylate synthetase 2 (rh-OAS2). (a) The healthy individuals' (HI) peripheral blood mononuclear cells (PBMCs) were treated with H_2O_2 (positive control for caspase-3 induction) or tumour supernatant (TSN of stage III patients) or left untreated. H_2O_2 used as positive control to induce caspase-3 levels showed higher levels of caspase-3 activation. The addition of TSN resulted in the increase of caspase-3 levels in the PBMCs. The data represented here are the mean of three independent experiments (*$P < 0{\cdot}05$). (b) Healthy individuals' (HI) PBMCs on incubation with tumour supernatant showed a drastic reduction in CD3-ζ chain expression (lane 3) compared with untreated (lane 1). On addition of caspase-3 inhibitor (DEVD-CHO), TSN was ineffective in inducing the CD3-ζ chain down-regulation (lane 2), i.e. caspase-3 inhibitor prevented TSN mediated down-regulation of CD3-ζ chain levels. The data shown are PBMCs of three HIs treated with TSN in the presence or absence of caspase-3 inhibitor (c) Addition of rh-OAS2 (6 and 12 ng/ml) on HI PBMCs resulted in the increase in caspase-3 levels. The data represented here are the mean of four independent experiments (**$P < 0{\cdot}005$ and *$P < 0{\cdot}05$). (d) Western blotting was performed on cell lysates prepared from PBMCs transfected with 100 nM control small interfering RNA (siRNA) (Lane 1) or PBMCs transfected with caspase-3-specific siRNA and incubated with rh-OAS2 (lane 3) or untransfected PBMCs stimulated with rh-OAS2 (lane 2) using antibodies for caspase-3 (detects total caspase along with cleaved caspase-3), CD3-ζ chain or *β*-actin (*β*-actin was used as loading control). Stimulation of PBMCs with rh-OAS2 induced caspase-3 activation causing CD3-ζ chain down-regulation in them. This down-regulation of CD3-ζ chain in PBMCs was rescued by the caspase-3 knockdown. (e) Western blotting was performed on cell lysates prepared from purified T cells transfected with 100 nM control siRNA (lane 1) or T cells transfected with caspase-3-specific siRNA and incubated with rh-OAS2 (lane 3) or untransfected T cells stimulated with rh-OAS2 (lane 2) using antibodies for caspase-3, CD3-ζ chain or *β*-actin. In purified T cells, a pronounced effect was observed on caspase-3 activation leading to CD3-ζ down-regulation on rh-OAS2 stimulation, which was recovered back to normal levels on caspase-3 knockdown. (f) PBMCs were treated with rh-OAS2 to examine the percentage of viable cells (annexin V/PI negative cells). The rh-OAS2 (6, 12 and 24 ng/ml) did not alter the viability of PBMCs in culture, indicating that increased caspase-3 levels were not associated with apoptosis.

Additionally, the effect of rh-OAS2 on apoptotic/necrotic cell death of PBMCs was analysed to rule out the possibility that the CD3-ζ chain down-regulation is not a result of apoptosis. To verify, the effect of rh-OAS2 stimulation on the frequency of apoptotic cells was compared with unstimulated cells. Results showed no significant differences in the frequency of early apoptotic (annexin V-positive), late apoptotic (annexin V-positive PI-positive) and necrotic (PI-positive) cells in untreated HI PBMCs or rh-OAS2-treated HI PBMCs (Fig. 6f). This shows that caspase-3 activity was not associated with the induction of cell death or apoptosis. It can be concluded that activation of caspase-3 and the associated CD3-ζ cleavage is a consequence of OAS2 signalling and not due to apoptosis of lymphocytes.

Discussion

Immunosuppression is a hallmark of oral cancer patients, a state in which established tumour escapes immune

attack. A number of phenotypic and functional alterations, including down-regulation of the CD3-ζ chain, contributes to immunosuppression.[23] It is well established that down-regulation of the CD3-ζ chain limits the favourable T helper type 1 response needed for controlling tumour growth.[9,24] Decreased or impaired CD3-ζ chain expression was also observed in other malignancies, such as lymphoma, ovarian cancer and gastric carcinomas, leading to ineffectiveness of the anti-tumour immune response against the autologous tumour.[25–27] Several distinct mechanisms that contribute to a rapid turnover of CD3-ζ chain have been proposed that may be responsible for differences observed in CD3-ζ chain expression in T cells of patients with cancer.[28–30] In the present study, it was demonstrated that treatment of HI PBMCs with cell-free supernatants derived from oral tumours led to the down-regulation of CD3-ζ chain expression in T cells without affecting the other T-cell signalling molecules (ZAP70, CD3-ε and $p56^{Lck}$). LC-MS/MS proteomic approach was therefore adopted and OAS2 (member of OAS gene family) was identified as one of the factors secreted by the oral tumour cells. The transcription of OAS genes is induced by both virus infection and interferon (IFN) stimulation conferring protective and anti-proliferative properties.[31,32] Among the OAS genes, OAS2 has the highest level of induction by interferons (IFN-α and IFN-β). Several studies have reported the presence of OAS2 in the sera of patients with viral infections and even after IFN treatment.[33–35] The success of IFN therapy was also correlated with levels of OAS gene activity in the sera of patients undergoing IFN therapy.[36,37] This correlation is interesting and suggests a biological relevance of extracellular OAS.

A variable number of factors, including tumour-secreted 14 000 MW protein, semaphorin A, NKG2D, are reported to be responsible for CD3-ζ chain down-regulation in different malignancies.[26,29,30] The present study reported how extrinsic soluble mediator OAS2 secreted by oral tumour cells modulate CD3-ζ chain expression in T cells.[38] The 2′5′-OAS, apart from its antiviral action is involved in other cellular processes such as cell growth and differentiation, gene regulation and apoptosis.[39] It has been demonstrated that the IFN-β-mediated signalling pathways as well as its upstream regulators are up-regulated in oral squamous cell carcinoma lesions.[40] It therefore appears that IFN-β signalling may act as a key factor responsible for OAS2 expression in oral tumours. Interferon-β signalling is also known for its immune regulatory properties, suggesting its importance in tumour progression either directly or through modulation of the immune system through its downstream mediators like OAS2.

The observation of OAS in the sera of patients with infection or undergoing IFN treatment[37] prompted us to look at the role of OAS in modulating the immune system. The differential expression of OAS2 has been reported in prostate and breast cancers.[41,42] Our study also confirmed the presence of OAS2 in oral tumours at both mRNA and protein levels. The significance of OAS2 in the tumour microenvironment can be appreciated by the observation that certain tumours exist in an antiviral state and hence are resistant to oncolytic-virus-based virotherapy used to obliterate tumour cells. These tumours are reported to have higher expression of the Janus kinase/signal transducer and activator of transcription pathway and IFN-stimulated genes (ISG).[43] It was also reported that over-expression of myxovirus resistance gene A (MxA) and OAS mRNAs were involved in the suppression of hepatitis B virus replication mediated by IL-17A in a non-cytopathic manner.[44] Hence, these tumours with a functional IFN-responsive pathway and expression of a downstream gene (OAS2) may be sensitive to IFN stimulation, thereby conferring the virus-resistant phenotype that shields tumours from virotherapy. The source of IFN in the tumour microenvironment is likely to be either the epithelial cells or the tumour-infiltrating immune cells, such as type 2 dendritic cells and macrophages.[40,45] The IFN secreted by these cells will activate the ISG genes and will execute their pro-tumour function either intracellularly or in secreted form by modulating the immune system. However, the source of OAS2 in oral tumours needs to be further investigated.

In our study, a surrogate model where OAS2 was over-expressed showed that secreted OAS2 could modulate the expression of CD3-ζ chain in T cells. This was further validated by stimulating HI PBMCs with rh-OAS2 which led to the specific down-regulation of CD3-ζ chain expression in T cells. Up-regulation of type I IFN response genes has also been reported in peripheral blood cells of patients with autoimmune diseases, like systemic lupus erythematosus, multiple sclerosis and rheumatoid arthritis and these diseases are associated with decreased CD3-ζ chain expression.[46–48] Type 1 IFNs have been shown to have anti-proliferative activity on activated $CD4^+$ and $CD8^+$ T cells.[49] Protein and mRNA levels of OAS genes are low in circulating mononuclear cells during clinically active, untreated multiple sclerosis. The IFN-β therapy treatment of multiple sclerosis and experimental autoimmune encephalomyelitis to decrease the levels of T helper type 17 cells is associated with increased expression of OAS2 protein.[37] Hence, OAS2 might be acting in a paracrine manner in the tumour microenvironment to decrease the expression of CD3-ζ chain in T lymphocytes.

The present data showed that on incubation of PBMCs with tumour supernatant or rh-OAS2 leads to increase in caspase-3 levels. Increased caspase-3 levels are observed in T cells under suboptimal T-cell stimulation leading to the induction of T-cell unresponsiveness without inducing cell death.[50] Caspase-3 activity is high in $CD3\zeta^{low/-}$ T

cells from a patient with systemic lupus erythematosus and treatment of these T cells with caspase inhibitors restores CD3-ζ chain expression.[51] 2′5′-OASs lead to cytochrome *c* release into the cytoplasm and then to caspase-3 activation.[52] Interferon-α also increases caspase-3 mRNA levels in activated T cells without modulating activation-induced cell death.[53] In our study, induction of caspase-3 in non-apoptotic cells was observed on stimulation with rh-OAS2. This observation supports that caspase-3-dependent proteolytic inactivation of CD3-ζ chain is essential to maintain T-cell tolerance in cancer patients.[53] The amino acid sequence of translated CD3-ζ chain contains putative caspase-3 cleavage sites DVLD and DTYD (five in number).[10] Hence, the selective loss of CD3-ζ chain and not of CD3-ε could be because of the caspase-3 activation, as observed upon stimulation with rh-OAS2 or tumour supernatant. However, the downstream consequence of CD3-ζ down-regulation on the rh-OAS2 challenge was not found to be dose-dependent but might depend on induction of caspase-3. To alter the expression of CD3-ζ chain on HI PBMCs in a dose-dependent manner, caspases are expected to vary in logarithmic values. In our study, such change in caspase-3 value was not observed upon rh-OAS2 stimulation. It has been shown that OAS that is exogenously added to cell cultures can be internalized to exert its effect. This exogenous OAS can exert its effect either by binding to the nucleotide-binding and oligomerization domain-2 to activate RNaseL or independent of this to induce caspase cascade.[36,54] Hence, the internalization of OAS2 inside the cells might be the limiting factor in inducing the caspase-3 levels in a dose-dependent manner.

Type I IFNs (IFN-α and IFN-β) could exert broad dual effects on the immune system, reflecting both immune stimulatory and immune-suppressive activities. Immune-stimulatory activities relate to the activation of myeloid dendritic cells, chemokines, chemokine receptors, co-stimulatory molecules (CD40, CD80 and CD86), and humoral responses. Immune-suppressive effects are reflected by Th2 cell skewing and anti-proliferative and pro-apoptotic effects. The role of IFNs in disease may range from beneficial to detrimental. However, it may be postulated that OAS2 plays a major immunoregulatory role based on its expression in oral tumours and autoimmune diseases where CD3-ζ chain expression is down-regulated.[9,55]

Type I IFNs are promising but incompletely understood anticancer agents. Clinical trials have demonstrated benefit in both haematological and solid tumours, although the effectiveness is somewhat modest in some cancers.[56,57] Therapy with IFN-α has been suggested to be effective in a subgroup of patients with oral cancer.[58,59] Further, it has also been demonstrated that the up-regulation of ISGs (Mx1, OAS3, IFI44, IFI44L, OAS2, USP18 and RSAD2) in the chronic hepatitis C virus-infected liver is related to a poor treatment response to pegylated IFN-α therapy.[60] Interferon-α may be important in the prevention of carcinogenesis and high expression of OAS2 might be impairing cellular response to IFN-α and promotes oral tumour progression by modulating anti-tumour immune response. However, the role of others ISGs in modulating the IFN-α response cannot be ruled out. Identification of molecular biomarkers like OAS2, which can also identify oral cancer patients sensitive to Type I IFN therapy would be very helpful in the clinic.

Hence, the data presented here indicate, for the first time, a potentially important function of tumour-derived OAS2 as a paracrine negative regulator of T-cell functions. Our data highlight OAS2 as a novel molecular target for the manipulation of T-cell-dependent immunity with important implications for cancer immunotherapy.

Acknowledgements

We are grateful to the Council for Scientific and Industrial research (CSIR), India for providing AAD with a fellowship. Help extended by Dr Robin Mukhopadhyaya and Dr Ajit Chande in cloning experiments is gratefully acknowledged.

Disclosures

The authors declare no conflict of interest.

References

1 Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011; **331**:1565–70.
2 Reichert TE, Strauss L, Wagner EM, Gooding W, Whiteside TL. Signaling abnormalities, apoptosis, and reduced proliferation of circulating and tumor-infiltrating lymphocytes in patients with oral carcinoma. *Clin Cancer Res* 2002; **8**:3137–45.
3 Zea AH, Curti BD, Longo DL, Alvord WG, Strobl SL, Mizoguchi H *et al.* Alterations in T cell receptor and signal transduction molecules in melanoma patients. *Clin Cancer Res* 1995; **1**:1327–35.
4 Whiteside TL. Immune cells in the tumor microenvironment. Mechanisms responsible for functional and signaling defects. *Adv Exp Med Biol* 1998; **451**:167–71.
5 Lockhart DC, Chan AK, Mak S, Joo HG, Daust HA, Carritte A *et al.* Loss of T-cell receptor-CD3ζ and T-cell function in tumor-infiltrating lymphocytes but not in tumor-associated lymphocytes in ovarian carcinoma. *Surgery* 2001; **129**:749–56.
6 Choi SH, Chung EJ, Whang DY, Lee SS, Jang YS, Kim CW. Alteration of signal-transducing molecules in tumor-infiltrating lymphocytes and peripheral blood T lymphocytes from human colorectal carcinoma patients. *Cancer Immunol Immunother* 1998; **45**:299–305.
7 Mizoguchi H, O'Shea JJ, Longo DL, Loeffler CM, McVicar DW, Ochoa AC. Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science* 1992; **258**:1795–8.
8 Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010; **140**:883–99.
9 Kulkarni DP, Wadia PP, Pradhan TN, Pathak AK, Chiplunkar SV. Mechanisms involved in the down-regulation of TCR ζ chain in tumor versus peripheral blood of oral cancer patients. *Int J Cancer* 2009; **124**:1605–13.
10 Gastman BR, Johnson DE, Whiteside TL, Rabinowich H. Caspase-mediated degradation of T-cell receptor ζ-chain. *Cancer Res* 1999; **59**:1422–7.
11 Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998; **281**:1312–6.
12 Rathmell JC, Thompson CB. The central effectors of cell death in the immune system. *Annu Rev Immunol* 1999; **17**:781–828.
13 Takahashi A, Kono K, Amemiya H, Iizuka H, Fujii H, Matsumoto Y. Elevated caspase-3 activity in peripheral blood T cells coexists with increased degree of T-cell apoptosis

and down-regulation of TCR ζ molecules in patients with gastric cancer. *Clin Cancer Res* 2001; **7**:74–80.

14 Maki A, Matsuda M, Asakawa M, Kono H, Fujii H, Matsumoto Y. Decreased expression of CD28 coincides with the down-modulation of CD3ζ and augmentation of caspase-3 activity in T cells from hepatocellular carcinoma-bearing patients and hepatitis C virus-infected patients. *J Gastroenterol Hepatol* 2004; **19**:1348–56.

15 Woo M, Hakem R, Furlonger C, Hakem A, Duncan GS, Sasaki T *et al.* Caspase-3 regulates cell cycle in B cells: a consequence of substrate specificity. *Nat Immunol* 2003; **4**:1016–22.

16 Alam A, Cohen LY, Aouad S, Sekaly RP. Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. *J Exp Med* 1999; **190**:1879–90.

17 Algeciras-Schimnich A, Barnhart BC, Peter ME. Apoptosis-independent functions of killer caspases. *Curr Opin Cell Biol* 2002; **14**:721–6.

18 Denis F, Rheaume E, Aouad SM, Alam A, Sekaly RP, Cohen LY. The role of caspases in T cell development and the control of immune responses. *Cell Mol Life Sci* 1998; **54**:1005–19.

19 Tatake RJ, Rajaram N, Damle RN, Balsara B, Bhisey AN, Gangal SG. Establishment and characterization of four new squamous cell carcinoma cell lines derived from oral tumors. *J Cancer Res Clin Oncol* 1990; **116**:179–86.

20 Rebouillat D, Hovnanian A, Marie I, Hovanessian AG. The 100-kDa 2′,5′-oligoadenylate synthetase catalyzing preferentially the synthesis of dimeric pppA2′p5′A molecules is composed of three homologous domains. *J Biol Chem* 1999; **274**:1557–65.

21 Ghosh A, Sarkar SN, Rowe TM, Sen GC. A specific isozyme of 2′-5′ oligoadenylate synthetase is a dual function proapoptotic protein of the Bcl-2 family. *J Biol Chem* 2001; **276**:25447–55.

22 Hovanessian AG. On the discovery of interferon-inducible, double-stranded RNA activated enzymes: the 2′-5′oligoadenylate synthetases and the protein kinase PKR. *Cytokine Growth Factor Rev* 2007; **18**:351–61.

23 Rapidis AD, Wolf GT. Immunotherapy of head and neck cancer: current and future considerations. *J Oncol* 2009; doi:10.1155/2009/346345.

24 Kiessling R, Wasserman K, Horiguchi S, Kono K, Sjöberg J, Pisa P *et al.* Tumor-induced immune dysfunction. *Cancer Immunol Immunother* 1999; **48**:353–62.

25 Frydecka I, Kaczmarek P, Bocko D, Kosmaczewska A, Morilla R, Catovsky D. Expression of signal-transducing ζ chain in peripheral blood T cells and natural killer cells in patients with Hodgkin's disease in different phases of the disease. *Leuk Lymphoma* 1999; **35**:545–54.

26 Taylor DD, Bender DP, Gercel-Taylor C, Stanson J, Whiteside TL. Modulation of TcR/CD3-ζ chain expression by a circulating factor derived from ovarian cancer patients. *Br J Cancer* 2001; **84**:1624–9.

27 Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Higashi H, Iwashige H *et al.* CD3-ζ chain expression of intratumoral lymphocytes is closely related to survival in gastric carcinoma patients. *Cancer* 2002; **94**:1437–42.

28 Ersek B, Molnar V, Balogh A, Matko J, Cope AP, Buzas EI *et al.* CD3ζ-chain expression of human T lymphocytes is regulated by TNF via Src-like adaptor protein-dependent proteasomal degradation. *J Immunol* 2012; **189**:1602–10.

29 Catalano A, Caprari P, Moretti S, Faronato M, Tamagnone L, Procopio A. Semaphorin-3A is expressed by tumor cells and alters T-cell signal transduction and function. *Blood* 2006; **107**:3321–9.

30 Hanaoka N, Jabri B, Dai Z, Ciszewski C, Stevens AM, Yee C *et al.* NKG2D initiates caspase-mediated CD3ζ degradation and lymphocyte receptor impairments associated with human cancer and autoimmune disease. *J Immunol* 2010; **185**:5732–42.

31 Benech P, Vigneron M, Peretz D, Revel M, Chebath J. Interferon-responsive regulatory elements in the promoter of the human 2′,5′-oligo(A) synthetase gene. *Mol Cell Biol* 1987; **7**:4498–504.

32 Melchjorsen J, Kristiansen H, Christiansen R, Rintahaka J, Matikainen S, Paludan SR *et al.* Differential regulation of the OASL and OAS1 genes in response to viral infections. *J Interferon Cytokine Res* 2009; **29**:199–207.

33 Mihm U, Ackermann O, Welsch C, Herrmann E, Hofmann WP, Grigorian N *et al.* Clinical relevance of the 2′-5′-oligoadenylate synthetase/RNase L system for treatment response in chronic hepatitis C. *J Hepatol* 2009; **50**:49–58.

34 Kim KI, Kim SR, Sasase N, Taniguchi M, Harada S, Kinoshita K *et al.* 2′-,5′-Oligoadenylate synthetase response ratio predicting virological response to PEG-interferon-α2b plus ribavirin therapy in patients with chronic hepatitis C. *J Clin Pharm Ther* 2006; **31**:441–6.

35 Shindo M, Hamada K, Morikawa T, Harano Y, Nakajima T, Okuno T. *In vivo* interferon system assessed by 2′-5′ oligoadenylate synthetase activity in chronic hepatitis C virus patients treated with pegylated interferon and ribavirin. *Hepatol Res* 2008; **38**:1213–20.

36 Kristiansen H, Scherer CA, McVean M, Iadonato SP, Vends S, Thavachelvam K *et al.* Extracellular 2′-5′ oligoadenylate synthetase stimulates RNase L-independent antiviral activity: a novel mechanism of virus-induced innate immunity. *J Virol* 2010; **84**:11898–904.

37 Feng X, Petraglia AL, Chen M, Byskosh PV, Boos MD, Reder AT. Low expression of interferon-stimulated genes in active multiple sclerosis is linked to subnormal phosphorylation of STAT1. *J Neuroimmunol* 2002; **129**:205–15.

38 Sanda C, Weitzel P, Tsukahara T, Schaley J, Edenberg HJ, Stephens MA *et al.* Differential gene induction by type I and type II interferons and their combination. *J Interferon Cytokine Res* 2006; **26**:462–72.

39 Hovanessian AG, Justesen J. The human 2′-5′oligoadenylate synthetase family: unique interferon-inducible enzymes catalyzing 2′-5′ instead of 3′-5′ phosphodiester bond formation. *Biochimie* 2007; **89**:779–88.

40 Chi LM, Lee CW, Chang KP, Hao SP, Lee HM, Liang Y *et al.* Enhanced interferon signaling pathway in oral cancer revealed by quantitative proteome analysis of microdissected specimens using 16O/18O labeling and integrated two-dimensional LC-ESI-MALDI tandem MS. *Mol Cell Proteomics* 2009; **8**:1453–74.

41 Bower JE, Greendale G, Crosswell AD, Garet D, Sternlieb B, Ganz PA *et al.* Yoga reduces inflammatory signaling in fatigued breast cancer survivors: a randomized controlled trial. *Psychoneuroendocrinology* 2014; **43**:20–9.

42 Vaarala MH, Porvari K, Kyllonen A, Vihko P. Differentially expressed genes in two LNCaP prostate cancer cell lines reflecting changes during prostate cancer progression. *Lab Invest* 2000; **80**:1259–68.

43 Liu YP, Suksanpaisan L, Steele MB, Russell SJ, Peng KW. Induction of antiviral genes by the tumor microenvironment confers resistance to virotherapy. *Sci Rep* 2013; **3**:2375.

44 Wang B, Zhao XP, Fan YC, Zhang JJ, Zhao J, Wang K. IL-17A but not IL-22 suppresses the replication of hepatitis B virus mediated by over-expression of MxA and OAS mRNA in the HepG2.2.15 cell line. *Antiviral Res* 2013; **97**:285–92.

45 Lallemand C, Lebon P, Rizza P, Blanchard B, Tovey MG. Constitutive expression of specific interferon isotypes in peripheral blood leukocytes from normal individuals and in promonocytic U937 cells. *J Leukoc Biol* 1996; **60**:137–46.

46 Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ *et al.* Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc Natl Acad Sci USA* 2003; **100**:2610–5.

47 van Baarsen LG, van der Pouw Kraan TC, Kragt JJ, Baggen JM, Rustenburg F, Hooper T *et al.* A subtype of multiple sclerosis defined by an activated immune defense program. *Genes Immun* 2006; **7**:522–31.

48 van der Pouw Kraan TC, Wijbrandts CA, van Baarsen LG, Voskuyl AE, Rustenburg F, Baggen JM *et al.* Rheumatoid arthritis subtypes identified by genomic profiling of peripheral blood cells: assignment of a type I interferon signature in a subpopulation of patients. *Ann Rheum Dis* 2007; **66**:1008–14.

49 Tough DF, Sun S, Zhang X, Sprent J. Stimulation of naive and memory T cells by cytokines. *Immunol Rev* 1999; **170**:39–47.

50 Puga I, Rao A, Macian F. Targeted cleavage of signaling proteins by caspase 3 inhibits T cell receptor signaling in anergic T cells. *Immunity* 2008; **29**:193–204.

51 Krishnan S, Kiang JG, Fisher CU, Nambiar MP, Nguyen HT, Kyttaris VC *et al.* Increased caspase-3 expression and activity contribute to reduced CD3ζ expression in systemic lupus erythematosus T cells. *J Immunol* 2005; **175**:3417–23.

52 Rusch L, Zhou A, Silverman RH. Caspase-dependent apoptosis by 2′,5′-oligoadenylate activation of RNase L is enhanced by IFN-β. *J Interferon Cytokine Res* 2000; **20**:1091–100.

53 Ludwiczek O, Kaser A, Koch RO, Vogel W, Cruikshank WW, Tilg H. Activation of caspase-3 by interferon α causes interleukin-16 secretion but fails to modulate activation induced cell death. *Eur Cytokine Netw* 2001; **12**:478–86.

54 Dugan JW, Albor A, David L, Fowlkes J, Blackledge MT, Martin TM *et al.* Nucleotide oligomerization domain-2 interacts with 2′-5′-oligoadenylate synthetase type 2 and enhances RNase-L function in THP-1 cells. *Mol Immunol* 2009; **47**:560–6.

55 Takeuchi T, Suzuki K, Kondo T, Yoshimoto K, Tsuzaka K. CD3 ζ defects in systemic lupus erythematosus. *Ann Rheum Dis* 2012; **71**(Suppl. 2):i78–81.

56 Clavien PA. Interferon: the magic bullet to prevent hepatocellular carcinoma recurrence after resection? *Ann Surg* 2007; **245**:843–5.

57 Sun HC, Tang ZY, Wang L, Qin LX, Ma ZC, Ye QH *et al.* Postoperative interferon α treatment postponed recurrence and improved overall survival in patients after curative resection of HBV-related hepatocellular carcinoma: a randomized clinical trial. *J Cancer Res Clin Oncol* 2006; **132**:458–65.

58 Urba SG, Forastiere AA, Wolf GT, Amrein PC. Intensive recombinant interleukin-2 and α-interferon therapy in patients with advanced head and neck squamous carcinoma. *Cancer* 1993; **71**:2326–31.

59 Mukherjee KK, Bose A, Ghosh D, Sarkar K, Goswami S, Pal S *et al.* IFNα2b augments immune responses of cisplatin+5-fluorouracil treated tongue squamous cell carcinoma patients – a preliminary study. *Indian J Med Res* 2012; **136**:54–9.

60 Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y *et al.* Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. *Gastroenterology* 2010; **139**:499–509.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 04 February 2015
doi: 10.3389/fimmu.2015.00037

The Jekyll and Hyde story of IL17-producing γδT cells

Rushikesh S. Patil, Sajad A. Bhat, Asif A. Dar and Shubhada V. Chiplunkar*

Chiplunkar Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, India

Edited by:
Julie Dechanet-Merville, Centre National de la Recherche Scientifique, France

Reviewed by:
Maria L. Toribio, Spanish Research Council (CSIC), Spain
Melanie Wencker, Institut National de la Santé et de la Recherche Médicale, France

***Correspondence:**
Shubhada V. Chiplunkar, Chiplunkar Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Sector 22, Navi Mumbai, Kharghar 410210, Maharashtra, India
e-mail: schiplunkar@actrec.gov.in

In comparison to conventional αβT cells, γδT cells are considered as specialized T cells based on their contributions in regulating immune response. γδT cells sense early environmental signals and initiate local immune-surveillance. The development of functional subtypes of γδT cells takes place in the thymus but they also exhibit plasticity in response to the activating signals and cytokines encountered in the extrathymic region. Thymic development of Tγδ1 requires strong TCR, CD27, and Skint-1 signals. However, differentiation of IL17-producing γδT cells (Tγδ17) is independent of Skint-1 or CD27 but requires notch signaling along with IL6 and TGFβ cytokines in the presence of weak TCR signal. In response to cytokines like IL23, IL6, and IL1β, Tγδ17 outshine Th17 cells for early activation and IL17 secretion. Despite expressing similar repertoire of lineage transcriptional factors, cytokines, and chemokine receptors, Tγδ17 cells differ from Th17 in spatial and temporal fashion. There are compelling reasons to consider significant role of Tγδ17 cells in regulating inflammation and thereby disease outcome. Tγδ17 cells regulate mobilization of innate immune cells and induce keratinocytes to secrete anti-microbial peptides thus exhibiting protective functions in anti-microbial immunity. In contrast, dysregulated Tγδ17 cells inhibit Treg cells, exacerbate autoimmunity, and are also known to support carcinogenesis by enhancing angiogenesis. The mechanism associated with this dual behavior of Tγδ17 is not clear. To exploit, Tγδ17 cells for beneficial use requires comprehensive analysis of their biology. Here, we summarize the current understanding on the characteristics, development, and functions of Tγδ17 cells in various pathological scenarios.

Keywords: γδT cell, IL17, Tγδ17, infection, inflammation, cancer

INTRODUCTION

Decades have passed since the accidental discovery of T cells expressing γ and δ chains (1), yet it is hard to define γδT cells like αβT cells. Ambiguity in understanding the functions of γδT cells is attributed to their unparalleled characteristics as compared to αβT cells. Current understanding of T cell biology has emerged extensively from studies on αβT cells; however, recent findings have underlined the crucial role of γδT cells in shaping the immune response in infections, inflammatory diseases, and cancer. They are involved in early immune response like innate cells, produce proinflammatory cytokines (IFNγ, IL17, and TNFα), and activate adaptive immune cells. The cytokines secreted by γδT cells determine their effector functions. In humans, the major cytokine produced by γδT cells is IFNγ, contributing to its role in antiviral, anti-bacterial, and anti-tumor immunity (2–4). However, upon activation γδT cells can be skewed toward IL17, IL4, or TGFβ producing phenotype governed by the polarizing cytokines present in the surrounding milieu (5). Recent investigations in mice and human have highlighted the role of IL17-producing γδT cells (hereafter referred as Tγδ17) in bacterial infection, inflammatory disease, and cancer (6–8). They are the primary source of IL17 in early disease condition and are pivotal in progression and disease outcome (9, 10). To understand the functional significance of Tγδ17 in pathological conditions, many efforts have made in mouse models but there is scanty literature available on human Tγδ17 cells. In this review, we will discuss the recent findings of Tγδ17 differentiation, mechanisms regulating IL17 production, and their relevance in pathological conditions.

γδT CELLS: UNIQUE BUT VERSATILE

Survival of γδT cells over strong evolutionary selection pressure highlights their exclusive importance and disparate properties from conventional αβT cells. Initially, γδT cells were considered as cells of innate immunity owing to their ability to recognize conserved non-peptide antigens expressed by stressed cells. In addition to this, they recognize pathogen-associated molecular pattern (PAMP) or danger-associated molecular pattern (DAMP) through pattern recognition receptors (PRR) expressed by them (11). Like adaptive immune cells, human γδT cells undergo clonal expansion and exhibit antigen-specific memory (12). Thus, γδT cells link innate and adaptive immunity thereby enhancing the immune response against invading pathogen or danger signal posed by "self" cells. Antigen recognition by murine or human γδT cells does not require antigen presentation by major histocompatibility complex (MHC) class I or class II (13) and the crystal structure of γδTCR has revealed its close homology with immunoglobulins suggesting that antigen recognition by γδT cells is similar to antigen–antibody interaction (14). However, diversity of antigens recognized by γδT cells brands it different from B cells. The antigens exclusively recognized by γδT cells are not peptides of protein antigens rather are small mono- and pyrophosphates of linear C5 isoprenoids called as phosphoantigens (13). These

prenyl pyrophospahtes are metabolites of cholesterol biosynthesis and are recognized through complementarity determining regions (CDRs) of γδT cells (15). In humans, during cholesterol biosynthesis, phosphorylated precursors such as isopentenyl pyrophosphate (IPP) and DMAPP (dimethylallyl pyrophosphate) are synthesized by mevalonate pathway (16). However, microbial pathogens use non-mevalonate pathway to produce these phosphorylated precursors (17). γδT cells respond to these natural or synthetic stimulators with varying degree. Based on this, stimulators are classified either as weak or potent stimulators. HMBPP [(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate], a metabolite of non-mevalonate pathway of bacteria *Mycobacterium tuberculosis* is 10^4 times more potent stimulator of human γδT cells than IPP (18). The exclusive response of γδT cells to these phosphoantigens has a potential therapeutic significance and synthetic pyrophosphates can be used to harness the cytotoxic potential of γδT cells.

Murine and human γδT cells also recognize phycoerythrin (PE) – fluorescent molecule of cyanobacteria and red algae. PE is directly recognized by γδT cells but there is no sequence similarity between PE-specific murine and human γδ TCR (19). Naturally occurring primary alkyl amines activate human Vγ2Vδ2 T cells and enhance immunity against certain microbes and plant-derived antigens (20, 21). Similar to natural killer (NK) cells, human γδT cells also recognize the stress-induced MHC class I-related molecules MICA, MICB, and the UL16-binding proteins that are upregulated on malignant or stressed cells (22, 23). The stress-related molecules are ligands for NKG2D expressed by γδT cells and this engagement also enhances γδT cells' response to non-peptide antigens (24). Human and murine γδT cells recognize lipid antigens presented by CD1 molecules, a classical ligand for NK T cell suggesting the phenomenon similar to MHC-restricted antigen recognition by αβT cells (25–27). The murine γδT cells also recognize non-classical MHC class I molecules like T10 and T22 (β2 microglobulin-associated molecules lacking peptide binding groove) (28, 29). In addition to non-protein and MHC related antigens, murine and human γδT cells also recognize small peptides such as heat shock proteins (HSPs) (30–32). However, they do not require antigen-presenting cells (APCs) and recognition of antigen is MHC unrestricted, resembling B cells (33). Thus, the broad spectrum antigen responsiveness of γδT cells helps them to mount faster immune response.

Like αβT cells, γδT cells develop in the thymus from $CD4^-CD8^-$ (double negative, DN) thymocytes (34); however, they precede αβT cells in T cells ontogeny. γδ TCR rearrangements can be traced in early embryonic stages in mice as well as in humans (35, 36). This highlights their role in neonatal protection as conventional T cells are functionally impaired and APCs are immature in newborns (37). During thymic development, the decision of γδ versus αβ T cell commitment is determined by TCR signal strength or notch signaling (38). In mice, the strong TCR signaling in absence of notch signal induces γδT cells lineage commitment whereas low TCR signal strength in presence of strong notch signaling promotes αβ T cell lineage (39–41). However, notch signaling alone is insufficient to decide γδ/αβ T cell commitment. The intrinsic signals from T cell receptor complex and trans-conditioning by different subsets of thymocytes also determine thymic development of γδT cells (42). In humans, notch has opposite role in αβ versus γδT cell lineage decision, sustained notch signaling is required for the development of γδT cells (43) which is determined by differential notch receptor–ligand interaction importantly Jagged2/Notch3 signaling (44). In human, γδT cells differentiate along two pathways, a notch-independent DN pathway, generating mature DN and $CD8\alpha\alpha^+$ SP (single positive) $TCR\gamma\delta^+$ cells. In the notch-dependent DP (double positive) pathway, immature $CD4^+$ SP, and subsequently DP $TCR\gamma\delta^+$ cells are generated. Human postnatal thymus thus exhibits a scenario of DN, DP, and SP $TCR\gamma\delta^+$ population, which highlights heterogeneity in human γδT cell development (45). The activated extrathymic γδT cells, in humans, express notch receptors, which regulate their effector functions. Inhibiting notch signaling in γδT cells dampened their anti-tumor cytotoxic potential (46). Thus, validates the requirement of notch signaling in both thymic development and functions of human γδT cells. The diversity of human γδ T cell repertoire at birth (majorly contributed by $V\delta1^+$ subset of γδT cells in cord blood) is restricted in adulthood especially to Vγ9Vδ2, a circulating subset of γδT cells. The absolute numbers of Vγ9Vδ2 T cells increase from minor population at birth to more than 75% of γδT cells pool in peripheral blood (35), which constitute around 1–10% of total T cells in humans. The γδT cells exit the thymus as mature T cells and express markers that are associated with antigen-experienced T cells (47).

The other important feature of γδT cells apart from antigen recognition is their tissue tropism. In humans, the first γδT cells to arise from thymus are $V\delta1^+$ (paired with various Vγ chains), which preferentially populate in epithelial tissue and constitute larger proportion of intraepithelial lymphocytes (IELs) (48). They rapidly and innately recognize stressed cells found to be enriched in various tumor tissues (4). The Vγ9Vδ2 is a lymphoid homing subset of γδT cells, which continually expand in response to microbial antigen in circulation and exhibit characteristics of adaptive immune system (49). These cells recognize, expand, and secrete cytokines in response to non-peptide antigens associated with microbes in circulation. In mouse, a substantial proportion of γδT cells reside as the IEL in the skin, intestine, and genitourinary tract. In response to the chemokine signals, $V\gamma5V\delta1^+$ T cells leave the fetal thymus, reside in the epidermis, and form dendritic-like network similar to Langerhans cells. These cells are called as dendritic epidermal T cells (DETCs) and constitute more than 90% of epidermal T cells (50). $V\gamma6^+$ T cells home to tongue and reproductive tract whereas $V\gamma7^+$ T cells home to intestinal tract suggesting that distinct TCR repertoire are present at different anatomical site and respond to antigens unique to their resident tissues (51–53). However, the functions of IELs are determined by the environment at the anatomical site (54) and hence specific γδ T cell subset could be used in tissue repair and generation of effective immune response at different epithelial sites.

γδT cells perform diverse effector functions determined by the TCR expressed, tissue localization, and activation status. Apart from these, MHC-independent recognition of antigens, production of IFNγ, and expression of cytotoxic granules classify γδT cells as potential cytotoxic cells (55). They can kill activated, infected, stressed, and transformed cells using various strategies such as engagement of death-inducing receptors, such as FAS

and TNF-related apoptosis-inducing ligand receptors (TRAILR) and the release of cytotoxic effector molecules such as perforin and granzyme (56, 57). Human γδT cells also recognize HSP (HSP60/70) expressed on tumor cells and enhance its cytolytic activity against the tumors (31, 58). γδT cells support the maturation and activation of other lymphocytes, NK cells, and macrophages with the help of secreted chemokines (CCL3, CCL4, CXCL10) (55). Another chemokine CXC–chemokine ligand 13 (CXCL13) produced by Vγ9Vδ2 cells can regulate B cell organization within lymphoid tissues and help B cells to produce antibodies (59). Human γδT cells can also crosstalk with dendritic cells (DCs) influencing each other functions like the antigen presentation by DCs, activation, and secretion of IL12 and IFNγ by γδT cells, which result in DC maturation (11, 60). These properties of γδT cells aid in generation of the effective immune response in the appropriate condition. Not only this, activated Vγ9Vδ2 cells can take up and process the soluble antigens, opsonize target cells, and can migrate to lymph nodes through CC-chemokine receptor 7 (CCR7) where they upregulate expression of MHCs and co-stimulatory receptors CD80 and CD86 (61, 62). Activated Vγ9Vδ2 cells has also been licensed to act as APC and activate CD4 and CD8 T cells (63). Collectively, these observations highlight the multi-talented role of γδT cells, having both Th- and Tc-like properties along with acting as APC. The special trait of γδT cells is their ability to recognize phosphorylated non-protein antigens and mediate its effector function in spatial and temporal manner making them a robust cell type, which can be manipulated to develop a promising tool for novel immunotherapies against certain types of diseases. However, care should be adapted while designing such immunotherapies because these cells have capacity to secrete various cytokines under different conditions.

Tγδ17: A SUBTYPE OF γδ T CELLS

Unlike αβ T cells, in mice, which leave thymus as naïve cells and are primed in the peripheral compartment, γδT cells undergo subset commitment in the thymus itself. However, in humans, upon activation with different cytokines, Vγ9Vδ2 cells can be polarized toward different effector subtypes like γδ1, γδ2 (64), γδ17 (65, 66), and γδTreg (67, 68). This functional plasticity of γδT cells assists them to tackle the distinct disease conditions and play important role in the early responses to invasive pathogens. The recent findings have stated that γδT cells are major IL17 producers and have shown their involvement in early onset of immune activation (69). Similar to Th17 cells, Tγδ17 cell express RORγt as a lineage determination transcriptional factor (70). Healthy adult human peripheral blood Vγ9Vδ2 T cells distinctively express Th1 signature and 50–80% produce IFNγ but <5% produce IL17 (6). However, Tγδ17cells have been demonstrated to be involved in the pathogenesis of transplantation rejection (71), autoimmune disease (72), allergy (73), and cancer (74) in humans. The biology of Tγδ17 is so naive that it compels us to cross-examine its genesis, functions, and clinical relevance to understand its therapeutic potential.

MOLECULAR EVIDENCES OF Tγδ17 GENESIS

The molecular mechanism of IL17-producing γδT cells remains an enigma. Most of the studies carried out to understand the differentiation mechanisms of Tγδ17cells are based on the murine models. γδT cells preferentially localized to barrier tissues are the initial source of IL17 and are likely to originate from the fetal thymus. These are called as the natural IL17-secreting γδT cells. γδT cells that make IL17 within 24 h fall in this category (75). γδT cells acquire IL17-secreting phenotype in secondary lymphoid tissues after antigen exposure, which is referred to as induced Tγδ17 cells (76, 77).

During development of T cells in thymus, murine γδT cells branch off at the transition of thymocytes from DN3 stage to DN4 stage (34). It is also reported that γδT cells develop from DN2 stage and specifically produce IL17 whereas IFNγ-producing γδT cells can develop from both DN2 and DN3 precursors (78) (**Figure 1**). This suggests that γδT cells do not develop like αβT cells and follow evolutionary ancient path of T cell development. However, the precise DN stage from which γδT cells develop is elusive (79). Fetal thymic γδ T-cell development occurs in successive waves by using the different Vγ and Vδ segments during the embryonic development (34, 80). Successful gene rearrangement of γδ T cells from early thymic precursors ($CD44^{hi}$) lead to the development of naïve γδ T cell characterized by $CD44^{lo}$ $CD27^{+}CD62L^{+}$ phenotype. This phenotype can either leave the thymus to populate in secondary lymphoid organs or it can undergo further intrathymic differentiation that results in the development of multiple γδ T cell subtypes such as dendritic epidermal γδT cell (DETCs), Tγδ17, or NK 1.1^{+} γδ cell (γδNKT cells) (80, 81). Recently, it was described that when thymic lobes of mice at E14 were colonized with DN1a cells from mice at E13 and E18, respectively. It was observed that although both populations (E13 DN1a cells and E18 DN1a cells) generated similar number of γδT cells, only E13 DN1a cells generated $V\gamma3^{+}$ DETCs. These observations indicate that precursor lineage of DETCs may be different and needs further investigation (82). DETCs develop at embryonic day 13 (E13) to approximately E17 and readily secrete IFNγ when activated. After the development of DETCs, the next functional developmental wave consists of Tγδ17 cells. Tγδ17 cells are heterogeneous in using TCR chains that mainly include $V\gamma6^{+}$ and $V\gamma4^{+}$ but also use $V\gamma1^{+}$ chain. $V\gamma6^{+}$ cells develop by E14 to around birth and finally Vγ1 and Vγ4 cells develop E16 onward (81). The other subtypes of γδ T cells, which develop in thymus, are γδ NKT cells, which are similar to invariant $TCR\alpha\beta^{+}$ NKT cells (83, 84).

There are different thymic signaling processes, which determine functional phenotype of γδT cells in thymus before migration to periphery and contribute to the balance between IFNγ committed versus IL17-commited subtypes (85). This biasness toward IL17 or IFNγ depends on the antigen experience in thymus. The γδ T cells that have encountered the cognate antigen interaction in thymus, gain the potential to differentiate into the IFNγ-producing functional phenotype while antigen naïve γδ T cells develop into IL17-producing γδT cells (86). This skewedness also reflects in their distribution outside the thymus. Most of Tγδ17 cells reside in lymph nodes whereas IFNγ-producing γδT cells are mainly found in the spleen and the mechanism for this distribution is not clear (86). Similar distribution is also found in αβT cells and it seems to be logical as the lymph nodes serve as the site of initial exposure to foreign antigens and propagate the wave of inflammation, thus are suited for the earliest source of the IL17 secretion (87).

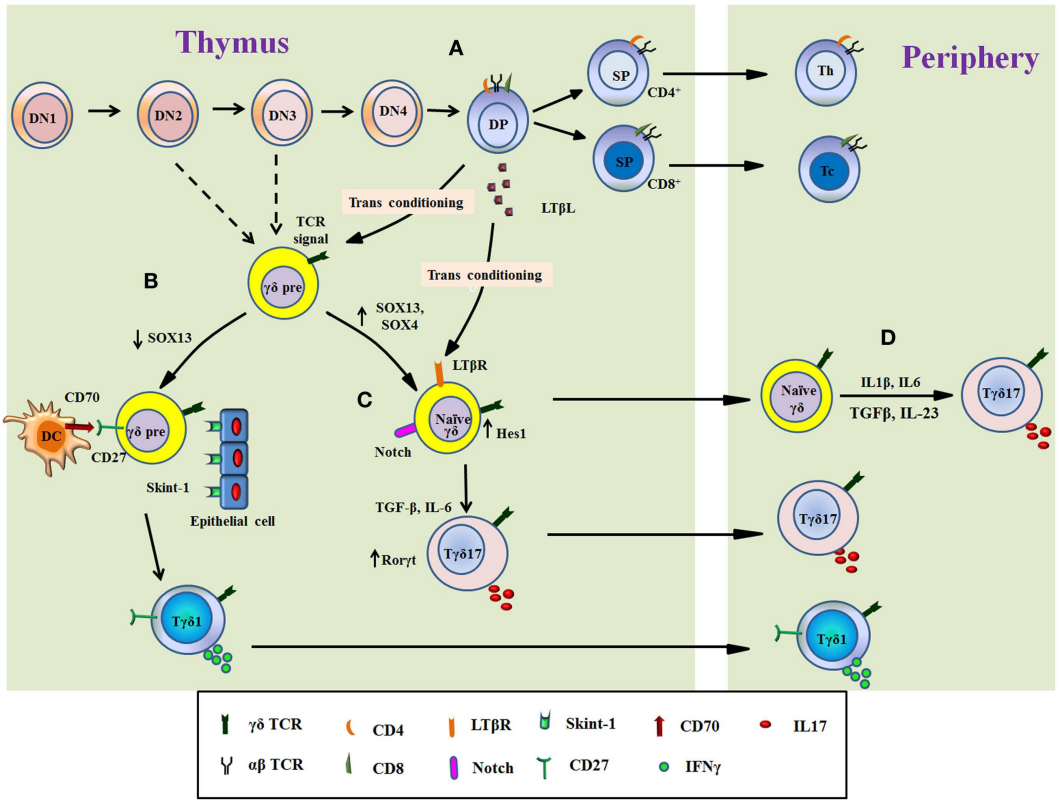

FIGURE 1 | Overview of Tγδ17 cells development. The figure illustrates the differentiation of Tγδ17 cells from T cell progenitors in the murine thymus **(A–C)** and from naïve γδT cells in periphery in human **(D)**. Progenitor T cells differentiate through double negative stage 1 (DN1) to DN stage 4 **(A)**. The decision of αβ or γδ TCR expression takes place at early T cells precursor (from DN2 or DN3 stage) as showed by dashed line. The thymocytes expressing αβ TCR develop into double-positive thymocytes, which support differentiation of functional subtypes of γδT cells called as transconditioning. DP thymocytes secrete LTβL, which support differentiation of Tγδ17. The DP αβ thymocytes then exit the thymus as mature single positive T cells (either $CD4^+$ or $CD8^+$ T cells) **(A)**. The functional programing of γδT cells is determined by TCR signal and/or other related signals. TCR signal, interaction with Skint-1 from epithelial cells, downregulation of SOX13, and signaling through CD27/CD70 divert γδ thymocytes toward IFNγ-producing phenotype (Tγδ1), which migrate to periphery **(B)**. Conversely, signaling through Notch receptor maintain Sox13 levels with increase in Hes1 and RORγt expression induce γδ thymocytes to produce IL17. Progression of γδ thymocytes to Tγδ17 cells is independent of signaling through Skint-1 and/or CD27 but require inputs from IL6 and TGFβ. The natural Tγδ17 cells developed in thymus migrate to tissue or periphery **(C)**. In human, naïve γδT cells, which exit thymus, can also differentiate into Tγδ17 cells in presence of TCR signal and cytokines such as IL6, IL1β, IL23, and TGFβ **(D)**.

Besides the γδ TCR signaling (86), expression of tumor necrosis factor receptor family member, CD27, determines the IL17 versus IFNγ production by γδT cells (88). $CD27^+$ γδT cells differentiate into IFNγ producing cells whereas IL17 production was restricted to $CD27^-$ T cells (89) (**Figure 1**). Thus thymic "imprinting" of the γδT cells as $CD27^+$ or $CD27^-$ regulates effector functions of γδT cells and is preserved in the periphery (89). CD27 is not only associated with IFNγ production but also aids γδT cells to interact with its ligand CD70 expressed on DCs, thymic epithelial cells, and double-positive thymocytes thus acting as a costimulatory receptor (89). Therefore, CD27 conveys an intrathymic message that licenses the $CD27^+$ γδ T cells for the production of IFNγ (47). Another signaling pathway that influences the differentiation of Tγδ17 is the signaling through lymphotoxin-β receptor (LTβR), a member of the tumor necrosis factor receptor family (90). Signaling through LTβR leads to the activation of the alternative nuclear factor (NF)-κB pathway via RelB. Ligands for LTβR regulating this developmental process are produced by $CD4^+CD8^+$ thymocytes (91). The homeostasis of this functional phenotypic differentiation, influenced by other thymic progenitors is known as transconditioning (91), which highlights coordination between different signaling pathways in thymus that occur in physically separate thymic niche (92). LTβR signaling pathway controls Tγδ17 development by regulating transcription factors RORγt and RORα4, required for IL17 expression in γδ thymocytes (93). The role of LTβR signaling, however, remains controversial as LTβR is present downstream to CD27 signaling, which is associated with the IFN-γ production (89).

The maturation of Tγδ17 cells from its precursors requires TCR signaling as mice with reduced ZAP70 show decreased number of Tγδ17 cells (94). However, TCR signaling alone is not sufficient as it also requires other signals (95). An src family kinase, Blk (B lymphoid kinase), is required for Tγδ17 cells development in thymus as Blk-deficient mice was reported to have less number of IL17-producing γδ T cells (96). Similarly, high-mobility group (HMG) box transcription factors, SOX4 and SOX13 are

positive regulators of Tγδ17 development (95, 97). These transcription factors expressed in immature T cells (98) highlight that the development of Tγδ17 is from early precursors (DN2) (78, 95). Other thymic determinant, which is responsible for the functional dichotomy in Tγδ17 and Tγδ1, is Skint-1, a thymic epithelial cell determinant. The interaction between Skint-1^+ cells and γδ thymocytes ($V\gamma5^+V\delta1^+$) induce an Egr3-mediated pathway, leading to differentiation toward IFNγ-producing γδ T cells. Further, it suppresses Sox13 and an RORγt transcription factor-associated Tγδ17 cells lineage differentiation suggesting that the functions of the earliest T cells are substantially preprogramed in the thymus (99). Notch signaling is known to be involved in thymic determination and development of Tγδ17 cells. Hes1, one of the basic helix–loop–helix (bHLH) proteins induced by Notch signaling is critical for the IL17 expression by γδ T cells and its thymic development (100–102). Further, the specific expression of Hes1 in $CD25^+$ and $CD27^-$ γδ T cells and decreased levels of Tγδ17 in Hes1-deficient mice highlights the critical role of Notch–Hes1 pathway in Tγδ17 development in thymus as well as in periphery (101). The thymic development of Tγδ17 is independent of STAT3 but partly dependent on RORγt (101) and most peripheral IL17-producing γδ cells express RORγt and respond rapidly to IL23 (103).

Developmental process of Tγδ17 also requires signaling through different cytokines. TGFβ signaling is necessary for Tγδ17 development (104). It has been shown that in absence of TGFβ1 or Smad3 (a component of the TGFβ signaling), the number of Tγδ17 thymocytes reduced drastically relative to that of wild-type mice (104). As compared to TGFβ, requirement of IL6 for Tγδ17 development is not well understood as there are contrasting reports on its role (72, 105). It is also reported that IL6 does not act directly on uncommitted γδ thymocytes but instead it acts indirectly by regulating the expression of Delta-like ligand 4, a ligand for notch receptor, expressed by thymic epithelial cells that promote the differentiation of Tγδ17 (101, 106). Moreover, IL23 and IL1 produced by DCs are crucial for IL17 production by γδT cells. $IL23^{-/-}$ and $IL23R^{-/-}$ mice showed the significant reduction in Tγδ17 cells after *L. monocytogenes* infection supporting earlier observation (107–110).

Thymic development of human Tγδ17 cells is poorly investigated. Around 80% circulating human Vγ9Vδ2 T cells are IFN-γ producers and express CD27 whereas CD27 negative cells are IL17-producing γδ T cells are <5% (65). Interaction of CD70 with CD27 promotes the expansion of Th1-biased Vγ9Vδ2 T cells in periphery (111). However, such role in their thymic development is unknown. Human Vγ9Vδ2 T cells can be polarized to Tγδ17 cells in periphery upon IPP activation and in the presence of cytokines like TGFβ, IL1β, IL6, and IL23, followed by a week of culture in differentiation medium supplemented with IL2 can induce IL17 in these cells (65, 66). In humans, there are contrasting reports on role of IL6 and IL23 in differentiation of Tγδ17. It has been shown that IL6 is required for differentiation of neonatal Tγδ17, and IL23 is required for the generation of adult IL17-producing γδT cells (65). In another study, it is reported that in the presence of TCR signaling, IL23 promotes the induction of IL17 in neonatal (but not adult) γδT cells (112). However, it appears that IL23 induces γδT cells to coproduce IL17 and IFNγ in adults but support development of Tγδ17 cells in neonates. In addition to the above-mentioned cytokines, IL7 selectively promotes the mouse and human IL17-producing γδT cells. IL7 activates STAT3 preferentially in γδT cells competent to produce IL17 (113). However, the increased IL17 production by γδT cells upon TCR stimulation in presence of IL7 is observed only in case of cord blood cells but not with peripheral lymphocytes. Thus, it is important to note that the antigen naïve γδT cells only can be reprogramed *in vitro* toward Tγδ17 phenotype (66, 113).

The kinetic study of IL17 production by γδT cells has shown that murine γδT cells secrete IL17 within few hours after stimulation (70). This phenomenon can be reasoned by the thymic development of murine Tγδ17 cells and constitutive presence of transcriptional regulators for IL17 production. However, human γδT cells in thymus are functionally immature and can attain their functional differentiation in periphery in presence of cytokines (114). This supports the kinetics of IL17 production by human γδT cells that mRNA expression of IL17 and RORγt peaks by day 3–6 and decrease by day 9 onward, after stimulation. The expression of cytokine receptors (IL1βR, IL6R, TGFβR, and IL23R) on Vγ9Vδ2 T cells peaks on day 3 and decrease by day 6 (66). Thus, coordinated combination of TCR and cytokine stimulation could be necessary for the sustained secretion of IL17 by γδT cells, which highlights the difference in kinetics of IL17 secretion by murine and human Tγδ17 cells. This underscores that human γδ T cells can be "reprogramed" in the periphery into different functional lineages.

Upon antigenic challenge, T cells differentiate to memory phenotype; either central memory (TCM) or effector memory (TEM) (115). Human Tγδ17 cells present in non-lymphoid environment belong to $CD27^-$ $CD45RA^{\pm}$ effector (74) or terminally differentiated (TEMRA) (66) memory phenotype. Similarly, murine Tγδ17 cells also show effector memory phenotype with $CD44^{high}$, $CD45RB^{low}$, and $CD62L^{low}$ (116). Thus, Tγδ17 cells differentiated either in thymus or in periphery, belong to memory phenotype, and licensed to patrol the blood, lymphoid organs, and peripheral tissues.

Tγδ17 IN MICROBIAL INFECTIONS

Tγδ17 cells can rapidly produce IL17 upon Toll-like receptors (TLR) or cytokine stimulation alone even in absence of antigen presentation. The general proinflammatory functions of IL17 [reviewed in Ref. (117, 118)] could be associated with γδT cells as they are major producers of IL17. Studies carried out in various infection models showed that Tγδ17 cells are protective against infection. During mycobacterial infection, IL17 produced by $V\gamma4^+$ and $V\gamma6^+$ cells induce pulmonary granuloma formation by recruitment of granulocytes and monocytes. The IL17 participates in maturation of granuloma by promoting tight cell to cell binding via ICAM1 and LFA1 induction (119). Mycobacteria-infected DCs secrete IL23, which regulate IL17 production by γδT cells emphasizing that the early activation of Tγδ17 cells is important for initiating inflammation and recruiting innate immune cells to the site of infection thereby enhancing bacterial clearance from host (120, 121). Tγδ17 cells also support cell-mediated immunity by inducing Th1cells against pulmonary mycobacterial infection (122).

In *Escherichia coli* infection model also, γδT cells were reported to be the major producers of IL17, which enhanced neutrophil infiltration to the peritoneum. The infiltration of cells diminished after antibody depletion of resident Vδ1$^+$ subtype of γδT cells highlighting its involvement in IL17 secretion in response to IL23 (9). Thus, IL23 and Tγδ17 cells play a dominant role as first line of defense in infection before CD4 T cell activation. In case of *L. monocytogenes* infection, a large number of γδ T cells accumulate in the lymph organs shortly after infection and begin to produce IL17A, signifying the role of Tγδ17 cells in the *Listeria* infection (123). IL17 was also shown to promote proliferation of CD8$^+$ cytotoxic T lymphocytes by enhancing DC cross-presentation *in vitro*. DCs stimulated with IL17 showed upregulation of MHC-I molecule H2Kb and enhanced secretion of cytokines (IL12, IL6, and IL1β). CD8α$^+$ DCs from *Il17a*$^{-/-}$ mice also produced less IL12 and are less potent in activating naive CD8$^+$ T cells (123). This indicate that Tγδ17 cells not only induce innate response but also critical for optimal adaptive cytotoxic response against intracellular bacterial infection. The alliance of IL23 and Tγδ17 is also demonstrated to have a protective role during infections such as *Klebsiella pneumonia* (124), *Citrobacter rodentium* (125, 126), *Salmonella enterica* (127, 128), and *Toxoplasma gondii* (129). The Tγδ17 cells also play a vital role in clearing fungal infections. The rapid production of IL17A was reported in the lungs at a very early stage after intravenous infection with *C. albicans*. Lung resident γδ T cells were the major source of early IL17A production regulated by IL23 and TLR2/MyD88-dependent pathway (130). Presence of Tγδ17 cells were also reported in the lungs of neutropenic mice during *C. neoformans* infection. These Tγδ17 cells played an important role in the chemotaxis of leukocytes and induction of protective immune response (131). Tγδ17 cells thus orchestrate the protective immunity by acting at the early onset in infection models (108).

Relatively few studies have evaluated the role of Tγδ17 cells in human microbial immunity. In patients with tuberculosis (TB), elevated levels of Tγδ17 cells were found in peripheral blood and were major producers of IL17 (6). As a protective role, in response to bacterial antigens, IL17-producing Vγ9Vδ2 T cells induce neutrophil migration through secretion of CXCL8 and promote their phagocytic activity (66). Tγδ17 cells also induce epithelial cells to secrete anti-microbial peptides like β-defensins in response to bacterial antigens (66). This signifies the modulatory effects of Tγδ17 cells on keratinocytes and other immune cells in anti-microbial defense. In children with bacterial meningitis, the population of IL17$^+$ Vγ9Vδ2 T cells significantly increase in peripheral blood and at the site of infection (cerebrospinal fluid). The reversal of this pattern after successful anti-bacterial therapy clearly suggests the anti-microbial role of Tγδ17 cells (66). Collectively, these studies provide new insight into the functions of γδ T cells as the first line of host defense against bacterial and fungal infection in human and may pave a path in designing newer treatment modalities.

TOLL-LIKE RECEPTORS REGULATE IL17 PRODUCTION IN Tγδ17 CELLS

γδT cells express various chemokine receptors, cytokine receptors, and PRRs, which regulate IL17 production. TLRs are the well-studied PRRs expressed by DCs, macrophages, and γδT cells. The unique microbial molecules called as PAMP are recognized by TLRs, which orchestrate the anti-microbial response in γδT cells (11). In malarial infection, MyD88 deficiency results in severe impairment of IL17A producing γδT cells levels, but not IFNγ producing γδT cells highlighting differential control by innate signaling through TLRs in infections (132). Murine Tγδ17 cells specifically express TLR1 and TLR2 but not TLR4. High number of Tγδ17 cells were induced upon *in vivo* stimulation with Pam3CSK4 (ligand for TLR2) but not with LPS (TLR4 ligand) or CpG (TLR9 ligand) (70). Interestingly, it has been shown that TLR4 indirectly controls IL17 generation by γδT cells through IL23 secreted by TLR4 expressing macrophages in response to HMG Box 1 (HMGB1, a damage-associated protein and TLR4 ligand) (133). Moreover, Tγδ17 cells promote experimental intraocular neovascularization (134) as well as early acute allograft rejection (135) in response to HMGB1. Signaling through TLR2 is indispensable for Tγδ17 in anti-microbial functions. Absence of TLR2 or MyD88 in cutaneous *Staphylococcus aureus* infection, or in *Candida albicans* infection, caused an impaired IL17 production and poor microbial clearance in the skin infiltrated with Vγ5$^+$ γδT cells (130, 136). Tγδ17 cells also express DC-associated C-type lectin 1 (dectin 1) and intraperitoneal injection of curdlan (dictin 1 ligand), induced IL17 production by γδT cells (70). In imiquimod (IMQ)-induced psoriasis-like model, dermal γδT cells spontaneously secreted a large amount of IL17 in IMQ-treated skin cells. Thus, it appears that TLR7/8 (receptor of IMQ) may regulate the IL17 production by γδT cells. It is important to note that the modulatory effects of TLRs on γδT cells as showed in *in vivo* murine models are mediated through IL23 and/or IL1β cytokines. The direct stimulation of CD27$^-$ γδT cells by TLR ligands (LPS or PAM) show no effect on IL17 production (132). This suggests that TLR signaling indirectly modulates Tγδ17 function.

RECEPTOR REPERTOIRE EXPRESSED BY Tγδ17 CELLS

The receptor profile of Tγδ17 cells is similar to Th17 cells. In mice, the majority of IL17-producing CD4 cells belong to CCR6$^+$ compartment compared to CCR6$^-$ (137). Sorted CCR6$^+$ γδT cells showed increased mRNA expression of IL17, IL22, IL23R, Rorγt, and aryl hydrocarbon receptor (AhR) compared to CCR6$^-$ γδT cells (70, 138). This suggests that CCR6 can be a phenotypic surface marker of Tγδ17 cells. Besides CCR6, Tγδ17 cells express various chemokine receptors including CCR1, CCR2, CCR4, CCR5, CCR7, CCR9, CXCR1, CXCR3, CXCR4, CXCR5, and CXCR6 (7). The early onset recruitment of Tγδ17 to the site of inflammation is determined by the type of chemokine receptor on them. Tγδ17 cells expressing CCR6 and CCR9 show selective migration toward allergic inflamed tissue in response to CCL25 (ligand for CCR9). α4β7 integrin expression is indispensable for this migration and transendothelial crossing of Tγδ17 cells. (139). Since migration through CCL2/CCR2 axis is determinant for total γδT cells, CCL25/CCR9-mediated migration seems to be specific for Tγδ17 subtype (140, 141).

In humans, Tγδ17 cells express CCR6 but not CXCR3, CXCR5, CCR3, CCR4, or CCR5. However, they express granzyme B, FASL, and TRAIL but not perforin (66). The lack of granzyme B and perforin coexpression may be responsible for absence of cytolytic activity of Tγδ17 cells. On the contrary, it has been shown that the

human colorectal tumor-infiltrating Tγδ17 cells do not express FASL or TRAIL but express CD161 and CCR6 (74). The inconsistency in expression of cytolytic markers and their relevance on Tγδ17 cells needs to be understood in detail. The AhR is indispensable for Tγδ17 cells as it promotes differentiation of naïve Vγ9Vδ2 T cells toward Tγδ17 phenotype (66).

In mouse model, it has been shown that $Ahr^{-/-}$ Tγδ17 cells express IL17 but fail to produce IL22 (70). Moreover, in mouse model of *Bacillus subtilis* induced pneumonitis, deficiency of Ahr resulted into low IL22 production but IL17 levels were maintained (142). Thus, although Ahr promotes IL17, it is indispensable for IL22 production by Tγδ17 cells.

INFLAMMATORY DISORDERS AND MANIA OF Tγδ17

Th17 cells and Tγδ17cells are essential in disease progression and are pathogenic in autoimmune disease. Dysregulated levels and sustained secretion of proinflammatory cytokines by γδ and/or CD4 T cells have devastating effects on autoimmune disease progression. In a collagen-induced arthritis (CIA) model (resembling human rheumatoid arthritis), IL17-producing $Vγ4/Vδ4^+$ T cells selectively increase in joints and lymph nodes. Depletion of γδ T cells by anti Vγ4 antibody, markedly reduced the disease severity score revealing its pathogenic nature (143). Interestingly, both Th17 and Tγδ17 are present in the joints but Th17 cells localize proximal to the bone, which facilitates its interaction with osteoclast. Selective depletion of Th17 cells abrogated the bone resorption suggesting that Th17 but not Tγδ17 cells are responsible for bone destruction. Thus, Tγδ17 cells may be responsible for enhancing joint inflammation and exacerbate CIA (144). In contrast, absence of Tγδ17 was reported in patients with rheumatoid arthritis and in murine model of autoimmune arthritis (SKG model) (145). The SKG mouse model has defects in the differentiation of Tγδ17 cells (94), which might result into low Tγδ17 cells in the inflamed joints. Thus, the role of Tγδ17 cells in autoimmune arthritis need to be evaluated comprehensively.

Tγδ17 also enhanced experimental autoimmune encephalomyelitis (EAE) (mouse model for human multiple sclerosis). Upon immunization of mice with myelin oligodendrocyte glycoprotein (MOG) peptide in complete Freund's adjuvant (CFA), $Vγ4^+CCR6^+IL23^+$ γδT cells accumulate in the central nervous system (CNS), which expand by 20-fold in absolute number during development of clinical signs of the disease (72). In contrast, IFNγ-producing γδT cells are low in CNS and marginally increase during course of EAE (103). The mechanism behind aggravation of EAE could be attributed to restraining the development of $Foxp3^+$ regulatory T cells (Tregs) functions by Tγδ17 cells. Supernatants from IL23-activated γδT cells inhibited the TGFβ driven conversion of naive $Foxp3^-$ αβ T cells into Foxp3 expressing T cells and also reversed the suppressive effect of Treg cells (72). Similar function of Tγδ17 was reported in cardiac transplantation in mice. IL17, majorly produced by γδT cells, accelerates acute rejection of transplanted heart but IL17 deficiency enhanced Treg expansion and prolonged allograft survival (71). In ischemic brain injury, Tγδ17 were reported to be present at the infract areas (146). Tγδ17 rather than Th17 was the major source of IL17 whereas IFNγ was majorly produced by Th1 cells. In mice, genetically deficient for IL17 or IL23, the infract areas were reduced suggesting a role of Tγδ17 as a key contributor of neuroinflammation (146). Overall, this suggests that in chronic inflammatory condition, innate cytokines IL23 and IL1β promote infiltration and generation of IL17-producing γδT cells, which aggravate the disease.

Experimental silicosis is a useful model for depicting chronic lung inflammation, tissue damage, and fibrosis. Tγδ17 along with Th17 accumulated in the lung in response to IL23 expressing macrophages by third day after silica treatment but interestingly did not induce lung fibrosis (73). On the contrary, in allergic lung inflammation, Tγδ17 cells are known to be protective (147, 148). Functional blockage of both IL17 and γδT cells impaired the resolution of airway lung inflammation (148). It is claimed that this protective role is mediated by prostaglandins (PGs), which are abundant at the site of inflammation. PGI2 analog iloprost enhanced IL17 production by γδT cells in the thymus, spleen, and lungs, reducing airway inflammation (147). This highlights the role of PGI2 analogs that can be exploited in the development of immune response in immunotherapeutic approaches. Age-related macular degeneration (AMD) is another chronic inflammation associated disease, characterized by choroidal neovascularization (CNV). In an experimental model, Tγδ17 cells along with $Thy\text{-}1^+$ ILCs (innate lymphoid cells) infiltrate the eye after laser treatment and promote neovascularization. This recruitment is in response to IL1β but not IL23 produced by macrophages (134).

Tγδ17 CELLS AS HEROES OR VILLAINS IN CANCER

The unmatched characteristics of human γδT cells to have MHC unrestricted tumor directed cytotoxicity, release of copious amounts of IFNγ, and recognition of cancer cells through variety of mechanisms render them as potential candidate for cancer immunotherapy (4, 149). Upon activation, γδT cells show cytotoxicity against myeloma (150), lymphoma (151), leukemia (152, 153), and other epithelial carcinomas (57, 154, 155) *in vitro*. Several clinical trials have been launched using γδT cells based therapies in cancer patients. The hallmark characteristic of γδT cells to be used for therapy is their ability to infiltrate tumors (156). *In vivo* activation by phosphoantigens or adaptive transfer of preactivated autologous γδT cells have proved successful in cancer treatment (157). However, the role of Tγδ17 cells as anticancer effector cells is not well defined.

In a chemotherapeutic approach, Tγδ17 cells are reported to play decisive role in several transplantable tumor models (EG7 thymoma, MCA205 sarcoma, CT26 colon cancer, and TS/A mammary carcinomas). Tγδ17 ($Vγ4^+/Vγ6^+$) cells were shown to invade the tumor bed early in response after drug treatment. This was followed by infiltration and induction of IFNγ-producing CD8 (Tc1) cells to the tumor bed. This infiltration of Tγδ17 and Tc1 cells was correlated and associated with tumor regression post radio or chemotherapy (158). Thus, IL17-producing $Vγ4^+/Vγ6^+$ cells are critical for the induction of Tc1 response in tumor tissue in response to drug treatment or radiation. Another study in bladder cancer supports the helper function of Tγδ17 cells in cancer treatment. Tγδ17 cells induce neutrophil infiltration to the tumor site and show anti-tumor effect upon *Mycobacterium bovis* BCG treatment (159).

In contrast to anti-tumor role of Tγδ17 cells, they also promote tumor development. With the notion that IL17 is a proangiogenic

cytokine (160), Tγδ17 cells promote angiogenesis in tumor model. In IL17$^{-/-}$ tumor bearing mice, the blood vessel density was markedly decreased compared to wild type. In addition, IL17 induced the expression of Ang-2 (angiopoietin) and VEGF (vascular endothelial growth factor) in tumor cells (8). In ovarian cancer model, it has been reported that CD27^{-} Vγ6^{+} cells produced higher IL17 and induce VEGF and Ang-2 in peritoneal exudates of tumor bearing mice after 6 weeks of post-tumor inoculation (161). Additionally, Tγδ17 cells induce mobilization of protumor small peritoneal macrophages (SPM) to the tumor bed, which express IL17-dependent proangiogenic profile (*Il1b, Il6, vegfa, tgfb, mif, cxcl1, cxcl8*, and *tie2*). SPMs also enhance ovarian cancer growth by stimulating tumor cell proliferation (161). In hepatocellular carcinoma mouse model, it was reported that IL17, majorly produced by Vγ4^{+}γδT cells, induced CXCL5 production by tumor cells, which enhance migration of MDSCs (myeloid-derived suppressor cells) expressing CXCR2 to the tumor site. In addition, IL17 also enhanced suppressive functions of MDSCs by inhibition of T cells proliferation and cytokine (IFNγ and TNFα) production (162). In return, MDSCs induced γδT cells to produce IL17 through IL23 and IL1β secretion forming positive feedback loop for Tγδ17 activation (162). Thus, Tγδ17 cells interact with myeloid cells and counteract tumor immune-surveillance.

In human colorectal cancer, IL8 and GM-CSF secreted by Tγδ17 promote migration of MDSCs while IL17 and GM-CSF enhanced their proliferation. Tγδ17 cells also support survival of MDSCs through IL17, IL8, and TNFα (74). Thus, it is possible to speculate that Tγδ17 cells might be responsible for gradual shift from initial inflammatory to immunosuppressive tumor environment in advanced stage cancer (163). In human colorectal carcinoma, Tγδ17 cells were positively correlated with advancing tumor stages as well as with clinicopathological features including tumor size, tumor invasion, lymphatic and vascular invasion, lymph node metastasis, and serum CEA (Carcinoembryonic antigen) levels suggesting their pathogenic role (74).

Collectively, these findings highlight the apparently opposite roles of Tγδ17 cells in cancer immunity. It seems that during tumor development, inflammatory environment (IL1β and IL23) modulate the cytokine profile of γδT cells from primary IFNγ toward proinflammatory IL17, which support tumor progression.

CONCLUDING REMARKS

Despite the small percentage in total T cell population, γδT cells have emerged as an important modulator of early immune responses. The development of functional subtypes of γδT cells require polarizing cues including molecular and cellular

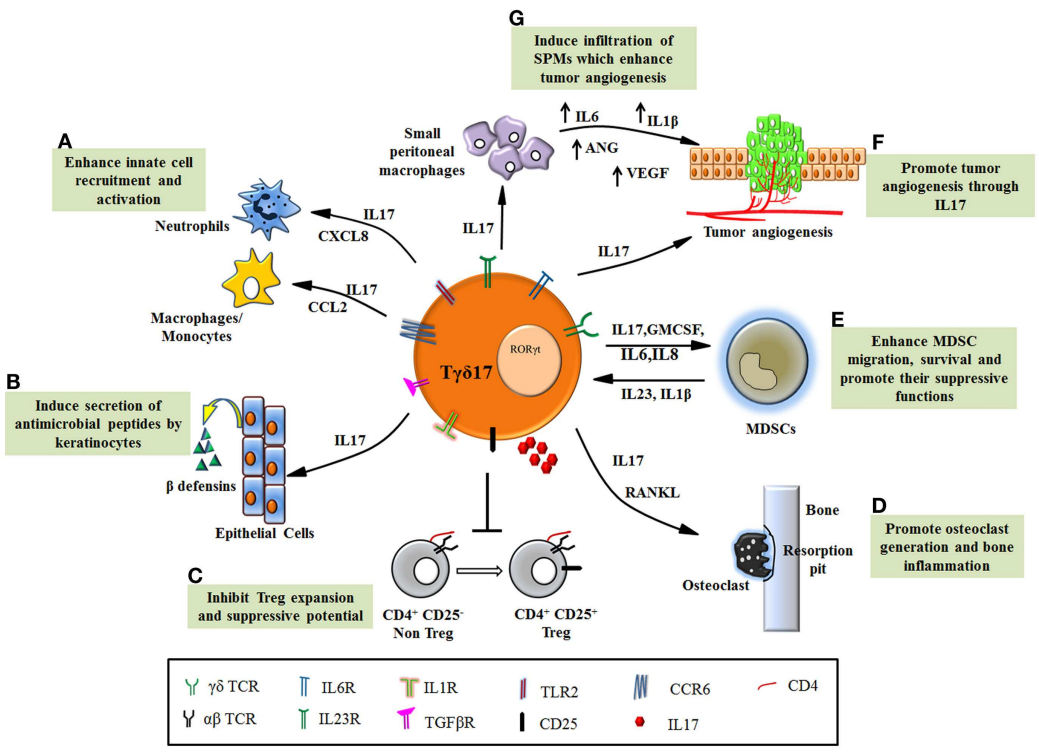

FIGURE 2 | Functions of Tγδ17 cells in pathological conditions. **(A)** Tγδ17 cells promote infiltration of neutrophils and monocytes/ macrophages to the site of inflammation through chemokines. **(B)** IL17 secreted by Tγδ17 cells induces keratinocytes to produce anti-microbial peptides such as β defensins and protect host in infections. **(C)** Dysregulated Tγδ17 cells in autoimmune diseases inhibit Treg expansion and its ability to suppress autoreactive cell, thereby exacerbating the disease. **(D)** The inflammatory condition in arthritis is worsened by IL17, which foster osteoclast formation through induction of RANKL. Tγδ17 cells are involved in bone resorption and enhance joint inflammation. **(E)** Human Tγδ17 cells support MDSC migration, survival, and promote their suppressive functions through IL17, GMCSF, and IL8. MDSCs also form feedback loop and promote Tγδ17 differentiation through IL23 and IL1β. **(F)** Tγδ17 cells secrete IL17 and induce tumorigenesis by their proangiogenic activity. **(G)** Murine Tγδ17 cells recruit small peritoneal macrophages to the tumor bed, which induce angiogenesis.

interaction and combination of multiple cytokines and chemokine receptors that regulate their distribution. This suggests that the functional determination of γδT cell subtypes is dictated by the local environment (thymus or peripheral blood or the inflamed tissue) in which they are present. Tγδ17 is a special γδT cell subset, distinctly present at early immune response in the tissue and can modulate the functions of other immune and epithelial cells but their relevance in disease outcome remains controversial. In response to microbial antigens, Tγδ17 cells promote infiltration of neutrophils and macrophages and induce production of antimicrobial peptides resulting in clearance of microbial load. Such protective behavior of Tγδ17 cells in infections can be exploited to develop newer approaches to tackle the microbial pathology (**Figure 2**).

The opposite side of Tγδ17 functions has revealed its detrimental role in enhancing inflammation in autoimmunity and cancer (**Figure 2**). The mechanism, which regulates such dual personality of Tγδ17 cells is unknown. It appears that the obvious common role executed by these cells is enhancement of inflammation but due to functional heterogeneity and their complex interdependency on other cells (innate and adaptive); the emerging scenario of their biology is far from complete. This provokes us to consider contextual behavior of Tγδ17 cells in disease pathology. Current progress in understanding the significance of Tγδ17 cells in inflammatory diseases has revealed their novel but debilitating functions such as suppression of Tregs in autoimmunity, induction of angiogenesis, and recruitment and activation of MDSCs in various malignancies. Thus, in inflammatory disorders, Tγδ17 cells can be targeted using various immunotherapeutic approaches. However, need of hour is to expand the understandings of Tγδ17 in humans and develop a protocol for their propagation and activation. The future therapies will rely on regulating the key transcription factor RORγt by designing suitable antagonists that will help in fine tuning Tγδ17 differentiation and eventually their function in chronic inflammation and infection.

ACKNOWLEDGMENTS

Department of Atomic Energy-Tata Memorial Centre (DAE-TMC), Council for Scientific and Industrial research (CSIR), and University Grants Commission (UGC) for providing the fellowships to Rushikesh S. Patil, Asif A. Dar, and Sajad A. Bhat, respectively. This work is supported by Department of Biotechnology Centre of Excellence grant, Govt. of India (DBT-COE) to Shubhada V. Chiplunkar (Grant No: BT/01/CEIB/09/V/06).

REFERENCES

1. Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature* (1984) **309**(5971):757–62. doi:10.1038/309757a0
2. Qin G, Liu Y, Zheng J, Xiang Z, Ng IH, Malik Peiris JS, et al. Phenotypic and functional characterization of human gammadelta T-cell subsets in response to influenza A viruses. *J Infect Dis* (2012) **205**(11):1646–53. doi:10.1093/infdis/jis253
3. Zheng J, Liu Y, Lau YL, Tu W. Gammadelta-T cells: an unpolished sword in human anti-infection immunity. *Cell Mol Immunol* (2013) **10**(1):50–7. doi:10.1038/cmi.2012.43
4. Hannani D, Ma Y, Yamazaki T, Dechanet-Merville J, Kroemer G, Zitvogel L. Harnessing gammadelta T cells in anticancer immunotherapy. *Trends Immunol* (2012) **33**(5):199–206. doi:10.1016/j.it.2012.01.006
5. Caccamo N, Todaro M, Sireci G, Meraviglia S, Stassi G, Dieli F. Mechanisms underlying lineage commitment and plasticity of human gammadelta T cells. *Cell Mol Immunol* (2013) **10**(1):30–4. doi:10.1038/cmi.2012.42
6. Peng MY, Wang ZH, Yao CY, Jiang LN, Jin QL, Wang J, et al. Interleukin 17-producing gamma delta T cells increased in patients with active pulmonary tuberculosis. *Cell Mol Immunol* (2008) **5**(3):203–8. doi:10.1038/cmi.2008.25
7. Cai Y, Shen X, Ding C, Qi C, Li K, Li X, et al. Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity* (2011) **35**(4):596–610. doi:10.1016/j.immuni.2011.08.001
8. Wakita D, Sumida K, Iwakura Y, Nishikawa H, Ohkuri T, Chamoto K, et al. Tumor-infiltrating IL-17-producing gammadelta T cells support the progression of tumor by promoting angiogenesis. *Eur J Immunol* (2010) **40**(7):1927–37. doi:10.1002/eji.200940157
9. Shibata K, Yamada H, Hara H, Kishihara K, Yoshikai Y. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after *Escherichia coli* infection via IL-17 production. *J Immunol* (2007) **178**(7):4466–72. doi:10.4049/jimmunol.178.7.4466
10. Kuang DM, Zhao Q, Wu Y, Peng C, Wang J, Xu Z, et al. Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. *J Hepatol* (2011) **54**(5):948–55. doi:10.1016/j.jhep.2010.08.041
11. Dar AA, Patil RS, Chiplunkar SV. Insights into the relationship between toll like receptors and gamma delta T cell responses. *Front Immunol* (2014) **5**:366. doi:10.3389/fimmu.2014.00366
12. Morita CT, Jin C, Sarikonda G, Wang H. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. *Immunol Rev* (2007) **215**:59–76. doi:10.1111/j.1600-065X.2006.00479.x
13. Born WK, Kemal Aydintug M, O'Brien RL. Diversity of gammadelta T-cell antigens. *Cell Mol Immunol* (2013) **10**(1):13–20. doi:10.1038/cmi.2012.45
14. Li H, Lebedeva MI, Llera AS, Fields BA, Brenner MB, Mariuzza RA. Structure of the Vdelta domain of a human gammadelta T-cell antigen receptor. *Nature* (1998) **391**(6666):502–6. doi:10.1038/35172
15. Wang H, Fang Z, Morita CT. Vgamma2Vdelta2 T cell receptor recognition of prenyl pyrophosphates is dependent on all CDRs. *J Immunol* (2010) **184**(11):6209–22. doi:10.4049/jimmunol.1000231
16. Buhaescu I, Izzedine H. Mevalonate pathway: a review of clinical and therapeutical implications. *Clin Biochem* (2007) **40**(9–10):575–84. doi:10.1016/j.clinbiochem.2007.03.016
17. Hunter WN. The non-mevalonate pathway of isoprenoid precursor biosynthesis. *J Biol Chem* (2007) **282**(30):21573–7. doi:10.1074/jbc.R700005200
18. Hintz M, Reichenberg A, Altincicek B, Bahr U, Gschwind RM, Kollas AK, et al. Identification of (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human gammadelta T cells in *Escherichia coli*. *FEBS Lett* (2001) **509**(2):317–22. doi:10.1016/S0014-5793(01)03191-X
19. Zeng X, Wei YL, Huang J, Newell EW, Yu H, Kidd BA, et al. gammadelta T cells recognize a microbial encoded B cell antigen to initiate a rapid antigen-specific interleukin-17 response. *Immunity* (2012) **37**(3):524–34. doi:10.1016/j.immuni.2012.06.011
20. Bukowski JF, Morita CT, Brenner MB. Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. *Immunity* (1999) **11**(1):57–65. doi:10.1016/S1074-7613(00)80081-3
21. Shanoj N, Banerjee G, Shinkar VP, Chiplunkar SV. Gamma delta T cell stimulatory activity of tea and Indian herb extracts. *Int J Tea Sci* (2013) **9**(2/3):26–35.
22. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. *Science* (1998) **279**(5357):1737–40. doi:10.1126/science.279.5357.1737
23. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc Natl Acad Sci U S A* (1999) **96**(12):6879–84. doi:10.1073/pnas.96.12.6879
24. Das H, Groh V, Kuijl C, Sugita M, Morita CT, Spies T, et al. MICA engagement by human Vgamma2Vdelta2 T cells enhances their antigen-dependent effector function. *Immunity* (2001) **15**(1):83–93. doi:10.1016/S1074-7613(01)00168-6
25. Russano AM, Agea E, Corazzi L, Postle AD, De Libero G, Porcelli S, et al. Recognition of pollen-derived phosphatidyl-ethanolamine by human

CD1d-restricted gamma delta T cells. *J Allergy Clin Immunol* (2006) **117**(5):1178–84. doi:10.1016/j.jaci.2006.01.001
26. Dieude M, Striegl H, Tyznik AJ, Wang J, Behar SM, Piccirillo CA, et al. Cardiolipin binds to CD1d and stimulates CD1d-restricted gammadelta T cells in the normal murine repertoire. *J Immunol* (2011) **186**(8):4771–81. doi:10.4049/jimmunol.1000921
27. Spada FM, Grant EP, Peters PJ, Sugita M, Melian A, Leslie DS, et al. Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. *J Exp Med* (2000) **191**(6):937–48. doi:10.1084/jem.191.6.937
28. Adams EJ, Chien YH, Garcia KC. Structure of a gammadelta T cell receptor in complex with the nonclassical MHC T22. *Science* (2005) **308**(5719):227–31. doi:10.1126/science.1106885
29. Crowley MP, Reich Z, Mavaddat N, Altman JD, Chien Y. The recognition of the nonclassical major histocompatibility complex (MHC) class I molecule, T10, by the gammadelta T cell, G8. *J Exp Med* (1997) **185**(7):1223–30. doi:10.1084/jem.185.7.1223
30. Laad AD, Thomas ML, Fakih AR, Chiplunkar SV. Human gamma delta T cells recognize heat shock protein-60 on oral tumor cells. *Int J Cancer* (1999) **80**(5):709–14. doi:10.1002/(SICI)1097-0215(19990301)80:5<709::AID-IJC14>3.0.CO;2-R
31. Thomas ML, Samant UC, Deshpande RK, Chiplunkar SV. Gammadelta T cells lyse autologous and allogenic oesophageal tumours: involvement of heat-shock proteins in the tumour cell lysis. *Cancer Immunol Immunother* (2000) **48**(11):653–9. doi:10.1007/s002620050014
32. O'Brien RL, Fu YX, Cranfill R, Dallas A, Ellis C, Reardon C, et al. Heat shock protein Hsp60-reactive gamma delta cells: a large, diversified T-lymphocyte subset with highly focused specificity. *Proc Natl Acad Sci U S A* (1992) **89**(10):4348–52. doi:10.1073/pnas.89.10.4348
33. Born WK, Zhang L, Nakayama M, Jin N, Chain JL, Huang Y, et al. Peptide antigens for gamma/delta T cells. *Cell Mol Life Sci* (2011) **68**(14):2335–43. doi:10.1007/s00018-011-0697-3
34. Prinz I, Sansoni A, Kissenpfennig A, Ardouin L, Malissen M, Malissen B. Visualization of the earliest steps of gammadelta T cell development in the adult thymus. *Nat Immunol* (2006) **7**(9):995–1003. doi:10.1038/ni1371
35. McVay LD, Carding SR. Generation of human gammadelta T-cell repertoires. *Crit Rev Immunol* (1999) **19**(5–6):431–60.
36. Carding SR, Kyes S, Jenkinson EJ, Kingston R, Bottomly K, Owen JJ, et al. Developmentally regulated fetal thymic and extrathymic T-cell receptor gamma delta gene expression. *Genes Dev* (1990) **4**(8):1304–15. doi:10.1101/gad.4.8.1304
37. Velilla PA, Rugeles MT, Chougnet CA. Defective antigen-presenting cell function in human neonates. *Clin Immunol* (2006) **121**(3):251–9. doi:10.1016/j.clim.2006.08.010
38. Washburn T, Schweighoffer E, Gridley T, Chang D, Fowlkes BJ, Cado D, et al. Notch activity influences the alphabeta versus gammadelta T cell lineage decision. *Cell* (1997) **88**(6):833–43. doi:10.1016/S0092-8674(00)81929-7
39. Haks MC, Lefebvre JM, Lauritsen JP, Carleton M, Rhodes M, Miyazaki T, et al. Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage. *Immunity* (2005) **22**(5):595–606. doi:10.1016/j.immuni.2005.04.003
40. Hayes SM, Li L, Love PE. TCR signal strength influences alphabeta/gamma delta lineage fate. *Immunity* (2005) **22**(5):583–93. doi:10.1016/j.immuni.2005.03.014
41. Zarin P, Wong GW, Mohtashami M, Wiest DL, Zuniga-Pflucker JC. Enforcement of gammadelta-lineage commitment by the pre-T-cell receptor in precursors with weak gammadelta-TCR signals. *Proc Natl Acad Sci U S A* (2014) **111**(15):5658–63. doi:10.1073/pnas.1312872111
42. Hayday AC, Pennington DJ. Key factors in the organized chaos of early T cell development. *Nat Immunol* (2007) **8**(2):137–44. doi:10.1038/ni1436
43. Garcia-Peydro M, de Yebenes VG, Toribio ML. Sustained Notch1 signaling instructs the earliest human intrathymic precursors to adopt a gammadelta T-cell fate in fetal thymus organ culture. *Blood* (2003) **102**(7):2444–51. doi:10.1182/blood-2002-10-3261
44. Van de Walle I, Waegemans E, De Medts J, De Smet G, De Smedt M, Snauwaert S, et al. Specific Notch receptor-ligand interactions control human TCR-alphabeta/gammadelta development by inducing differential Notch signal strength. *J Exp Med* (2013) **210**(4):683–97. doi:10.1084/jem.20121798
45. Van Coppernolle S, Vanhee S, Verstichel G, Snauwaert S, van der Spek A, Velghe I, et al. Notch induces human T-cell receptor gammadelta+ thymocytes to differentiate along a parallel, highly proliferative and bipotent CD4 CD8 double-positive pathway. *Leukemia* (2012) **26**(1):127–38. doi:10.1038/leu.2011.324
46. Gogoi D, Dar AA, Chiplunkar SV. Involvement of Notch in activation and effector functions of gammadelta T cells. *J Immunol* (2014) **192**(5):2054–62. doi:10.4049/jimmunol.1300369
47. Korn T, Petermann F. Development and function of interleukin 17-producing gammadelta T cells. *Ann N Y Acad Sci* (2012) **1247**:34–45. doi:10.1111/j.1749-6632.2011.06355.x
48. Hayday A, Theodoridis E, Ramsburg E, Shires J. Intraepithelial lymphocytes: exploring the third way in immunology. *Nat Immunol* (2001) **2**(11):997–1003. doi:10.1038/ni1101-997
49. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol* (2010) **10**(7):467–78. doi:10.1038/nri2781
50. Bergstresser PR, Sullivan S, Streilein JW, Tigelaar RE. Origin and function of Thy-1+ dendritic epidermal cells in mice. *J Invest Dermatol* (1985) **85**:85s–90s. doi:10.1111/1523-1747.ep12275516
51. Itohara S, Nakanishi N, Kanagawa O, Kubo R, Tonegawa S. Monoclonal antibodies specific to native murine T-cell receptor gamma delta: analysis of gamma delta T cells during thymic ontogeny and in peripheral lymphoid organs. *Proc Natl Acad Sci U S A* (1989) **86**(13):5094–8. doi:10.1073/pnas.86.13.5094
52. Girardi M. Immunosurveillance and immunoregulation by gammadelta T cells. *J Invest Dermatol* (2006) **126**(1):25–31. doi:10.1038/sj.jid.5700003
53. Janeway CA Jr, Jones B, Hayday A. Specificity and function of T cells bearing gamma delta receptors. *Immunol Today* (1988) **9**(3):73–6. doi:10.1016/0167-5699(88)91267-4
54. Goodman T, Lefrancois L. Intraepithelial lymphocytes. Anatomical site, not T cell receptor form, dictates phenotype and function. *J Exp Med* (1989) **170**(5):1569–81. doi:10.1084/jem.170.5.1569
55. Vantourout P, Hayday A. Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat Rev Immunol* (2013) **13**(2):88–100. doi:10.1038/nri3384
56. Qin G, Mao H, Zheng J, Sia SF, Liu Y, Chan PL, et al. Phosphoantigen-expanded human gammadelta T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses. *J Infect Dis* (2009) **200**(6):858–65. doi:10.1086/605413
57. Dhar S, Chiplunkar SV. Lysis of aminobisphosphonate-sensitized MCF-7 breast tumor cells by Vgamma9Vdelta2 T cells. *Cancer Immun* (2010) **10**:10.
58. Zhang H, Hu H, Jiang X, He H, Cui L, He W. Membrane HSP70: the molecule triggering gammadelta T cells in the early stage of tumorigenesis. *Immunol Invest* (2005) **34**(4):453–68. doi:10.1080/08820130500265349
59. Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* (2000) **406**(6793):309–14. doi:10.1038/35018581
60. Conti L, Casetti R, Cardone M, Varano B, Martino A, Belardelli F, et al. Reciprocal activating interaction between dendritic cells and pamidronate-stimulated gammadelta T cells: role of CD86 and inflammatory cytokines. *J Immunol* (2005) **174**(1):252–60. doi:10.4049/jimmunol.174.1.252
61. Brandes M, Willimann K, Bioley G, Levy N, Eberl M, Luo M, et al. Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses. *Proc Natl Acad Sci U S A* (2009) **106**(7):2307–12. doi:10.1073/pnas.0810059106
62. Himoudi N, Morgenstern DA, Yan M, Vernay B, Saraiva L, Wu Y, et al. Human gammadelta T lymphocytes are licensed for professional antigen presentation by interaction with opsonized target cells. *J Immunol* (2012) **188**(4):1708–16. doi:10.4049/jimmunol.1102654
63. Brandes M, Willimann K, Moser B. Professional antigen-presentation function by human gammadelta T Cells. *Science* (2005) **309**(5732):264–8. doi:10.1126/science.1110267
64. Wesch D, Glatzel A, Kabelitz D. Differentiation of resting human peripheral blood gamma delta T cells toward Th1- or Th2-phenotype. *Cell Immunol* (2001) **212**(2):110–7. doi:10.1006/cimm.2001.1850
65. Ness-Schwickerath KJ, Jin C, Morita CT. Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells. *J Immunol* (2010) **184**(12):7268–80. doi:10.4049/jimmunol.1000600

66. Caccamo N, La Mendola C, Orlando V, Meraviglia S, Todaro M, Stassi G, et al. Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. *Blood* (2011) **118**(1):129–38. doi:10.1182/blood-2011-01-331298
67. Casetti R, Agrati C, Wallace M, Sacchi A, Martini F, Martino A, et al. Cutting edge: TGF-beta1 and IL-15 Induce FOXP3+ gammadelta regulatory T cells in the presence of antigen stimulation. *J Immunol* (2009) **183**(6):3574–7. doi:10.4049/jimmunol.0901334
68. Li X, Kang N, Zhang X, Dong X, Wei W, Cui L, et al. Generation of human regulatory gammadelta T cells by TCRgammadelta stimulation in the presence of TGF-beta and their involvement in the pathogenesis of systemic lupus erythematosus. *J Immunol* (2011) **186**(12):6693–700. doi:10.4049/jimmunol.1002776
69. Keijsers RR, Joosten I, van Erp PE, Koenen HJ, van de Kerkhof PC. Cellular sources of il-17: a paradigm shift? *Exp Dermatol* (2014) **23**(11):799–803. doi:10.1111/exd.12487
70. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* (2009) **31**(2):321–30. doi:10.1016/j.immuni.2009.06.020
71. Itoh S, Kimura N, Axtell RC, Velotta JB, Gong Y, Wang X, et al. Interleukin-17 accelerates allograft rejection by suppressing regulatory T cell expansion. *Circulation* (2011) **124**(11 Suppl):S187–96. doi:10.1161/CIRCULATIONAHA.110.014852
72. Petermann F, Rothhammer V, Claussen MC, Haas JD, Blanco LR, Heink S, et al. gammadelta T cells enhance autoimmunity by restraining regulatory T cell responses via an interleukin-23-dependent mechanism. *Immunity* (2010) **33**(3):351–63. doi:10.1016/j.immuni.2010.08.013
73. Lo Re S, Dumoutier L, Couillin I, Van Vyve C, Yakoub Y, Uwambayinema F, et al. IL-17A-producing gammadelta T and Th17 lymphocytes mediate lung inflammation but not fibrosis in experimental silicosis. *J Immunol* (2010) **184**(11):6367–77. doi:10.4049/jimmunol.0900459
74. Wu P, Wu D, Ni C, Ye J, Chen W, Hu G, et al. Gammadeltat17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. *Immunity* (2014) **40**(5):785–800. doi:10.1016/j.immuni.2014.03.013
75. Price AE, Reinhardt RL, Liang HE, Locksley RM. Marking and quantifying IL-17A-producing cells in vivo. *PLoS One* (2012) **7**(6):e39750. doi:10.1371/journal.pone.0039750
76. Chien YH, Zeng X, Prinz I. The natural and the inducible: interleukin (IL)-17-producing gammadelta T cells. *Trends Immunol* (2013) **34**(4):151–4. doi:10.1016/j.it.2012.11.004
77. Prinz I, Fohse L. Gammadelta T cells are not alone. *Immunol Cell Biol* (2012) **90**(4):370–1. doi:10.1038/icb.2011.61
78. Shibata K, Yamada H, Nakamura M, Hatano S, Katsuragi Y, Kominami R, et al. IFN-gamma-producing and IL-17-producing gammadelta T cells differentiate at distinct developmental stages in murine fetal thymus. *J Immunol* (2014) **192**(5):2210–8. doi:10.4049/jimmunol.1302145
79. Pennington DJ, Silva-Santos B, Hayday AC. Gammadelta T cell development – having the strength to get there. *Curr Opin Immunol* (2005) **17**(2):108–15. doi:10.1016/j.coi.2005.01.009
80. Turchinovich G, Pennington DJ. T cell receptor signalling in gammadelta cell development: strength isn't everything. *Trends Immunol* (2011) **32**(12):567–73. doi:10.1016/j.it.2011.09.005
81. Prinz I, Silva-Santos B, Pennington DJ. Functional development of gammadelta T cells. *Eur J Immunol* (2013) **43**(8):1988–94. doi:10.1002/eji.201343759
82. Ramond C, Berthault C, Burlen-Defranoux O, de Sousa AP, Guy-Grand D, Vieira P, et al. Two waves of distinct hematopoietic progenitor cells colonize the fetal thymus. *Nat Immunol* (2014) **15**(1):27–35. doi:10.1038/ni.2782
83. Vicari AP, Mocci S, Openshaw P, O'Garra A, Zlotnik A. Mouse gamma delta TCR+NK1.1+ thymocytes specifically produce interleukin-4, are major histocompatibility complex class I independent, and are developmentally related to alpha beta TCR+NK1.1+ thymocytes. *Eur J Immunol* (1996) **26**(7):1424–9. doi:10.1002/eji.1830260704
84. Azuara V, Levraud JP, Lembezat MP, Pereira P. A novel subset of adult gamma delta thymocytes that secretes a distinct pattern of cytokines and expresses a very restricted T cell receptor repertoire. *Eur J Immunol* (1997) **27**(2):544–53. doi:10.1002/eji.1830270228
85. Haas JD, Ravens S, Duber S, Sandrock I, Oberdorfer L, Kashani E, et al. Development of interleukin-17-producing gammadelta T cells is restricted to a functional embryonic wave. *Immunity* (2012) **37**(1):48–59. doi:10.1016/j.immuni.2012.06.003
86. Jensen KD, Su X, Shin S, Li L, Youssef S, Yamasaki S, et al. Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma. *Immunity* (2008) **29**(1):90–100. doi:10.1016/j.immuni.2008.04.022
87. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* (1999) **99**(1):23–33. doi:10.1016/S0092-8674(00)80059-8
88. Ribot JC, Silva-Santos B. Differentiation and activation of gammadelta T Lymphocytes: focus on CD27 and CD28 costimulatory receptors. *Adv Exp Med Biol* (2013) **785**:95–105. doi:10.1007/978-1-4614-6217-0_11
89. Ribot JC, deBarros A, Pang DJ, Neves JF, Peperzak V, Roberts SJ, et al. CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. *Nat Immunol* (2009) **10**(4):427–36. doi:10.1038/ni.1717
90. Muller JR, Siebenlist U. Lymphotoxin beta receptor induces sequential activation of distinct NF-kappa B factors via separate signaling pathways. *J Biol Chem* (2003) **278**(14):12006–12. doi:10.1074/jbc.M210768200
91. Silva-Santos B, Pennington DJ, Hayday AC. Lymphotoxin-mediated regulation of gammadelta cell differentiation by alphabeta T cell progenitors. *Science* (2005) **307**(5711):925–8. doi:10.1126/science.1103978
92. Hayes SM, Laird RM. Genetic requirements for the development and differentiation of interleukin-17-producing gammadelta T cells. *Crit Rev Immunol* (2012) **32**(1):81–95. doi:10.1615/CritRevImmunol.v32.i1.50
93. Powolny-Budnicka I, Riemann M, Tanzer S, Schmid RM, Hehlgans T, Weih F. RelA and RelB transcription factors in distinct thymocyte populations control lymphotoxin-dependent interleukin-17 production in gammadelta T cells. *Immunity* (2011) **34**(3):364–74. doi:10.1016/j.immuni.2011.02.019
94. Wencker M, Turchinovich G, Di Marco Barros R, Deban L, Jandke A, Cope A, et al. Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor responsiveness. *Nat Immunol* (2014) **15**(1):80–7. doi:10.1038/ni.2773
95. Malhotra N, Narayan K, Cho OH, Sylvia KE, Yin C, Melichar H, et al. A network of high-mobility group box transcription factors programs innate interleukin-17 production. *Immunity* (2013) **38**(4):681–93. doi:10.1016/j.immuni.2013.01.010
96. Laird RM, Laky K, Hayes SM. Unexpected role for the B cell-specific Src family kinase B lymphoid kinase in the development of IL-17-producing gammadelta T cells. *J Immunol* (2010) **185**(11):6518–27. doi:10.4049/jimmunol.1002766
97. Gray EE, Ramirez-Valle F, Xu Y, Wu S, Wu Z, Karjalainen KE, et al. Deficiency in IL-17-committed Vgamma4(+) gammadelta T cells in a spontaneous Sox13-mutant CD45.1(+) congenic mouse substrain provides protection from dermatitis. *Nat Immunol* (2013) **14**(6):584–92. doi:10.1038/ni.2585
98. Narayan K, Sylvia KE, Malhotra N, Yin CC, Martens G, Vallerskog T, et al. Intrathymic programming of effector fates in three molecularly distinct gammadelta T cell subtypes. *Nat Immunol* (2012) **13**(5):511–8. doi:10.1038/ni.2247
99. Turchinovich G, Hayday AC. Skint-1 identifies a common molecular mechanism for the development of interferon-gamma-secreting versus interleukin-17-secreting gammadelta T cells. *Immunity* (2011) **35**(1):59–68. doi:10.1016/j.immuni.2011.04.018
100. Maekawa Y, Tsukumo S, Chiba S, Hirai H, Hayashi Y, Okada H, et al. Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. *Immunity* (2003) **19**(4):549–59. doi:10.1016/S1074-7613(03)00270-X
101. Shibata K, Yamada H, Sato T, Dejima T, Nakamura M, Ikawa T, et al. Notch-Hes1 pathway is required for the development of IL-17-producing gammadelta T cells. *Blood* (2011) **118**(3):586–93. doi:10.1182/blood-2011-02-334995
102. Keerthivasan S, Suleiman R, Lawlor R, Roderick J, Bates T, Minter L, et al. Notch signaling regulates mouse and human Th17 differentiation. *J Immunol* (2011) **187**(2):692–701. doi:10.4049/jimmunol.1003658
103. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KH. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* (2009) **31**(2):331–41. doi:10.1016/j.immuni.2009.08.001
104. Do JS, Fink PJ, Li L, Spolski R, Robinson J, Leonard WJ, et al. Cutting edge: spontaneous development of IL-17-producing gamma delta T cells in the

thymus occurs via a TGF-beta 1-dependent mechanism. *J Immunol* (2010) **184**(4):1675–9. doi:10.4049/jimmunol.0903539
105. Lochner M, Peduto L, Cherrier M, Sawa S, Langa F, Varona R, et al. In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. *J Exp Med* (2008) **205**(6):1381–93. doi:10.1084/jem.20080034
106. Suzuki M, Yamamoto M, Sugimoto A, Nakamura S, Motoda R, Orita K. Delta-4 expression on a stromal cell line is augmented by interleukin-6 via STAT3 activation. *Exp Hematol* (2006) **34**(9):1143–50. doi:10.1016/j.exphem.2006.04.027
107. Meeks KD, Sieve AN, Kolls JK, Ghilardi N, Berg RE. IL-23 is required for protection against systemic infection with *Listeria* monocytogenes. *J Immunol* (2009) **183**(12):8026–34. doi:10.4049/jimmunol.0901588
108. Riol-Blanco L, Lazarevic V, Awasthi A, Mitsdoerffer M, Wilson BS, Croxford A, et al. IL-23 receptor regulates unconventional IL-17-producing T cells that control bacterial infections. *J Immunol* (2010) **184**(4):1710–20. doi:10.4049/jimmunol.0902796
109. McGeachy MJ, Chen Y, Tato CM, Laurence A, Joyce-Shaikh B, Blumenschein WM, et al. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol* (2009) **10**(3):314–24. doi:10.1038/ni.1698
110. Doisne JM, Soulard V, Becourt C, Amniai L, Henrot P, Havenar-Daughton C, et al. Cutting edge: crucial role of IL-1 and IL-23 in the innate IL-17 response of peripheral lymph node NK1.1- invariant NKT cells to bacteria. *J Immunol* (2011) **186**(2):662–6. doi:10.4049/jimmunol.1002725
111. DeBarros A, Chaves-Ferreira M, d'Orey F, Ribot JC, Silva-Santos B. CD70-CD27 interactions provide survival and proliferative signals that regulate T cell receptor-driven activation of human gammadelta peripheral blood lymphocytes. *Eur J Immunol* (2011) **41**(1):195–201. doi:10.1002/eji.201040905
112. Moens E, Brouwer M, Dimova T, Goldman M, Willems F, Vermijlen D. IL-23R and TCR signaling drives the generation of neonatal Vgamma9Vdelta2 T cells expressing high levels of cytotoxic mediators and producing IFN-gamma and IL-17. *J Leukoc Biol* (2011) **89**(5):743–52. doi:10.1189/jlb.0910501
113. Michel ML, Pang DJ, Haque SF, Potocnik AJ, Pennington DJ, Hayday AC. Interleukin 7 (IL-7) selectively promotes mouse and human IL-17-producing gammadelta cells. *Proc Natl Acad Sci U S A* (2012) **109**(43):17549–54. doi:10.1073/pnas.1204327109
114. Ribot JC, Ribeiro ST, Correia DV, Sousa AE, Silva-Santos B. Human gammadelta thymocytes are functionally immature and differentiate into cytotoxic type 1 effector T cells upon IL-2/IL-15 signaling. *J Immunol* (2014) **192**(5):2237–43. doi:10.4049/jimmunol.1303119
115. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* (2004) **22**:745–63. doi:10.1146/annurev.immunol.22.012703.104702
116. Roark CL, Simonian PL, Fontenot AP, Born WK, O'Brien RL. Gammadelta T cells: an important source of IL-17. *Curr Opin Immunol* (2008) **20**(3):353–7. doi:10.1016/j.coi.2008.03.006
117. Sabat R, Witte E, Witte K, Wolk K. IL-22 and IL-17: an overview. In: Quesniaux V, Ryffel B, Padova F, editors. *IL-17, IL-22 and Their Producing Cells: Role in Inflammation and Autoimmunity. Progress in Inflammation Research*. Basel: Springer (2013). p. 11–35.
118. Eyerich S, Eyerich K, Cavani A, Schmidt-Weber C. IL-17 and IL-22: siblings, not twins. *Trends Immunol* (2010) **31**(9):354–61. doi:10.1016/j.it.2010.06.004
119. Okamoto Yoshida Y, Umemura M, Yahagi A, O'Brien RL, Ikuta K, Kishihara K, et al. Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. *J Immunol* (2010) **184**(8):4414–22. doi:10.4049/jimmunol.0903332
120. Lockhart E, Green AM, Flynn JL. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during *Mycobacterium tuberculosis* infection. *J Immunol* (2006) **177**(7):4662–9. doi:10.4049/jimmunol.177.7.4662
121. Curtis MM, Way SS. Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. *Immunology* (2009) **126**(2):177–85. doi:10.1111/j.1365-2567.2008.03017.x
122. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, et al. IL-17-mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium* bovis *bacille* Calmette-Guerin infection. *J Immunol* (2007) **178**(6):3786–96. doi:10.4049/jimmunol.178.6.3786
123. Xu S, Han Y, Xu X, Bao Y, Zhang M, Cao X. IL-17A-producing gammadeltaT cells promote CTL responses against *Listeria* monocytogenes infection by enhancing dendritic cell cross-presentation. *J Immunol* (2010) **185**(10):5879–87. doi:10.4049/jimmunol.1001763
124. Happel KI, Dubin PJ, Zheng M, Ghilardi N, Lockhart C, Quinton LJ, et al. Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*. *J Exp Med* (2005) **202**(6):761–9. doi:10.1084/jem.20050193
125. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* (2006) **441**(7090):231–4. doi:10.1038/nature04754
126. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med* (2008) **14**(3):282–9. doi:10.1038/nm1720
127. Schulz SM, Kohler G, Schutze N, Knauer J, Straubinger RK, Chackerian AA, et al. Protective immunity to systemic infection with attenuated *Salmonella enterica* serovar enteritidis in the absence of IL-12 is associated with IL-23-dependent IL-22, but not IL-17. *J Immunol* (2008) **181**(11):7891–901. doi:10.4049/jimmunol.181.11.7891
128. Godinez I, Raffatellu M, Chu H, Paixao TA, Haneda T, Santos RL, et al. Interleukin-23 orchestrates mucosal responses to *Salmonella enterica* serotype *Typhimurium* in the intestine. *Infect Immun* (2009) **77**(1):387–98. doi:10.1128/IAI.00933-08
129. Kelly MN, Kolls JK, Happel K, Schwartzman JD, Schwarzenberger P, Combe C, et al. Interleukin-17/interleukin-17 receptor-mediated signaling is important for generation of an optimal polymorphonuclear response against *Toxoplasma gondii* infection. *Infect Immun* (2005) **73**(1):617–21. doi:10.1128/IAI.73.1.617-621.2005
130. Dejima T, Shibata K, Yamada H, Hara H, Iwakura Y, Naito S, et al. Protective role of naturally occurring interleukin-17A-producing gammadelta T cells in the lung at the early stage of systemic candidiasis in mice. *Infect Immun* (2011) **79**(11):4503–10. doi:10.1128/IAI.05799-11
131. Wozniak KL, Kolls JK, Wormley FL Jr. Depletion of neutrophils in a protective model of pulmonary cryptococcosis results in increased IL-17A production by gammadelta T cells. *BMC Immunol* (2012) **13**:65. doi:10.1186/1471-2172-13-65
132. Ribot JC, Chaves-Ferreira M, d'Orey F, Wencker M, Goncalves-Sousa N, Decalf J, et al. Cutting edge: adaptive versus innate receptor signals selectively control the pool sizes of murine IFN-gamma- or IL-17-producing gammadelta T cells upon infection. *J Immunol* (2010) **185**(11):6421–5. doi:10.4049/jimmunol.1002283
133. Wang X, Sun R, Wei H, Tian Z. High-mobility group box 1 (HMGB1)-Toll-like receptor (TLR)4-interleukin (IL)-23-IL-17A axis in drug-induced damage-associated lethal hepatitis: interaction of gammadelta T cells with macrophages. *Hepatology* (2013) **57**(1):373–84. doi:10.1002/hep.25982
134. Hasegawa E, Sonoda KH, Shichita T, Morita R, Sekiya T, Kimura A, et al. IL-23-independent induction of IL-17 from gammadeltaT cells and innate lymphoid cells promotes experimental intraocular neovascularization. *J Immunol* (2013) **190**(4):1778–87. doi:10.4049/jimmunol.1202495
135. Xia Q, Duan L, Shi L, Zheng F, Gong F, Fang M. High-mobility group box 1 accelerates early acute allograft rejection via enhancing IL-17+ gammadelta T-cell response. *Transpl Int* (2014) **27**(4):399–407. doi:10.1111/tri.12264
136. Cho JS, Pietras EM, Garcia NC, Ramos RI, Farzam DM, Monroe HR, et al. IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. *J Clin Invest* (2010) **120**(5):1762–73. doi:10.1172/JCI40891
137. Hirota K, Yoshitomi H, Hashimoto M, Maeda S, Teradaira S, Sugimoto N, et al. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J Exp Med* (2007) **204**(12):2803–12. doi:10.1084/jem.20071397
138. Haas JD, Gonzalez FH, Schmitz S, Chennupati V, Fohse L, Kremmer E, et al. CCR6 and NK1.1 distinguish between IL-17A and IFN-gamma-producing gammadelta effector T cells. *Eur J Immunol* (2009) **39**(12):3488–97. doi:10.1002/eji.200939922
139. Costa MF, Bornstein VU, Candea AL, Henriques-Pons A, Henriques MG, Penido C. CCL25 induces alpha(4)beta(7) integrin-dependent migration of IL-17(+) gammadelta T lymphocytes during an allergic reaction. *Eur J Immunol* (2012) **42**(5):1250–60. doi:10.1002/eji.201142021
140. Penido C, Costa MF, Souza MC, Costa KA, Candea AL, Benjamim CF, et al. Involvement of CC chemokines in gammadelta T lymphocyte trafficking

during allergic inflammation: the role of CCL2/CCR2 pathway. *Int Immunol* (2008) **20**(1):129–39. doi:10.1093/intimm/dxm128
141. Kabelitz D, Wesch D. Features and functions of gamma delta T lymphocytes: focus on chemokines and their receptors. *Crit Rev Immunol* (2003) **23**(5–6):339–70. doi:10.1615/CritRevImmunol.v23.i56.10
142. Simonian PL, Wehrmann F, Roark CL, Born WK, O'Brien RL, Fontenot AP. Gammadelta T cells protect against lung fibrosis via IL-22. *J Exp Med* (2010) **207**(10):2239–53. doi:10.1084/jem.20100061
143. Roark CL, French JD, Taylor MA, Bendele AM, Born WK, O'Brien RL. Exacerbation of collagen-induced arthritis by oligoclonal, IL-17-producing gamma delta T cells. *J Immunol* (2007) **179**(8):5576–83. doi:10.4049/jimmunol.179.8.5576
144. Pollinger B, Junt T, Metzler B, Walker UA, Tyndall A, Allard C, et al. Th17 cells, not IL-17+ gammadelta T cells, drive arthritic bone destruction in mice and humans. *J Immunol* (2011) **186**(4):2602–12. doi:10.4049/jimmunol.1003370
145. Ito Y, Usui T, Kobayashi S, Iguchi-Hashimoto M, Ito H, Yoshitomi H, et al. Gamma/delta T cells are the predominant source of interleukin-17 in affected joints in collagen-induced arthritis, but not in rheumatoid arthritis. *Arthritis Rheum* (2009) **60**(8):2294–303. doi:10.1002/art.24687
146. Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, et al. Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. *Nat Med* (2009) **15**(8):946–50. doi:10.1038/nm.1999
147. Jaffar Z, Ferrini ME, Shaw PK, FitzGerald GA, Roberts K. Prostaglandin I(2) promotes the development of IL-17-producing gammadelta T cells that associate with the epithelium during allergic lung inflammation. *J Immunol* (2011) **187**(10):5380–91. doi:10.4049/jimmunol.1101261
148. Murdoch JR, Lloyd CM. Resolution of allergic airway inflammation and airway hyperreactivity is mediated by IL-17-producing {gamma}{delta}T cells. *Am J Respir Crit Care Med* (2010) **182**(4):464–76. doi:10.1164/rccm.200911-1775OC
149. Gogoi D, Chiplunkar SV. Targeting gamma delta T cells for cancer immunotherapy: bench to bedside. *Indian J Med Res* (2013) **138**(5):755–61.
150. von Lilienfeld-Toal M, Nattermann J, Feldmann G, Sievers E, Frank S, Strehl J, et al. Activated gammadelta T cells express the natural cytotoxicity receptor natural killer p 44 and show cytotoxic activity against myeloma cells. *Clin Exp Immunol* (2006) **144**(3):528–33. doi:10.1111/j.1365-2249.2006.03078.x
151. Zhou J, Kang N, Cui L, Ba D, He W. Anti-gammadelta TCR antibody-expanded gammadelta T cells: a better choice for the adoptive immunotherapy of lymphoid malignancies. *Cell Mol Immunol* (2012) **9**(1):34–44. doi:10.1038/cmi.2011.16
152. Lanca T, Correia DV, Moita CF, Raquel H, Neves-Costa A, Ferreira C, et al. The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to gammadelta T-cell cytotoxicity. *Blood* (2010) **115**(12):2407–11. doi:10.1182/blood-2009-08-237123
153. Siegers GM, Felizardo TC, Mathieson AM, Kosaka Y, Wang XH, Medin JA, et al. Anti-leukemia activity of in vitro-expanded human gamma delta T cells in a xenogeneic Ph+ leukemia model. *PLoS One* (2011) **6**(2):e16700. doi:10.1371/journal.pone.0016700
154. Kong Y, Cao W, Xi X, Ma C, Cui L, He W. The NKG2D ligand ULBP4 binds to TCRgamma9/delta2 and induces cytotoxicity to tumor cells through both TCRgammadelta and NKG2D. *Blood* (2009) **114**(2):310–7. doi:10.1182/blood-2008-12-196287
155. Viey E, Fromont G, Escudier B, Morel Y, Da Rocha S, Chouaib S, et al. Phosphostim-activated gamma delta T cells kill autologous metastatic renal cell carcinoma. *J Immunol* (2005) **174**(3):1338–47. doi:10.4049/jimmunol.174.3.1338
156. Viey E, Lucas C, Romagne F, Escudier B, Chouaib S, Caignard A. Chemokine receptors expression and migration potential of tumor-infiltrating and peripheral-expanded Vgamma9Vdelta2 T cells from renal cell carcinoma patients. *J Immunother* (2008) **31**(3):313–23. doi:10.1097/CJI.0b013e3181609988
157. Fournie JJ, Sicard H, Poupot M, Bezombes C, Blanc A, Romagne F, et al. What lessons can be learned from gammadelta T cell-based cancer immunotherapy trials? *Cell Mol Immunol* (2013) **10**(1):35–41. doi:10.1038/cmi.2012.39
158. Ma Y, Aymeric L, Locher C, Mattarollo SR, Delahaye NF, Pereira P, et al. Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy. *J Exp Med* (2011) **208**(3):491–503. doi:10.1084/jem.20100269
159. Takeuchi A, Dejima T, Yamada H, Shibata K, Nakamura R, Eto M, et al. IL-17 production by gammadelta T cells is important for the antitumor effect of *Mycobacterium* bovis *bacillus* Calmette-Guerin treatment against bladder cancer. *Eur J Immunol* (2011) **41**(1):246–51. doi:10.1002/eji.201040773
160. Yang B, Kang H, Fung A, Zhao H, Wang T, Ma D. The role of interleukin 17 in tumour proliferation, angiogenesis, and metastasis. *Mediators Inflamm* (2014) **2014**:623759. doi:10.1155/2014/623759
161. Rei M, Goncalves-Sousa N, Lanca T, Thompson RG, Mensurado S, Balkwill FR, et al. Murine CD27(-) Vgamma6(+) gammadelta T cells producing IL-17A promote ovarian cancer growth via mobilization of protumor small peritoneal macrophages. *Proc Natl Acad Sci U S A* (2014) **111**(34):E3562–70. doi:10.1073/pnas.1403424111
162. Ma S, Cheng Q, Cai Y, Gong H, Wu Y, Yu X, et al. IL-17A produced by gammadelta T cells promotes tumor growth in hepatocellular carcinoma. *Cancer Res* (2014) **74**(7):1969–82. doi:10.1158/0008-5472.CAN-13-2534
163. Fialova A, Partlova S, Sojka L, Hromadkova H, Brtnicky T, Fucikova J, et al. Dynamics of T-cell infiltration during the course of ovarian cancer: the gradual shift from a Th17 effector cell response to a predominant infiltration by regulatory T-cells. *Int J Cancer* (2013) **132**(5):1070–9. doi:10.1002/ijc.27759

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 October 2014; accepted: 20 January 2015; published online: 04 February 2015.
*Citation: Patil RS, Bhat SA, Dar AA and Chiplunkar SV (2015) The Jekyll and Hyde story of IL17-producing γδT cells. Front. Immunol. **6**:37. doi: 10.3389/fimmu.2015.00037*
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 31 July 2014
doi: 10.3389/fimmu.2014.00366

Insights into the relationship between toll like receptors and gamma delta T cell responses

Asif Amin Dar, Rushikesh Sudam Patil and Shubhada Vivek Chiplunkar*

Chiplunkar Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Navi Mumbai, India

Edited by:
Anton G. Kutikhin, Russian Academy of Medical Sciences, Russia

Reviewed by:
Isaias Glezer, Universidade Federal de São Paulo, Brazil
Rajesh Kumar Sharma, University of Louisville, USA

Correspondence:
Shubhada Vivek Chiplunkar, Chiplunkar Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Sector 22 Kharghar, Navi Mumbai, Maharashtra 410210, India
e-mail: schiplunkar@actrec.gov.in

The tumor microenvironment is an important aspect of cancer biology that contributes to tumor initiation, tumor progression and responses to therapy. The composition and characteristics of the tumor microenvironment vary widely and are important in determining the anti-tumor immune response. Successful immunization requires activation of both innate and adaptive immunity. Generally, immune system is compromised in patients with cancer due to immune suppression, loss of tumor antigen expression and dysfunction of antigen presenting cells (APC). Thus, therapeutic immunization leading to cancer regression remains a significant challenge. Certain cells of the immune system, including dendritic cells (DCs) and gamma delta (γδ) T cells are capable of driving potent anti-tumor responses. The property of MHC-unrestricted cytotoxicity, high potential of cytokine release, tissue tropism and early activation in infections and malignant disease makes γδ T cells as an emerging candidate for immunotherapy. Various strategies are being developed to enhance anti-tumor immune responses of γδ T cells and DCs one of them is the use of novel adjuvants like toll like receptors (TLR) agonists, which enhance γδ T cell function directly or through DC activation, which has ability to prime γδ T cells. TLR agonists are being used clinically either alone or in combination with tumor antigens and has shown initial success in both enhancing immune responses and eliciting anti-tumor activity. TLR activated γδ T cells and DCs nurture each other's activation. This provides a potent base for first line of defense and manipulation of the adaptive response against pathogens and cancer. The available data provides a strong rationale for initiating combinatorial therapy for the treatment of diseases and this review will summarize the application of adjuvants (TLRs) for boosting immune response of γδ T cells to treat cancer and infectious diseases and their use in combinatorial therapy.

Keywords: immunotherapy, γδ T cells, toll like receptors, tumors, dendritic cells

INTRODUCTION

Innate and adaptive immune responses are sentinels of host against the diverse repertoire of infectious agents (viruses and bacteria) and cancer. Both components of immune system identify invading microorganisms or damaged tissues as non-self and activate immune responses to eliminate them. Efficient immune responses depend upon how close an interaction is between the innate and adaptive immune system. γδ T cells and toll like receptors (TLR) serve as an important link between the innate and adaptive immune responses (1–3). Extensive studies have suggested that γδ T cells play important roles in host defense against microbial infections, tumorigenesis, immunoregulation and development of autoimmunity. γδ T cells also have several innate cell-like characters that allow their early and rapid activation following recognition of cellular stress and infection (4, 5). However to accomplish these functions, γδ T cells use both the T cell receptor (TCR) and additional activating receptors (notably NKG2D, NOTCH, and TLR) to respond to stress-induced ligands and infection. γδ T cells express TLRs and modulate early immune responses against different pathogens (6). In this review, we summarize and discuss some of the recent advances of the γδ T cell biology and how direct control of γδ T lymphocyte function and activation is monitored by TLR receptors and ligands. The review highlights involvement of TLR signaling in γδ T cell functions and their implications in harnessing γδ T cells for cancer immunotherapy.

γδ T CELLS, ANATOMICAL DISTRIBUTION AND ANTIGENIC DIVERSITY

Based on the type of TCR they express, T lymphocytes can be divided into two major subsets, αβ and γδ T cells. γδ T cell represents a small subset of T lymphocytes (1–10%) in peripheral blood. While in anatomical locations like small intestine, γδ T cells comprise a major bulk of T cells (25–60% in human gut) (7). γδ T cells are the first T cells to appear in thymus during T cell ontogeny in every vertebrate (8), which suggests that their primary contribution could be neonatal protection because at this point conventional αβ T cell responses are severely functionally impaired and DCs are immature (9). In neonates, the $V\delta2^{+}$ cells derived from human cord blood showed early signs of activation. These cells secrete IFN-γ and express perforin after short-term *in vitro* stimulation (10). In comparison to the neonate derived αβ T cells of peripheral blood, γδ T cell subset produces copious amount of IFN-γ and are precociously active (11). Hence, γδ

T cells are well engaged in newborns to contribute to immune-protection, immune-regulation and compensate for impaired αβ T cell compartment.

γδ T cells are unconventional $CD3^+$ T cells and differ from the conventional αβ T cells in their biology and function (**Table 1**). Although a sizeable fraction of γδ T cells in the intraepithelial lymphocyte compartments of human and mice are $CD8\alpha\alpha^+$ but the peripheral blood γδ T cells are predominantly double negative ($CD4^-CD8^-$) T cells. The absence of CD4 or CD8 expression on majority of the circulating γδ T cells is well in line with the fact that antigen recognition is not MHC restricted (12, 13). Crystal structure analysis of the γδ TCR revealed that γδ TCR is highly variable in length resembling immuno-globulins (Ig) more than the αβ TCR. The antigen recognition property of γδ T cells is fundamentally different from αβ T cells but similar to antigen–antibody binding, which is more likely to occur independent of MHC cross presentation (14). However, recently butyrophilin BTN3A1, a non-polymorphic ubiquitously expressed molecule was identified as an antigen presenting molecule of Vγ9Vδ2 T cells. Soluble BTN3A1 binds (Isopentenyl diphosphate) IPP and (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) with different affinities in 1:1 ratio to stimulate γδ T cells (15).

The important feature of γδ T cells is their tropism to epithelial tissues. With respect to anatomical localization, γδ T cell population can be divided into two groups: lymphoid-homing γδ T cells that can be primed in the circulation and clonally expand in a conventional "adaptive" manner; and innate-like cells that respond rapidly and at a relatively high frequency in many tissue sites. Migration and anatomical localization of T lymphocytes is crucial for their antigen specificity and maintaining homeostasis in the mammalian immune system. Although γδ T cells are well represented among peripheral blood mononuclear cells (PBMC) and in afferent and efferent lymph, they are rarely found in lymph node parenchyma, spleen, Peyer's patches and thymus. Moreover, unlike αβ T cells, splenic γδ T cells, if present, are not confined to the lymphoid areas (the white pulp) but are also found throughout the red pulp of spleen and marginal zones of cell trafficking (16). γδ T cells are abundantly present in the epithelia of skin, genital and intestinal tract (17). In the small intestines of humans, mice, chickens and cattle, γδ T cells comprise a substantial fraction of intestinal intraepithelial lymphocytes (IELs); in mice $\gamma\delta^+$ IELs constitute 50–60% of the IEL pool (18–20). The epidermal $\gamma\delta^+$ IELs of mice and cattle (but not humans) have a marked dendritic morphology and are hence known as dendritic epidermal T cells (DETCs) (21). DETCs are maintained at steady state in normal adult murine skin but on activation execute specialized functions like tissue repair (22). DETCs also maintain keratinocyte homeostasis, which along with Langerhan cells forms its neighborhood (23). Under pathological conditions, γδ T cells quickly expand and infiltrate into lymphoid compartments and other tissues.

Another striking difference between αβ and γδ T cells is the range of antigens or ligands that are recognized by the respective TCRs. Unlike αβ T cells, which recognize protein antigen processed inside the cell and presented by MHC molecules, γδ T cells recognize antigens like B cells as revealed by structural and functional studies (24).γδ T cells can respond to a variety of stimuli irrespective of their molecular or genetic nature. In mice, the non-classical MHC class I molecules T10 and T22 are recognized by γδ T cells (25–28). Similar to T10 and T20, murine class II MHC (IA) antigens IE and IA are identified to act as ligands for γδ T cell clones (29, 30). In addition, herpes glycoprotein GI-reactive γδ T cell clones protect mice from herpes simplex virus (HSV) induced lethal encephalitis (31, 32). γδ TCRs can also bind to an algal molecule, phycoerythrin inducing upregulation of CD44 and downregulation of CD62L in γδ T cells (33). B6 murine splenic and hepatic γδ T cells respond to cardiolipin (bacterial cell-wall phospholipid and endogenous component of mitochondria) presented by CD1d molecules (34). Insulin derived peptide B:9–23 is also recognized by the γδ T cell clones derived from non-obese diabetic mice (NOD mice) (35). SKINT1, a mouse immunoglobulin superfamily member, bears structural similarity to human CD277 (butyrophilin 3A1) and is expressed by medullary thymic epithelial cells (mTECs) and keratinocytes that is crucial for the development of $V\gamma5V\delta1^+$ DETCs (36).

In humans, majority of γδ T cells express a rearranged T cell receptor (TCR) composed of Vγ9 and Vδ2 domains; thus, this population is referred to as Vγ9Vδ2. The Vγ9Vδ2 T cells recognize self and microbial phosphorylated metabolites generated in eukaryotic mevalonate pathway and in the microbial 2-C-methyl-derythritol 4-phosphate (MEP) pathway (37). Initially, it was reported that the non-peptidic ligands isolated from mycobacterial cell lysates were

Table 1 | Comparison between αβ and γδ T cells.

S.No.	αβ T cells	γδ T cells
1	Constitutes about 65–70% of total PBMCs	Constitutes about 1–10% of total PBMCs
2	Recognize the processed peptide antigen with the help antigen presenting molecule MHC1 and MHC II	Do not show MHC restriction but may require the antigen presenting molecule Butyrophilin 3A1 molecule
3	Express either $CD8^+$ or $CD4^+$	Mostly double negative, murine intestinal IELs may be $CD8\alpha\alpha^+$
4	TCR junctional diversity is very diverse	TCR junctional diversity is small
5	Do not show tissue tropism	Show tissue tropism
6	αβ T Cells response is late	γδ T cells respond earlier
7	Regulatory phenotype is attributed to $CD4^+CD25^+$ T cells	Regulatory phenotype is attributable to various subsets, including murine $V\gamma5^+$ DETCs and human $V\gamma1^+$ peripheral cells

stimulatory for Vγ9Vδ2 T cell clones. Later, IPP, an intermediate metabolite of the mevalonate pathway, was isolated and identified as a stimulatory molecule. Characterization of the microbial antigens recognized by human γδ T cells predicted that these are non-proteinaceous in nature and have critical phosphate residues (37, 38). Subsequent studies, conducted with *M. tuberculosis*, identified HMBPP, an intermediate metabolite of the MEP pathway, as a strong agonist of γδ TCR. The measured potencies of IPP and HMBPP show an enormous difference. The ED50 of IPP is ~20 μM, whereas that of HMBPP is ~70 pM, i.e., more than 105 times lower (38).

Another stimulatory molecule is *Staphylococcus aureus* enterotoxin A (SEA) that directly interacts with the TCR Vγ9 chain independently of the paired Vδ chain. The mechanism of recognition of this superantigen is different from that of phosphorylated metabolites and requires the interaction with MHC class II molecules. γδ T cells kill target cells and release cytokines upon interaction with SEA but do not proliferate (39).

Recently, the TCR from a γδ T cell clone derived from a cytomegalovirus (CMV)-infected transplant patient was shown to directly bind to endothelial protein C receptor (EPCR), which is a lipid carrier with a similar structure to CD1, showing again that γδ TCR engagement is cargo independent (40). ATP F1 synthase has been identified as stimulatory ligand of the TCR Vγ9Vδ2. ATP F1 synthase is an intracellular protein complex involved in ATP generation. However, optimal responses of Vγ9Vδ2 T cells by tumor target cell lines expressing F1-ATPase requires apolipoprotein A1. A monoclonal antibody interacting with apolipoprotein A1 was shown to inhibit TCR γδ activation as it disrupted the trimolecular complex of ApoA1, ATP F1 synthase, and γδ TCR required for optimal response (41).

The second major population of human γδ T cells utilizes the Vδ1 chain, which pairs with a variety of Vγ chains. This subset of $V\delta1^+$ T cells is mainly found in tissues and is activated by CD1c and CD1d-expressing cells. The group 1 CD1 molecules have ability to present lipid A to human γδ T cells. The human γδ T cells also recognize the related group 2 CD1 molecule as CD1d/lipid complex. Phosphatidyl ethanol amine (PE), a phospholipid, activates γδ T cells in a CD1d manner dependent suggesting its CD1d restricted recognition (42). In addition, some populations of γδ T cells in normal human PBMCs also recognize lipid molecules such as cardiolipin (a marker of damaged mitochondria), sulfatide (a myelin glycosphingolipid), or α-galactosylceramide (α-GalCer) in association with CD1d, which are noted ligands of natural killer T (NKT) cells (34, 43–45). Human γδ T cells also recognize the stress-induced MHC class I-related MICA/MICB molecules and the UL16-binding proteins that are upregulated on malignant or stressed cells (46–48). Heat shock proteins (HSPs) expressed on the cell membrane play an important role in cancer immunity. Hsp60 expressed on oral tumors act as ligand for Vγ9Vδ2 T cells (49, 50). Hsp60 and Hsp70 expressing human oral and esophageal tumors are lysed by Vγ9Vδ2 T cells (49–51). Hsp72 expressing neutrophils were rapidly killed by γδ T cells through direct cell to cell contact, indicating that hsp72 expression on cell surface pre-disposes inflamed neutrophils to killing by γδ T cells (52). In Another study, hsp90 expression on EBV infected B cells rapidly promoted γδ T cell proliferation (53). This confirms that γδ T cells recognize qualitatively distinct antigens, which are profoundly regulated by their anatomical localization.

CO-RECEPTORS AND γδ T CELL ACTIVATION

Most γδ T cells respond to non-peptidic antigens even in the absence of antigen presenting cells (APCs). However, the presence of APCs can greatly enhance the γδ T cell response (54). This suggests that accessory molecules/receptors may be involved in effector functions of these cells. Some of important co-receptors used by γδ T cells include NOTCH, NKG2D, and TLR (55).

Our study has identified Notch as an additional signal contributing to antigen specific effector functions of γδ T cells. We have shown that γδ T cells express Notch1 and Notch2 at both mRNA and protein level. Inhibition of Notch signaling in anti-CD3 MAb stimulated γδ T cells resulted in marked decrease in proliferation, cytotoxic potential, and cytokine production by γδ T cells confirming the involvement of Notch signaling in regulating antigen specific responses of γδ T cells (55).

γδ T cells express NKG2D on their cell surface resulting in their activation. Treatment of PBMC with immobilized NKG2D-specific mAb or NKG2D ligand MHC class I related protein A (MICA) resulted in the up-regulation of CD69 and CD25 on Vγ9Vδ2. Furthermore, NKG2D increased the production of TNF-alpha and release of cytolytic granules by Vγ9Vδ2 T cells (56). Later, it was shown that the protein kinase C transduction pathway as a main regulator of the NKG2D-mediated costimulation of anti-tumor Vγ9Vδ2 T cell cytolytic response (57).

TLR agonists are also known to trigger the early activation and the IFN-γ secretion by Vγ9Vδ2T cells (58). TLR ligands indirectly increase the anti-tumoricidal activity of Vγ9Vδ2T cells (59). In this review, we will focus on TLR as an additional co-receptor modulating the function of immune cells with special focus on γδ T cells.

TOLL LIKE RECEPTOR AND IMMUNE CELLS

The immune system functions in anti-microbial defense by recognizing groups of molecules unique to microorganisms (60). These unique microbial molecules are called pathogen-associated molecular patterns (PAMPs) and are recognized by a family of cellular receptors called pattern recognition receptors (PRRs) (61). TLRs along with retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptor (NLRs) are prototype PPRs, which recognize pathogen-associated molecular patterns (PAMPs) from microorganisms or danger-associated molecular patterns (DAMPs) from damaged tissues (62). Recognition of PAMPs by TLRs trigger release of inflammatory cytokines and type 1 interferon's (IFN) for host defense (60, 63–65). The adaptive immune system, on the other hand, is responsible for elimination of pathogens in the late phase of infection and in the generation of immunological memory mediated by B and T cells (66).

TLRs derived their name from *Drosophila melanogaster* Toll protein based on their homology (67). In mammals, till date 13 members of TLR family has been identified (63, 68–71). TLR1-9 is conserved in humans and mice while TLR10 is non-functional in mice because of a retroviral insertion while TLR11-13 is lost from the human genome. The first TLR identified was TLR4

and recognizes bacterial lipopolysaccharide (LPS) from Gram-negative bacteria (67, 72, 73). TLRs are classified into several groups based on the types of PAMPs they recognize. TLR1, 2, 4 and 6 recognize lipids whereas the highly related TLR7, TLR8 and TLR9 recognize nucleic acids. Murine TLR11 recognizes a protozoan derived profilin-like protein while TLR13 recognizes *Vesicular stomatitis virus* (63). TLRs are localized in the distinct cellular compartments, for example; TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are expressed on the cell surface whereas TLR3, TLR7, TLR8 TLR9, TLR11, TLR12 and TLR13 are expressed in intracellular vesicles such as the endosome and ER. The intracellular TLRs are transported to the intracellular vesicles via UNC93B1, a trans-membrane protein, which is localized in the ER of the cell (70, 71, 74–77). TLR family receptors have a common structural architecture. TLRs are type I integral membrane glycoproteins characterized by multiple extracellular leucine-rich repeats (LRRs) and a single intracellular Toll/interleukin-1 (IL-1) receptor (TIR). TLRs mostly form homo-dimers with a few exceptions, which form heterodimers to trigger a signal. For example, TLR2 forms heterodimers with TLR1 or TLR6 enabling differential recognition of lipopeptides. The TIR domain of TLRs is required for the interaction and recruitment of various adaptor molecules to activate downstream signaling pathway. After recognizing PAMPs, TLRs activate intracellular signaling pathways that lead to the induction of inflammatory cytokine genes such as TNF-α, IL-6, IL-1β and IL-12 through the recruitment of adaptors such as MyD88, TRIF, TRAM, TIRAP and SARM1 (78). MyD88 is a universal adaptor used by all TLRs, except TLR3, to induce inflammatory pathways through activation of MAP Kinases (ERK, JNK, p38) and transcriptional factor NF-κB (63, 79). TLR3 and TLR4 use TRIF to bring activation of alternative pathway (TRIF-dependent pathway) through transcription factors IRF3 and NF-κB to induce type 1 IFN and inflammatory cytokines (80–82). TRAM selectively participates in the activation of the TRIF-dependent pathway downstream of TLR4, but not TLR3 (83, 84). TIRAP functions to recruit MyD88 leading to activation of MyD88-dependent pathway downstream of TLR2 and TLR4 (85, 86).Sterile-α- and armadillo-motif-containing protein 1 (SARM1), was shown to inhibit TRIF and is also critical for TLR-independent innate immunity (87). Thus, signaling pathways can be broadly classified as either MyD88-dependent pathway or TRIF-dependent pathway.

Hornung et al. have showed differential expression of TLR1-10 on human APCs and lymphocytes including T cells and their functional discrepancy in recognition of specific TLR ligands (88). $CD4^+$ T cells express almost all TLRs at mRNA levels but may not express all as functional protein (89, 90). Moreover, they do not respond to all TLR ligands. Stimulation with TLR5, 7, or 8 agonists combined with TCR activation of $CD4^+$T cells resulted in increased proliferation and production of IL-2, IL-8, IL-10, IFN-γ and TNFα (91). There are other reports as well suggesting the functional modulation of subtypes of $CD4^+$ T cells by TLR ligands. The mouse Th1 but not Th2 cells responded to TLR2 agonist and resulted in enhanced proliferation and IFN-γ production independent of TCR stimulation (92). This work validated that the TLR can regulate function of $CD4^+$ T cells even in absence of TCR engagement. $CD4^+CD25^+$ regulatory T cells (Tregs) express majority of TLRs with selectively higher expression of TLR2, 4, 5, 7/8, and 10 compared to $CD4^+CD25^-$ conventional T cells (93). Liu et al. showed that $CD4^+CD25^+$ regulatory T cells and $CD4^+CD25^-$ conventional T cells express TLR2 and proliferated upon stimulation with its agonist. TLR2 stimulation also led to transient loss of Treg suppressive potential through suppression of FOXP3 (94, 95). However, Tregs also express TLR5 but upon stimulation with flagellin (ligand of TLR5), do not proliferate rather showed increased suppressive capacity and enhanced expression of FOXP3 (96). These reports suggest that the suppressive function of Treg can be either enhanced or dampened by the type of TLR ligand engaged. TLR2 stimulation not only abrogates suppressive functions of $CD4^+$ Tregs but also drives naïve as well as effector Treg population toward IL17 producing Th17 phenotype (97). Th17 cells express TLR2 along with TLR6 compared to Th1 and Th2 subsets and promote Th17 differentiation upon Pam3Cys stimulation and accelerates experimental autoimmune encephalomyelitis (98). Like TLR2, TLR4 also regulate the functions of $CD4^+$ T cells. In a mouse model of arthritis, mice lacking TLR2 showed enhanced histopathological scores of arthritis by a shift in T cell balance from Th2 and T regulatory cells toward pathogenic Th1 cells. TLR4, in contrast, contributes to more severe disease by modulating the Th17 cell population and IL-17 production (99, 100). Recently, Li et al. showed that high-mobility group box 1 (HMGB1) proteins decrease Treg/Th17 ratio by inhibiting FOXP3 and enhancing RORγt in $CD4^+$ T cells via TLR4–IL6 axis in patients with chronic hepatitis B infections (101). This shows that HMGB1 (TLR4 ligand) act as a modulator of $CD4^+$ T cells responses in chronic viral inflammation. $CD4^+$ T cells also express intracellular TLRs such as TLR9 and TLR3. Both these TLRs promote T cell survival via activation of NF-κB and MAPK signaling (102). Although the effector functions of $CD4^+$ T cells are regulated by TLRs but the molecular pathway involved in skewing of $CD4^+$ T cell function is poorly understood.

Like $CD4^+$ T cells, $CD8^+$ T cells also show differential expression of TLRs with high expression of TLR3 but lower expression of TRL1,2,5,9,10 compared to $CD4^+$ T cells at mRNA level. It is important to note that the expression of TLR2, TLR3 and TLR5 increases on CD8 T cells in infected tonsils compared to controls (89) indicating immune activating role of TLRs in infections. Stimulation of $CD8^+$ T cells through TLR2 agonists enhances their proliferation and IFN-γ production (103, 104). It also promotes cytolytic activity of $CD8^+$ T cells and enhances anti-tumor response mediated through MyD88-dependent TLR1/2 pathway (105). Recently, Mercier et al. showed that TLR2 cooperate with NOD-containing protein 1 (NOD1) to enhance TCR mediated activation and can serve as alternative co-stimulatory receptor in $CD8^+$ T cells (106). $CD8^+$ T cells also express intracellular TLRs such as TLR3, TLR9 which are more potent in inducing $CD8^+$ T cell activation *in vivo* (107).

Natural killer (NK) cell is a vital player in innate immune system. They recognize infected and transformed cells with down-regulated major histocompatibility complex (MHC) class 1 molecules. They are the primary producers of IFN-γ and are protective against infections. Unlike CD4 and CD8 T cells NK cells as well as $CD56^+CD3^+$ NKT cells constitutively express TLR 1–8 with high expression of TLR2 and 3 at mRNA level. They recognize

bacterial PAMPs and respond by producing α-defensins (108–111). Human NK cells can also directly recognize *Mycobacterium bovis* via TLR2 and enhance their cytolytic activity against tumor cells (112). Tumor-associated macrophages induce NK cell IFN-γ production and cytolytic activity upon TLR engagement (113). TLRs modulate NK cell function directly or indirectly to promote antibody dependent cell mediated cytotoxicity and cross presentation of viral antigens to T lymphocytes (114, 115). This highlights that the cells of adaptive immune system do express TLRs and their function can be directly or indirectly modulated by TLR ligands.

ACTIVATION OF γδ T CELLS BY TLR LIGANDS

In 1997, the first human homolog of *Drosophila* Toll protein was cloned and characterized. It was also established that γδ T cells also express hToll mRNA (67). Purified γδ T cells were found to respond to the *E. coli* native lipid A in a TCR-independent fashion and the LPS/lipid A-reactive γδ T cells strongly expressed TLR2 mRNA. TLR2 antisense oligonucleotide inhibited the proliferation of γδ T cells in response to the native lipid A as well as the TLR2-deficient mice showed an impaired response of the γδ T cells following injection of native lipid A. These results suggest that TLR2 is involved in the activation of canonical Vγ6/Vδ1 T cells by native lipid A (116). Again, functional presence of TLR2 on Vγ2Vδ2 T cells (also known as Vγ9Vδ2 T cells) was reported when the dual stimulation of Vγ2Vδ2 T cells with anti-TCR antibody and Pam_3Cys increased synthesis and secretion of IFN-γ and elevated the levels of CD107a expression. IFN-γ secretion and cell surface CD107a levels are markers of increased effector function in Vγ2Vδ2 T cells (117). Similarly, Bruno et al. reported that IL-23 and TLR2 co-stimulation induces IL17 expression in γδ T cells. However, TLR1 and TLR2 expression was found only on $CCR6^+$ IL-17 producing murine peritoneal γδ T cells but not others. Thus, γδ T cells with innate receptor expression coupled with IL-17 production establishes them as first line of defense that can orchestrate an inflammatory response to pathogen-derived and environmental signals long before Th17 can sense the bacterial invasion (118). Pam3CSK4, TLR2 agonist was able to stimulate only splenic γδ T cell proliferation but not the dermal γδ T cells demonstrating that TLR2 signaling shows tissue tropism. (19). Furthermore, a profound change in the circulating γδ T-cell population was observed in early burn injury (24 h). These γδ T-cells showed TLR2 and TLR4 expression, priming them for TLR reactivity, However TLR expression was specific to circulatory γδ T cell subset and was transient, since it was not observed after post-injury (7 days). Transient nature of the post-burn increase in γδ T-cell TLR expression is likely to be protective to the host, most likely via regulation of inflammation and initiation of healing processes (119).Mitochondrial danger-associated molecular patterns (MTDs) induce TLR2 and TLR4 expression on γδ T cells in dose dependent manner. MTDs also induced the production of IL-1β, IL-6, IL-10, RANTES, and vascular endothelial growth factor by γδ T-cells thereby resulting in initiation of sterile inflammation leading to tissue/cellular repair (120).

Different studies have reported that γδ T cells express TLR3 (121, 122). TLR3 recognizes viral dsRNA, synthetic analogs of dsRNA, polyinosinic–polycytidylic acid [poly (I:C)] and small interfering (si) RNA. The direct stimulation of freshly isolated γδ T cells via TCR and surrogate TLR3 ligand poly (I:C) dramatically increased IFN-γ production. Addition of neutralizing anti-TLR3 mAb inhibited the co-stimulatory effect of poly (I:C), presumably by antagonizing the TLR3 signaling (122). Thus, the integrated signals of TLR3 and TCR induce a strong antiviral effector function in γδ T cells supporting the decisive role of γδ T cells in early defense against viral infection. In other study, it has been reported that γδ cells of term babies and of adults express TLR3 and TLR7 while the preterm babies have reduced levels. The greater levels of IFN-γ protein was observed in adult and cord blood cells co-stimulated with anti-CD3 and poly(I:C) whereas this was not seen in γδ T cell clones of preterm babies. Thus, reduced level of TLR3 expression by preterm-derived clones had an overt functional consequence on IFN-γ levels (11). Interestingly, a primary role of TLR3 in humans appears to mediate resistance to HSV-induced encephalitis (123). Hence, premature babies are particularly susceptible to HSV infection because of reduced levels of TLR3 on γδ T cells.

TLR4 was reported to be absent in the γδ T cells but can become functional in γδ T cells depending on localization, environmental signals, or γδ TCR usage (19, 118, 124). However, our own data has shown that TLR4 is expressed on human γδ T cells. Stimulation of γδ T cells with LPS (TLR4 ligand) increased their proliferation, IFN-γ release, and cytotoxic potential (125). DETCs lack cell surface expression of TLR4–MD2. MD-2 physically associates with TLR4 on the cell surface and is required for LPS signaling. However, TLR4–MD2 expression was upregulated when DETCs emigrated from the epidermis during cutaneous inflammation. The migration signals of DETCs may promote the TLR4–MD2 expression (126). Cairns et al. showed that late post-burn injury increased expression of TLR-4 on splenic T-cells (127). However, Martin et al. reported transient TLR-4 expression post-burn in the circulation or spleen but were specific for the γδ T-cell subset (119). Several evidences suggest that murine γδ T cells recognize LPS/LA through TLR2 or TLR4 (128, 129). Importantly activated γδ T cells, especially Vδ2 T cells, in peripheral blood cells recognize LA, a major component of LPS, via TLR4 resulting in extensive proliferation and production of IFN-γ and TNF-α *in vitro* (130). The data suggest that γδ T cells play an important role in the control of infection induced by gram negative bacteria. Reynolds et al. showed that a heterogeneous population of γδ T cells responds to LPS via TLR4 dependent manner and demonstrate the crucial and innate role of TLR4 in promoting the activation of γδ T cells, which contributes to the initiation of autoimmune inflammation (100). Another study showed the indirect role of TLR4 in HMGB–TLR4–IL-23–IL17A axis between macrophages and γδ T cells, which contribute to the accumulation of neutrophils and liver inflammation. Necrotic hepatocytes release HMGB1, a damage-associated molecule or TLR4 ligand, which increased IL-23 production of macrophages in a TLR4 dependent manner. IL-23 aids γδ T cells in liver in the generation of IL-17A, which then recruits hepatic neutrophils (131).

Human γδ T cells were found to express appreciable levels of TLR7. Costimulation with poly I:C upregulated the TLR7 expression in TCR-cross linked freshly isolated γδ T cells (124). In addition, tumor-infiltrating γδ T cells also express TLR7 (132). In case of mouse dermal γδ T cells, both TLR7 and

TLR9 signaling promoted IL-17 production, which could be synergistically enhanced with the addition of IL-23 (19).

The identification of dominant γδ T cells in the total population of tumor-infiltrating lymphocytes (TILs) in renal, breast, and prostate cancer suggested that these cells might have the potent negative immune regulatory function (132, 133). The breast tumor-derived bulk γδ T cell lines and clones efficiently suppressed the proliferation and IL-2 secretion of naïve/effector T cells and inhibited DC maturation and function. Hence, their depletion or the reversal of their suppressive function could enhance anti-tumor immune responses against breast cancer. Indeed as in $CD4^+$ regulatory T cells (Tregs), the immunosuppressive activity of γδ T cells could be reversed by human TLR8 ligands both *in vitro* and *in vivo*. Study revealed that MyD88, TRAF6, IKKα, IKKβ and p38α molecules in γδ1 cells were required for these cells to respond to TLR8 ligands (132, 134, 135). **Table 2** shows expression and co-stimulatory effects mediated by TLR activation of γδ T cells

TLRs MODULATE CROSSTALK BETWEEN γδ T AND DENDRITIC CELLS

The functional fate of effector T cells is governed by antigen presentation and the cytokine milieu in the local environment. Dendritic cells (DCs) being professional APCs, recognize the danger signal, process it, and present it to the T lymphocytes thereby modulate adaptive immune response. γδ T cells influence the antigen presenting property of DCs. DCs pre-incubated with activated γδ T cells enhance the production of IFN-γ by alloreactive T cells in mixed lymphocyte reaction (136). Moreover, γδ T cells not only upregulated CD86 and MHC I expression on DC but themselves get activated, leading to up-regulation of CD25, CD69, and cytokine production (137). These studies showed how γδ T cell and DCs regulate each other's function. There are reports, which have shown how γδ T cells interact with DC or *vice versa* via TLR ligands. Leslie et al. reported that stimulation with TLR ligands in γδ/DC cocultures enhanced the maturation and production of IL12p70 by DCs (138). TLR also regulate the γδ T cells and DC crosstalk in microbial context. TLR2-stimulated DCs enhanced IFN-γ production by Vδ2 T cells; conversely, phosphoantigen activated Vδ2 T cells enhanced TLR2-induced DC maturation via IFN-γ, which co-stimulated interleukin-12 (IL-12) p70 secretion by DCs (139). Further, γδ T cells stimulated with TLR7 (CL097) or TLR3 (poly I: C) agonists produce IFN-γ, TNFα and/or IL-6 thereby inducing DC maturation, which prime effector T cells against West Nile Virus (WNV) infection (140). This study confirmed that the antiviral effector immunity may be regulated by interplay of DCs, γδ T cells and TLRs. Similarly, in human's γδ T cells and DCs regulate each other's immunostimulatory functions. TLR3 and TLR4 ligands stimulation of human PBMCs induced a rapid and exclusive IFN-γ production by Vγ9Vδ2 subset dependent on type 1 IFN secreted by monocytic DC. TLR-induced IFN-γ response of Vγ9Vδ2 T cells led to efficient DC polarization into IL-12p70-producing cells (58). In another study, it was reported that Vδ2 cells are indirectly activated by BCG and IL-12p70 secreted by DCs. IL-12p70 production by DC is modulated by Toll like receptor 2/4 ligands from BCG and IFN-γ secreted by memory CD4 T cells (141). This study portrayed the complex interplay between cells of the innate and adaptive immune response in contributing to immunosurveillance against pathogenic infections.

TLRs COMPLEMENT CYTOTOXIC POTENTIAL OF γδ T CELLS AGAINST TUMOR CELLS

γδ T cells have capability to lyse different types of tumors and tumor-derived cell lines (49, 50, 142–145). Circulating as well as tumor-infiltrating γδ T cells have the ability to produce abundant proinflammatory cytokines like IFN-γ and TNF-α, cytotoxic mediators and MHC-independent recognition of antigens, render them as important players in cancer immunotherapy (143, 145). In addition to TCR, γδ T cells use additional stimulatory co-receptors or ligands including TLRs to execute effector functions and TLR agonists are considered as adjuvants in clinical trial of cancer immunotherapy (146). Kalyan et al. even quoted that "TLR signaling may perfectly complement the anti-tumor synergy of aminobisphosponates and activated γδ T cells and this combined innate artillery could provide the necessary ammunition to topple malignancy's stronghold on the immune system" (147). Paradoxically, TLR agonists execute dual role of enhancing immune response (148) as well as increasing invasiveness of tumor cells (149–152). Hence, the tripartite cooperation of tumor cell, TLRs, and γδ T cells should be carefully analyzed. In concordance to this, Shojaei et al. reported that Toll like receptor 3 and 7 agonists enhanced the tumor cell lysis by human γδ T cells. The enhanced capability of γδ T cells to lyse tumor cells was attributed to increased expression of CD54 and downregulation of MHC class 1 on tumor cells. Poly(I:C) treatment of pancreatic adenocarcinomas resulted in overexpression of CD54 and concomitant coculture of tumor cells with γδ T cells led to interaction between CD54 and its ligand CD11a/CD18 triggering effector function in γδ T cells. However, TLR7 surrogate ligand induced

Table 2 | Expression and functions mediated by TLRs on γδ T cells.

TLR	Functions	References
TLR 2	Recognize LPS, enhance proliferation, induce IFNγ and CD107a expression, enhance IL17 secretion, expression transiently increases after burn injury, mitochondrial danger-associated molecular patterns (MTDs) induce expression and production of IL-1β, IL-6, IL-10, RANTES, and VEGF	(19, 116–120)
TLR3	Induce IFNγ production in conjunction with TCR stimulation, resistance to HSV induced encephalitis	(11, 121–123)
TLR4	Increases proliferation, IFN-γ release, and cytotoxic potential, activation following burn injury	(100, 125, 127, 130)
TLR7/9	Upregulate upon poly I:C costimulation, promote IL-17 production	(19, 124, 132)
TLR8	Reversal of immunosuppressive activity	(132, 134, 135)

downregulation of MHC class 1 molecule on tumor cells resulting in a reduced affinity for inhibitory receptor NKG2A on γδ T cells (59). Manipulation of TLR signaling by using TLR8 agonists reversed the suppressive potential of γδ Tregs found elevated in breast cancer (132). Polysaccharide K (PSK) known for its anti-tumor and immuno-modulatory function can also activate TLR2 leading to increased secretion of IFN-γ by γδ T cells on stimulation. The cell–cell contact between γδ T cells and DC was required for optimal activation of γδ T cells. However, PSK along with anti-TCR could co-activate γδ T cells even in the absence of DC. The study confirmed that the anti-tumor effect of PSK was through activation of γδ T cells (153).

Studies from our lab have shown that the TLR signaling in γδ T cells derived from the oral cancer (OC) patients may be dysfunctional. We reported that γδ T cells from healthy individuals (HI) and OC patients express higher levels of TLR2, TLR3, TLR4, and TLR9 than in αβT cells. Higher TLR expression was observed in HI compared to OC patients. Stimulation with IL2 and TLR agonists (Pam3CSK, Poly I:C, LPS, and CpG ODN) resulted in higher proliferative response of peripheral blood lymphocytes from HI compared to OC patients. However, the role of other immune cells that may influence the TLR ligand stimulation induced activation status of lymphocytes cannot be ignored (125). Impairment in TLR expression/signaling can be viewed as a strategy employed by tumor cells to avoid immune recognition.

TLRs AND γδ T CELLS IN DISEASES

Studies have demonstrated the protective role of γδ T cells in infection and inflammation (154–157). Inoue et al. showed that during mycobacterial infection, γδ T cells precedes the αβ T cells, indicating role of γδ T cells as first line of defense against infections (158). The conserved molecular patterns associated with pathogens are directly recognized by γδ T cells leading to rapid protective response against the danger signal. Unlike αβ TCR, γδ TCR acts as pattern recognition receptor providing advantage in anti-infection immunity by directly initiating cytotoxicity against infected cells or through production of cytokine to involve multiple immune system components to combat infection (159, 160). Activated γδ T cells through TLR3 and TLR4 ligands rescue the repressed maturation of virus-infected DCs and mount a potent antiviral response (58, 140). Malarial infection in MyD88 deficient mice resulted in impairment in $CD27^{-}$IL-17A-producing γδ T cell without affecting the IFN-γ producing γδ T cells (161). This study specifies the role of TLR in promoting proliferation

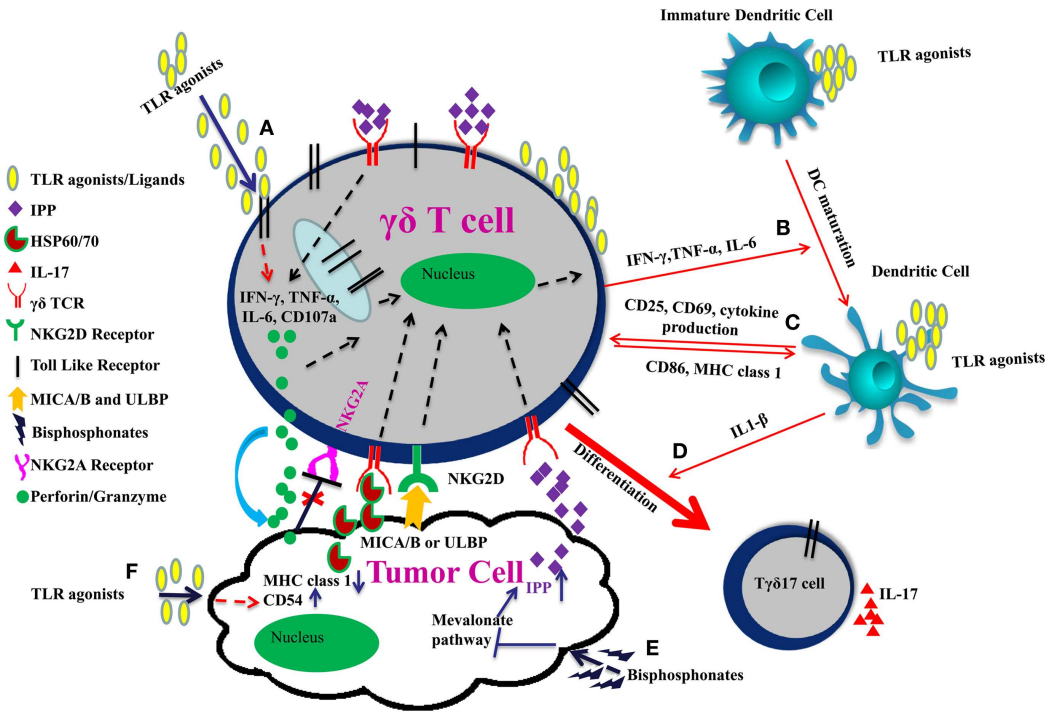

FIGURE 1 | Improving γδ T cell functions by TLRs in combinatorial therapy. **(A)** TLR agonists induce effector function of γδ T cells through IFN-γ, TNF-α, IL-6 secretion, and increased expression of CD107a. **(B)** IFN-γ, TNF-α, and IL-6 secreted by γδ T cells and TLR agonists promote the maturation of dendritic cell. **(C)** γδ T cells upregulate CD86 and MHC I expression on DCs and are themselves activated through up-regulation of CD25, CD69, and cytokine production thereby modulating each other's function. **(D)** Co-stimulation of γδ T cells with TLR agonists and IL-1β secreted by dendritic cells promote their polarization toward IL17 producing cells. **(E)** γδ TCR also recognizes the specific molecular patterns such as IPP, which are induced upon inhibition of mevalonate pathway by bisphosphonates. Moreover, NKG2D receptor on γδ T cells recognizes MICA/B or ULBP expressed on tumor cells. This binding enhances release of perforins and granzymes by the γδ T cells leading to tumor cell lysis. **(F)** TLR agonists act as adjuvants and can induce CD54 expression and downregulation of MHC class 1 on tumor cells. Interaction between CD54 and its ligand CD11a/CD18 trigger effector functions in γδ T cells. Downregulation of MHC class 1 molecule on tumor cells result in reduced signaling through the inhibitory receptor NKG2A on γδ T cells, which enhances the cytotoxic potential of γδ T cell.

of proinflammatory γδ T cells. Another study by Martin et al. showed that IL17 producing γδ T cells express TLR1 and TLR2 and expand in response to their ligands and mount an adequate response against heat-killed *M. tuberculosis* or *C. albicans* infection (118). However, γδ T cell are also known to directly recognize the pathogen-derived molecules and mediate cytotoxic effector function either through secretion of perforin and granzyme B or by secretion of proinflammatory cytokine IL17 (162–164). The involvement of TLRs in regulating anti-microbial γδ T cell function should be investigated in depth to exploit it as a cell based therapy for infectious diseases.

CONCLUDING REMARKS

The characteristic copious IFN-γ or IL17 secretion, MHC-independent antigen recognition, tissue tropism, and potent cytotoxicity make γδ T cells promising targets for immunotherapy. Similar to αβ T cells, γδ T cells exhibit functional and phenotypic plasticity, which influences the nature of the downstream adaptive immune response. The adoptive transfer of *ex vivo* expanded Vγ9Vδ2 T cells or *in vivo* activation of Vγ9Vδ2 T cells (phosphoantigens or amino-bisphosphonates) can be utilized as adjuvant to conventional therapies. Clinical trials of Vγ9Vδ2 T cells as immunotherapeutic agents have shown encouraging results that could be attributed to its low toxicity grade. Combinations of cellular immune-based therapies with chemotherapy and other anti-tumor agents may be of clinical benefit in the treatment of malignancies. Combinatorial treatment using, chemotherapeutic agents or bisphosphonate zoledronate (ZOL) sensitizes tumor-derived cell lines to rapid γδ T cells killing. Vγ9Vδ2 T cell triggering may be also enhanced by combining TCR stimulation with engagement of TLRs. Various TLR agonists are currently under investigation in clinical trials for their ability to orchestrate anti-tumor immunity. In one study, simultaneous use of both Imiquimod (TLR7 agonist) and CpG–ODN (TLR9 agonist) loaded onto virus like nanoparticles was found to be effective in triggering effector and memory $CD8^+$ T cell response (165). Similarly, combination of γδ T cells and DCs along with nanoparticle loaded TLR agonists can be employed for developing effective immunotherapeutic strategies. The direct or indirect stimulation of γδ T cells by TLR agonists could be a strategy to optimize Th1-mediated immune responses as adjuvant in vaccines against infectious or malignant diseases.

Administration of an "immunogenic chemotherapy" (such as oxaliplatin or anthracycline or an X-ray-based regimen) or local delivery of TLR surrogates in the tumor microenvironment (which stimulate local DCs and provides a source of IL-1β) may be also instrumental in polarization of γδ TILs into IL17 producing cells. Tγδ17 cells play a crucial role in anti-microbial immunity but their role in tumor immunity remains controversial. Tγδ17 have both pro and anti-tumor properties. TLR use in combinatorial therapy, therefore, could be a double edged sword. Careful use of TLR agonists in combinatorial γδ T cell based therapy is needed to strike the balance between pro and anti-tumor effects (**Figure 1**).

REFERENCES

1. Tipping PG. Toll-like receptors: the interface between innate and adaptive immunity. *J Am Soc Nephrol* (2006) **17**(7):1769–71. doi:10.1681/ASN.2006050489
2. Holtmeier W, Kabelitz D. Gammadelta T cells link innate and adaptive immune responses. *Chem Immunol Allergy* (2005) **86**:151–83. doi:10.1159/000086659
3. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* (2001) **2**(8):675–80. doi:10.1038/90609
4. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol* (2010) **10**(7):467–78. doi:10.1038/nri2781
5. Hayday AC. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* (2009) **31**(2):184–96. doi:10.1016/j.immuni.2009.08.006
6. Wesch D, Peters C, Oberg HH, Pietschmann K, Kabelitz D. Modulation of gammadelta T cell responses by TLR ligands. *Cell Mol Life Sci* (2011) **68**(14):2357–70. doi:10.1007/s00018-011-0699-1
7. Hayday AC. [Gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol* (2000) **18**:975–1026. doi:10.1146/annurev.immunol.18.1.975
8. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Annu Rev Immunol* (2003) **21**:139–76. doi:10.1146/annurev.immunol.21.120601.141107
9. Velilla PA, Rugeles MT, Chougnet CA. Defective antigen-presenting cell function in human neonates. *Clin Immunol* (2006) **121**(3):251–9. doi:10.1016/j.clim.2006.08.010
10. De Rosa SC, Andrus JP, Perfetto SP, Mantovani JJ, Herzenberg LA, Herzenberg LA, et al. Ontogeny of gamma delta T cells in humans. *J Immunol* (2004) **172**(3):1637–45. doi:10.4049/jimmunol.172.3.1637
11. Gibbons DL, Haque SF, Silberzahn T, Hamilton K, Langford C, Ellis P, et al. Neonates harbour highly active gammadelta T cells with selective impairments in preterm infants. *Eur J Immunol* (2009) **39**(7):1794–806. doi:10.1002/eji.200939222
12. Shin S, El-Diwany R, Schaffert S, Adams EJ, Garcia KC, Pereira P, et al. Antigen recognition determinants of gammadelta T cell receptors. *Science* (2005) **308**(5719):252–5. doi:10.1126/science.1106480
13. Kalyan S, Kabelitz D. Defining the nature of human gammadelta T cells: a biographical sketch of the highly empathetic. *Cell Mol Immunol* (2013) **10**(1):21–9. doi:10.1038/cmi.2012.44
14. Li H, Lebedeva MI, Llera AS, Fields BA, Brenner MB, Mariuzza RA. Structure of the Vdelta domain of a human gammadelta T-cell antigen receptor. *Nature* (1998) **391**(6666):502–6. doi:10.1038/35172
15. Vavassori S, Kumar A, Wan GS, Ramanjaneyulu GS, Cavallari M, El Daker S, et al. Butyrophilin 3A1 binds phosphorylated antigens and stimulates human gammadelta T cells. *Nat Immunol* (2013) **14**(9):908–16. doi:10.1038/ni.2665
16. Bordessoule D, Gaulard P, Mason DY. Preferential localisation of human lymphocytes bearing gamma delta T cell receptors to the red pulp of the spleen. *J Clin Pathol* (1990) **43**(6):461–4. doi:10.1136/jcp.43.6.461
17. Itohara S, Farr AG, Lafaille JJ, Bonneville M, Takagaki Y, Haas W, et al. Homing of a gamma delta thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. *Nature* (1990) **343**(6260):754–7. doi:10.1038/343754a0
18. Goodman T, Lefrancois L. Intraepithelial lymphocytes. Anatomical site, not T cell receptor form, dictates phenotype and function. *J Exp Med* (1989) **170**(5):1569–81. doi:10.1084/jem.170.5.1569
19. Cai Y, Shen X, Ding C, Qi C, Li K, Li X, et al. Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity* (2011) **35**(4):596–610. doi:10.1016/j.immuni.2011.08.001
20. Gray EE, Suzuki K, Cyster JG. Cutting edge: identification of a motile IL-17-producing gammadelta T cell population in the dermis. *J Immunol* (2011) **186**(11):6091–5. doi:10.4049/jimmunol.1100427
21. Havran WL, Allison JP. Origin of Thy-1+ dendritic epidermal cells of adult mice from fetal thymic precursors. *Nature* (1990) **344**(6261):68–70. doi:10.1038/344068a0
22. Jameson J, Ugarte K, Chen N, Yachi P, Fuchs E, Boismenu R, et al. A role for skin gammadelta T cells in wound repair. *Science* (2002) **296**(5568):747–9. doi:10.1126/science.1069639
23. Sharp LL, Jameson JM, Cauvi G, Havran WL. Dendritic epidermal T cells regulate skin homeostasis through local production of insulin-like growth factor 1. *Nat Immunol* (2005) **6**(1):73–9. doi:10.1038/ni1152
24. Chien YH, Jores R, Crowley MP. Recognition by gamma/delta T cells. *Annu Rev Immunol* (1996) **14**:511–32. doi:10.1146/annurev.immunol.14.1.511
25. Adams EJ, Chien YH, Garcia KC. Structure of a gammadelta T cell receptor in complex with the nonclassical MHC T22. *Science* (2005) **308**(5719):227–31. doi:10.1126/science.1106885

26. Bluestone JA, Cron RQ, Cotterman M, Houlden BA, Matis LA. Structure and specificity of T cell receptor gamma/delta on major histocompatibility complex antigen-specific CD3+, CD4-, CD8- T lymphocytes. *J Exp Med* (1988) **168**(5):1899–916. doi:10.1084/jem.168.5.1899
27. Crowley MP, Reich Z, Mavaddat N, Altman JD, Chien Y. The recognition of the nonclassical major histocompatibility complex (MHC) class I molecule, T10, by the gammadelta T cell, G8. *J Exp Med* (1997) **185**(7):1223–30. doi:10.1084/jem.185.7.1223
28. Bonneville M, Ito K, Krecko EG, Itohara S, Kappes D, Ishida I, et al. Recognition of a self major histocompatibility complex TL region product by gamma delta T-cell receptors. *Proc Natl Acad Sci U S A* (1989) **86**(15):5928–32. doi:10.1073/pnas.86.15.5928
29. Matis LA, Fry AM, Cron RQ, Cotterman MM, Dick RF, Bluestone JA. Structure and specificity of a class II MHC alloreactive gamma delta T cell receptor heterodimer. *Science* (1989) **245**(4919):746–9. doi:10.1126/science.2528206
30. Schild H, Mavaddat N, Litzenberger C, Ehrich EW, Davis MM, Bluestone JA, et al. The nature of major histocompatibility complex recognition by gamma delta T cells. *Cell* (1994) **76**(1):29–37. doi:10.1016/0092-8674(94)90170-8
31. Sciammas R, Kodukula P, Tang Q, Hendricks RL, Bluestone JA. T cell receptor-gamma/delta cells protect mice from herpes simplex virus type 1-induced lethal encephalitis. *J Exp Med* (1997) **185**(11):1969–75. doi:10.1084/jem.185.11.1969
32. Johnson RM, Lancki DW, Sperling AI, Dick RF, Spear PG, Fitch FW, et al. A murine CD4-, CD8- T cell receptor-gamma delta T lymphocyte clone specific for herpes simplex virus glycoprotein I. *J Immunol* (1992) **148**(4):983–8.
33. Zeng X, Wei YL, Huang J, Newell EW, Yu H, Kidd BA, et al. Gammadelta T cells recognize a microbial encoded B cell antigen to initiate a rapid antigen-specific interleukin-17 response. *Immunity* (2012) **37**(3):524–34. doi:10.1016/j.immuni.2012.06.011
34. Dieudé M, Striegl H, Tyznik AJ, Wang J, Behar SM, Piccirillo CA, et al. Cardiolipin binds to CD1d and stimulates CD1d-restricted gammadelta T cells in the normal murine repertoire. *J Immunol* (2011) **186**(8):4771–81. doi:10.4049/jimmunol.1000921
35. Zhang L, Jin N, Nakayama M, O'Brien RL, Eisenbarth GS, Born WK. Gamma delta T cell receptors confer autonomous responsiveness to the insulin-peptide B:9-23. *J Autoimmun* (2010) **34**(4):478–84. doi:10.1016/j.jaut.2009.12.008
36. Havran WL, Chien YH, Allison JP. Recognition of self antigens by skin-derived T cells with invariant gamma delta antigen receptors. *Science* (1991) **252**(5011):1430–2. doi:10.1126/science.1828619
37. Tanaka Y, Sano S, Nieves E, De Libero G, Rosa D, Modlin RL, et al. Nonpeptide ligands for human gamma delta T cells. *Proc Natl Acad Sci U S A* (1994) **91**(17):8175–9. doi:10.1073/pnas.91.17.8175
38. Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR. Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. *Nature* (1995) **375**(6527):155–8. doi:10.1038/375155a0
39. Rust CJ, Verreck F, Vietor H, Koning F. Specific recognition of staphylococcal enterotoxin A by human T cells bearing receptors with the V gamma 9 region. *Nature* (1990) **346**(6284):572–4. doi:10.1038/346572a0
40. Willcox CR, Pitard V, Netzer S, Couzi L, Salim M, Silberzahn T, et al. Cytomegalovirus and tumor stress surveillance by binding of a human gammadelta T cell antigen receptor to endothelial protein C receptor. *Nat Immunol* (2012) **13**(9):872–9. doi:10.1038/ni.2394
41. Scotet E, Martinez LO, Grant E, Barbaras R, Jenö P, Guiraud M, et al. Tumor recognition following Vgamma9Vdelta2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I. *Immunity* (2005) **22**(1):71–80. doi:10.1016/j.immuni.2004.11.012
42. Russano AM, Agea E, Corazzi L, Postle AD, De Libero G, Porcelli S, et al. Recognition of pollen-derived phosphatidyl-ethanolamine by human CD1d-restricted gamma delta T cells. *J Allergy Clin Immunol* (2006) **117**(5):1178–84. doi:10.1016/j.jaci.2006.01.001
43. Bai L, Picard D, Anderson B, Chaudhary V, Luoma A, Jabri B, et al. The majority of CD1d-sulfatide-specific T cells in human blood use a semiinvariant Vdelta1 TCR. *Eur J Immunol* (2012) **42**(9):2505–10. doi:10.1002/eji.201242531
44. Uldrich AP, Le Nours J, Pellicci DG, Gherardin NA, McPherson KG, Lim RT, et al. CD1d-lipid antigen recognition by the gammadelta TCR. *Nat Immunol* (2013) **14**(11):1137–45. doi:10.1038/ni.2713
45. Luoma AM, Castro CD, Mayassi T, Bembinster LA, Bai L, Picard D, et al. Crystal structure of Vdelta1 T cell receptor in complex with CD1d-sulfatide shows MHC-like recognition of a self-lipid by human gammadelta T cells. *Immunity* (2013) **39**(6):1032–42. doi:10.1016/j.immuni.2013.11.001
46. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. *Science* (1998) **279**(5357):1737–40. doi:10.1126/science.279.5357.1737
47. Wu J, Groh V, Spies T. T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial gamma delta T cells. *J Immunol* (2002) **169**(3):1236–40. doi:10.4049/jimmunol.169.3.1236
48. Kong Y, Cao W, Xi X, Ma C, Cui L, He W. The NKG2D ligand ULBP4 binds to TCRgamma9/delta2 and induces cytotoxicity to tumor cells through both TCRgammadelta and NKG2D. *Blood* (2009) **114**(2):310–7. doi:10.1182/blood-2008-12-196287
49. Laad AD, Thomas ML, Fakih AR, Chiplunkar SV. Human gamma delta T cells recognize heat shock protein-60 on oral tumor cells. *Int J Cancer* (1999) **80**(5):709–14. doi:10.1002/(SICI)1097-0215(19990301)80:5<709::AID-IJC14>3.0.CO;2-R
50. Thomas ML, Samant UC, Deshpande RK, Chiplunkar SV. Gammadelta T cells lyse autologous and allogenic oesophageal tumours: involvement of heat-shock proteins in the tumour cell lysis. *Cancer Immunol Immunother* (2000) **48**(11):653–9. doi:10.1007/s002620050014
51. Zhang H, Hu H, Jiang X, He H, Cui L, He W. Membrane HSP70: the molecule triggering gammadelta T cells in the early stage of tumorigenesis. *Immunol Invest* (2005) **34**(4):453–68. doi:10.1080/08820130500265349
52. Hirsh MI, Hashiguchi N, Chen Y, Yip L, Junger WG. Surface expression of HSP72 by LPS-stimulated neutrophils facilitates gammadeltaT cell-mediated killing. *Eur J Immunol* (2006) **36**(3):712–21. doi:10.1002/eji.200535422
53. Kotsiopriftis M, Tanner JE, Alfieri C. Heat shock protein 90 expression in Epstein-Barr virus-infected B cells promotes gammadelta T-cell proliferation in vitro. *J Virol* (2005) **79**(11):7255–61. doi:10.1128/JVI.79.11.7255-7261.2005
54. Morita CT, Beckman EM, Bukowski JF, Tanaka Y, Band H, Bloom BR, et al. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells. *Immunity* (1995) **3**(4):495–507. doi:10.1016/1074-7613(95)90178-7
55. Gogoi D, Dar AA, Chiplunkar SV. Involvement of Notch in activation and effector functions of gammadelta T cells. *J Immunol* (2014) **192**(5):2054–62. doi:10.4049/jimmunol.1300369
56. Rincon-Orozco B, Kunzmann V, Wrobel P, Kabelitz D, Steinle A, Herrmann T. Activation of V gamma 9V delta 2 T cells by NKG2D. *J Immunol* (2005) **175**(4):2144–51. doi:10.4049/jimmunol.175.4.2144
57. Nedellec S, Sabourin C, Bonneville M, Scotet E. NKG2D costimulates human V gamma 9V delta 2 T cell antitumor cytotoxicity through protein kinase C theta-dependent modulation of early TCR-induced calcium and transduction signals. *J Immunol* (2010) **185**(1):55–63. doi:10.4049/jimmunol.1000373
58. Devilder MC, Allain S, Dousset C, Bonneville M, Scotet E. Early triggering of exclusive IFN-gamma responses of human Vgamma9Vdelta2 T cells by TLR-activated myeloid and plasmacytoid dendritic cells. *J Immunol* (2009) **183**(6):3625–33. doi:10.4049/jimmunol.0901571
59. Shojaei H, Oberg HH, Juricke M, Marischen L, Kunz M, Mundhenke C, et al. Toll-like receptors 3 and 7 agonists enhance tumor cell lysis by human gammadelta T cells. *Cancer Res* (2009) **69**(22):8710–7. doi:10.1158/0008-5472.CAN-09-1602
60. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* (2002) **20**:197–216. doi:10.1146/annurev.immunol.20.083001.084359
61. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* (2009) **22**(2):240–73. doi:10.1128/CMR.00046-08
62. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* (1989) **54**(Pt 1):1–13. doi:10.1101/SQB.1989.054.01.003
63. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* (2006) **124**(4):783–801. doi:10.1016/j.cell.2006.02.015
64. Beutler BA. TLRs and innate immunity. *Blood* (2009) **113**(7):1399–407. doi:10.1182/blood-2008-07-019307

65. Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature* (2007) **449**(7164):819–26. doi:10.1038/nature06246
66. Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. *Microbes Infect* (2004) **6**(15):1382–7. doi:10.1016/j.micinf.2004.08.018
67. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the *Drosophila* toll protein signals activation of adaptive immunity. *Nature* (1997) **388**(6640):394–7. doi:10.1038/41131
68. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* (2005) **17**(1):1–14. doi:10.1093/intimm/dxh186
69. Shi Z, Cai Z, Sanchez A, Zhang T, Wen S, Wang J, et al. A novel toll-like receptor that recognizes vesicular stomatitis virus. *J Biol Chem* (2011) **286**(6):4517–24. doi:10.1074/jbc.M110.159590
70. Oldenburg M, Krüger A, Ferstl R, Kaufmann A, Nees G, Sigmund A, et al. TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. *Science* (2012) **337**(6098):1111–5. doi:10.1126/science.1220363
71. Koblansky AA, Jankovic D, Oh H, Hieny S, Sungnak W, Mathur R, et al. Recognition of profilin by toll-like receptor 12 is critical for host resistance to *Toxoplasma gondii*. *Immunity* (2013) **38**(1):119–30. doi:10.1016/j.immuni.2012.09.016
72. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to *Drosophila* toll. *Proc Natl Acad Sci U S A* (1998) **95**(2):588–93. doi:10.1073/pnas.95.2.588
73. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* (1998) **282**(5396):2085–8. doi:10.1126/science.282.5396.2085
74. Tabeta K, Georgel P, Janssen E, Du X, Hoebe K, Crozat K, et al. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc Natl Acad Sci U S A* (2004) **101**(10):3516–21. doi:10.1073/pnas.0400525101
75. Brinkmann MM, Spooner E, Hoebe K, Beutler B, Ploegh HL, Kim YM. The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. *J Cell Biol* (2007) **177**(2):265–75. doi:10.1083/jcb.200612056
76. Kim YM, Brinkmann MM, Paquet ME, Ploegh HL. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. *Nature* (2008) **452**(7184):234–8. doi:10.1038/nature06726
77. Ewald SE, Lee BL, Lau L, Wickliffe KE, Shi GP, Chapman HA, et al. The ectodomain of toll-like receptor 9 is cleaved to generate a functional receptor. *Nature* (2008) **456**(7222):658–62. doi:10.1038/nature07405
78. O'Neill LA, Golenbock D, Bowie AG. The history of toll-like receptors – redefining innate immunity. *Nat Rev Immunol* (2013) **13**(6):453–60. doi:10.1038/nri3446
79. Wesche H, Henzel WJ, Shillinglaw W, Li S, Cao Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* (1997) **7**(6):837–47. doi:10.1016/S1074-7613(00)80402-1
80. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al. Cutting edge: a novel toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the toll-like receptor signaling. *J Immunol* (2002) **169**(12):6668–72. doi:10.4049/jimmunol.169.12.6668
81. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* (2003) **4**(2):161–7. doi:10.1038/ni886
82. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* (2003) **301**(5633):640–3. doi:10.1126/science.1087262
83. Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med* (2003) **198**(7):1043–55. doi:10.1084/jem.20031023
84. Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, et al. TRAM is specifically involved in the toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol* (2003) **4**(11):1144–50. doi:10.1038/ni986
85. Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, et al. Mal (MyD88-adapter-like) is required for toll-like receptor-4 signal transduction. *Nature* (2001) **413**(6851):78–83. doi:10.1038/35092578
86. Horng T, Barton GM, Medzhitov R. TIRAP: an adapter molecule in the toll signaling pathway. *Nat Immunol* (2001) **2**(9):835–41. doi:10.1038/ni0901-835
87. Carty M, Goodbody R, Schröder M, Stack J, Moynagh PN, Bowie AG. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent toll-like receptor signaling. *Nat Immunol* (2006) **7**(10):1074–81. doi:10.1038/ni1382
88. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdörfer B, Giese T, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* (2002) **168**(9):4531–7. doi:10.4049/jimmunol.168.9.4531
89. Mansson A, Adner M, Cardell LO. Toll-like receptors in cellular subsets of human tonsil T cells: altered expression during recurrent tonsillitis. *Respir Res* (2006) **7**:36. doi:10.1186/1465-9921-7-36
90. Kabelitz D. Expression and function of toll-like receptors in T lymphocytes. *Curr Opin Immunol* (2007) **19**(1):39–45. doi:10.1016/j.coi.2006.11.007
91. Caron G, Duluc D, Frémaux I, Jeannin P, David C, Gascan H, et al. Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-gamma production by memory CD4+ T cells. *J Immunol* (2005) **175**(3):1551–7. doi:10.4049/jimmunol.175.3.1551
92. Imanishi T, Hara H, Suzuki S, Suzuki N, Akira S, Saito T. Cutting edge: TLR2 directly triggers Th1 effector functions. *J Immunol* (2007) **178**(11):6715–9. doi:10.4049/jimmunol.178.11.6715
93. Dai J, Liu B, Li Z. Regulatory T cells and toll-like receptors: what is the missing link? *Int Immunopharmacol* (2009) **9**(5):528–33. doi:10.1016/j.intimp.2009.01.027
94. Liu H, Komai-Koma M, Xu D, Liew FY. Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. *Proc Natl Acad Sci U S A* (2006) **103**(18):7048–53. doi:10.1073/pnas.0601554103
95. Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. *J Clin Invest* (2006) **116**(2):485–94. doi:10.1172/JCI25439
96. Crellin NK, Garcia RV, Hadisfar O, Allan SE, Steiner TS, Levings MK. Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells. *J Immunol* (2005) **175**(12):8051–9. doi:10.4049/jimmunol.175.12.8051
97. Nyirenda MH, Sanvito L, Darlington PJ, O'Brien K, Zhang GX, Constantinescu CS, et al. TLR2 stimulation drives human naive and effector regulatory T cells into a Th17-like phenotype with reduced suppressive function. *J Immunol* (2011) **187**(5):2278–90. doi:10.4049/jimmunol.1003715
98. Reynolds JM, Pappu BP, Peng J, Martinez GJ, Zhang Y, Chung Y, et al. Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. *Immunity* (2010) **32**(5):692–702. doi:10.1016/j.immuni.2010.04.010
99. Abdollahi-Roodsaz S, Joosten LA, Koenders MI, Devesa I, Roelofs MF, Radstake TR, et al. Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. *J Clin Invest* (2008) **118**(1):205–16. doi:10.1172/JCI32639
100. Reynolds JM, Martinez GJ, Chung Y, Dong C. Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. *Proc Natl Acad Sci U S A* (2012) **109**(32):13064–9. doi:10.1073/pnas.1120585109
101. Li J, Wang FP, She WM, Yang CQ, Li L, Tu CT, et al. Enhanced high-mobility group box 1 (HMGB1) modulates regulatory T cells (Treg)/T helper 17 (Th17) balance via toll-like receptor (TLR)-4-interleukin (IL)-6 pathway in patients with chronic hepatitis B. *J Viral Hepat* (2014) **21**(2):129–40. doi:10.1111/jvh.12152
102. Reynolds JM, Dong C. Toll-like receptor regulation of effector T lymphocyte function. *Trends Immunol* (2013) **34**(10):511–9. doi:10.1016/j.it.2013.06.003
103. Mercier BC, Cottalorda A, Coupet CA, Marvel J, Bonnefoy-Bérard N. TLR2 engagement on CD8 T cells enables generation of functional memory cells in response to a suboptimal TCR signal. *J Immunol* (2009) **182**(4):1860–7. doi:10.4049/jimmunol.0801167
104. Cottalorda A, Mercier BC, Mbitikon-Kobo FM, Arpin C, Teoh DY, McMichael A, et al. TLR2 engagement on memory CD8(+) T cells improves their cytokine-mediated proliferation and IFN-gamma secretion in the absence of Ag. *Eur J Immunol* (2009) **39**(10):2673–81. doi:10.1002/eji.200939627
105. Asprodites N, Zheng L, Geng D, Velasco-Gonzalez C, Sanchez-Perez L, Davila E. Engagement of toll-like receptor-2 on cytotoxic T-lymphocytes occurs in vivo and augments antitumor activity. *FASEB J* (2008) **22**(10):3628–37. doi:10.1096/fj.08-108274

106. Mercier BC, Ventre E, Fogeron ML, Debaud AL, Tomkowiak M, Marvel J, et al. NOD1 cooperates with TLR2 to enhance T cell receptor-mediated activation in CD8 T cells. *PLoS One* (2012) **7**(7):e42170. doi:10.1371/journal.pone.0042170
107. Mandraju R, Murray S, Forman J, Pasare C. Differential ability of surface and endosomal TLRs to induce CD8 T cell responses in vivo. *J Immunol* (2014) **192**(9):4303–15. doi:10.4049/jimmunol.1302244
108. Chalifour A, Jeannin P, Gauchat JF, Blaecke A, Malissard M, N'Guyen T, et al. Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers alpha-defensin production. *Blood* (2004) **104**(6):1778–83. doi:10.1182/blood-2003-08-2820
109. Sivori S, Falco M, Della Chiesa M, Carlomagno S, Vitale M, Moretta L, et al. CpG and double-stranded RNA trigger human NK cells by toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc Natl Acad Sci U S A* (2004) **101**(27):10116–21. doi:10.1073/pnas.0403744101
110. Hart OM, Athie-Morales V, O'Connor GM, Gardiner CM. TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFN-gamma production. *J Immunol* (2005) **175**(3):1636–42. doi:10.4049/jimmunol.175.3.1636
111. Souza-Fonseca-Guimaraes F, Adib-Conquy M, Cavaillon JM. Natural killer (NK) cells in antibacterial innate immunity: angels or devils? *Mol Med* (2012) **18**:270–85. doi:10.2119/molmed.2011.00201
112. Marcenaro E, Ferranti B, Falco M, Moretta L, Moretta A. Human NK cells directly recognize *Mycobacterium bovis* via TLR2 and acquire the ability to kill monocyte-derived DC. *Int Immunol* (2008) **20**(9):1155–67. doi:10.1093/intimm/dxn073
113. Bellora F, Castriconi R, Dondero A, Pessino A, Nencioni A, Liggieri G, et al. TLR activation of tumor-associated macrophages from ovarian cancer patients triggers cytolytic activity of NK cells. *Eur J Immunol* (2014) **44**(6):1814–22. doi:10.1002/eji.201344130
114. Kim M, Osborne NR, Zeng W, Donaghy H, McKinnon K, Jackson DC, et al. Herpes simplex virus antigens directly activate NK cells via TLR2, thus facilitating their presentation to CD4 T lymphocytes. *J Immunol* (2012) **188**(9):4158–70. doi:10.4049/jimmunol.1103450
115. Lu H, Dietsch GN, Matthews MA, Yang Y, Ghanekar S, Inokuma M, et al. VTX-2337 is a novel TLR8 agonist that activates NK cells and augments ADCC. *Clin Cancer Res* (2012) **18**(2):499–509. doi:10.1158/1078-0432.CCR-11-1625
116. Mokuno Y, Matsuguchi T, Takano M, Nishimura H, Washizu J, Ogawa T, et al. Expression of toll-like receptor 2 on gamma delta T cells bearing invariant V gamma 6/V delta 1 induced by Escherichia coli infection in mice. *J Immunol* (2000) **165**(2):931–40. doi:10.4049/jimmunol.165.2.931
117. Deetz CO, Hebbeler AM, Propp NA, Cairo C, Tikhonov I, Pauza CD. Gamma interferon secretion by human Vgamma2Vdelta2 T cells after stimulation with antibody against the T-cell receptor plus the toll-like receptor 2 agonist Pam3Cys. *Infect Immun* (2006) **74**(8):4505–11. doi:10.1128/IAI.00088-06
118. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* (2009) **31**(2):321–30. doi:10.1016/j.immuni.2009.06.020
119. Schwacha MG, Daniel T. Up-regulation of cell surface toll-like receptors on circulating gammadelta T-cells following burn injury. *Cytokine* (2008) **44**(3):328–34. doi:10.1016/j.cyto.2008.09.001
120. Schwacha MG, Rani M, Zhang Q, Nunez-Cantu O, Cap AP. Mitochondrial damage-associated molecular patterns activate gammadelta T-cells. *Innate Immun* (2014) **20**(3):261–8. doi:10.1177/1753425913488969
121. Hedges JF, Lubick KJ, Jutila MA. Gamma delta T cells respond directly to pathogen-associated molecular patterns. *J Immunol* (2005) **174**(10):6045–53. doi:10.4049/jimmunol.174.10.6045
122. Wesch D, Beetz S, Oberg HH, Marget M, Krengel K, Kabelitz D. Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes. *J Immunol* (2006) **176**(3):1348–54. doi:10.4049/jimmunol.176.3.1348
123. Zhang SY, Jouanguy E, Ugolini S, Smahi A, Elain G, Romero P, et al. TLR3 deficiency in patients with herpes simplex encephalitis. *Science* (2007) **317**(5844):1522–7. doi:10.1126/science.1139522
124. Pietschmann K, Beetz S, Welte S, Martens I, Gruen J, Oberg HH, et al. Toll-like receptor expression and function in subsets of human gammadelta T lymphocytes. *Scand J Immunol* (2009) **70**(3):245–55. doi:10.1111/j.1365-3083.2009.02290.x
125. Paleja B, Anand A, Chaukar D, D'Cruz A, Chiplunkar S. Decreased functional response to toll like receptor ligands in patients with oral cancer. *Hum Immunol* (2013) **74**(8):927–36. doi:10.1016/j.humimm.2013.04.018
126. Shimura H, Nitahara A, Ito A, Tomiyama K, Ito M, Kawai K. Up-regulation of cell surface toll-like receptor 4-MD2 expression on dendritic epidermal T cells after the emigration from epidermis during cutaneous inflammation. *J Dermatol Sci* (2005) **37**(2):101–10. doi:10.1016/j.jdermsci.2004.11.006
127. Cairns B, Maile R, Barnes CM, Frelinger JA, Meyer AA. Increased toll-like receptor 4 expression on T cells may be a mechanism for enhanced T cell response late after burn injury. *J Trauma* (2006) **61**(2):293–8; discussion 298–9. doi:10.1097/01.ta.0000228969.46633.bb
128. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* (1999) **274**(16):10689–92. doi:10.1074/jbc.274.16.10689
129. Yang RB, Mark MR, Gray A, Huang A, Xie MH, Zhang M, et al. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* (1998) **395**(6699):284–8. doi:10.1038/26239
130. Cui Y, Kang L, Cui L, He W. Human gammadelta T cell recognition of lipid A is predominately presented by CD1b or CD1c on dendritic cells. *Biol Direct* (2009) **4**:47. doi:10.1186/1745-6150-4-47
131. Wang X, Sun R, Wei H, Tian Z. High-mobility group box 1 (HMGB1)-toll-like receptor (TLR)4-interleukin (IL)-23-IL-17A axis in drug-induced damage-associated lethal hepatitis: interaction of gammadelta T cells with macrophages. *Hepatology* (2013) **57**(1):373–84. doi:10.1002/hep.25982
132. Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang RF. Tumor-infiltrating gammadelta T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway. *Immunity* (2007) **27**(2):334–48. doi:10.1016/j.immuni.2007.05.020
133. Choudhary A, Davodeau F, Moreau A, Peyrat MA, Bonneville M, Jotereau F. Selective lysis of autologous tumor cells by recurrent gamma delta tumor-infiltrating lymphocytes from renal carcinoma. *J Immunol* (1995) **154**(8):3932–40.
134. Peng G, Guo Z, Kiniwa Y, Voo KS, Peng W, Fu T, et al. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. *Science* (2005) **309**(5739):1380–4. doi:10.1126/science.1113401
135. Ye J, Ma C, Hsueh EC, Eickhoff CS, Zhang Y, Varvares MA, et al. Tumor-derived gammadelta regulatory T cells suppress innate and adaptive immunity through the induction of immunosenescence. *J Immunol* (2013) **190**(5):2403–14. doi:10.4049/jimmunol.1202369
136. Ismaili J, Olislagers V, Poupot R, Fournié JJ, Goldman M. Human gamma delta T cells induce dendritic cell maturation. *Clin Immunol* (2002) **103**(3 Pt 1):296–302. doi:10.1006/clim.2002.5218
137. Conti L, Casetti R, Cardone M, Varano B, Martino A, Belardelli F, et al. Reciprocal activating interaction between dendritic cells and pamidronate-stimulated gammadelta T cells: role of CD86 and inflammatory cytokines. *J Immunol* (2005) **174**(1):252–60. doi:10.4049/jimmunol.174.1.252
138. Leslie DS, Vincent MS, Spada FM, Das H, Sugita M, Morita CT, et al. CD1-mediated gamma/delta T cell maturation of dendritic cells. *J Exp Med* (2002) **196**(12):1575–84. doi:10.1084/jem.20021515
139. Shrestha N, Ida JA, Lubinski AS, Pallin M, Kaplan G, Haslett PA. Regulation of acquired immunity by gamma delta T-cell/dendritic-cell interactions. *Ann N Y Acad Sci* (2005) **1062**:79–94. doi:10.1196/annals.1358.011
140. Fang H, Welte T, Zheng X, Chang GJ, Holbrook MR, Soong L, et al. Gammadelta T cells promote the maturation of dendritic cells during West Nile virus infection. *FEMS Immunol Med Microbiol* (2010) **59**(1):71–80. doi:10.1111/j.1574-695X.2010.00663.x
141. Fowler DW, Copier J, Dalgleish AG, Bodman-Smith MD. Tripartite immune cell co-operation in the bacillus Calmette Guerin-induced activation of gammadelta T cells. *Immunol Cell Biol* (2013) **91**(7):461–8. doi:10.1038/icb.2013.30
142. Dhar S, Chiplunkar SV. Lysis of aminobisphosphonate-sensitized MCF-7 breast tumor cells by Vgamma9Vdelta2 T cells. *Cancer Immun* (2010) **10**:10.
143. D'Asaro M, La Mendola C, Di Liberto D, Orlando V, Todaro M, Spina M, et al. V gamma 9V delta 2 T lymphocytes efficiently recognize and kill zoledronate-sensitized, imatinib-sensitive, and imatinib-resistant chronic myelogenous leukemia cells. *J Immunol* (2010) **184**(6):3260–8. doi:10.4049/jimmunol.0903454
144. Chargui J, Combaret V, Scaglione V, Iacono I, Péri V, Valteau-Couanet D, et al. Bromohydrin pyrophosphate-stimulated Vgamma9delta2 T cells expanded

ex vivo from patients with poor-prognosis neuroblastoma lyse autologous primary tumor cells. *J Immunother* (2010) **33**(6):591–8. doi:10.1097/CJI.0b013e3181dda207
145. Gogoi D, Chiplunkar SV. Targeting gamma delta T cells for cancer immunotherapy: bench to bedside. *Indian J Med Res* (2013) **138**(5):755–61.
146. Weeratna RD, Makinen SR, McCluskie MJ, Davis HL. TLR agonists as vaccine adjuvants: comparison of CpG ODN and resiquimod (R-848). *Vaccine* (2005) **23**(45):5263–70. doi:10.1016/j.vaccine.2005.06.024
147. Kalyan S, Wesch D, Kabelitz D. Aminobisphosphonates and toll-like receptor ligands: recruiting Vgamma9Vdelta2 T cells for the treatment of hematologic malignancy. *Curr Med Chem* (2011) **18**(34):5206–16. doi:10.2174/092986711798184280
148. Paulos CM, Kaiser A, Wrzesinski C, Hinrichs CS, Cassard L, Boni A, et al. Toll-like receptors in tumor immunotherapy. *Clin Cancer Res* (2007) **13**(18 Pt 1):5280–9. doi:10.1158/1078-0432.CCR-07-1378
149. Smits EL, Ponsaerts P, Berneman ZN, Van Tendeloo VF. The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy. *Oncologist* (2008) **13**(8):859–75. doi:10.1634/theoncologist.2008-0097
150. Yu L, Chen S. Toll-like receptors expressed in tumor cells: targets for therapy. *Cancer Immunol Immunother* (2008) **57**(9):1271–8. doi:10.1007/s00262-008-0459-8
151. Conroy H, Marshall NA, Mills KH. TLR ligand suppression or enhancement of Treg cells? A double-edged sword in immunity to tumours. *Oncogene* (2008) **27**(2):168–80. doi:10.1038/sj.onc.1210910
152. Ridnour LA, Cheng RY, Switzer CH, Heinecke JL, Ambs S, Glynn S, et al. Molecular pathways: toll-like receptors in the tumor microenvironment – poor prognosis or new therapeutic opportunity. *Clin Cancer Res* (2013) **19**(6):1340–6. doi:10.1158/1078-0432.CCR-12-0408
153. Inatsuka C, Yang Y, Gad E, Rastetter L, Disis ML, Lu H. Gamma delta T cells are activated by polysaccharide K (PSK) and contribute to the anti-tumor effect of PSK. *Cancer Immunol Immunother* (2013) **62**(8):1335–45. doi:10.1007/s00262-013-1436-4
154. Hiromatsu K, Yoshikai Y, Matsuzaki G, Ohga S, Muramori K, Matsumoto K, et al. A protective role of gamma/delta T cells in primary infection with *Listeria monocytogenes* in mice. *J Exp Med* (1992) **175**(1):49–56. doi:10.1084/jem.175.1.49
155. Hara T, Mizuno Y, Takaki K, Takada H, Akeda H, Aoki T, et al. Predominant activation and expansion of V gamma 9-bearing gamma delta T cells in vivo as well as in vitro in *Salmonella* infection. *J Clin Invest* (1992) **90**(1):204–10. doi:10.1172/JCI115837
156. Kühl AA, Pawlowski NN, Grollich K, Loddenkemper C, Zeitz M, Hoffmann JC. Aggravation of intestinal inflammation by depletion/deficiency of gammadelta T cells in different types of IBD animal models. *J Leukoc Biol* (2007) **81**(1):168–75. doi:10.1189/jlb.1105696
157. Poccia F, Agrati C, Martini F, Capobianchi MR, Wallace M, Malkovsky M. Antiviral reactivities of gammadelta T cells. *Microbes Infect* (2005) **7**(3):518–28. doi:10.1016/j.micinf.2004.12.009
158. Inoue T, Yoshikai Y, Matsuzaki G, Nomoto K. Early appearing gamma/delta-bearing T cells during infection with Calmette Guerin bacillus. *J Immunol* (1991) **146**(8):2754–62.
159. Beetz S, Wesch D, Marischen L, Welte S, Oberg HH, Kabelitz D. Innate immune functions of human gammadelta T cells. *Immunobiology* (2008) **213**(3–4):173–82. doi:10.1016/j.imbio.2007.10.006
160. Zheng J, Liu Y, Lau YL, Tu W. Gammadelta-T cells: an unpolished sword in human anti-infection immunity. *Cell Mol Immunol* (2013) **10**(1):50–7. doi:10.1038/cmi.2012.43
161. Ribot JC, Chaves-Ferreira M, d'Orey F, Wencker M, Gonçalves-Sousa N, Decalf J, et al. Cutting edge: adaptive versus innate receptor signals selectively control the pool sizes of murine IFN-gamma- or IL-17-producing gammadelta T cells upon infection. *J Immunol* (2010) **185**(11):6421–5. doi:10.4049/jimmunol.1002283
162. Born WK, Zhang L, Nakayama M, Jin N, Chain JL, Huang Y, et al. Peptide antigens for gamma/delta T cells. *Cell Mol Life Sci* (2011) **68**(14):2335–43. doi:10.1007/s00018-011-0697-3
163. Nedellec S, Bonneville M, Scotet E. Human Vgamma9Vdelta2 T cells: from signals to functions. *Semin Immunol* (2010) **22**(4):199–206. doi:10.1016/j.smim.2010.04.004
164. Qin G, Mao H, Zheng J, Sia SF, Liu Y, Chan PL, et al. Phosphoantigen-expanded human gammadelta T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses. *J Infect Dis* (2009) **200**(6):858–65. doi:10.1086/605413
165. Goldinger SM, Dummer R, Baumgaertner P, Mihic-Probst D, Schwarz K, Hammann-Haenni A, et al. Nano-particle vaccination combined with TLR-7 and -9 ligands triggers memory and effector CD8(+) T-cell responses in melanoma patients. *Eur J Immunol* (2012) **42**(11):3049–61. doi:10.1002/eji.201142361

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 May 2014; accepted: 15 July 2014; published online: 31 July 2014.
Citation: Dar AA, Patil RS and Chiplunkar SV (2014) Insights into the relationship between toll like receptors and gamma delta T cell responses. Front. Immunol. 5:366. doi: 10.3389/fimmu.2014.00366
This article was submitted to Tumor Immunity, a section of the journal Frontiers in Immunology.

The Journal of Immunology

Involvement of Notch in Activation and Effector Functions of γδ T cells

Dimpu Gogoi, Asif A. Dar and Shubhada V. Chiplunkar

J Immunol 2014; 192:2054-2062; Prepublished online 31 January 2014;
doi: 10.4049/jimmunol.1300369
http://www.jimmunol.org/content/192/5/2054

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Involvement of Notch in Activation and Effector Functions of γδ T cells

Dimpu Gogoi, Asif A. Dar, and Shubhada V. Chiplunkar

Notch signaling plays a pivotal role in cell fate decision and lineage commitment of lymphocytes. Although the role of Notch in $CD4^+$ and $CD8^+$ αβ T cells is well documented, there are no reports on how Notch signaling regulates effector functions of γδ T cells. γδ T cells are a minor fraction in the peripheral blood but are known to play a major role in defense against pathogens and tumors. In this study, we show that Notch receptors (mRNA and protein) are expressed in peripheral γδ T cells. Inhibition of Notch signaling by γ-secretase inhibitor inhibited the proliferation and IFN-γ secretion of γδ T cells in response to stimulation with phosphoantigens and anti-CD3 mAb. In the presence of γ-secretase inhibitor, the antitumor cytolytic ability of γδ T cells was inhibited with a decreased CD107a expression. Knockdown of Notch1 and Notch2 genes in γδ T cells using small interfering RNA inhibited their antitumor cytotoxic potential. Our study describes for the first time, to our knowledge, the role of Notch as an additional signal contributing to Ag-specific effector functions of γδ T cells. ***The Journal of Immunology*****, 2014, 192: 2054–2062.**

Compared with αβ T cells (>90%), γδ T cells are a minor fraction of T lymphocytes in the peripheral blood (<10%). γδ T cells differ from classical αβ T cells with respect to Ag recognition, tissue localization, and the use of TCR gene repertoire (1). Vγ9Vδ2 represents the dominant subset of the peripheral blood in humans (2). γδ T lymphocytes play a major role in defense against pathogens and tumors (3–5). γδ T lymphocytes are activated by phosphoantigens-isopentyl pyrophosphate (IPP) or 4-hydroxy-3-methyl-but-2-eneyl pyrophosphate, which is produced through the mevalonate pathway in mammalian cells or nonmevalonate/rohmer pathway in non-mammalian cells, respectively (6). Aminobisphosphonates are synthetic analogs of inorganic pyrophosphates and are widely used in the treatment of skeletal disorders (7). Nitrogen-containing bisphosphonates such as risedronate and zoledronate inhibit farnesyl pyrophosphate synthase, a key enzyme of the mevalonate pathway leading to accumulation of IPP pool in the cells (8). Upon activation, γδ T cells release copious amounts of IFN-γ and TNF-α (2, 5, 9). Earlier data from our own laboratory and others have shown that γδ T cells isolated from cancer patients can mediate potent antitumor immunity (10–12). Tumor cells treated with bisphosphonate zoledronate are actively lysed by activated γδ T cells (13, 14).

The Notch signaling pathway, originally described in drosophila, controls the development and activation of a variety of immune cells (15). Notch signaling is suggested to play a role in cell fate decisions and has been implicated in γδ versus αβ lineage decisions (16, 17). The molecular events triggering T cell development (γδ versus αβ lineage) are essentially different in human and mice. In mice, it was reported that the development of γδ T cells from γδ TCR-expressing T cell progenitors requires the absence of Notch ligand interaction (16–18). In contrast, there is an opposing role for Notch signal in human αβ/γδ lineage decision. The induction of γδ-lineage precursors to split off from the αβ T cell program by Notch1 activity was observed in humans (19). It was also reported that high level of Notch activation generates T lineage precursors and γδ T cells but inhibits differentiation toward αβ lineage (20).

The role of Notch in regulating effector functions of $CD8^+$ T cells and NK cells have been described (21, 22). Signaling through the TCR in both $CD4^+$ and $CD8^+$ T cells induces the activation of Notch1 (22, 23). However, the role of Notch in regulating the effector functions of human γδ T cells has not been reported earlier.

Notch proteins are single-pass transmembrane receptors that require multiple enzymatic cleavages to produce the full-length heterodimer expressed on the cell surface. In mammals, there are four Notch receptors (Notch1-4) and five Notch ligands, three delta-like (Dll1, Dll3, and Dll4) and two Jagged (Jag1 and Jag2) (24). The interaction between Notch receptor and ligand pair cleaves the extracellular and transmembrane domain of Notch through activation of ADAM proteases and γ-secretase, respectively (25). The γ-secretase–induced cleavage generates Notch intracellular domain (NICD), which translocates to the nucleus. In the nucleus, NICD binds to cofactors like CBF-1/suppressor of hairless/Lag1, mastermind like1, and p300/CBP to create a complex that acts as a transcriptional coactivator. Notch signaling then induces the expression of target genes, for example, HES1 (hairy and enhancer of split-1), HES-related repressor protein, and so on (26).

In this report, we describe the expression of Notch receptors on human γδ T cells in the peripheral blood of healthy individuals and further demonstrate the importance of Notch pathway in Ag-specific responses of γδ T cells. Our data demonstrate that Notch pathway also regulates the cytotoxic effector functions of γδ T cells against tumor cells. These results suggest that Notch signaling can be viewed as an additional mechanism regulating antitumor effector functions of γδ T cells.

Chiplunkar Laboratory, Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre, Kharghar, Navi Mumbai, Maharashtra 410210, India

Received for publication February 5, 2013. Accepted for publication December 25, 2013.

Address correspondence and reprint requests to Prof. Shubhada V. Chiplunkar, Chiplunkar Laboratory, Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre, Kharghar, Navi Mumbai, Maharashtra 410210, India. E-mail address: schiplunkar@actrec.gov.in

Abbreviations used in this article: BrHPP, bromohydrin pyrophosphate; Dll, delta-like; GSI-X, γ-secretase inhibitor-X; HES1, hairy and enhancer of split-1; IPP, isopentyl pyrophosphate; Jag, Jagged; LAMP1, lysosome-associated membrane protein-1; NICD, Notch intracellular domain; N1ICD, Notch1 intracellular domain; PI, propidium iodide; siRNA, small interfering RNA.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1300369

Materials and Methods

γδ T cell expansion and purification

Blood samples were collected from healthy individuals. The study was approved by the institutional Ethics Committee, and written informed consent was obtained from the donor before collection of blood samples. PBMCs were isolated by differential density gradient centrifugation (Ficoll Hypaque; Sigma-Aldrich, St. Louis, MO), and γδ T cells were enriched from peripheral blood using plate-bound anti-CD3 (OKT3) mAb and rIL-2 (Peprotech, Rocky Hill, NJ). In brief, lymphocytes were cultured with RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum plus rIL-2 (100 U/ml), 2 mM glutamine, and antibiotics. Five milliliters of cell suspension (1×10^6/ml) was added to 25-cm^2 culture flasks (Nunc, Roskilde, Denmark) precoated with 1 mg/ml anti-CD3 mAb, as described in earlier study (27). Cells were incubated at 37˚C and fed daily with 1 ml growth medium containing 100 U/ml rIL-2. On the fifth day, cells were transferred to 75-cm^2 culture flask containing10 ml growth medium containing 500 U/ml IL-2. Cells were then subcultured after every 2 d with the addition of fresh growth medium until day 12. γδ T cells were purified from the expanded PBMCs using MicroBeads (Miltenyi Biotec, Bergish Gladbach, Germany). The separation procedure was conducted according to the manufacturer's instructions. The purity of separated cells was >95% as determined by flow cytometry (BD Biosciences, San Jose, CA).

Quantitative RT-PCR

RNA was extracted from immunomagnetically purified γδ T cells using TRIzol reagent (Invitrogen Life Technologies, Grand Island NY) in accordance with the company's instructions. Quantitative RT-PCR for different Notch receptor isoforms, ligands, and its target genes was performed with PRISM 7700 (PE Applied Biosystems, Foster City, CA). Samples were analyzed using TaqMan primer sets purchased from Applied Biosystems (NOTCH1 Hs01062011_m1, NOTCH2 Hs01050719_m1, NOTCH3 Hs01128541_m1, NOTCH4 Hs00270200_m1, DLL1 Hs00194509_m1, DLL3 Hs00213561_m1, DLL4 Hs00184092_m1, JAG1 Hs01070036_m1, JAG2 Hs00171432_m1, HES1 Hs00172878_m1, NF-κB Hs00765730_m1, ACTB [β-actin] Hs99999903_m1). All values were normalized to the expression of the housekeeping gene β-actin.

Western blotting

A total of 1×10^6 γδ T cells was incubated with rIL-2 (100 U/ml; Peprotech) and bromohydrin pyrophosphate (BrHPP/IPH1101), which was kindly provided by Innate Pharma (Marseille, France) at a concentration of 200 nM for 24 h. These cells were pretreated for 30 min at 37˚C with γ-secretase inhibitor-X, L-685,458 (GSI-X) (Calbiochem, La Jolla, CA) at a concentration of 15 μM, or left untreated, before stimulation. The expression of Notch1 intracellular domain (N1ICD) and c-Myc were analyzed by Western blot analysis. Whole-cell lysates (1×10^6 cells) were prepared in SDS sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue), vortexed to reduce sample viscosity, denatured by boiling, and then cooled on ice. Samples were resolved on 8% SDS-PAGE gels, transferred onto Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The primary Abs to N1ICD (R&D Systems, Minneapolis, MN), c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma-Aldrich) as loading control were added at 1:5000, 1:1000 and 1:1000 dilution, respectively. Immunostaining was performed using appropriate secondary Ab at a dilution of 1:1000 and developed with ECL plus Western blot detection system (Amersham Pharmacia).

Flow cytometry

Purified γδ T cells were rested overnight at 37˚C. Next day, these cells were rinsed in cold PBS and cold-fixed in 1% paraformaldehyde in PBS for 10 min at 4˚C. The cells were washed and permeabilized for 15 min with 0.1% saponin in PBS. Cells were stained with allophycocyanin-labeled mouse anti-human γδ TCR Ab (BD Bioscience, San Diego, CA), sheep anti-human N1ICD Ab, or goat anti-human Notch2 intracellular domain Ab (R&D Systems) for 45 min at 4˚C. Thereafter, cells were washed and incubated with FITC-labeled donkey anti-sheep IgG or FITC-labeled rabbit anti-goat IgG, respectively, for another 45 min at 4˚C. For cell-surface markers, nonpermeabilized cells were stained with labeled Abs for CD14-PerCP, CD15-Pacific Blue, CD19-PE, CD33-PECF594, and CD56-FITC (BD Biosciences, San Diego, CA). γδ T cells were also stained with rabbit anti-human Dll1 and Jag1 ligands (Calbiochem) for 45 min at 4˚C. Thereafter, cells were washed and incubated with FITC-labeled goat anti-rabbit IgG (Sigma Aldrich) for another 45 min at 4˚C. Appropriate isotype controls were used. The Annexin propidium iodide (PI) staining was performed to determine the effect of GSI-X on the cell viability. In brief, γδ T cells were left untreated or were stimulated with rIL-2 alone or rIL-2 and BrHPP for 48 h at 37˚C in round-bottomed, 96-well plates (Nunc). GSI-X was added as described earlier. Cells were then harvested, suspended in binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.25 mM $CaCl_2$), and incubated with PI and FITC-conjugated Annexin V (BD Biosciences) in dark for 15 min at room temperature. After incubation, 400 μl binding buffer was added and cells were analyzed. For analyzing cell-surface expression of activation markers, γδ T cells were left untreated or treated with rIL-2 and BrHPP for 24 h with GSI-X or left untreated as previously described. Cells were then incubated with FITC-conjugated CD69 or PE-conjugated CD25 (BD Biosciences) for 45 min in dark and subsequently washed with FACS buffer (0.01 M PBS pH 7.4, 1% FCS, 0.01% sodium azide), fixed with 1% paraformaldehyde, and the intensity of fluorescence was measured using flow cytometer (FACSAria; BD Biosciences).

For degranulation assay, purified γδ T cells were incubated alone or with rIL-2 (0.1 U; Peprotech) overnight at 37˚C in round-bottom, 96-well plates (Nunc) and were taken as effector cells. The target cells were oral cancer cell line, AW13516 (28), and were treated for 18 h with zoledronate (100 μM; Panacea Biotech, New Delhi, India). Cells were cocultured at an E/T ratio of 4:1 in the presence of monensin (5 μg/ml; Sigma-Aldrich). Anti–CD107a-PE Ab (BD Biosciences) was added at the start of coculture assay. After 4 h, cells were washed and γδ T cells were then stained using anti-human TCR-γδ FITC Ab (BD Biosciences), and were acquired and analyzed on flow cytometer for the expression of CD107a on γδ T cells.

For the above experiments, γδ T cells were gated on the basis of their forward and side scatter characteristics and the fluorescence intensity was measured. Cells were analyzed using FlowJo software (Tree Star, Ashland, OR).

Proliferation assays and cytokine ELISA

Proliferation of γδ T cells was assayed by [^{3}H]thymidine uptake assay. A total of 5×10^4 γδ T cells was pretreated for 30 min at 37˚C with GSI-X (Calbiochem, La Jolla, CA) at different concentration ranging from 2.5 to 15 μM, or left untreated, before cells were stimulated in round-bottom, 96-well tissue culture plates with rIL-2 (0.1 IU/ml; Peprotech) and plate-bound anti-CD3 mAb (1 μg/well; BD Biosciences) for 72 h.

Similarly, γδ T cells were also incubated in round-bottom, 96-well tissue culture plates with rIL-2 (0.1 IU/ml; Peprotech) plus BrHPP (200 nM; Innate Pharma) or IPP (40 μM; Sigma-Aldrich) or 20 nM c-HDMAPP (IPH1201/picostim; Innate Pharma) for 72 h. For experiments using GSI-X, cells were pretreated with 15 μM GSI-X. The cultures were pulsed with 1 μCi [^{3}H]thymidine (Board of Radiation and Isotope Technology, Mumbai, India) during the last 18 h of the assay. The radioactivity incorporated in the DNA was measured in a liquid beta scintillation counter (Packard, Meriden, CT).

For cytokine ELISA, γδ T cells were stimulated with anti-CD3 mAb with or without GSI-X as described earlier. Likewise, γδ T cells were treated with different phosphoantigens (IPP, BrHPP, and c-HDMAPP). After 24 h, supernatants were collected, and IFN-γ concentration was assayed with an ELISA-based assay using anti–IFN-γ purchased from BD Biosciences.

Cytotoxicity assay

[^{51}Cr] release assay was used to measure the cytotoxicity of γδ T cells against oral cancer cell line (AW13516) as target cells. γδ T cells were left alone or treated with rIL-2 (0.1 U; Peprotech) overnight at 37˚C, and AW13516 cells were treated for 18 h with zoledronate (100 μM; Panacea Biotech). For experiments using GSI-X, cells were pretreated as described earlier. Standard 4 h [^{51}Cr] release assay was performed as previously described (13). AW13516 cells were labeled with [^{51}Cr] for 90 min at 37˚C. Labeled target cells (AW13516) were incubated with effector cells (γδ T cells) at 40:1 E/T ratio at 37˚C in 5% CO_2 for 4 h. After incubation, plates were centrifuged, supernatants were collected, and radioactive chromium release was measured using 1470 Wallac automated gamma counter (Perkin-Elmer, Downers Grove, IL). Spontaneous release was determined by incubating the target cells with medium alone, and maximum release was determined by incubating target cells with 10% Triton X-100. The percent specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Small interfering RNA

γδ T cells isolated by MACS column (as described earlier) were transfected with small interfering RNA (siRNA) specific for NOTCH1, NOTCH2

genes, and fluorescent oligonucleotide SiGLO (transfection indicator; Thermo Fisher Scientific, Waltham, MA). siRNA oligos were transfected at a concentration of 50 nM using X-tremeGENE HP transfection reagent (Roche Diagnostics, Indianapolis, IN). The inhibition of Notch1 and Notch2 expression were assessed at 48 h.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0. The Student *t* test was used as the test of significance.

Results

Expression of Notch receptors and ligands in human peripheral blood γδ T cells

The mRNA expression of Notch genes (Notch1-4) and its ligands Dll1, Dll3, and Dll4 and Jag1 and Jag2 were quantitated in ex vivo expanded and purified γδ T cells. The mRNA expression of Notch2 gene was higher than Notch1 (Fig. 1A). Relatively very low expression of Dll1 and Jag1 mRNA was observed. mRNA of Notch3, Notch4, Dll3, Dll4, and Jag2 was not detected in γδ T cells. After Notch activation, NICD enters the nucleus and then regulates the expression of target genes (25). Flow cytometry showed that Notch receptors (Notch1 and Notch2) are dominantly expressed on γδ T cells (Fig. 1B), although a low-level expression of ligands Dll1 and Jag1 are observed. The purity of isolated γδ T cells was analyzed by flow cytometry (95 ± 5%) and further, the contamination of non–T cell was ruled out by staining isolated γδ T cells for CD14, CD15, CD19, CD33, and CD56 markers (Fig. 1C). Cells were positive for CD56, which is a marker expressed by γδ T cells. The expression of NICD in γδ T cells further confirmed that Notch signaling may be active in γδ T cells.

Disruption of Notch signaling in activated γδ T cells reduces expression of Notch receptor and target genes

The release of NICD mediated by γ-secretase activity is required by all Notch receptors (Notch1-4) to initiate downstream signaling (29). In this study, we used GSI-X to block γ-secretase activity in γδ T cells (30). γδ T cells were stimulated with rIL-2 and BrHPP in the presence and absence of GSI-X. Stimulation of γδ T cells with BrHPP and rIL-2 triggered the activation of Notch signaling, which can be observed by abundant release of N1ICD by Western blot analysis (Fig. 2A). Treatment of BrHPP and rIL-2 activated γδ T cells with GSI-X decreased release of processed Notch1 (decreased NICD expression) compared with γδ T cells stimulated with BrHPP and rIL-2 alone (Fig. 2A). Stimulation of the Notch signaling pathway leads to the induction of c-Myc expression (31). Finally, we assessed whether activation of γδ T cells with BrHPP and rIL-2 was necessary for the Notch-mediated induction of cell-cycle regulator, c-Myc. We observed that Notch1 activation governs the downstream induction of c-Myc expression, which was abrogated upon GSI-X treatment (Fig. 2A). Simultaneously, we also monitored expression of mRNA for Notch receptors (1–4) and Notch ligands (Dll1, Dll3, Dll4, Jag1, and Jag2) in γδ T cells stimulated with BrHPP and rIL-2 in the presence and absence of GSI-X (Fig. 2B). After treatment with

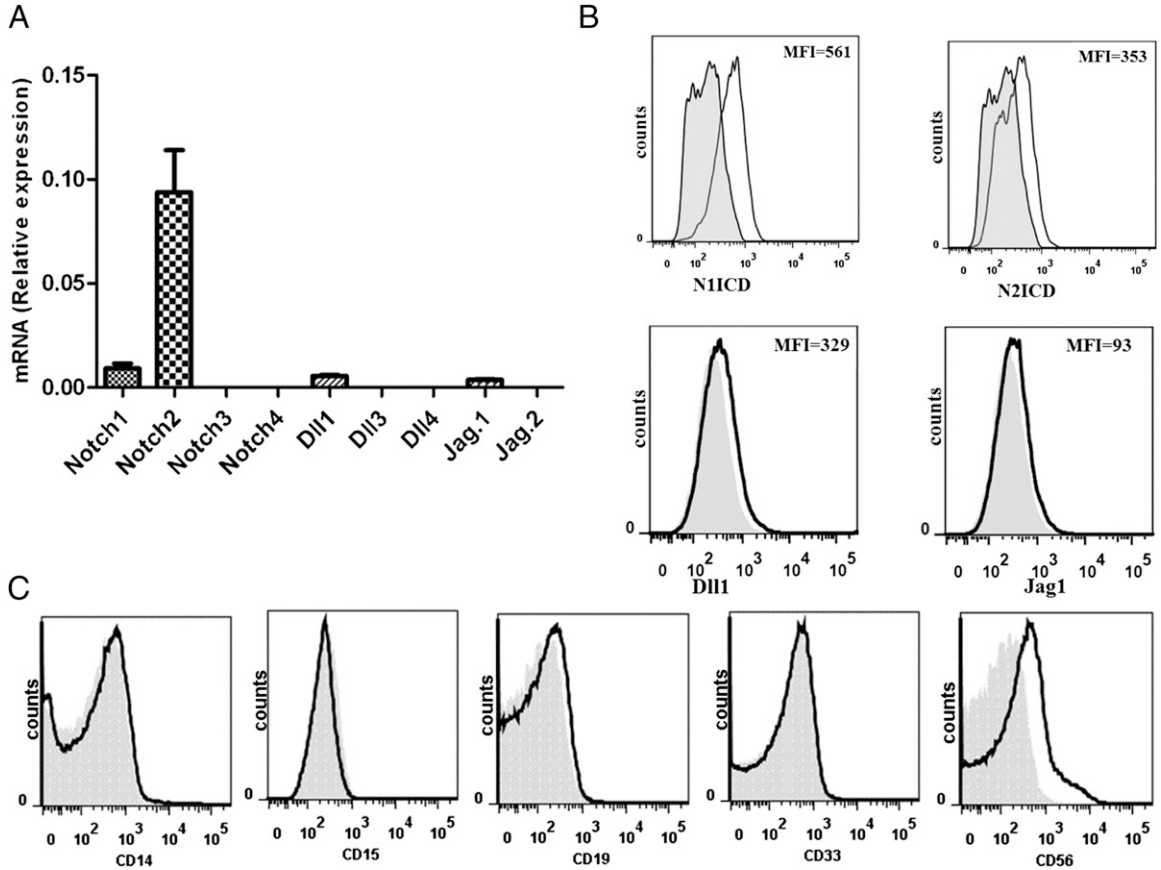

FIGURE 1. Notch receptors are expressed on peripheral γδ T cells. γδ T cells were MACS purified from the peripheral blood. (**A**) Real-time quantitative PCR showed mRNA expression of Notch1, Notch2, Dll1, and Jag1 on γδ T cells. Data were normalized to expression of GAPDH. Data represent mean of four independent experiments. (**B**) Expression of N1ICD, Notch2 intracellular domain, Dll1, and Jag1 on γδ T cells. The MFI in each case has been corrected for the MFI of the isotype control. (**C**) Purified γδ T cells were negative for CD14, CD15, CD19, and CD33 receptors, and were positive for CD56 marker. Filled histogram indicates the isotype control.

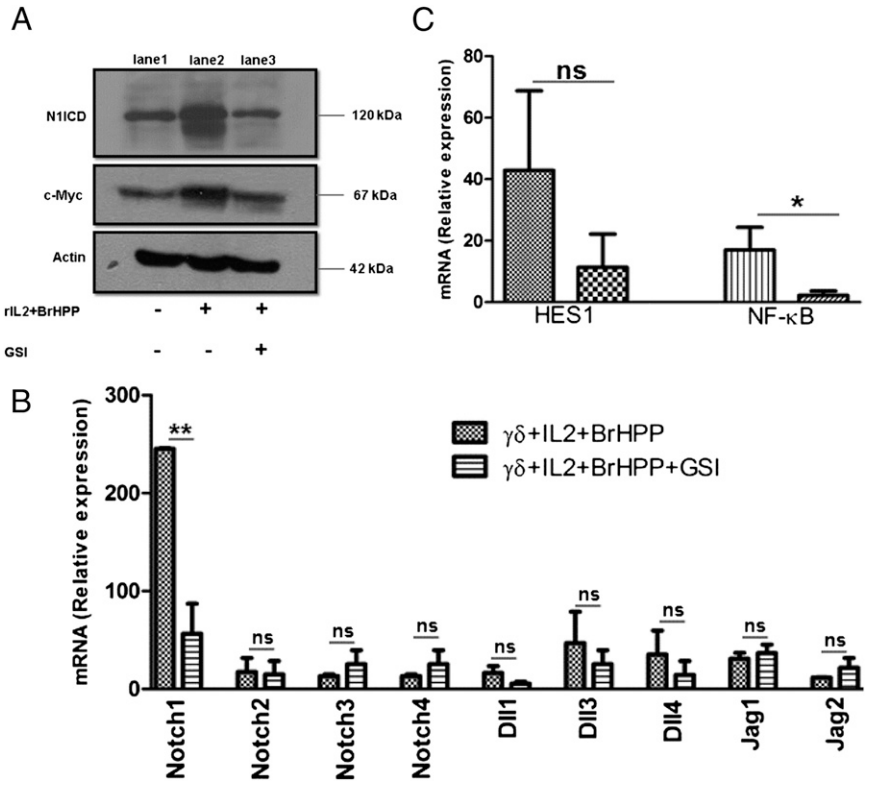

FIGURE 2. GSI-X treatment leads to decreased expression of Notch receptors and its target genes in Ag-activated γδ T cells. (**A**) Western blotting for detection of 120-kDa NICD and 67-kDa c-Myc in unstimulated (*lane 1*), BrHPP, and rIL-2–activated (24 h) γδ T cells in the presence (*lane 3*) or absence of GSI-X (*lane 2*), using Abs that recognize the cleaved active form of Notch1 (N1ICD) and c-Myc. (**B**) Real-time PCR was performed for all the notch receptor isoforms (Notch1-4) and its ligands (Dll1, Dll3, and Dll4; Jag1 and Jag2) on γδ T cells. (**C**) γδ T cells were analyzed for both canonical (HES1) and noncanonical (NF-κB) target gene expression. In both (B) and (C), total RNA was extracted from BrHPP and IL-2–stimulated γδ T cells (4 h) with or without GSI-X treatment. The expression of specific mRNA is relative to GAPDH and is normalized to that same ratio in unstimulated cells. The Student *t* test was used as the test of significance (*$p < 0.05$, **$p < 0.005$).

GSI-X, a marked decrease in the expression of mRNA for Notch1 receptor was observed in Ag-activated γδ T cells (Fig. 2B).

In addition to the well-known canonical Notch signaling pathway, evidence has emerged that noncanonical Notch signaling may also play an important role in T cells (32). We therefore investigated the expression of two target genes Hes1 and NF-κB in γδ T cells, which are representatives of canonical and noncanonical Notch signaling pathway, respectively. Using real-time PCR, we demonstrated that relative expression of mRNA for Hes1 and NF-κB were reduced in BrHPP and rIL-2 activated γδ T cells upon treatment with GSI-X (Fig. 2C).

Inhibition of Notch signaling blocks γδ T cell activation and proliferation

To determine the functional consequences of Notch signal in activated γδ T cells, we assessed γδ TCR-mediated proliferation response by [^{3}H]thymidine incorporation assay. Purified γδ T cells were pretreated with different concentrations of GSI-X (15, 10, 5, and 2.5 μM) or left untreated, before cells were activated with plate-bound anti-CD3 mAb and rIL-2. A marked increase in the proliferation of γδ T cells was observed in the presence of anti-CD3 mAb and rIL-2. However, blocking of Notch signal by GSI-X leads to decreased proliferative response

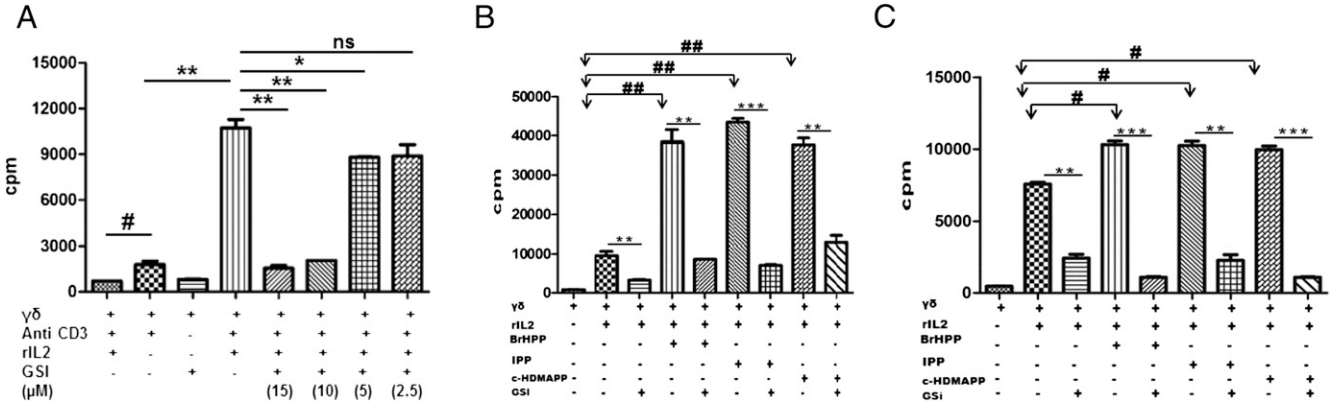

FIGURE 3. Inhibition of Ag-driven proliferation of γδ T cells by GSI-X. (**A**) Ex vivo–expanded γδ T cells were stimulated with anti-CD3 mAb in the presence of rIL-2, for 72 h, with or without GSI-X treatment. Addition of GSI-X leads to decrease in the proliferative response of γδ T cells in a concentration-dependent manner (15, 10, 5, and 2.5 μM). (**B**) Freshly isolated γδ T cells were stimulated with phosphoantigens (BrHPP, IPP, and c-HDMAPP), for 72 h, in the presence of rIL-2 with or without GSI-X treatment (**C**) Ex vivo–expanded γδ T cells were stimulated with BrHPP, IPP, and c-HDMAPP, for 72 h, in the presence of rIL-2 with or without GSI-X treatment. Addition of GSI-X leads to decrease in the proliferative response of γδ T cells. Proliferation was determined by [^{3}H]thymidine incorporation assay. Results represent the mean ± SE of cpm of three replicates studied in each experiment. Data shown are representative figures from three independent experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, compared with cpm of the cells without GSI-X treatment. $^{\#}p < 0.05$, $^{\#\#}p < 0.005$, compared cpm of cells treated with rIL-2 alone.

in a concentration-dependent manner (Fig. 3A). We also used freshly isolated, as well as ex vivo–stimulated and expanded, γδ T cells to compare their ability to respond to phosphoantigens in the presence and absence of GSI-X (15 μM). We observed a significantly increased proliferative response of γδ T cells to phosphoantigens (BrHPP, IPP, and c-HDMAPP) in the presence of rIL-2 (Fig. 3B, 3C). Freshly isolated γδ T cells showed robust proliferative responses to Ags compared with ex vivo–expanded γδ T cells. However, in the presence of GSI-X, the proliferative responses of γδ T cells to Ags were significantly reduced in both sets of isolated γδ T cells (Fig. 3B, 3C).

The expression of early and late activation markers, CD69 and CD25, respectively, were analyzed on unstimulated and Ag-stimulated

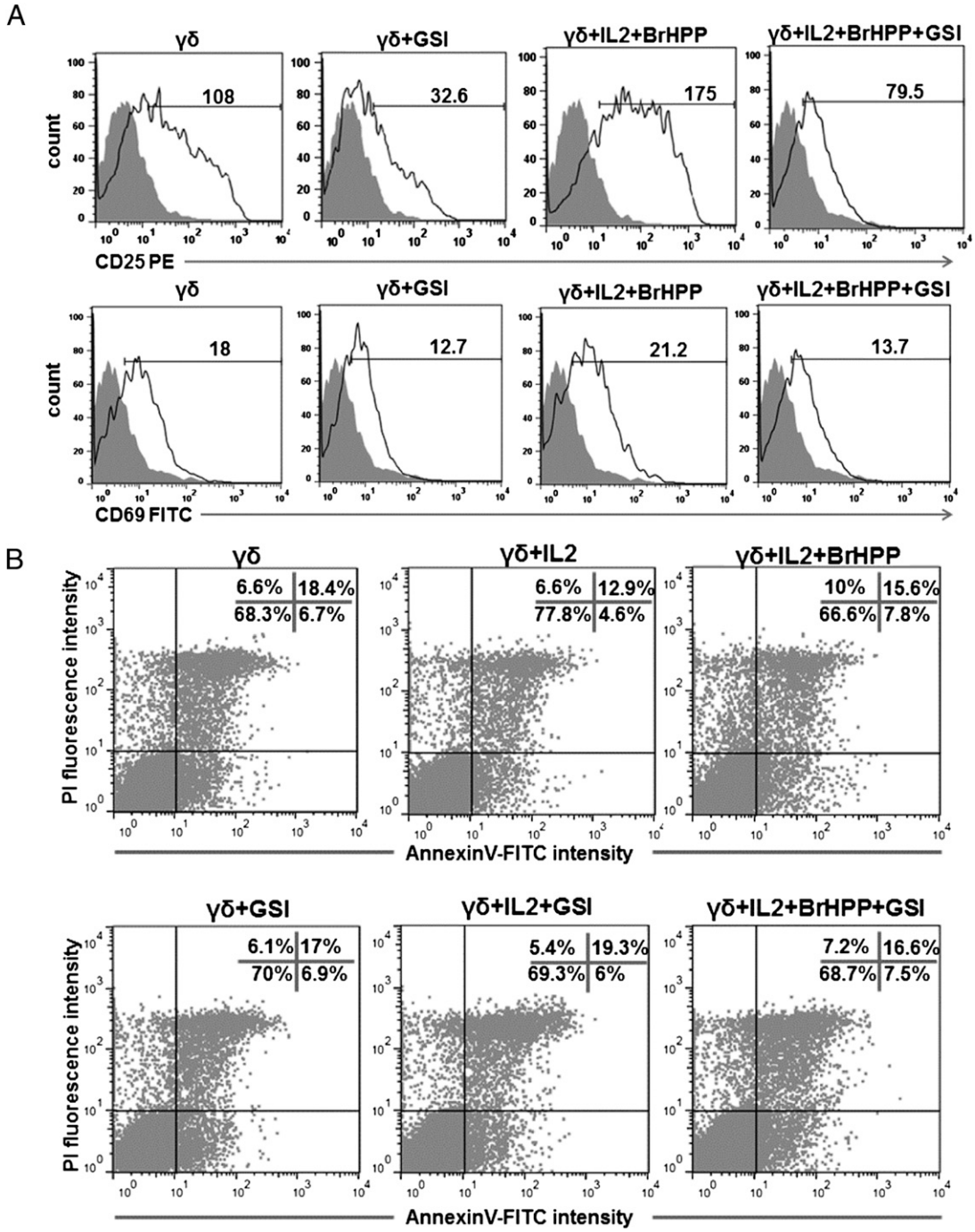

FIGURE 4. GSI-X inhibits the expression of activation markers on γδ T cells but does not induce apoptotic or necrotic cell death in γδ T cells. (**A**) The effect of GSI-X on cell-surface expression of late (CD25) and early (CD69) activation markers on unstimulated and BrHPP-stimulated (24 h) γδ T cells was analyzed by flow cytometry. Blocking of Notch signaling by GSI-X inhibits the surface expression of activation markers. Data indicate the MFI of the activation markers. Dark shaded histogram indicates isotype control. (**B**) Annexin V and PI staining of unstimulated γδ T cells (γδ), rIL-2–activated γδ T cells (γδ+IL-2), and γδ T cells stimulated with BrHPP in the presence of rIL-2 (γδ+IL-2+BrHPP) without GSI-X (*upper panel*) or with GSI-X (*lower panel*). These cells were stained after culturing for 24 h and analyzed by flow cytometry. The unaffected, early apoptotic, late apoptotic, and necrotic cells are present in the lower left, lower right, upper right, and upper left quadrant, respectively. Dot plots show the mean percentage of positive cells. Results shown are representative of three independent experiments.

γδ T cells with or without GSI-X treatment. Ex vivo–expanded γδ T cells alone (unstimulated) or after stimulation with BrHPP and rIL-2 showed higher expression of CD25 compared with CD69. Treatment with GSI-X showed a moderate reduction in CD69 expression. A marked reduction in the expression of late activation marker CD25 was observed on both unstimulated and BrHPP-stimulated γδ T cells (Fig. 4A).

We then investigated whether treatment with GSI-X results in apoptotic/necrotic cell death of Ag-activated γδ T cells. To verify, we compared the effects of GSI-X on the frequency of apoptotic cells in unstimulated γδ T cells and after stimulation with rIL-2 alone or with both rIL-2 and BrHPP. We did not observe differences in the frequency of early apoptotic (Annexin V^+), late apoptotic (apoptotic V^+ PI^+), and necrotic (PI^+) γδ T cells in untreated compared with GSI-X–treated cells (Fig. 4B).

Notch regulates cytolytic potential of γδ T lymphocytes

Notch signaling has been reported to regulate cytotoxic responses in both CTL and NK cells (33, 34). The effect of Notch signaling in cytolytic potential of γδ T lymphocytes was examined. Purified γδ T cells were coincubated for 4 h with zoledronate-treated oral cancer cells. We evaluated CD107a (lysosome-associated membrane protein-1 [LAMP1]) expression, a marker of degranulation in unstimulated and rIL-2–stimulated γδ T cells in the presence and absence of GSI-X. Upon coincubation with the oral cancer cells, few γδ T cells showed surface expression of CD107a (18.4%). However, in the presence of rIL-2, the proportion of $CD107a^+$ γδ T cells increased (40.9%; Fig. 5A). Addition of GSI-X leads to reduction in the percentage of $CD107a^+$ in both unstimulated γδ T lymphocytes (13.1%) and rIL-2–stimulated γδ T cells (12.4%). The cytotoxic potential of γδ T cells against zoledronate-treated tumor cells (AW13516) was determined by

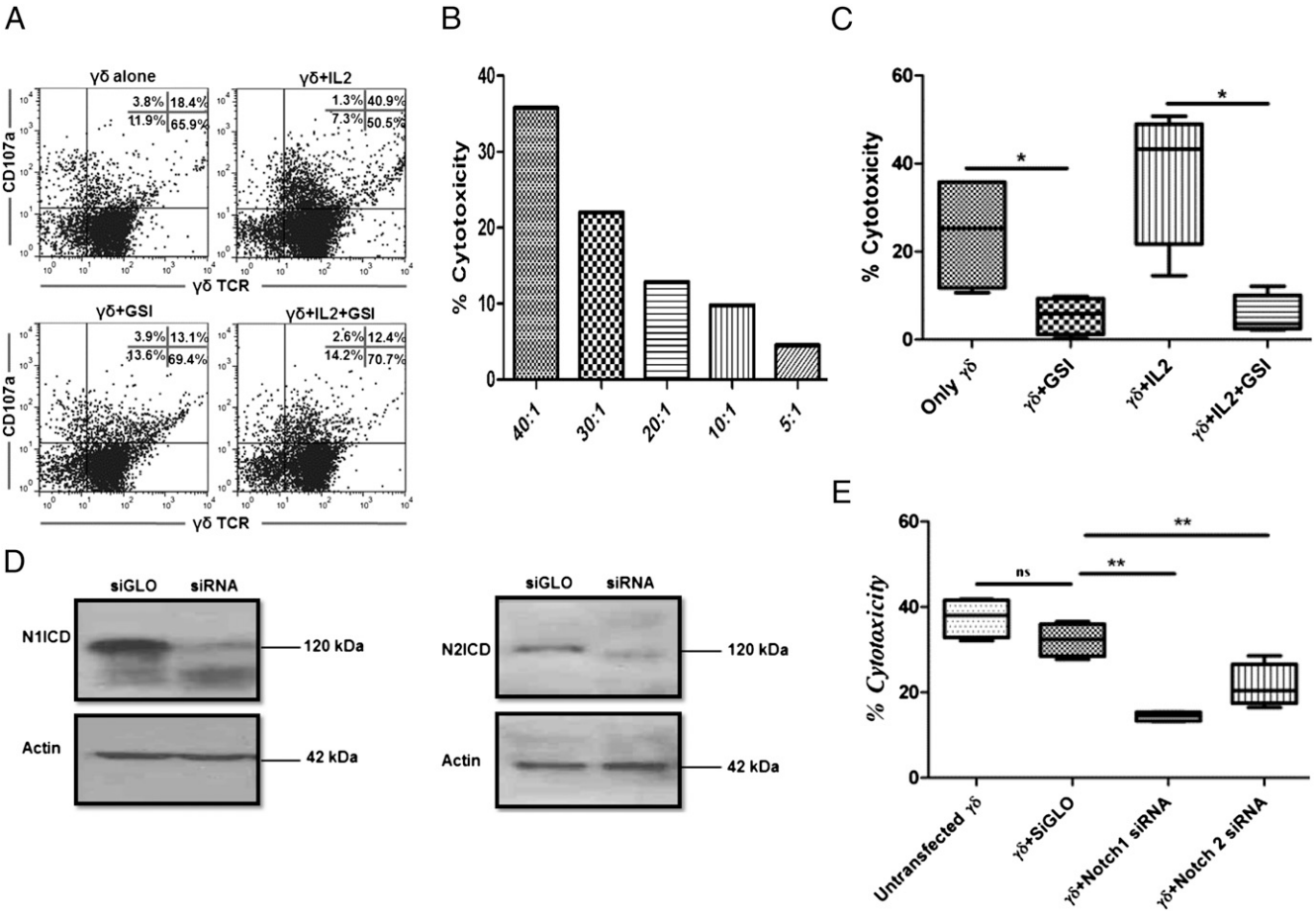

FIGURE 5. Notch signaling regulates cytolytic effector functions of γδ T cells. (**A**) Expression of CD107a or LAMP1 (marker for degranulation) on γδ T cells. Effector γδ T cells alone or in the presence of rIL-2 were cocultured with target oral cancer cell line (AW13516) at the ratio of 4:1 and incubated for 4 h. AW13516 were previously treated with zoledronate (100 μM) for 16 h. Decreased expression of CD107a was observed when the cells were treated with GSI-X (*lower panel*). Panel is representative of three experiments. (**B**) Zoledronate-treated AW13516 cells labeled with [^{51}Cr] used as target cells were cocultured with γδ T cells at different E/T ratios in a standard 4-h [^{51}Cr] release assay. Data represent percentage cytotoxicity as described in *Materials and Methods*. Representative data of an independent experiment. (**C**) Cytolytic ability of γδ T cells alone or in the presence of rIL-2 against zoledronate-treated AW13516 was determined by [^{51}Cr] release assay at the ratio of 40:1. Treatment of γδ T cells with GSI-X blocked the target cell lysis. *$p < 0.05$ compared with percentage cytotoxicity of both γδ T cells and γδ T cells with rIL-2 without GSI-X. Results are mean percentage cytotoxicity of γδ T cells from five healthy individuals. (**D**) γδ cells were transfected with 50 nM control (SiGLO) or different Notch-specific SiRNA duplexes. Forty-eight hours post-transfection, cells were harvested and samples were subjected to Western blot analysis for Notch1 and Notch2 (*top panel*) or β-Actin (*bottom panel*). The latter was included as loading/normalization control. (**E**) γδ T cells were transfected with 50 nM control (SiGLO) or specific Notch1 and Notch2 siRNA duplexes, Forty-eight hours posttransfection, the cytolytic effector function of rIL-2–activated γδ T cells against zoledronate-treated AW13516 was determined by [^{51}Cr] release assay at the ratio of 40:1. Silencing of either Notch1 or Notch2 inhibits target cell lysis by γδ T cells. **$p < 0.005$ compared with percentage cytotoxicity of Notch-specific siRNA with SiGLO-treated γδ T cells. The results are mean percentage cytotoxicity of γδ T cells from five healthy individuals.

titration at different E/T ratio ranging from 5:1 to 40:1. As seen in Fig. 5B, maximum cytotoxicity of γδ T cells was observed at E/T ratio of 40:1. This ratio of E/T was used in further experiments where effect of GSI-X on cytolytic ability of γδ T cells was examined. At E/T ratio of 40:1, ex vivo–expanded γδ T cells in the presence of rIL-2 efficiently lysed zoledronate-treated oral tumor cells compared with untreated cells. Addition of GSI-X significantly reduced the cytotoxic ability of γδ T cells against zoledronate-treated tumor targets (Fig. 5C).

Because pharmacological inhibition of the γ-secretase complex may have nonspecific effects, we assessed the contribution of the Notch pathway in regulating the cytotoxic potential using siRNA-mediated knockdown of individual Notch receptors. The synthetic siRNA sequences targeting the Notch1 and Notch2 were transfected into the γδ T cells. γδ T cells were transfected with 50 nM control (SiGLO) or different Notch-specific siRNA duplexes. After 48 h, cells were harvested and lysates were prepared. Western blot analysis for Notch1 and Notch2 showed that siRNA transfection led to the reduced expression of Notch1 and Notch2 receptors compared with fluorescent oligonucleotide SiGLO (Fig. 5D). Next, we compared antitumor cytotoxic ability of γδ T cells transfected with siRNA specific for Notch1 and Notch2 against zoledronate-treated tumor cells (AW13516). Cytotoxicity of γδ T cells was observed at E/T ratio of 40:1 and consistent with GSI-X treatment data (Fig. 5C); silencing of both Notch1 and Notch2 led to significant reduction in cytotoxic potential of γδ T cells (Fig. 5E). This observation confirms that cytotoxic potential of γδ T cells is regulated by Notch signaling.

Notch signaling regulates cytokines production in activated γδ T cells

To address whether Notch signal has any role in the effector functions of activated γδ T cells, we examined the effect of GSI-X on cytokine production. The consequence of GSI-X treatment on the concentration of TH1 (IL-2, IFN-γ, and TNF-α), TH2 (IL-4, IL-6, and IL-10), and TH17 (IL-17) production by γδ T cells treated with rIL-2 alone or along with BrHPP was assayed. It was observed that inhibition of Notch signaling leads to marked reduction in TNF-α, IFN-γ, and IL-17 production by activated γδ T cells (data not shown). The inhibition was more pronounced in IFN-γ production. We therefore confirmed the effect of GSI-X on IFN-γ production by ELISA. Activated human γδ T cells secrete IFN-γ, and Notch signaling regulates IFN-γ secretion in activated $CD4^+$ T cells (5, 23). γδ T cells were stimulated with c-HDMAPP, IPP, and BrHPP, which leads to increased production of IFN-γ. The amount of IFN-γ decreased when the cells were pretreated with GSI-X (Fig. 6A). Like phosphoantigens, anti-CD3 mAb stimulation of γδ T cells was also inhibited by GSI-X, which is depicted by dose-dependent decrease in IFN-γ production (Fig. 6B). Thus, a role of Notch in regulating IFN-γ production in γδ T cells is unraveled, which suggests that Notch plays a role in effector functions of γδ T cells.

Discussion

The role of Notch signaling in T cell differentiation, activation, and effector functions have been well documented in CD4 and CD8 T cells (22, 23). However, there is a lacuna of studies addressing the role of notch signaling in effector functions of human γδ T cells. A recent report showed that Hes1 is critically involved in the development of IL-17–producing γδ T cells (35). In these investigations, we report for the first time, to our knowledge, that peripheral human γδ T cells express notch receptors and notch signaling is required in Ag-specific responses of γδ T cells. We report Notch1 and Notch2 expression in γδ T cells at mRNA and protein level as analyzed by real-time PCR and flow cytometry. γδ T cells are known to be activated by phosphoantigens, for example, IPP and 4-hydroxy-3-methyl-but-2-eneyl pyrophosphate, which are intermediates of the mevalonate pathway of cholesterol metabolism in eukaryotic cells and rohmer pathway in prokaryotic cells (6). Using BRHPP, a synthetic analog of IPP in the presence of pharmacological GSI-X, we show that loss of Notch signaling in γδ T cells results in downregulation of mRNA for Notch1 and Notch2 receptors. Reduction in the expression of NICD by GSI-X in BrHPP-stimulated γδ T cells further confirmed the involvement of Notch signaling in regulating Ag-specific responses of γδ T cells. Activation of the Notch signaling pathway leads to the induction of c-Myc expression in immune cells (36), which was also observed in γδ T cells. Recent reports suggest that Notch signaling involves both canonical and noncanonical pathways in T cells, and these interactions will influence cell fate decisions and functions (32, 37). We therefore analyzed the expression of

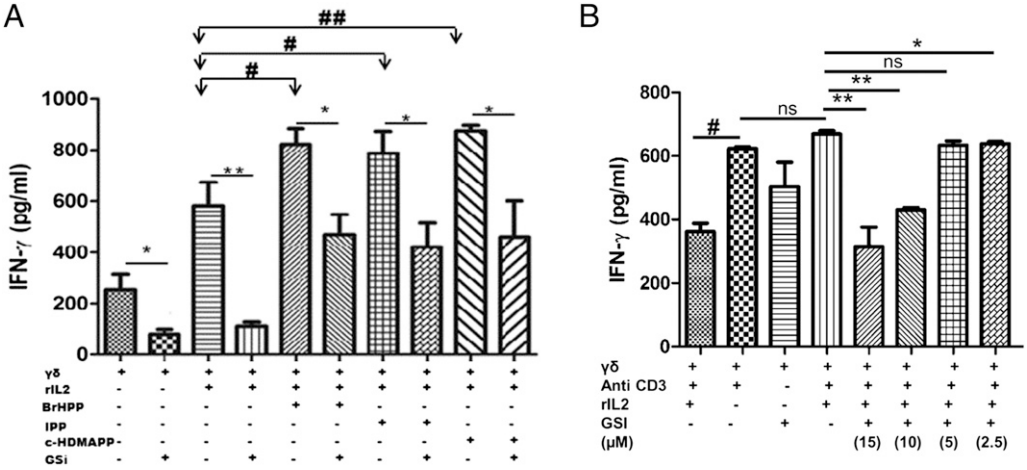

FIGURE 6. Treatment with GSI-X blocks IFN-γ production by γδ T cells. (**A**) γδ T cells alone, γδ T cells with rIL-2, or along with three different phosphoantigens (BrHPP, IPP, and c-HDMAPP) were cultured in the presence or absence of GSI-X for 24 h in 96-well plates. (**B**) γδ T cells were either cultured alone, or in the presence of anti-CD3 mAb or with rIl2 and in the presence or absence of different concentration of GSI-X (15, 10, 5, and 2.5 μM) for 24 h in 96-well plates. Supernatants were collected, and level of IFN-γ was measured by sandwich ELISA. GSI-X treatment decreases IFN-γ production by both unstimulated and stimulated γδ T cells. Results shown are mean of three experiments. $^*p < 0.05$, $^{**}p < 0.005$, compared with IFN-γ production of the cells without GSI-X treatment. $^{\#}p < 0.5$, $^{\#\#}p < 0.05$, compared with cpm of cells treated with rIL-2 alone.

mRNA for Hes1 and NF-κB in BrHPP-stimulated γδ T cells after inhibiting the Notch signaling by GSI-X. A decrease in the Notch target gene NF-κB indicates that noncanonical Notch signaling pathway is active in activated γδ T cells.

Further, inhibiting the Notch signaling in anti-CD3 mAb-stimulated γδ T cells resulted in marked decrease in proliferation of γδ T cells, confirming TCR engagement as a key initiating event affected by GSI-X treatment. Similarly, γδ T cells (freshly isolated and ex vivo expanded) activated with BrHPP, IPP, and c-HDMAPP as Ags resulted in a significantly decreased proliferation of γδ T cells in response to these Ags. The observation that Notch signaling is involved in regulating Ag-specific proliferative responses of γδ T cells prompted us to look at the expression of early and late activation markers CD69 and CD25 on γδ T cells stimulated with the Ag (BrHPP) in the presence and absence of GSI-X. Reduction in CD69 expression and a marked decrease in CD25 (IL-2R) expression on Ag-stimulated γδ T cells was observed when Notch signaling was inhibited. Various reports have shown that Notch signaling induces the expression of CD25 in immune cells (38–40). NF-κB, which is downstream of Notch signaling, also regulates the expression of CD25 (41). γδ T cells are dependent on IL-2 for their growth and survival, and express high-affinity CD25 (42). It is therefore not surprising that CD25 expression in γδ T cells is also regulated by Notch signaling, and IL-2 stimulation will thereby enhance the proliferative response and cytokine production of γδ T cells via IL-2R (CD25).

The importance of Notch signaling in mediating cytotoxic responses in immune cells has been well documented. Earlier studies carried out in murine $CD8^+$ T lymphocytes and NK cells have demonstrated the importance of Notch signaling in regulating their effector functions (22, 33). Notch signaling was shown to directly regulate granzyme B expression in $CD8^+$ cytotoxic T lymphocytes (34). The involvement of Notch in antitumor immunity was further supported by studies that showed that deficiency of Notch2 decreased the antitumor responses of $CD8^+$ T cells in mice models (43). Notch signaling also contributes to dendritic cell–mediated NK cell activation, which enhanced the killing activity of NK cells. Murine NK cells exhibit enhanced cytokine expression and cytotoxic function in response to signaling from tumor cells or dendritic cells transduced with Jag2 (33).

We therefore expected that Notch signaling may similarly regulate cytotoxic effector functions of γδ T cells. We showed that blocking of Notch signaling in γδ T cells by GSI-X inhibits the ability of γδ T cells to lyse tumor targets. We used oral tumor cell line AW13516 treated with zoledronate as target cell line. Earlier data from our laboratory have demonstrated that tumor cells treated with zoledronate are aggressively killed by γδ T cells (10, 13). Our data demonstrate that treatment with GSI-X blocks the ability of γδ T cells to lyse both untreated and zoledronate-treated tumor cells. Moreover, we found that specific silencing of either Notch1 or Notch2 by siRNA led to the reduced cytotoxic potential of γδ T cells. This result suggests that both Notch1 and Notch2 are involved in the cytolytic activity of γδ T cells. Cho and colleagues (22) demonstrated that Notch1 regulates expression of eomesodermin, perforin, and granzyme B through direct binding to the promoters of these effector molecules. In $CD8^+$ T cells, Notch2 signaling was shown to directly control CTL effector molecules including granzyme B, by integrating RBP-J and CREB1 (40). We observed that GSI-X treatment of γδ T cells alone or γδ T cells activated with IL-2 results in a reduction of CD107a expression in these cells. CD107a or LAMP1 is a marker of degranulation in cytotoxic T lymphocytes (44–46).

γδ T cells are known to secrete copious amounts of IFN-γ. IFN-γ plays a crucial role in protective immune response against certain pathogens and tumors (47–49). We showed that blocking Notch signaling with GSI-X inhibited the IFN-γ secretion by γδ T cells stimulated with phosphoantigens BrHPP, IPP, and c-HDMAPP. Likewise, we observed that GSI-X inhibits IFN-γ production by anti-CD3 mAb–activated γδ T cells. γδ T cells provide an early source of IFN-γ in tumor immunosurveillance and against viral challenge (47, 50). Epigenetic program that regulates IFN-γ gene transcription in γδ T cells is different from $CD4^+$ and $CD8^+$ T cells (51). Eomesodermin contributes to T bet–independent IFN-γ production in γδ T cells (51). We observed that BrHPP-stimulated γδ T cells produced IL-17, and its secretion was inhibited in the presence of GSI-X (data not shown). γδ T cells have been reported to be a potent source of IL-17 (52, 53). It has been reported that DLL4 upregulates RORC expression in T cells, and both RORC and IL-17 gene promoters are direct transcriptional notch targets and enhance Th17 cell population (54).

In this study, we describe for the first time, to our knowledge, the role of Notch in regulating the effector functions of human γδ T cells. Notch signaling appears to play an important role in modulating the Ag-specific proliferation of γδ T cells, their ability to lyse tumor targets and secrete IFN-γ upon stimulation. Taken together, these studies identify Notch as an additional signal contributing to Ag-specific effector functions of γδ T cells. These studies may have important implications in clinical situations where new strategies for the clinical manipulation of γδ T cells for cancer immunotherapy are being investigated.

Acknowledgments

We thank Innate Pharma (Marseilles, France) for providing the reagents and Shamal Vatale for helping in analysis of flow experiments.

Disclosures

The authors have no financial conflicts of interest.

References

1. Hayday, A. C. 2000. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu. Rev. Immunol.* 18: 975–1026.
2. Haas, W., P. Pereira, and S. Tonegawa. 1993. Gamma/delta cells. *Annu. Rev. Immunol.* 11: 637–685.
3. Chien, Y. H., R. Jores, and M. P. Crowley. 1996. Recognition by gamma/delta T cells. *Annu. Rev. Immunol.* 14: 511–532.
4. Gober, H. J., M. Kistowska, L. Angman, P. Jenö, L. Mori, and G. De Libero. 2003. Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. *J. Exp. Med.* 197: 163–168.
5. Wang, L., H. Das, A. Kamath, and J. F. Bukowski. 2001. Human V gamma 2V delta 2 T cells produce IFN-gamma and TNF-alpha with an on/off/on cycling pattern in response to live bacterial products. *J. Immunol.* 167: 6195–6201.
6. Morita, C. T., C. Jin, G. Sarikonda, and H. Wang. 2007. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. *Immunol. Rev.* 215: 59–76.
7. Santini, D., S. Galluzzo, B. Vincenzi, G. Schiavon, E. Fratto, F. Pantano, and G. Tonini. 2007. New developments of aminobisphosphonates: the double face of Janus. *Ann. Oncol.* 18(Suppl. 6): vi164–vi167.
8. Russell, R. G. 2006. Bisphosphonates: from bench to bedside. *Ann. N. Y. Acad. Sci.* 1068: 367–401.
9. García, V. E., P. A. Sieling, J. Gong, P. F. Barnes, K. Uyemura, Y. Tanaka, B. R. Bloom, C. T. Morita, and R. L. Modlin. 1997. Single-cell cytokine analysis of gamma delta T cell responses to nonpeptide mycobacterial antigens. *J. Immunol.* 159: 1328–1335.
10. Laad, A. D., M. L. Thomas, A. R. Fakih, and S. V. Chiplunkar. 1999. Human gamma delta T cells recognize heat shock protein-60 on oral tumor cells. *Int. J. Cancer* 80: 709–714.
11. Chargui, J., V. Combaret, V. Scaglione, I. Iacono, V. Péri, D. Valteau-Couanet, M. Dubrel, E. Angevin, A. Puisieux, F. Romagne, and C. Bergeron. 2010. Bromohydrin pyrophosphate-stimulated Vgamma9delta2 T cells expanded ex vivo from patients with poor-prognosis neuroblastoma lyse autologous primary tumor cells. *J. Immunother.* 33: 591–598.
12. D'Asaro, M., C. La Mendola, D. Di Liberto, V. Orlando, M. Todaro, M. Spina, G. Guggino, S. Meraviglia, N. Caccamo, A. Messina, et al. 2010. V gamma 9V delta 2 T lymphocytes efficiently recognize and kill zoledronate-sensitized, imatinib-sensitive, and imatinib-resistant chronic myelogenous leukemia cells. *J. Immunol.* 184: 3260–3268.

13. Dhar, S., and S. V. Chiplunkar. 2010. Lysis of aminobisphosphonate-sensitized MCF-7 breast tumor cells by Vγ9Vδ2 T cells. *Cancer Immun.* 10: 10.
14. Mattarollo, S. R., T. Kenna, M. Nieda, and A. J. Nicol. 2007. Chemotherapy and zoledronate sensitize solid tumour cells to Vgamma9Vdelta2 T cell cytotoxicity. *Cancer Immunol. Immunother.* 56: 1285–1297.
15. Yuan, J. S., P. C. Kousis, S. Suliman, I. Visan, and C. J. Guidos. 2010. Functions of notch signaling in the immune system: consensus and controversies. *Annu. Rev. Immunol.* 28: 343–365.
16. Tanigaki, K., M. Tsuji, N. Yamamoto, H. Han, J. Tsukada, H. Inoue, M. Kubo, and T. Honjo. 2004. Regulation of alphabeta/gammadelta T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling. *Immunity* 20: 611–622.
17. Washburn, T., E. Schweighoffer, T. Gridley, D. Chang, B. J. Fowlkes, D. Cado, and E. Robey. 1997. Notch activity influences the alphabeta versus gammadelta T cell lineage decision. *Cell* 88: 833–843.
18. Ciofani, M., G. C. Knowles, D. L. Wiest, H. von Boehmer, and J. C. Zúñiga-Pflücker. 2006. Stage-specific and differential notch dependency at the alphabeta and gammadelta T lineage bifurcation. *Immunity* 25: 105–116.
19. García-Peydró, M., V. G. de Yébenes, and M. L. Toribio. 2003. Sustained Notch1 signaling instructs the earliest human intrathymic precursors to adopt a gammadelta T-cell fate in fetal thymus organ culture. *Blood* 102: 2444–2451.
20. Van de Walle, I., G. De Smet, M. De Smedt, B. Vandekerckhove, G. Leclercq, J. Plum, and T. Taghon. 2009. An early decrease in Notch activation is required for human TCR-alphabeta lineage differentiation at the expense of TCR-gammadelta T cells. *Blood* 113: 2988–2998.
21. Bachanova, V., V. McCullar, T. Lenvik, R. Wangen, K. A. Peterson, D. E. Ankarlo, A. Panoskaltsis-Mortari, J. E. Wagner, and J. S. Miller. 2009. Activated notch supports development of cytokine producing NK cells which are hyporesponsive and fail to acquire NK cell effector functions. *Biol. Blood Marrow Transplant.* 15: 183–194.
22. Cho, O. H., H. M. Shin, L. Miele, T. E. Golde, A. Fauq, L. M. Minter, and B. A. Osborne. 2009. Notch regulates cytolytic effector function in CD8+ T cells. *J. Immunol.* 182: 3380–3389.
23. Palaga, T., L. Miele, T. E. Golde, and B. A. Osborne. 2003. TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells. *J. Immunol.* 171: 3019–3024.
24. Artavanis-Tsakonas, S., and M. A. Muskavitch. 2010. Notch: the past, the present, and the future. *Curr. Top. Dev. Biol.* 92: 1–29.
25. Bray, S. J. 2006. Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.* 7: 678–689.
26. Fiúza, U. M., and A. M. Arias. 2007. Cell and molecular biology of Notch. *J. Endocrinol.* 194: 459–474.
27. Yamaguchi, T., Y. Fujimiya, Y. Suzuki, R. Katakura, and T. Ebina. 1997. A simple method for the propagation and purification of gamma delta T cells from the peripheral blood of glioblastoma patients using solid-phase anti-CD3 antibody and soluble IL-2. *J. Immunol. Methods* 205: 19–28.
28. Tatake, R. J., N. Rajaram, R. N. Damle, B. Balsara, A. N. Bhisey, and S. G. Gangal. 1990. Establishment and characterization of four new squamous cell carcinoma cell lines derived from oral tumors. *J. Cancer Res. Clin. Oncol.* 116: 179–186.
29. Mizutani, T., Y. Taniguchi, T. Aoki, N. Hashimoto, and T. Honjo. 2001. Conservation of the biochemical mechanisms of signal transduction among mammalian Notch family members. *Proc. Natl. Acad. Sci. USA* 98: 9026–9031.
30. Bheeshmachar, G., D. Purushotaman, H. Sade, V. Gunasekharan, A. Rangarajan, and A. Sarin. 2006. Evidence for a role for notch signaling in the cytokine-dependent survival of activated T cells. *J. Immunol.* 177: 5041–5050.
31. Guy, C. S., K. M. Vignali, J. Temirov, M. L. Bettini, A. E. Overacre, M. Smeltzer, H. Zhang, J. B. Huppa, Y. H. Tsai, C. Lobry, et al. 2013. Distinct TCR signaling pathways drive proliferation and cytokine production in T cells. *Nat. Immunol.* 14: 262–270.
32. Minter, L. M., and B. A. Osborne. 2012. Canonical and non-canonical Notch signaling in CD4$^+$ T cells. *Curr. Top. Microbiol. Immunol.* 360: 99–114.
33. Kijima, M., T. Yamaguchi, C. Ishifune, Y. Maekawa, A. Koyanagi, H. Yagita, S. Chiba, K. Kishihara, M. Shimada, and K. Yasutomo. 2008. Dendritic cell-mediated NK cell activation is controlled by Jagged2-Notch interaction. *Proc. Natl. Acad. Sci. USA* 105: 7010–7015.
34. Maekawa, Y., Y. Minato, C. Ishifune, T. Kurihara, A. Kitamura, H. Kojima, H. Yagita, M. Sakata-Yanagimoto, T. Saito, I. Taniuchi, et al. 2008. Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. *Nat. Immunol.* 9: 1140–1147.
35. Shibata, K., H. Yamada, T. Sato, T. Dejima, M. Nakamura, T. Ikawa, H. Hara, S. Yamasaki, R. Kageyama, Y. Iwakura, et al. 2011. Notch-Hes1 pathway is required for the development of IL-17-producing γδ T cells. *Blood* 118: 586–593.
36. Radtke, F., N. Fasnacht, and H. R. Macdonald. 2010. Notch signaling in the immune system. *Immunity* 32: 14–27.
37. Auderset, F., S. Schuster, M. Coutaz, U. Koch, F. Desgranges, E. Merck, H. R. MacDonald, F. Radtke, and F. Tacchini-Cottier. 2012. Redundant Notch1 and Notch2 signaling is necessary for IFNγ secretion by T helper 1 cells during infection with Leishmania major. *PLoS Pathog.* 8: e1002560.
38. Deftos, M. L., E. Huang, E. W. Ojala, K. A. Forbush, and M. J. Bevan. 2000. Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes. *Immunity* 13: 73–84.
39. Fowlkes, B. J., and E. A. Robey. 2002. A reassessment of the effect of activated Notch1 on CD4 and CD8 T cell development. *J. Immunol.* 169: 1817–1821.
40. Adler, S. H., E. Chiffoleau, L. Xu, N. M. Dalton, J. M. Burg, A. D. Wells, M. S. Wolfe, L. A. Turka, and W. S. Pear. 2003. Notch signaling augments T cell responsiveness by enhancing CD25 expression. *J. Immunol.* 171: 2896–2903.
41. Bellavia, D., A. F. Campese, E. Alesse, A. Vacca, M. P. Felli, A. Balestri, A. Stoppacciaro, C. Tiveron, L. Tatangelo, M. Giovarelli, et al. 2000. Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. *EMBO J.* 19: 3337–3348.
42. Kjeldsen-Kragh, J., A. J. Quayle, B. S. Skålhegg, M. Sioud, and O. Førre. 1993. Selective activation of resting human gamma delta T lymphocytes by interleukin-2. *Eur. J. Immunol.* 23: 2092–2099.
43. Sugimoto, K., Y. Maekawa, A. Kitamura, J. Nishida, A. Koyanagi, H. Yagita, H. Kojima, S. Chiba, M. Shimada, and K. Yasutomo. 2010. Notch2 signaling is required for potent antitumor immunity in vivo. *J. Immunol.* 184: 4673–4678.
44. Kwon, C., P. Cheng, I. N. King, P. Andersen, L. Shenje, V. Nigam, and D. Srivastava. 2011. Notch post-translationally regulates β-catenin protein in stem and progenitor cells. *Nat. Cell Biol.* 13: 1244–1251.
45. Raghu, H., C. S. Gondi, D. H. Dinh, M. Gujrati, and J. S. Rao. 2011. Specific knockdown of uPA/uPAR attenuates invasion in glioblastoma cells and xenografts by inhibition of cleavage and trafficking of Notch -1 receptor. *Mol. Cancer* 10: 130.
46. van Tetering, G., N. Bovenschen, J. Meeldijk, P. J. van Diest, and M. Vooijs. 2011. Cleavage of Notch1 by granzyme B disables its transcriptional activity. *Biochem. J.* 437: 313–322.
47. Gao, Y., W. Yang, M. Pan, E. Scully, M. Girardi, L. H. Augenlicht, J. Craft, and Z. Yin. 2003. Gamma delta T cells provide an early source of interferon gamma in tumor immunity. *J. Exp. Med.* 198: 433–442.
48. Schoenborn, J. R., and C. B. Wilson. 2007. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv. Immunol.* 96: 41–101.
49. Skeen, M. J., and H. K. Ziegler. 1995. Activation of gamma delta T cells for production of IFN-gamma is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. *J. Immunol.* 154: 5832–5841.
50. Wang, T., E. Scully, Z. Yin, J. H. Kim, S. Wang, J. Yan, M. Mamula, J. F. Anderson, J. Craft, and E. Fikrig. 2003. IFN-gamma-producing gamma delta T cells help control murine West Nile virus infection. *J. Immunol.* 171: 2524–2531.
51. Chen, L., W. He, S. T. Kim, J. Tao, Y. Gao, H. Chi, A. M. Intlekofer, B. Harvey, S. L. Reiner, Z. Yin, et al. 2007. Epigenetic and transcriptional programs lead to default IFN-gamma production by gammadelta T cells. *J. Immunol.* 178: 2730–2736.
52. Lockhart, E., A. M. Green, and J. L. Flynn. 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during *Mycobacterium tuberculosis* infection. *J. Immunol.* 177: 4662–4669.
53. Umemura, M., A. Yahagi, S. Hamada, M. D. Begum, H. Watanabe, K. Kawakami, T. Suda, K. Sudo, S. Nakae, Y. Iwakura, and G. Matsuzaki. 2007. IL-17-mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium* bovis bacille Calmette-Guerin infection. *J. Immunol.* 178: 3786–3796.
54. Mukherjee, S., M. A. Schaller, R. Neupane, S. L. Kunkel, and N. W. Lukacs. 2009. Regulation of T cell activation by Notch ligand, DLL4, promotes IL-17 production and Rorc activation. *J. Immunol.* 182: 7381–7388.