Understanding pathogenesis of gallbladder cancer: Role of Th17 and Regulatory T cells

By
Rushikesh Sudam Patil
(LIFE09201004010)

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

December, 2016

Homi Bhabha National Institute

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As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Mr. Rushikesh Sudam Patil entitled "**Understanding pathogenesis of gall bladder cancer: Role of TH17 and Regulatory T cells**" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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Date: 30/12/2016
Place: Navi Mumbai

Rajesh Dikshit
Dr. Rajesh Dikshit
Co-Guide

S. Chiplunkar
Dr. Shubhada V. Chiplunkar
Guide

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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: 06/01/2017

Rushikesh Sudam Patil

LIST OF PUBLICATIONS ARISING FROM THE THESIS

Journal

1. **Patil RS,** Shalini KS, Chiplunkar SV, **"**Immunosuppressive role of γδ T cells in cancer: The other side of the coin", **Translational Cancer Research**. 2016 Dec (Manuscript accepted for publication)

2. **Patil RS,** Shah SU, Shrikhande SV, Goel M, Dikshit R, and Chiplunkar SV, "IL17 producing γδ T cells induce angiogenesis and are associated with poor survival in gallbladder cancer patients", **International Journal of Cancer.** 2016 Apr 8. doi: 10.1002/ijc.30134

3. **Patil RS**, Bhat SA, Dar AA, Chiplunkar SV, "The Jekyll and Hyde story of IL17 producing γδT (Tγδ17) cells", Feb4 2015, **Frontiers in Immunology.** 2015 Feb 4;6:37. doi: 10.3389/fimmu.2015.00037

4. Dar AA, **Patil RS**, Chiplunkar SV, "Insights into the relationship between toll like receptors and gamma delta T cells responses", **Frontiers in Immunology**. 2014 Jul 31;5:366. doi: 10.3389/fimmu.2014.00366.

Conferences (Abstracts published)

1. **Patil RS,** Shah SU, Shrikhande SV, Goel M, Dikshit R, and Chiplunkar SV, "Pro-tumor role of IL17 producing γδT cells in gallbladder cancer", **European Journal of Cancer** Volume 54, Supplement 1, Pages S1-S78 (February 2016). doi: 10.1016/S0959-8049(16)31954-2.

2. **Patil RS**, Shah SU, Shrikhande SV, Goel M, Dikshit R, and Chiplunkar SV, "Role of IL17 producing γδ T cells (Tγδ17) in gall bladder cancer pathogenesis", Feb10 2015, ***J Carcinog***,14:21.

Presentations at conferences

1. Oral presentation on "Tγδ17 is a proangiogenic subtype of γδT cells and associates with poor survival of gallbladder cancer patients" at "**13th FIMSA advanced Immunology Course"** held at 'Department of Immunopathology, PGIMER, Chandigarh from 17-19 March 2016.

2. Oral presentation on "Paradox of immune system in Gallbladder Cancer: Role of Tγδ17 cells" at **'Society of biological chemists (India) Mumbai chapter meeting'** held at NIRRH Mumbai (22 August 2015).

3. Oral presentation on "Role of IL17 producing γδT cells (Tγδ17) in gallbladder cancer pathogenesis" in international conference **"Carcinogenesis-2015"** on 'Molecular pathways to therapeutics: Paradigms and challenges in oncology' at ACTREC, Tata Memorial Centre, Navi Mumbai from 11-13 February 2015.

4. Oral presentation on "IL17 producing γδT cells (Tγδ17) contribute to tumorigenesis of gallbladder cancer" at **"41st annual conference of Indian Immunology Society-Immunocon-2014"**at Madurai Kamaraj University, Madurai, from 12-14 December 2014.

5. Poster was presented on "Dynamics of Tγδ17 and Regulatory T cells in Gallbladder cancer" in international conference **"Keystone Symposium on Infection**

inflammation and cancer joint with immune evolution in cancer" at Fairmont Chateau Whistler, Whistler, British Columbia, Canada from 9-14 March 2014.

6. Poster was presented on "Immune dysfunctions in Gallbladder cancer : Imbalance of Tγδ17 and Regulatory T cells" at **"IX DAE-BRNS Life Sciences Symposium (LSS-2013) on Current advances in immunobiology and cancer"** at BARC, Anushaktinagar, Mumbai from 28-30 November 2013.

7. Poster was presented on "Dynamics of Th17 and regulatory T cells in pathogenesis of Gall Bladder cancer" at **" 39th Annual Conference of Indian Immunology Society-IMMUNOCON 2012"** at Banaras Hindu University, Varanasi from 9-11 November 2012.

8. Poster was presented on "Imbalance of Th17 and regulatory T cells in gall bladder cancer: implications in pathogenesis" at **"National Education Day"** SVKM's NMIMS School of Science, Mithibai College, Mumbai on 1 November, 2012.

9. Poster was presented on "Imbalance of Th17 and regulatory T cells in gall bladder cancer : implications in pathogenesis" at **"31st Annual Convention of Indian Association for Cancer Research & International Symposium on Cancer Genomics and its Impact in the Clinics"** at ACTREC, Navi Mumbai from 26-29 January 2012.

Awards

- **Travel award** for oral presentation at 13th FIMSA advanced Immunology Course at PGIMER, Chandigarh (March 2016)
- **Dr. G.P. Talwar Young Scientist Award** in 41st Annual Conference of Indian

Immunology Society (IMMUNOCON-2014), Madurai Kamaraj University. (December 2014)

- **Travel fellowship** from Homi Bhabha National Institute, (February 2014)
- Sam Mistry **travel fellowship** award from Sam Mistry Foundation, Mumbai (February 2014)
- 2nd Poster presentation award in conference on "National Education Day", NMIMS School of Science, Mumbai. (2012)

Navi Mumbai

Date: 06/01/2017 Rushikesh Sudam Patil

I dedicate this dissertation to my late brother and my parents. The unconditional support provided by them made my PhD journey easy and successful.

Acknowledgements

It gives me an immense pleasure to express my gratitude to all of them who directly or indirectly contributed for successful completion of my PhD dissertation work. After an exhaustive tenure of almost six years, when I recollect the memories, it has been a period of intense learning for me, not only in the scientific arena, but also on a personal level. This journey has a big impact on me. I have received a great deal of help from many individuals during the course of this study, but several are noteworthy of special attention.

First of all I pay my sincere reverence to my mentor Professor Shubhada V. Chiplunkar. This work would not have been possible without her guidance, support and encouragement. Her continuous support, patience, encouragement, enthusiasm and immense knowledge were the key motivations during my PhD. Her valuable suggestions during tough times and the freedom she gave to conduct experiments helped me to grow intellectually and develop my research skills throughout this journey. Her scientific and ethical approach towards research has influentially shaped my interest in my work. I am truly thankful to her for my personal and academic development. Her selfless dedication towards research, unflinching courage and assertive conviction will always inspire me for my future endeavours. I feel privileged to have her as my mentor.

I have also honoured greatly to have eminent scientists as my Doctoral Committee members. I am thankful to Dr. Dalal, Dr. Gupta, Dr. Dikshit, Dr. Sainis, and Dr. (late) Kalraiya for their criticisms and valuable suggestions.

I am indebted to our clinical collaborators Dr. Shrikhande, Dr Goel and Dr. Dikshit for providing the clinical samples of gallbladder cancer patients. I am also thankful to Dr. Baretto for his help during initial phase of the study.

Special thanks to Dr. Dikshit lab members at Tata Memorial Hospital for the help extended by them for patient sample collection. I also thank Dr. Indap and her lab members at Ruparel College Mumbai; especially Dr. Shweta Agarwal and Mr. Sachin Chaugule for chick chorioallantoic membrane assay.

I would like to thank Advanced Centre for Treatment Research and Education in Cancer (ACTREC) and Department of Atomic Energy (DAE) for providing research scholarship to me during this tenure. I acknowledge Department of Biotechnology (DBT), India for

providing the financial support to conduct this research. For scholarships and conference funding, I would like to thank DBT project grant, Homi Bhabha National Institute, Sam Mistry foundation, Mumbai.

I appreciate the generosity and cooperation shown by all the healthy volunteers and gallbladder cancer patients for donating their blood and consenting to be a part of the study. Special thanks to Mr. Anand and Ms. Lalita, Tumor Tissue Repository TMH, for their help and support during sample collection.

I acknowledge the rich and supportive environment provided by Chiplunkar Lab members. I feel lucky to be a part of "Chiplunkar lab" group. The friendly and healthy atmosphere in the lab nourished me to grow and develop my personality. I would like to thank Dr. Jyoti Kode, Trupti Pradhan, Shamal Vetale, Meena Patkar, Rekha Gour, Dakaveji, Desaiji, Sawantji, Kalpesh, Ram, Preeta Menon, Lata, Maya Dolas, and Sujata Hake. Special thanks to Shamal Vetale for introducing me to the field of flowcytometry in a friendly atmosphere. Thanks to Meena Patkar for being a great help to withdraw blood from healthy donors and for placing purchase orders.

I found students in Chiplunkar Lab group immensely knowledgeable and dedicated. I am thankful to them for friendly and collegial atmosphere in the lab. They were wonderful colleagues in every sense. In person, I would like to thank Bhairav, Nirmala, Dimpu, Babita, Swati, Aparna, Asif, Gauri, Sajad, Sachin, Shalini, Neha, Siddhesh, Sagar, Pradeep, Atul, Devanand, Priyanka, Pravin, Prasad, for all the happy moments. I thank Bhairav, Dimpu and Asif for their brotherly support and guidance as philosopher in hard and good times.

I specially thank Sagar for collecting patient samples from TMH and initial standardization experiments. It was my benefit to have Sagar as my colleague on the project. Special thanks to Saleha who worked with me as summer trainee.

I acknowledge the academic environment of ACTREC, supporting the scientific careers of young minds. I thank people from Flowcytometry, Microscopy, Genomics, 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 a wonderful host for diverse lab parties at his home or at outside venues. I would also like to thank the group of Shamal, Meena, Asif, Sachin, Sajad, Dakave, Desai, Ram and Siddhesh for making my time outside the lab memorable by arranging picnics and parties.

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

I want to thank those who took the time to finalize the drafts of this thesis and paper manuscript. I thank Asif, Shalini, Sandeepa and Sachin who took their time to read and re-read very early drafts of the thesis/manuscripts and provided helpful comments.

Eventually I am thankful to my parents for their sacrifices for me and being a constant source of love. Without them I am nobody. I am grateful to them for the values they taught me to live as human. I also pay sincere respect to my late brother who always used to wish for my best. I thank my uncle, aunt and my cousins where I stayed for long during PhD tenure. I thank them for all the happy time I spent. I would also like to thank my wife Sandeepa for her love and constant moral support during thesis writing.

Last but not the least I am grateful to the Almighty, for this wonderful life!

Rushikesh Sudam Patil

CONTENTS

Synopsis

Homi Bhabha National Institute

Ph. D. PROGRAMME

1. Name of the Student:	Rushikesh Sudam Patil
2. Name of the Constituent Institution:	Tata Memorial Centre**,** Advanced Centre for Treatment, Research and Education in Cancer
3. Enrolment No. :	LIFE09201004010
4. Title of the Thesis:	**"**Understanding pathogenesis of gall bladder cancer: Role of TH17 and Regulatory T cells"
5. Board of Studies:	Life Sciences

SYNOPSIS

Introduction: Gallbladder Cancer (GBC) is a relatively uncommon but lethal biliary tract related cancer. Its occurrence shows ethnic and geographical variations and is prevalent in Peru, Ecuador, Poland, Chile, Pakistan, Japan and northern India [1]. GBC is 2-3 times common in women than men and highest incidences are reported in north Indian women [2]. Its anatomic location, elusive symptoms and diagnosis at advanced stage renders 5 year survival less than 5% [1]. Cholelithiasis (gallstone) is a major risk factor of GBC. 70-80% patients are associated with gallstone disease. Chronic inflammation associated with cholelithiasis leads to dysplastic changes in gallbladder resulting into high grade premalignant carcinoma *in situ* [3]. Complete surgical resection is the only curative option available, but more than 90% GBC patients are with un-resectable disease. Despite improved results of chemotherapy and surgery, the long term outcome remains disappointing [4].

Therefore, efforts are needed to identify other etiological factors contributing to pathogenesis of GBC.

The inflammatory microenvironment is an essential component of a tumor and plays a decisive role at different stages of tumor development. It modulates host immune response to facilitate tumor growth [5]. Interleukin-17 (IL17) is a potent proinflammatory cytokine and its elevated levels have been found to be detrimental in autoimmune diseases and cancers [6]. IL17 is known to induce, chemoresistance, neoangiogenesis and activation of matrix metalloproteinases which in turn enhances tumor progression [7, 8]. However, the cellular source of IL17 and its clinical relevance in GBC is not well studied.

$CD4^+IL17^+$ (Th17) is a subset of $CD4^+$ T cell, characterized by the production of IL-17A. Th17 cells and its related cytokines are reported to be present in tumor environment of prostate, ovarian, colorectal, head and neck, gastric cancer and other malignancies [9]. The proinflammatory cytokines such as IL6, IL1β, IL23 and TGFβ induce the differentiation and/or stabilization of Th17 cells [10]. Th17 generation is controlled by the master transcription factors retinoic acid-related orphan receptor (ROR)γt, RORα, aryl hydrocarbon receptor (AHR) and interferon regulatory factor 4 (IRF4). Increased intra-tumor Th17 density associates with higher blood vessel density and poor prognosis of cancer patients [11].

In contrast, another subset of $CD4^+$ T cells, characterized as $CD4^+CD127^{low/-}CD25^+Foxp3^+$ regulatory T cells (Treg), are known to play a critical role in immune tolerance and control of autoimmunity [12]. They express Foxp3 as lineage determining transcription factor and actively engage in inhibiting the activation of tumor specific CD4 and CD8 T cells [13]. Treg cells suppress target cell types by applying various mechanisms including secretion of suppressor cytokines (TGFβ, IL10), IL2 sequestration, expression of co-inhibitory molecules (CTLA4, PDL1, TIM3) or cytolysis [13]. The elevated proportions of Tregs present in peripheral blood and tumor microenvironment are associated with poor prognosis of head and

neck, lung, liver, gastrointestinal tracts, pancreas, ovary, breast and several other cancer patients [12].

The tumor infiltrating immune cells are engaged in extensive crosstalk with cancer cells. The type, functional orientation, density and location of infiltrating immune cells determine the fate of tumor progression and response to anti-tumor therapy. An expanding body of literature has highlighted the functional relevance of Th17and Treg cells in tumor environment [14]. The crosstalk of pro and anti-inflammatory immune response mediated by Th17 and Treg cells, determining disease outcome, is not investigated in GBC. The present prospective study aims at understanding the dynamics of Th17 and Treg contributing to inflammation and thereby progression of GBC.

Aims and Objectives:

1. Analysis of the immunophenotypes and effector functions of peripheral blood lymphocytes of GBC patients in comparison to healthy individuals
2. Understanding the functional dynamics of pro-inflammatory (Th17, Tc17 and Tγδ17) and anti-inflammatory regulatory T cells ($CD4^{+}CD127^{low/-}CD25^{+}Foxp3^{+}$; Tregs) in peripheral blood and tumor microenvironment of GBC patients
3. Protumor role of IL17 producing γδ T (Tγδ17) cells in GBC

Methodology

Patient samples: Heparinised peripheral blood was collected from GBC patients (n=52) prior to chemotherapy/radiotherapy or surgery after obtaining written informed consent. The study protocol was approved by ACTREC-TMC Institutional review board. The patients were grouped according to the TNM classification as stage II, stage III and stage IV. Tumor tissues (n=17) were obtained from GBC patients undergoing cholecystectomy. Heparinised blood and clotted blood was collected from healthy individuals (n=30) who participated voluntarily.

Cell isolation and culture: Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood using Ficoll Hypaque (Sigma-Aldrich, St. Louis, MO). Single cell suspension of surgically resected tumor was prepared by enzyme digestion (0.05%

collagenase, 0.02% DNase and 5U/ml hyaluronidase [Sigma-Aldrich]) and the cells were cultured in serum-free medium and tumor supernatants were collected after 24 h. To isolate Tγδ17 cells, purified γδT cells were stimulated with PMA and Ionomycin for 5 h and labelled with IL17 catch reagent followed by secretion phase of 45 minutes at 37^0C. After labelling with anti-IL17 detection antibody, cells were sorted for IL17-PE positive cells (purity > 90%). Gallbladder cancer cell lines [OCUG-1 (poorly differentiated) and NOZ (moderately differentiated)] were purchased from Japan Health Science Research Resources Bank (Osaka, Japan). Cells were cultured in William's E medium (Sigma Aldrich, USA) supplemented with 10% FBS (37^0C, 5% CO2).

Flow cytometry: Flow cytometric analysis was performed using FACS Aria flow cytometer (Becton Dickinson, CA, USA) and analysed by FlowJo software (Tree Star, Ashland, OR). Fluorescence minus one control was used in all experiments to determine background fluorescence. For intracellular cytokines staining, PBMCs were stimulated with PMA (50 ng/ml) and ionomycin (1μg/ml) for 5 h in presence of Brefeldin A (5 μg/ml). Minimum 50,000 events were acquired on FACS Aria flow cytometer and analyzed by FlowJo.

Regulatory T cells suppression assay: Tregs were isolated from PBMCs using immunomagnetic separation kit (BD Biosciences). $CD4^+CD25^-$ T cells were used as responder T cells (Tres) and labelled with carboxyfluorescein succinimidyl ester (CFSE; 5μM) (Life technologies). Tres cells ($1x10^4$) were co-cultured with Tregs for 5 days at different ratios (Tres: Treg = 1:2, 1:1, 1:0.5, 1:0). Co-cultures were stimulated with anti-CD3/anti-CD28 coated beads (1bead: 1cell) (Treg suppression inspector, Miltenyi biotech). Cells were acquired on FACS Aria and analyzed by FlowJo software.

Cytokines and chemokines measurement: Cytokines and chemokines in sera samples of GBC patients and culture supernatants were measured by Th1/Th2/Th17 cytometric bead array kit and flex sets for IL8, IL1β, IL12p70, CXCL9, CCL5, CXCL10, CCL2 (BD

Biosciences) as per manufacturer's instructions. Samples were acquired on FACS Aria and analysed using BD FCAP Array (BD Biosciences). TGFβ (BD Biosciences) and IL23 (eBiosciences, CA, USA) were determined by ELISA.

Cell migration assay: Migration was studied by trans-well assay using Millicell cell-culture inserts (Merck Millipore, MA) with pore size 8.0µm. OCUG-1 cells ($5x10^4$) were cultured in 600µl serum-free William's E medium. In some experiments rhCXCL9 (100ng/ml; PeproTech, NJ, USA) or tumor supernatants were added to the lower chamber. Isolated Tγδ17 cells ($5x10^4$/100µl medium) or γδT cells ($1x10^5$/100µl medium) were added onto the trans-well filter. Migrated cells from lower chamber were counted using haemocytometer after 7 h. For blocking experiments, Tγδ17/γδT cells were incubated with anti-CXCR3 antibody (10µg/ml; R&D Systems, USA), 30 minutes before trans-well co-culture.

Angiogenesis array: Cell-free supernatant of Tγδ17 was incubated with OCUG-1 cells ($2x10^4$/well) in presence / absence of neutralizing anti-IL17 antibody (10µg/ml; R&D Systems, MN, USA). After 48 h, supernatants were collected and analysed for vascular endothelial growth factor (VEGF) by ELISA (R&D systems, MN, USA) or angiogenesis related proteins by human proteome profiler angiogenesis array (R&D systems, MN, USA). The data was evaluated using Image J 1.48V software (NIH, USA) and expressed as mean pixel density.

Chorioallantoic membrane (CAM) assay: On embryonic day 5 of fertilized chicken eggs, a small window was made in the shell and 200µl medium/ Tγδ17 supernatant/ rhIL17 (100ng/ml, R&D systems, MN, USA) was added onto the CAM of growing embryo. After 48 h, eggs were cracked open and embryos were carefully transferred to 100 mm petri dish and images were captured. Angiogenesis was quantitatively evaluated by scoring number of branching points in control and treated CAMs.

Cell proliferation assay: OCUG-1 cells ($1x10^4$) were cultured in serum-free medium with rhIL17 (R&D systems, MN, USA in different concentrations. 0.5µCi/10µl/well ^{3}H-Thymidine (specific activity240GBq/mmol; Radiation and Isotype Technology, India) was added during last 18 h of the assay. After 72 h, cells were harvested and radioactivity was measured on liquid β-scintillation counter (Packard USA) as counts per minute (CPM).

Wound healing assay: OCUG-I and NOZ GBC cell lines were treated with Mitomycin C (10µg/ml) for 2 h. Three scratches were made precisely with the help of T-10 micro tips. The rhIL-17 (50ng/ml, 100ng/ml) was added and cell migration was monitored for 21 h using time lapse inverted microscope. Images were analysed using Image J1.48V software.

Matrigel invasion assay: In a 24 well transwell assay (8.0 µm pore size), the upper chambers were coated with the matrigel (BD Biosciences, USA).OCUG-1 ($5X10^4$) and NOZ ($3X10^4$) cells were suspended in 200µl plain William's E medium containing IL17 (50ng/ml or 100ng/ml). The invasion of cells was monitored towards William's E medium with 10% FBS added to the lower chamber. Unmigrated cells were removed using cotton swabs. The cells were stained by 1% crystal violet and observed using 10 X objective.

Statistical analysis: Statistical significances were calculated by two-tailed student's t-test or Mann-Whitney test (GraphPad Prism software, Lake Forest, CA). Overall patient survival was calculated by Kaplan-Meier curve and compared by Log-rank test. Survival time was defined as the interval between date of diagnosis and date of death or last follow-up, whichever occurred earlier. $p< 0.05$ was considered statistically significant.

<u>Results</u>: 1. Immunophenotyping and effector functions of peripheral blood lymphocytes of GBC patients

The prevalence of immune infiltrates in the tumor microenvironment has significant influence on development of cancer. The focus of the present prospective study was to investigate immune infiltrates of GBC and to study the functional characteristics of adaptive

and innate immune cells contributing to tumor progression. It was observed that the immune cells contributing to adaptive immunity ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$, $CD3^+CD56^+$) were decreased in the peripheral blood of GBC patients compared to HI. The expression of CD3ξ chain, vital signalling molecule in T cell receptor (TCR) mediated signalling of T cells, was downregulated in PBMCs of GBC patients compared to healthy individuals (HI). The compromised signalling through TCR resulted in low lymphocyte proliferative response to anti CD3mAb or mitogen (phytohaemagglutinin; PHA) stimulation as studied by ^{3}H-Thymidine incorporation assay. Moreover, the PBMCs of GBC patients showed decreased secretion of effector cytokines upon stimulation anti-CD3 mAb or PHA. Thus the data suggests that the systemic immune dysfunction may result in insufficient activation of anti-tumor immune response in GBC patients.

2. **Dynamics of pro inflammatory (Th17, Tc17 and Tγδ17) and anti-inflammatory (Tregs) immune cells in peripheral blood and tumor environment of GBC patients**

Chronic inflammatory condition of cholelithiasis is a vital risk factor of GBC. However, the crosstalk of immune cells contributing to inflammation is not well understood in GBC. Using multicolour flowcytometry, it was observed that IL17 producing $CD4^+$ (Th17), $CD8^+$ (Tc17), and $\gamma\delta^+$ (Tγδ17) cells were significantly increased in PBMCs of GBC patients compared to HI. Levels of these cells were further elevated in tumor compartment than peripheral blood of GBC patients. Interestingly, the relative percentages of Tγδ17 appeared to be higher in tumor infiltrating lymphocytes (TILs) compared to Th17 and Tc17. In contrast, $\gamma\delta^+IFN\gamma^+$ cells were significantly decreased in TILs compared to PBMCs of GBC patients and HI. A significant decrease was also observed in $CD8^+IFN\gamma^+$ cells in TILs compared to PBMCs of GBC patients. However, no changes were observed in $CD4^+IFN\gamma^+$ cells in GBC patients compared to HI. Interestingly, it was observed that the Tγδ17 and $\gamma\delta^+IFN\gamma^+$ cells showed a negative

correlation. It is reported that γδT cells expressing CD27 produce IFNγ and $CD27^-$ γδT cells secrete IL17 [15]. In GBC patients, the levels of $CD27^-$ γδT cells were significantly elevated than $CD27^+$γδT cells. Overall the data suggests that the cytokine profile of γδT cells in GBC patients is skewed towards production of IL17.

To study the anti-inflammatory and immunosuppressive arm of immune response in GBC patients we investigated the levels of regulatory T cells (Tregs; $CD4^+CD25^+CD127^{low/-}$ expressing Foxp3 more than 80%). Studies demonstrated that Tregs were significantly decreased in peripheral blood of GBC patients than HI. However, the percentages of Tregs in TILs were higher than PBMCs of GBC patients but comparable to HI. The median fluorescence intensity (MFI) of Foxp3 expression on Tregs was significantly increased in TILs than PBMCs of GBC patients. Moreover, the suppressive potential of Tregs, as analyzed by CFSE dye dilution method, in peripheral blood of GBC patients was comparable to HI. This indicates that although Tregs were decreased in PBMCs of GBC patients, their suppressive potential was not compromised. Study of dynamics of these cells revealed that the ratios of Tγδ17/Treg, Th17/Treg and Tc17/Treg were significantly increased in PBMCs as well as in TILs of GBC patients indicating an inverse correlation of IL17 producing cells and Tregs. Tγδ17, Th17 and Tc17 cells showed no correlation with clinical stage (II to IV) of GBC patients. However, their levels remained high in all stages of GBC patients compared to HI. In contrast, the levels of Tregs in GBC patients of all stages (II to IV) remained lower than HI. This clearly indicates that the immune response is skewed towards IL17 producing cells in GBC patients.

As increased levels of Tγδ17 were observed in GBC patients, we reasoned that the cytokine milieu promoting Tγδ17 differentiation should be present in sera and tumor environment. Cytokine profile of GBC patients revealed that the cytokines (IL6, IL23, IL1β) required for polarization and/or stabilization of IL17 producing cells were elevated in sera and tumor

environment of GBC patients thus making the environment conducive for differentiation of Tγδ17 cells.

To investigate the clinical significance of Tγδ17, Th17, Tc17 and Tregs, the survival time of patients was analyzed with frequency of these cells in peripheral blood of GBC patients. Cox proportional regression analysis revealed that patients with high Tγδ17 showed poor overall survival than patients with low levels of Tγδ17. In contrast, the patients with high levels of $\gamma\delta^{+}IFN\gamma^{+}$ had longer overall survival than patients with low levels of $\gamma\delta^{+}IFN\gamma^{+}$ cells. Similarly, Th17 and Treg cells in peripheral blood were associated with poor survival of GBC patients. Tc17 were not associated with survival of patients. Altogether, the data suggests that Tγδ17, Th17 and Treg cells might serve as biomarkers for prediction of risk and prognosis of GBC. Interestingly, our data revealed that in GBC patients, Tγδ17 cells are emerging as an important phenotype in cancer progression.

3. Protumor role of Tγδ17 cells in GBC patients

The increased levels of Tγδ17 cells observed in tumor environment of GBC patients suggest that these cells have the propensity to migrate to the tumor. To test this hypothesis, expression of chemokine receptors (CCR6, CCR7, CXCR4 and CXCR3) on Th17, Tc17 and Tγδ17 cells were analyzed in peripheral blood of GBC patients. It was observed that Tγδ17 expressed elevated levels of CXCR3 than Th17 or Tc17. However, CCR6, CCR7 and CXCR4 were expressed at comparable levels by Tγδ17, Th17 and Tc17. Next, to investigate migration of Tγδ17 cells, purified Tγδ17 cells were cultured with GBC cells in transwell assay. Enhanced migration of Tγδ17cells was observed towards OCUG-1 or rhCXCL9 or tumor supernatants. In the presence of neutralizing anti-CXCR3 antibody, the migration of Tγδ17 cells was significantly curtailed. The data suggests that tumor environment induces infiltration of Tγδ17 cells to the tumor bed through CXCL9-CXCR3 axis. Similar results

were obtained with purified γδT cells suggesting total γδT cells are also recruited towards tumor environment using CXCL9 as chemoattractant. In order to investigate whether the elevated levels of Tγδ17 in GBC patients, contribute to tumor progression, the cytokine profile of Tγδ17 was analysed. It was observed that Tγδ17 primarily secretes high levels of IL17 and low levels of IL2 and TNFα but did not produce other cytokines (IL4, IL6, IL10 and IFNγ). The presence of IL17 receptor on the GBC cells (OCUG-1 and NOZ) was confirmed by flow cytometry and immunofluorescence staining. The data showed that more than 80% of GBC cells (OCUG-1 and NOZ cell lines) expressed IL17 receptor on the surface.

The proangiogenic action of Tγδ17 cells on GBC was studied by human protein profiler angiogenesis array. Cell-free supernatants from Tγδ17 cells significantly upregulated secretion of angiogenesis promoting factors from OCUG-1 cells such as VEGF, uPA, MMP9, MCP1, GM-CSF, CXCL16, coagulation factor III, angiogenin, etc. compared to OCUG-1 cells cultured with medium alone. This effect was abrogated by addition of anti-IL17 mAb. Further, the proangiogenic effects of Tγδ17 were validated by chorioallantoic membrane (CAM) assay. Cell-free supernatants of Tγδ17 enhanced vascularization of CAM compared to medium alone. The effect was curtailed after addition of anti-IL17 mAb to the assay. Collectively, the data suggests that Tγδ17 cells are proangiogenic subtype of γδT cells and may contribute to carcinogenesis of GBC.

Next, to study whether IL17 has direct role in GBC promotion, poorly differentiated (OCUG1) and moderately differentiated (NOZ) cell lines of GBC were treated with rhIL17 and analysed for their proliferation, migration and invasion potential. It was observed that addition of recombinant IL17 to GBC cells induced proliferation, migration, matrigel invasion and VEGF production in concentration dependent manner. However, the effect was

more pronounced on poorly differentiated GBC cells. In conclusion, the data suggests that the cytokine IL17 significantly contributes to progression of GBC.

Summary and Conclusion

Despite conventional treatments modalities, GBC remains a highly lethal malignancy. 70-80 % GBC patients are associated with inflammatory condition of cholelithiasis. However, the mediators of inflammatory circuits in GBC remain unexplored. The current study has shown that low lymphocyte proliferative response, decreased cytokine secretion upon stimulation with anti CD3 mAb and down regulated CD3ξ chain expression in PBMCs demonstrate immune-dysfunction and incapability to mount optimum antitumor immune response in GBC patients. The increased levels of Tregs were reported in cancer patients as strategy of tumor immune evasion [16]. In GBC patients, the multicolour flowcytometry showed that Tregs were decreased in peripheral blood of GBC patients at all stages of disease compared to HI. However, their suppressive potential was not compromised suggesting that Tregs in GBC patients are functionally normal. We observed that GBC patients with high peripheral blood Treg cells have decreased survival compared to those with low levels. In addition, Tregs were increased in tumor compartment and express elevated levels of Foxp3 compared to peripheral blood of GBC patients. In contrast, the analysis of inflammatory immune cells revealed that compared to HI, Th17, Tγδ17 and Tc17 cells were increased in PBMCs and TILs of GBC patients. Serum cytokines profile of GBC patients showed elevated levels of cytokines (IL6, IL23 and IL1β) that are required for polarization and/or stabilization of IL17 producing cells. It was shown that the tumor environment of GBC recruits Tγδ17 cells to the tumor bed through CXCL9-CXCR3 axis and Tγδ17cells induce angiogenesis in GBC cells by secretion of IL17 as confirmed by CAM assay. Further, the data provide evidence that rhIL17 enhance proliferation, migration and invasion potential of GBC. Thus the present study provides

strong evidence that IL17 and Tγδ17 cells are emerging as important signatures of GBC and holds significance for targeted therapies.

Currently there are many drugs under clinical trial targeting IL17 inflammatory axis including monoclonal antibodies specific for IL17 (Secukinumab), IL17R (brodalumab), IL23 p40 subunit (ustekinumab), IL23 p19 subunit and small molecules with inverse agonist activity against RORγt, etc.[17]. Moreover, the increased levels of Tregs observed in GBC tumors could also be targeted using depleting antibodies or their migration to the tumor can be inhibited.

In conclusion, Tγδ17 and Th17 are emerging as predictive markers in GBC. Our data suggests that Tγδ17 mediated angiogenesis and Treg cells mediated immunosuppression may contribute to the negative clinical outcome of GBC patients. Thus, future immunotherapeutic treatment modality for GBC may use a combined approach to block the trafficking of Tγδ17 cells to the tumor, inhibit functions of IL17 and reverse the immunosuppression mediated by Treg cells.

References

1. Dutta, U., *Gallbladder cancer: can newer insights improve the outcome?* J Gastroenterol Hepatol, 2012. **27**(4): p. 642-53.
2. Randi, G., S. Franceschi, and C. La Vecchia, *Gallbladder cancer worldwide: geographical distribution and risk factors.* Int J Cancer, 2006. **118**(7): p. 1591-602.
3. Wistuba, II and A.F. Gazdar, *Gallbladder cancer: lessons from a rare tumour.* Nat Rev Cancer, 2004. **4**(9): p. 695-706.
4. Sirohi, B., et al., *Neoadjuvant chemotherapy in patients with locally advanced gallbladder cancer.* Future Oncol, 2015. **11**(10): p. 1501-9.
5. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-99.
6. Iwakura, Y., et al., *Functional specialization of interleukin-17 family members.* Immunity, 2011. **34**(2): p. 149-62.
7. Zarogoulidis, P., et al., *Interleukin-8 and interleukin-17 for cancer.* Cancer Invest, 2014. **32**(5): p. 197-205.
8. Cochaud, S., et al., *IL-17A is produced by breast cancer TILs and promotes chemoresistance and proliferation through ERK1/2.* Sci Rep, 2013. **3**: p. 3456.
9. Wilke, C. and W. Zou, *TH17 Cells in the Tumor Micro-environment*, in *TH17 Cells in Health and Disease*, S. Jiang, Editor 2011, Springer New York. p. 481-496.
10. Kesselring, R., et al., *Human Th17 cells can be induced through head and neck cancer and have a functional impact on HNSCC development.* Br J Cancer, 2010. **103**(8): p. 1245-54.

11. Chen, X., et al., *Increased IL-17-producing cells correlate with poor survival and lymphangiogenesis in NSCLC patients.* Lung Cancer, 2010. **69**(3): p. 348-54.
12. Nishikawa, H. and S. Sakaguchi, *Regulatory T cells in cancer immunotherapy.* Curr Opin Immunol, 2014. **27**: p. 1-7.
13. Shevach, E.M., *Mechanisms of foxp3+ T regulatory cell-mediated suppression.* Immunity, 2009. **30**(5): p. 636-45.
14. Chen, X. and J.J. Oppenheim, *Th17 cells and Tregs: unlikely allies.* J Leukoc Biol, 2014.
15. Ribot, J.C., 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): p. 427-36.
16. Nishikawa, H. and S. Sakaguchi, *Regulatory T cells in tumor immunity.* Int J Cancer, 2010. **127**(4): p. 759-67.
17. Miossec, P. and J.K. Kolls, *Targeting IL-17 and TH17 cells in chronic inflammation.* Nat Rev Drug Discov, 2012. **11**(10): p. 763-76.

Publications in Refereed Journal

A) Published manuscripts

5. Dar AA, **Patil RS**, Chiplunkar SV, "Insights into the relationship between toll like receptors and gamma delta T cells responses", **Front Immunol**. 2014 Jul 31;5:366. doi: 10.3389/fimmu.2014.00366.
6. **Patil RS**, Bhat SA, Dar AA, Chiplunkar SV, "The Jekyll and Hyde story of IL17 producing γδT (Tγδ17) cells", Feb4 2015, **Front Immunol.** 2015 Feb 4;6:37. doi: 10.3389/fimmu.2015.00037
7. **Patil RS,** Shah SU, Shrikhande SV, Goel M, Dikshit R, and Chiplunkar SV, "IL17 producing γδ T cells induce angiogenesis and are associated with poor survival in gallbladder cancer patients", **Int J Cancer.** 2016 Apr 8. doi: 10.1002/ijc.30134

B) Communicated Manuscript

C) Manuscripts in preparation

1. **Patil RS** and Chiplunkar SV, "Interleukin 17 promotes proliferation, invasion and angiogenesis of gallbladder cancer"

D) Other Publications / Conference Presentations:

Conference Proceedings:

3. **Patil RS,** Shah SU, Shrikhande SV, Goel M, Dikshit R, and Chiplunkar SV, "Pro-tumor role of IL17 producing γδT cells in gallbladder cancer", **European Journal of Cancer** Volume 54, Supplement 1, Pages S1-S78 (February 2016). doi: 10.1016/S0959-8049(16)31954-2.
4. **Patil RS**, Shah SU, Shrikhande SV, Goel M, Dikshit R, and Chiplunkar SV, "Role of IL17 producing γδ T cells (Tγδ17) in gall bladder cancer pathogenesis", Feb10 2015, ***J Carcinog***,14:21.

Awards:

- Received **Travel award** for oral presentation at 13th FIMSA advanced Immunology Course at PGIMER, Chandigarh (March 2016)
- **Dr. G.P. Talwar Young Scientist Award** in 41st Annual Conference of Indian Immunology Society (IMMUNOCON-2014), Madurai Kamaraj University. (December 2014)
- Received **travel fellowship** from Homi Bhabha National Institute, (February 2014)
- Received Sam Mistry **travel fellowship** award from Sam Mistry Foundation, Mumbai (February 2014)
- 2nd Poster presentation award in conference on "National Education Day", NMIMS School of Science, Mumbai. (2012)

Presentations:

1. Oral presentation on "Tγδ17 is a proangiogenic subtype of γδT cells and associates with poor survival of gallbladder cancer patients" at "**13th FIMSA advanced Immunology Course"** held at 'Department of Immunopathology, PGIMER, Chandigarh from 17-19 March 2016.
2. Oral presentation on "Paradox of immune system in Gallbladder Cancer: Role of Tγδ17 cells" at **'Society of biological chemists (India) Mumbai chapter meeting'** held at

NIRRH Mumbai (22 August 2015).

3. Oral presentation on "Role of IL17 producing γδT cells (Tγδ17) in gallbladder cancer pathogenesis" in international conference **"Carcinogenesis-2015"** on 'Molecular pathways to therapeutics: Paradigms and challenges in oncology' at ACTREC, Tata Memorial Centre, Navi Mumbai from 11-13 February 2015.
4. Oral presentation on "IL17 producing γδT cells (Tγδ17) contribute to tumorigenesis of gallbladder cancer" at **"41st annual conference of Indian Immunology Society-Immunocon-2014"**at Madurai Kamaraj University, Madurai, from 12-14 December 2014.
5. Poster was presented on "Dynamics of Tγδ17 and Regulatory T cells in Gallbladder cancer" in international conference **"Keystone Symposium on Infection inflammation and cancer joint with immune evolution in cancer"** at Fairmont Chateau Whistler, Whistler, British Columbia, Canada from 9-14 March 2014.
6. Poster was presented on "Immune dysfunctions in Gallbladder cancer : Imbalance of Tγδ17 and Regulatory T cells" at **"IX DAE-BRNS Life Sciences Symposium (LSS-2013**) **on Current advances in immunobiology and cancer"** at BARC, Anushaktinagar, Mumbai from 28-30 November 2013.
7. Poster was presented on "Dynamics of Th17 and regulatory T cells in pathogenesis of Gall Bladder cancer" at **" 39th Annual Conference of Indian Immunology Society-IMMUNOCON 2012"** at Banaras Hindu University, Varanasi from 9-11 November 2012.
8. Poster was presented on "Imbalance of Th17 and regulatory T cells in gall bladder cancer: implications in pathogenesis" at **"National Education Day"** SVKM's NMIMS School of Science, Mithibai College, Mumbai on 1 November, 2012.

9. Poster was presented on "Imbalance of Th17 and regulatory T cells in gall bladder cancer : implications in pathogenesis" at **"31st Annual Convention of Indian Association for Cancer Research & International Symposium on Cancer Genomics and its Impact in the Clinics"** at ACTREC, Navi Mumbai from 26-29 January 2012.

Signature of Student:

Date: 30/06/2016

Doctoral Committee:

S. No.	Name	Designation	Signature	Date
1	Dr. S. N. Dalal	Chairperson		1/7/16
2	Dr. S. V. Chiplunkar	Guide/Convener		1/7/16
3	Dr. Rajesh Dikshit	Co-guide		1/7/16
4	Dr. Sanjay Gupta	Member		11/7/16

Forwarded through:

1/7/16

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

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

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

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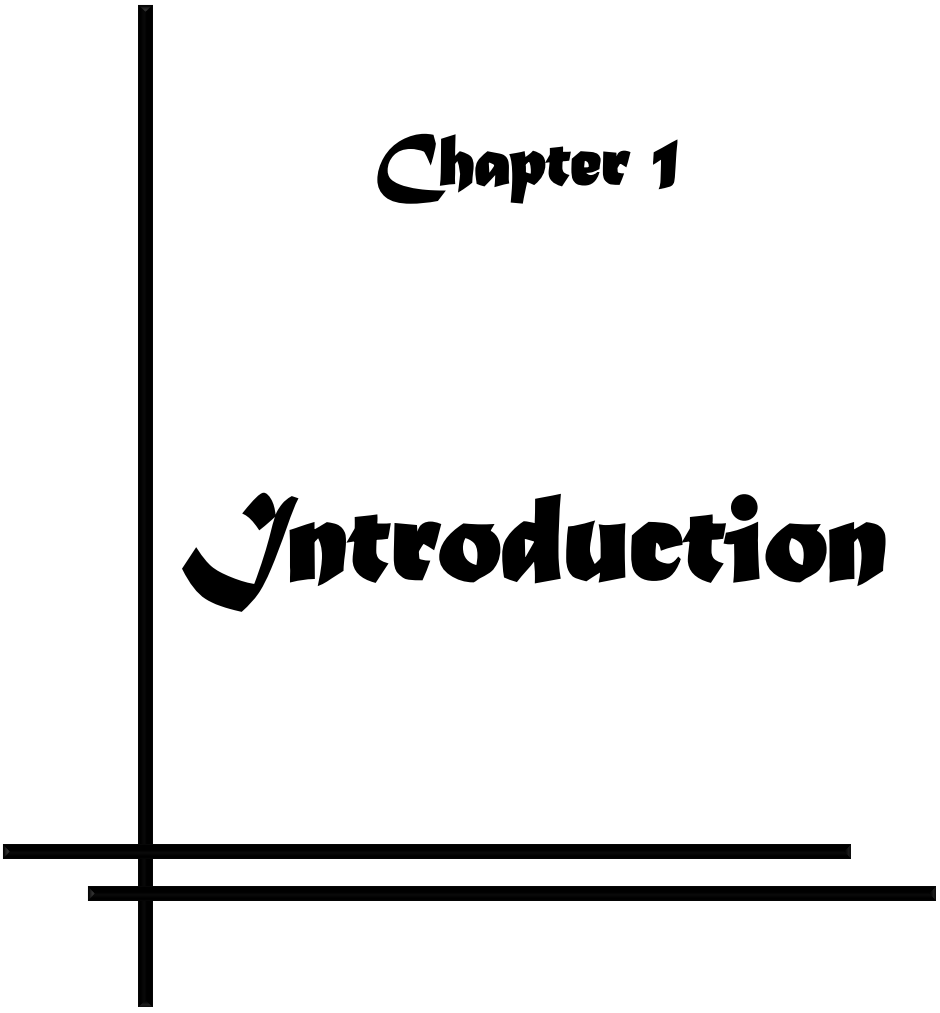
Abbreviations

^{3}H-TdR	Tritiated Thymidine
ADCC	Antibody dependent cellular cytotoxicity
Ag	Antigen
Act1	NF-κB activator 1
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
APC	Antigen Presenting Cell
APC	Allophycocyanin
AP-1	Activator protein 1
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
AID	Activation-induced cytidine deaminase
BTLA	B and T lymphocyte attenuator
BSA	Bovine serum Albumin
Bcl-6	B-cell lymphoma 6 protein
GBq	Giga Becquerel
CBA	Cytometric Bead Array
Cbl-b	Casitas B cell lymphoma-b
CCL	Chemokine Ligand
CCR	C-C motif chemokine receptor
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CAM	Chorioallantoic membrane
cm	Centimeter
COX	Cyclooxygenase
cpm	Counts per minute
C/EBPβ	CCAAT-enhancer-binding protein β
CXC	Chemokine (C-X-C motif)
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte associated protein 4
CTLA8	cytotoxic T lymphocyte associated antigen 8
DAMP	Damage associated molecular patterns
DAPI	4,6-diamidino-2-phenylindole
DC	Dendritic Cell
DEPC	Diethyl Pyrocarbonate
DN	Double negative
DMSO	Dimethyl sulphoxide
DP	Double positive
SP	Single positive
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol

EAE	experimental autoimmune encephalomyelitis
EDTA	Ethylene Diamine tetra acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FBS	Fetal bovine serum
FCS	Fetal calf serum
FH	Ficoll Hypaque
FITC	Fluorescein isothiocyanate
5-FU	5-Fluorouracil
FSC	Forward Scatter
Foxp3	Forkhead box P3
GBC	Gallbladder cancer
GMCSF	Granulocyte macrophage colony-stimulating factor
G-CSF	Granulocyte colony-stimulating factor
GRAIL	gene related to anergy in lymphocytes
GWAS	genome-wide association study
GSK-3β	Glycogen synthase kinase-3β
HRP	Horseradish peroxidase
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
h	Hours
HRPO	Horse radish peroxidase
HMGB1	High mobility group box 1
HSP	Heat shock proteins
IDO	Indoleamine 2,3-dioxygenase
IFN-α/β	Interferon alpha/beta
IFN-γ	Interferon gamma
iNOS	inducible nitric oxide synthase
IL	Interleukin
IPP	Isopentenyl pyrophosphate
IKKi	I kappa B kinase I
JAK	Janus tyrosine kinase
kDa	Kilo Dalton
LXRβ	Liver X receptor β
LTβR	lymphotoxin-β receptor
mAb	Monoclonal antibody
MACS	Magnetically activated cell sorting
MAPK	Mitogen Activated Protein Kinase
MCA	methylcholanthrene
MCP-1	monocyte chemoattractant protein 1
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 molar
mm	milimeter
MMP	matrix metalloproteinases
Mo-MDSC	monocytic MDSC
mRNA	Messenger Ribonucleic acid
NF-κB	Nuclear factor kappa B
NKT	Natural killer T
NK	Natural killer
NKG2D	Natural Killer Group 2D
nM	Nano Moles
OGG1	8-Oxoguanine glycosylase
OD	Optical Density
PAMP	Pathogen-associated molecular pattern
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
PTGS	Prostaglandin-endoperoxide synthase
PRR	pattern recognition receptors
PMA	Phorbol 12-myristate 13-acetate
PTK	protein tyrosine kinases
rIL-2	Recombinant Interleukin 2
RANTES	regulated on activation, normal T cell expressed and secreted
Rag	Recombination activating gene
RNA	Ribonucleic acid
RNAase	Ribonuclease
ROS	Reactive Oxygen Species
RNI	Reactive nitrogen intermediates
rpm	Revolutions per minute
RPMI	Roswell Park memorial Institute
RT	Room temperature
RT-PCR	Real Time-Polymerase chain reaction
ROR	retinoid-related orphan receptor
SCC	Squamous cell carcinoma
SEFIR	Similar Expression of Fibroblast growth factor genes and IL17Rs
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error mean
SLAP	Src-like adaptor protein

Sec	Seconds
SSC	Side scatter
STAT	signal transducer and activator of transcription
TAA	Tumor associated antigen
TAK1	TRAF6 dependent TGFβ activated kinase
TAMs	tumor-associated macrophages
TCR	T-cell receptor
TLR	Toll like receptor
TGF-β	Transforming growth factor beta
Th	T helper
Tc	T cytotoxic
TIL	Tumor infiltrating lymphocytes
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
TIM-3	T-cell immunoglobulin and mucin-domain
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
TRAF	TNF receptor associated factor
Tregs	Regulatory T cells
Tres	Responder T cells
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
αβ	Alpha beta
γδ	Gamma delta
µCi	Micro-Curie
µg	Microgram
µl	Microliter
UICC	International Union Against Cancer



Chapter 1

Introduction

Introduction

In India, more than 1 million new cancer cases are diagnosed every year and this number is expected to double in next 20 years [1]. Nearly 0.7 million deaths occurred in India due to cancer, which is home to about 17% of the global population with mortality to incidence ratio 68.6 [2]. Over the years, the burden of cancer has shifted to less developed countries, which currently account for about 57% of cases and 65% of cancer deaths worldwide. Although incidence rates for all cancers combined are twice in more developed compared with developing countries, mortality rates are only 8% to 15% in more developed countries [3]. This disparity highlights the lack of sophisticated cancer care system in developing countries and the need to pursue comprehensive understanding of cancer in local setting. The burden of cancer is linked to socioeconomic inequalities to access health care thus it is a major health care challenge in India. It leads to catastrophic healthcare expenditure and push entire family below poverty line [2]. Thus, the current cancer care system in India demands extensive research focusing on basic understanding of molecular mechanisms of cancer progression as well as technological improvements providing affordable, equitable, and universal cancer care for the entire population.

Gallbladder cancer (GBC) is one of the uncommon types of cancer but is highly lethal with 5 year survival of patients less than 5%. The increasing incidences of GBC in India are giving alarming signals that the search for new therapeutic targets with the hope of improving survival in patients with GBC is much needed. First described by Maximillian de Stoll in 1777, it is the fifth most common malignancy of gastrointestinal cancer worldwide and 4th most common cancer (following breast, cervix and ovary) and commonest gastro-intestine related cancer in India (accounting for 80 to 90% of biliary tract cancer) [4, 5]. The incidence of GBC displays striking differences on the basis of gender, ethnicity and geography. Though it is common in the Indian subcontinent (India, Pakistan), South America (Chile, Bolivia,

Columbia), East Asia (Korea, Japan) and central Europe (Slovakia, Poland, and Czech Republic), it is relatively infrequent in most parts of Europe, North America and Australia/New Zealand. The prevalence of GBC is three times higher among women than men in almost all populations (7.5 per 100,000 for men and 23 per 100,000 for women). The highest GBC incidence rate worldwide is reported for women in north India followed by Pakistan, Ecuador and Chile [6]. The incidence of GBC varies within India. It is much higher in northern parts specifically in gangetic belts than in southern parts of India. The risk of GBC increases with increase in age. The incidence increases after age of 45 and peaks after age of 65 years [4, 7].

The major risk factor of GBC is cholelithiasis. More than 70% of GBC patients are associated with gall stone disease. However, a very small fraction of patients with cholelithiasis develop GBC. Increasing stone size (>3 cm), number, volume, and weight, all are associated with an increased risk of cancer [8]. Cholelithiasis and GBC frequently coexist in the same population suggesting gallstones may function as cofactor in GBC. It is hypothesized that gallstones might cause direct mechanical irritation to the surrounding mucosal surface or they might affect gallbladder function, leading to delayed or incomplete emptying of bile with subsequent bile stasis and dilation of the gallbladder, which predisposes to inflammation. The chronic inflammation associated with cholelithiasis leads to dysplastic changes in gallbladder wall resulting into high grade premalignant carcinoma *in situ* [9].

Patients diagnosed with GBC show dismal prognosis with median survival of 3 to 6 months irrespective of treatment [10]. Anatomical location of gallbladder in the abdomen and elusive early symptoms, render diagnosis of these patients at an advanced stage. Complete surgical removal of gallbladder is the only curative treatment available [11]. However, more than 90% of patients present with an unresectable advanced disease. In addition, among patients undergoing “curative” resection, recurrence rates are high since GBC represents most

aggressive type of cancer among biliary tract related cancers. There is lack of suitable biomarkers for early diagnosis of the disease and established adjuvant treatments are unavailable in this setting [12, 13]. Despite improved results of chemotherapy and surgery, the long term outcome remains disappointing [14]. Therefore, for successful management of GBC, efforts are needed to identify other etiological factors contributing to pathogenesis of the disease.

The inflammatory microenvironment is an essential component of a tumor and plays a decisive role at different stages of tumor development. In GBC, chronic persistence of gallstones leads to inflammatory condition of chronic cholecystitis [15]. In murine models of gallstone disease, chronic gallbladder inflammation occurs at early stages as a local response to the presence of lithogenic bile (i.e. cholesterol supersaturated bile) [16]. Although the histological changes in gallbladder wall are induced by chemical stimulus (lithogenic bile), presence of microbiota of intestinal origin is also reported in gallbladder milieu [17]. The epidemiological evidence indicates a definite relation of GBC with infection of *Salmonella typhi* and *Helicobacter pylori* which result in chronic inflammation [10]. Liver flukes, particularly *Clonorchis sinensis* and *Opisthorchis viverrini*, have been implicated in patients of biliary tree cancer [18, 19]. The persistent inflammation in gallbladder could promote epithelial hyperplasia, dysplasia and ultimately progression to carcinoma [20]. Polymorphisms in genes related to the immune system, inflammation and oxidative stress namely PTGS2 (Prostaglandin-endoperoxide synthase 2 / cyclooxygenase 2), TLR2 (Toll like receptor 2), TLR4, IL1RN (IL1 receptor antagonist), IL1B, IL10, IL8, CCR5 (C-C motif chemokine receptor 5), LXRβ (Liver X receptor β) and OGG1 (8-Oxoguanine glycosylase) have been associated with increased risk of GBC. Enhanced expression of COX2 and VEGF in GBC tissue associates with poor prognosis of GBC patients [21, 22]. A study on immune infiltrates in GBC analysed by immunohistochemistry showed that enhanced expression of

BTLA (B and T lymphocyte attenuator; co-inhibitory receptor) and Cbl-b (Casitas–B-lineage lymphoma protein-b; the anergy cell marker) are involved in inhibition of antitumor immunity and associates with unfavourable outcome in GBC patients [23]. Thus the inflammation-related genes, under the stimulus of gallstones or other insults, may accelerate the development of GBC [20]. The gathered evidence strongly links chronic inflammation with GBC progression. However, the immune players contributing to tumor associated inflammation in GBC are elusive.

Inflammatory cytokines have been observed to increase in pathogenic condition and are associated with disease exacerbation. Interleukin 17 (IL17), a 32 kilo Dalton dimeric protein, is a potent proinflammatory cytokine. [24]. It is predominately produced by $CD4^+$ (Th17) cells, but also by $CD8^+$ T cells (Tc17), Natural Killer T cells (NKT cells), macrophages, neutrophils and γδ T cells [24, 25]. Originally called cytotoxic T lymphocyte associated antigen 8 (CTLA8), induces the expression of proinflammatory cytokines including Tumor Necrosis Factor (TNF), IL1, IL6, Colony Stimulating Factors, chemokines, antimicrobial peptides and matrix metalloproteinases from endothelial cells, epithelial cells and fibroblasts. IL17 increases the immigration of neutrophils, macrophages and monocytes to inflamed tissues [25]. It acts through the IL17 receptor expressed on nearly every cell type of body [26]. It is shown that fibroblasts, epithelial cells and endothelial cells are the major targets of IL17. The contribution of IL17 to disease progression is thus linked to the role of these cells in disease pathology [26]. IL17 has a protective role against fungi and bacteria but when dysregulated, its elevated levels have been found in many autoimmune diseases and cancers [24]. Several human studies have highlighted the correlation between the level of IL17 and poor prognosis in cancer patients [27, 28]. Increased levels of IL17 in tumor tissue participate in neoangiogenesis and associate with microvessel density in tumors [29]. IL17 has been shown to induce, chemoresistance, neoangiogenesis and activation of matrix

metalloproteinases which in turn enhances tumor progression [30, 31]. However, the cellular source of IL17 and its clinical relevance in GBC is not well studied. Additionally, how IL17 augments progression of GBC is not well understood.

Initial studies on IL17 at mRNA level showed that it is expressed in memory $CD4^+$ T cells [32]. Later it was shown that IL17 is produced by distinct subset of $CD4^+$ T cells different from IFNγ producing subset called as Th17 ($CD4^+IL17^+$) cells. The proinflammatory cytokines such as IL6, IL1β, IL23 and TGFβ induce the differentiation and/or stabilization of Th17cells [33]. Analogous to the differentiation of Th1 and Th2 cell lineages, Th17 cells require both retinoid-related orphan receptor-α (RORα) and RORγ as lineage-specific transcription factors [34]. Ubiquitin mediated degradation of RORγt results into impairment of Th17 differentiation suggesting critical requirement of RORγt for Th17 functions [35]. IL6 plays an essential role by activating STAT3 (Signal transducer and activator of transcription 3), which directly drives transcription of Th17 lineage-specific genes including Rorc, IL17, and IL23R [36]. IL21 and IL23 induce RORγt, which in synergy with STAT3 promote IL17 expression [37]. Th17 cells migrate to the tumor environment via CCR6/CCL20 chemokine axis [38]. RANTES and MCP-1 secreted by tumor cells and tumor-derived fibroblasts also mediate the recruitment of Th17 cells to the tumor bed [39]. Th17 cells and its related cytokines are reported to be present in tumor environment of hepatocellular, prostate, ovarian, colorectal, head and neck, breast, gastric cancer and various other malignancies [40, 41]. Th17 cells expressing CCR4 and CCR6 suppress lytic function, proliferation, and cytokine secretion of $CD8^+$ T cells. Additionally IL17 secreted by Th17 cells promotes not only regular angiogenesis but also lymphangiogenesis and development of lymphatic vessels by inducing expression of the pro-angiogenic factors including VEGF-D [42-44]. In contrast, it was demonstrated that increased levels of Th17 correlated with the number of NK cells, $CD4^+IFN\gamma^+$, $IL\text{-}17^+IFN\gamma^+$, $CD8^+IFN\gamma^+$ T cells in tumor tissue and were associated with

improved survival of patients with ovarian, oesophageal and lung cancer [38, 45, 46]. Th17 cells can also undergo lineage conversion into regulatory T cells (Treg) which suppress antitumor immune response [47, 48]. Moreover, Th17 cells cultured with TGF-β/IL-6 co-express CD39 and CD73 ectonucleotidases on their surface which transforms ATP or ADP into immunosuppressive adenosine hampering antitumor immune response [49]. Thus, although the accumulation of Th17 cells into tumors is commonly observed, their effect on cancer progression remains controversial [50].

In contrast to the proinflammatory subtypes of $CD4^+$ T cells, another subset characterized as $CD4^+CD127^{low/-}CD25^+Foxp3^+$ regulatory T cells (Treg), are known to play a critical role in immune tolerance and control of autoimmunity [51]. They express Foxp3 as lineage determining transcription factor and are actively engaged in inhibiting activation of CD4 and CD8 T cells thereby impairing antitumor immune responses [52]. Treg cells suppress target cell types by using various mechanisms including secretion of suppressor cytokines (TGFβ, IL10), IL2 sequestration, expression of co-inhibitory molecules (CTLA4, PDL1, TIM3) or cytolysis [52]. The tumor environment secretes large amounts of CC-chemokine ligand 22 (CCL22) which recruit Treg cell through CCR4 to the tumor bed [53]. Consequently, elevated proportions of Tregs have been identified in peripheral blood as well as in tumor microenvironment and are associated with poor prognosis of head and neck, lung, liver, gastrointestinal tract, pancreas, ovary, breast and several other cancer patients [51].

An expanding body of literature has highlighted the importance of balance between destructive inflammation and protective immunity determining the direction of malignant progression [54, 55]. The tumor infiltrating immune cells are engaged in extensive crosstalk with cancer cells. The type, functional orientation, density and location of infiltrating immune cells determine the fate of tumor progression and response to anti-tumor therapy [56]. Th17 and Treg are mutually contradictory subtypes of $CD4^+$ T cells mediating inflammation and

immunosuppression respectively. The cytokines present in tumor environment play decisive role in determining the balance of Th17 and Treg [57]. The altered balance of Th17/Treg is associated with disease progression in patients with lung cancer, pancreatic cancer, cervical cancer, etc. suggesting that interaction of Th17 and Tregs represent important regulatory mechanism in cancer pathogenesis [58-60]. Maintaining an appropriate balance of Th17 and Treg cells may ensure effective immunity against tumor. The molecular mechanisms underlying the involvement and regulation of these two subsets in cancer immunopathology remain largely unknown. Thus understanding the functional relevance of IL17 producing T cells and Treg cells in GBC tumor environment holds significance.

The present prospective study is aimed at investigating how the dynamics of proinflammatory (IL17 producing T cells) and immunosuppressive (Treg cells) contributes to inflammation and thereby progression of GBC.

On the basis of available information as described above, we addressed following objectives in the current thesis

1. Analysis of the immune scenario in peripheral blood of GBC patients
2. Understanding the functional dynamics of pro-inflammatory (Th17, Tc17 and Tγδ17) and anti-inflammatory regulatory T cells ($CD4^{+}CD127^{low/-}CD25^{+}Foxp3^{+}$; Tregs) in peripheral blood and tumor microenvironment of GBC patients
3. Exploring the pro-tumor role of IL17 producing γδT (Tγδ17) cells in GBC

Chapter 2

Review of literature

2.1 Gallbladder Cancer

Gallbladder cancer (GBC) is the most common malignancy of the biliary tract, representing 80-95% of biliary tract cancers worldwide [4]. The cancers of biliary tract, importantly GBC, are relatively infrequent but highly lethal diseases that are notoriously difficult to diagnose and treat. The biliary tract consists of an interconnected system of intra- and extrahepatic ducts that transport bile secreted from the liver to the digestive tract. Gallbladder is a sac-like structure situated beneath the right lobe of the liver (Figure 2.1 A). It is a vital component of the biliary system that receives bile produced in the liver, modifies its composition and then releases it into the duodenum [9]. The GB has a thin wall (< 3 mm) composed of a single layered columnar lining, a thin lamina propria, a thin muscle layer and serosa (Figure 2.1 B) [61]. The bile secreted into the duodenum facilitates the intestinal absorption of dietary lipids across the absorptive cells of the brush-border membrane of the intestine, either through passive diffusion or by a carrier-mediated mechanism. Filling of the gallbladder with bile is promoted by contraction of the sphincter of Oddi (a junction where common bile duct meets duodenum). Bile, secreted from liver is an isotonic fluid consisting of an electrolyte resembling blood plasma in composition. The gallbladder epithelium is one of the most absorptive epithelial surfaces. The trans-mucosal absorption of water and electrolytes by gallbladder wall concentrates the bile. The evacuation of the gallbladder contents into the duodenum through contraction of the muscular layer is regulated by a peptide hormone cholecystokinin, which is released from the duodenal mucosa in response to the ingestion of fats and amino acids [9].

2.2 Anatomical and Physiological Considerations of GBC

GBC originates from the mucosal epithelial lining (adenocarcinoma) of the gallbladder and the cystic duct [12]. Eighty to ninety percent of gallbladder cancers are adenocarcinomas. The remainder are papillary, squamous cell, adenosquamous, undifferentiated or small cell

carcinomas [62]. The development of gallbladder cancer is proposed to occur over a span of 5–15 years, with tissue alterations including metaplasia, dysplasia, carcinoma *in situ*, and invasive cancer [4]. GBC is characterized by local invasion, extensive regional lymph node metastasis, vascular encasement, and distant metastases. It is the most aggressive of the biliary cancers and neither radiation nor conventional chemotherapy significantly improves survival or quality of life [9, 12]. Gallbladder is an out pouching from the gastrointestinal tract and regularly empties more viscous bile material against gravity to prevent stagnation. Thus gallbladder mucosa is constantly exposed to various metabolites excreted by the liver. Progressive physiological concentration of toxic materials in the bile, promote chronic injury and mutagenesis [11]. Thus majority of GBC originate from the fundus (60%) and remaining from body (30%) or neck (10%) of the gallbladder [62].

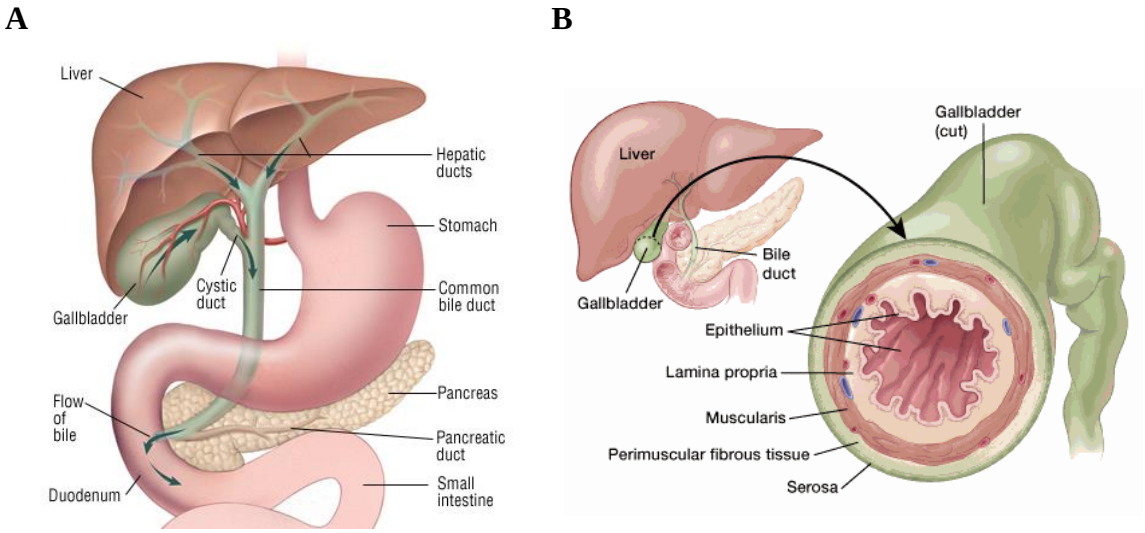


Figure 2.1 : (A) *A representative diagram depicting anatomical location of gallbladder along with other visceral organs.* ***(B)*** *A representative image of cut section of gallbladder wall. The images were adapted from google images.*

Gallbladder cancer spreads in four modes: 1) local invasion to liver or adjacent organs; 2) lymphatic spread; 3) peritoneal dissemination; 4) haematogenous spread. The propensity of local invasion and lymphatic spread is the highest [62]. The lymphatic drainage follows four

routes: cholecysto-retropancreatic to the retropancreatic group, cholecysto-celiac to the celiac group, cholecysto-mesenteric to the superior mesenteric group and the ascending lymphatics to the hilar group of lymph nodes. The lymphatic drainage of gallbladder is extensive and in multiple directions, facilitating early lymphatic spread [61]. The gallbladder is in an anatomically "busy" area due to presence of adjoining bile duct, portal vein, liver, duodenum and colon, making surgical resection and radiotherapy difficult [11]. Diagnosis of GBC commonly occurs as an incidental finding in the setting of surgical intervention for cholelithiasis (gall stone disease), resulting in advanced disease at the time of initial diagnosis [63]. The local and metastatic spread of GBC is evaluated by surgical staging which helps in planning the treatment.

2.3 Surgical staging of GBC

The staging of GBC is based on the depth of penetration and extent of spread. Multiple tumour staging systems (Nevin staging, Japanese Biliary Surgical Society staging system, TNM staging system of the American Joint Committee on Cancer [64]/International Union Against Cancer [UICC]) have been described for gallbladder cancer [5]. In 1976, Nevin *et al*. originally classified patients into five stages combining staging and histological grading of this cancer: Stage 1–*in situ* cancer; Stage 2–cancer not yet transmural; Stage 3–transmural direct liver invasion; Stage 4–lymph node metastases; Stage 5–distant metastases [65].

Table 2.1 Nevin staging system for gallbladder cancer

Stage Grouping	***Characteristics***
Stage I	Intramucosal tumor
Stage II	Tumor extends to muscularis
Stage III	Tumor extends to serosa
Stage IV	Transmural involvement and cystic lymph node involvement

Stage V	Direct extension to the liver and or distant metastasis

The Japanese Biliary Surgical Society staging system classified tumours into four stages: Stage I- Cancer spread confined to gallbladder capsule; Stage II– positive N1 lymph nodes and/or minimal liver/bile duct invasion; Stage III– positive N2 lymph nodes and/or marked liver/ bile duct invasion; Stage IV–distant metastases [66]. However, this system is rarely used outside Japan. In 2010, the seventh edition of the American Joint Committee on Cancer has published a simplified tumor node metastasis (TNM) classification [67]. It is presently the most widely used system. This system is also used to stage cancers that start in the cystic duct (the tube that carries bile away from the gallbladder). The TNM system is based on 3 key pieces of information:

- **T :** describes how far the primary tumor has grown into the wall of the gallbladder and if it has grown into other nearby organs or tissues.
- **N :** describes whether the cancer has spread to regional lymph nodes
- **M :** indicates whether the cancer has metastasized to the distant other organs of the body. The most common sites of gallbladder cancer spread are the liver, peritoneum, and the lungs.

Table 2.2 Staging of GBC according to AJCC 7th edition

Stage	***Characteristics***
Primary Tumor (T)	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*

T1a	Tumor invades lamina propria		
T1b	Tumor invades muscle layer		
T2	Tumor invades perimuscular connective tissue; no extension beyond serosa or into liver		
T3	Tumor perforates the serosa and/or directly invades liver and/or 1 adjacent organ/structure		
T4	Tumor invades main portal vein or hepatic artery or invades 2 or more extrahepatic organs		
Regional Lymph Nodes (N)			
N0	No regional lymph node metastasis		
N1	Metastasis to nodes along cystic duct, common bile duct, hepatic artery and or portal vein		
N2	Metastasis to periaortic, pericaval, superior mesenteric artery and/or celiac artery lymph nodes		
Distant metastasis (M)			
M0	No distant metastasis		
M1	Distant metastasis		
Stage Grouping			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage IIIA	T3	N0	M0
Stage IIIB	T1-3	N1	M0
Stage IVA	T4	N0-1	M0
Stage IVB	Any T	N2	M0
	Any T	Any N	M1

GBC categorized as stages I or II are potentially resectable with curative intent; stage III generally indicates locally unresectable disease as a consequence of vascular invasion or involvement of multiple adjacent organs; stage IV represents unresectability with distant metastases. Histologically, GBC is graded according to its cellular differentiation into four grades. The scale used for grading gallbladder cancers goes from G1 (where the cancer looks much like normal gallbladder tissue) to G4 (where the cells are poorly differentiated). The grades G2 and G3 fall somewhere in between. The majority of GBC patients presented with grade 3, exhibit poorly differentiated tumors [68].

2.4 Epidemiology of GBC

Gallbladder cancer (GBC) was first described by Maximillian de Stoll in two autopsy cases in 1777, with the first documented cancer resection performed by Keen in 1891 [5]. GBC is the fifth most common cancer of biliary tract. The overall mean survival rate for patients with advanced gallbladder cancer is 6 months, with a 5-year survival rate of less than 5% [8]. Worldwide, GBC has a low occurrence, < 2 per 100,000, but there is a widely variable geographic pattern for GBC occurrence. Prevalence of GBC is rare in developed countries but prevalent in developing countries. Asia is a high risk continent, while the United States and most western and Mediterranean European countries (e.g., UK, France, and Norway) represent low risk areas [62]. Annual incidence rate of GBC is high in north and South American Indians, particularly amongst women: 15.5 per 100,000 in women (vs 7.5/100,000 in men) from La Paz, Bolivia, and 11.3 per 100,000 in women (vs 4/100,000 in men) from New Mexico. The increasing rate of GBC is the leading cause of cancer death in Chilean women, exceeding even breast, lung and cervical cancers [69]. The other high risk regions include Poland (incidence rate is 14/100,000 for women), northern India (21.5/100,000 for women), Ecuador (12.9/100,000) and south Pakistan (11.3/ 100,000 for women) [6, 62].

Other South American countries, Israel, Japan and China show intermediate incidence of GBC.

2.4.1 GBC : Indian scenario

In India, the incidence of GBC is increasing [7]. A comparative study of different cancer registries in India has shown that the Delhi registry has more number of GBC cases but incidence rate of GBC is high in Kamrup urban district (Assam) followed by Imphal district (Nagaland) [7]. Interestingly the prevalence of GBC in India shows a peculiar north-south distribution [70]. The age standardised GBC incidence is high in north and eastern India among both genders compared to south India. Residence in Gangetic belt is one of the important risk factor identified in the epidemiological studies [71]. The presence of low levels of selenium and zinc and high levels of copper, cadmium, nickel, chromium, and lead in the bile and gallbladder tissue may be associated with increased risk of GBC in patients residing in Gangetic belt [72]. The incidence of GBC is higher in rural India compared to urban area [73, 74]. The lower socioeconomic status, illiteracy, poor nutritional diet, tobacco habit and joint family are identified risk factors which may be associated with rural and urban distribution of GBC occurrence [71, 74, 75]. The incidence of GBC cases in India are projected to increase in near future in both males and females [76]. Therefore, the important inputs are required from various health agencies for management of GBC.

2.5 Risk factors of GBC progression

Risk factors responsible for GBC include genetic predisposition, geographic variation, ethnicity, increasing age, female gender, chronic inflammation, congenital developmental abnormalities, low socio-economic status, low frequency of cholecystectomy for gallbladder diseases and exposure to certain chemicals. The most relevant risk factors are discussed here.

2.5.1 Age

The incidence of GBC increases with advancing age. The age-adjusted incidence rates of GBC is reported as 0.16/100,000 (for those 20−49 years), 1.47/100,000 (for those 50−64 years), 4.91/100,000 (65−74 years), and 8.69/100,000 for individuals over the age of 75 years [64]. The highest mortality rate was 5.05/100,000, for individuals over the age of 75.

In India, the age standardised incidence rate of GBC revealed an increasing trend after age of 45 years and peaks after age of 65 years [7]. The various epidemiological studies shown that the GBC incidence rate increases with higher age. However, the mean ages of GBC patients (51.5 years in males and 55.5 years in females) in India are lower compared to those of the world population[4, 7, 75]. This might be a reflection of the average low life expectancy in India.

2.5.2 Ethnicity

The geographic pattern of GBC occurrence varies widely across the globe. The prevalence also varies in different ethnic populations. The highest rate of GBC incidence is exhibited by Mapuche Indians from Valdivia, Chile, South America (12.3/100,000 for males and 27.3/100,000 for females). American Indians in New Mexico, USA, follow, with an average annual rate of 8.9/100,000 [4]. Asia is a high risk continent, where an increased frequency of GBC occurs in northern Indian females, Pakistani females and Korean males. In India there exist differences in the GBC occurrence between north and south India which can be related to different ethnic and cultural background [70]. The Korean males have highest incidence rate in Asia than females (8.1 for males and 5.6 for females per 100,000) [4]. The mortality rate of GBC follows the incidence. Worldwide, the burden peaks in the Mapuche Indians of Chile (35 per 100,000 each year), closely followed by Hispanics and North American Indians. However, mortality is declining in some countries, like the USA, Canada, Australia, and parts of Europe (the UK and Hungary), but increasing in others, including Chile and

Japan [77]. The availability of standard healthcare and treatment for GBC may be responsible for the lower mortality in developed countries.

2.5.3 Gender

Frequency of GBC occurrence shows a marked predominance in women over men worldwide. The incidence is highest in north Indian women followed by Pakistan and Chile [6]. Women are affected two to six times more often than men [4]. This bias varies greatly in different parts of the world, being highest in countries and regions with the highest rates of GBC. The main victims of the disease are elderly postmenopausal females as compared to the males with median age of 51 to 60 years [78]. The role of estrogen receptor and progesterone receptor expression in gallbladder cancer is not significantly different between men and women [79]. The co-expression of both receptors is increased in females with gallbladder cancer as compared with males. There are contradictory reports on involvement of estrogen and progesterone responsible for high risk of GBC in females [79]. However, greater number of pregnancies in women is one of the risk factors of GBC [78]. The increased use of oral contraceptives among premenopausal female is another serious risk factor for the high occurrence of GBC in females [78].

2.5.4 Cholelithiasis (gallstone disease)

Cholelithiasis is a major risk factor of GBC. More than 80% of GBC patients are diagnosed with gallstones [8]. Epidemiological data showed that the occurrence of GBC is high in area prevalent for gallstone disease. The coexistence of GBC and gallstone disease is observed in Pima Indian females (21/100,000 cancer incidence and 75.8% gallstone prevalence), North American Indian females (7.1/100,000 and 64.1%), Chilean Mapuche Indian females (27.3/100,000 and 49.4%), and East Indian females (22/100,000 and 21.6%) [4]. GBC and gallstone disease often share common risk factors including age, gender, parity and ethnicity [8].

Gallstones are formed by the concentration of normal or abnormal bile constituents. There are three main types: cholesterol gallstones, mixed gallstones and pigment gallstones [8]. Cholesterol stones seem to be more common than pigment stones in GBC patients. Cholesterol gallstones contain more than 50% cholesterol monohydrate and a mixture of calcium salts, bile pigments, proteins and fatty acids. In the presence of an excess of cholesterol in relation to phospholipids and bile acids, the unstable, cholesterol-rich vesicles aggregate into larger multi-lamellar vesicles, from which cholesterol crystals precipitate [9]. These cholesterol crystals transform into stones by aggregation and compaction of cholesterol and calcium bilirubinate. Thus excessive cholesterol secretion and gallstone synthesis is associated with obesity, ageing, pregnancy, hyperlipidaemia and use of oral contraceptive and hypolipodaemic agents [9]. It has been suggested that increase in number, volume, and weight of stones have a greater impact on the risk of developing gallbladder cancer [80, 81]. In case-control studies, the relative risk for gallbladder cancer in patients with gallstones ≥ 3cm was shown to be 9-10 times higher, compared with patients with stones smaller than 1cm [81-83]. A polymorphism at the ABCG5/G8 (hepatocanalicular cholesterol transporter) heterodimer partner was the first genetic risk factor of cholesterol gallstone disease discovered by a GWAS study [84]. This polymorphism allows increased sterol secretion leading to cholesterol hyper saturation and thereby promoting gallstone formation [85]. Thus the genetic predisposition to the gallstone formation increases the risk of GBC incidence.

It is suggested that chronic persistence of gallstones might cause direct mechanical irritation to the surrounding mucosal surface which leads to inflammation. Chronic inflammation leads to intermediate low-grade dysplastic changes in the gallbladder epithelium followed by increased stratification of the atypical epithelium, results in late high-grade premalignant changes, called as carcinoma *in situ* [10]. Gallstones may also obstruct the release of bile leading to delayed or incomplete emptying with subsequent bile stasis and dilation of the

gallbladder, which predisposes to inflammation. It is reported that in gallbladder specimens excised for cholelithiasis or cholecystitis, 83% exhibited epithelial hyperplasia, 13.5% atypical hyperplasia and 3.5% *in situ* carcinoma, suggesting cholelithiasis and cholecystitis could produce a series of epithelial pathologic changes [86]. Therefore, cholelithiasis is a well-established risk factor for GBC although only 1–3% of patients with gallstones develop GBC.

2.6 Inflammation and GBC

2.6.1 Cholelithiasis induced inflammation

Given the association between chronic cholecystitis and gallbladder cancer, chronic inflammation is probably the most common causative factor for GBC. Gallstone related gallbladder carcinogenesis occurs mainly through the metaplasia–dysplasia–carcinoma pathway. Epithelial metaplasia is characterised by a transformation of a differentiated epithelium and is associated with tissue damage and chronic inflammation [87]. The metaplastic gallbladder epithelium is often infiltrated with denser populations of T and B lymphocytes and macrophages correlated with an increase in the average gallbladder wall thickness [20, 88]. Cyclooxygenase-2 (COX2) is an enzyme involved in prostaglandin biosynthesis and inflammation is overexpressed in high grade lesions of GBC [89]. Treatment of patients with aspirin showed reduced risk of GBC suggesting the vital role of inflammation in GBC development [90].

Polymorphisms in genes related to the immune system, inflammation and oxidative stress namely PTGS2 (Prostaglandin-endoperoxide synthase 2 / cyclooxygenase 2), TLR2 (Toll like receptor 2), TLR4, IL1RN (IL1 receptor antagonist), IL1B, IL10, IL8, CCR5 (C-C motif chemokine receptor 5), LXRβ (Liver X receptor β) and OGG1 (8-Oxoguanine glycosylase) have been reported to be associated with increased risk of GBC. Thus, variants in inflammation related genes may, under the stimulus of gallstones or other insults, accelerate

the development of GBC [20]. In a murine study, presence of cholesterol crystals in gallbladder developed local changes in the gallbladder characterized by increased mucus layer thickness, IL1 and myeloperoxidase activity [91]. In another study, mice on lithogenic diet showed infiltration of inflammatory cells composed of eosinophils, macrophages, neutrophils and lymphocytes within the lamina propria and granulocyte infiltration in the gallbladder with progressive impairment of gallbladder emptying [16, 92]. Interestingly, it is shown that $Rag2^{-/-}$ mice (deficient for B and T cells) were resistant for development of gallstones [93]. In addition, gallbladder tissue from patients with gallstones has been observed to contain higher levels of infiltration of COX-2/iNOS positive macrophages, granulocytes and mast cells compared to controls (patients without gallstones) suggesting the role of inflammatory immune infiltrates in gallstone formations [94]. The ethnic differences in the population also contribute to the gallstone formation and inflammation. The bile samples of patients from Chile and The Netherlands showed the differences in lithogenic process and degree of gallbladder inflammatory response [95]. Thus the chronic inflammatory infiltrates are involved in the early stages of gallstone formation and seem to contribute to GBC.

2.6.2 Infection associated inflammation in GBC

Salmonella infection in gallbladder

Another important risk factor for GBC is the chronic carriage of infectious microorganisms in the gallbladder. Up to 20% of cases of cancer worldwide are associated with microbial infection [96]. The bacterial genera identified by culture or by PCR in the gallbladders of patients with cholecystitis and cholelithiasis, include *Salmonella, Escherichia, Klebsiella* and *Helicobacter* [97]. The epidemiological evidence indicates a definite relationship between *S. typhi* and GBC. About 3–5% of GBC patients are chronic carriers of *S. typhi* infection as the bacteria are known to persist in the biliary system [98]. Studies performed in north India, for

both typhoid fever and GBC, have reported odds ratios for GBC of 8.5 [99], 9.2 [100], 14 [98] and 22.8 [101] among chronic typhoid carriers, supporting the strong association between these pathologies. Recently, molecular evidence linking Salmonella infection with GBC development has shown that infection with *Salmonella enterica* induced malignant transformation in predisposed mice, murine gallbladder organoids, and fibroblasts, with TP53 mutations and c-MYC amplification. It involved the activation of MAPK and AKT pathways mediated by *Salmonella enterica* effectors secreted during infection [102]. This suggests the importance of *Salmonella* infection as significant risk factor of GBC.

Helicobacter infection in gallbladder

Infection of *Helicobacter* species is another risk factor of GBC. Several PCR-based studies showed that *Helicobacter* species were identified in resected gallbladder tissue and bile collected from patients with cholecystitis or cholelithiasis [103-105]. The infection rate of *H. bilis* is 2-3 times higher in patients with biliary tract cancer compared to healthy individuals. *H. bilis* infection activates transcription factor nuclear factor-kappa B (NF-κB) in human bile duct cancer cells and stimulates production of vascular endothelial growth factor (VEGF) and leads to enhancement of angiogenesis [106]. *H. pylori*, classified as a class I carcinogen by the World Health Organization, is a well-established cause of gastric cancer and recently proved to be associated with GBC. The presence of *H. pylori* in the gallbladder tissue was more frequent in patients with biliary tract carcinoma compared with the control group [105]. Another study showed the association of *H. pylori* infection and increase in serum inflammatory cytokines in patients with GBC [107]. However, other studies failed to demonstrate any increase in risk of GBC in presence of *H. bilis* [108] or *H. pylori* [109]. The disparities in the reports associating *Helicobacter* with GBC may be due to small sample sizes and various detecting methods that have different sensitivity and specificity.

Parasite infection in gallbladder

The association of biliary tract cancers and infection of parasite liver fluke are also reported. High incidence of cholangiocarcinoma is well correlated with the presence of the liver fluke, *Opisthorchis viverrini* and *Clonorchis sinensis* [18]. Liver fluke infection causes pathological changes mainly to the bile ducts where the worms can be found, as well as to the liver and gall bladder in both humans and animals. In the chronic phase of infection, when the parasites develop into the adult stage leads to hyperplasia and adenomatous formations of the bile duct epithelium resulting into periductal fibrosis and scarring. The inflammatory responses become less severe in chronic infection than in the acute phase, suggesting that immune modulation may occur and there is marked humoral immunity in *O. viverrini*-infected humans. However, there is scanty literature detailing the relationship of liverfluke infection and GBC. Thus, the infection by liver fluke is one of the important risk factor of biliary tract cancer [19].

2.7 Clinical management of GBC

GBC is highly aggressive disease. The diagnosis and pre-operative assessment of the extent of gallbladder cancer is important in determining the management plan. Early gallbladder carcinoma does not have any specific symptoms. Patients with early disease are operated for acute cholecystetitis and have improved survival. The patients with biliary-tract disease show symptoms of jaundice, weight loss, general weakness, and pain in the right upper quadrant. The patients with malignant tumours outside the biliary tract, exhibit symptoms like anorexia, weight loss, general weakness, and local complications of the tumour such as a fistula or invasion of adjacent organs. Jaundice is common and is an indicator of poor prognosis [110]. Complete surgical resection (cholecystectomy) remains the only potentially curative treatment for primary adenocarcinoma of the gallbladder. However, only 10% of patients present with early stage disease and are considered surgical candidates. Ultrasonography,

computed tomography scan, magnetic resonance imaging with magnetic resonance cholangiography or endoscopic retrograde cholangiopancreatography provides information for tumour staging and resectability. Patients with T1 disease have a disease-specific survival rate of 100% at 5 years after simple cholecystectomy. Unfortunately, few cases of GBC are identified at these early, curable stages [111]. Systemic therapy is the mainstay of treatment for patients who present recurrent or metastatic disease. Gallbladder cancer is likely to have spread to loco-regional sites at the time of diagnosis. It is therefore necessary to administer adjuvant chemotherapy in all patients with advanced GBC. Gemcitabine, cisplatin and 5-FU are the widely prescribed chemotherapeutic drugs to GBC patients [12]. Various randomised trials using combined treatment of cisplatin, 5-FU, Oxaliplatin and gemcitabine have improved benefits to the patients. However, the long term survival is disappointing [112, 113].

Advances in the understandings of molecular pathogenesis of cancers led to the development of targeted therapies. In recent past, target specific antibodies alone or in combination with chemotherapy were evaluated in biliary tract cancer patients in clinical trials. Use of the molecular inhibitors /antibodies targeting EGFR pathway (Erlotinib, Lapatinib, Cetuximab) or VEGF pathway (Bevacizumab, Sorafenib, Sunitinib and Vandetanib) alone or in combination with chemotherapeutic drugs (Oxaliplatin, gemcitabine, etc) are under clinical trials [113]. However, the success rate of these drugs is not promising. Therefore, the discoveries of newer targets are necessary for management of GBC.

2.8 Cancer and immune system

For years it was believed that the development of cancer is restricted to the transformed cells and thus the scientific efforts were focused on understanding the biology exclusively of tumor cells. However, the emerging data in past two decades has unequivocally established the considerable involvement of non-neoplastic cells present in tumor environment such as

cells of immune system and cells of mesenchymal origin [114]. The significance of immune response in cancer was first envisaged by Burnet and Thomas as an "Immunosurveillance hypothesis", stating that adaptive immunity was responsible for preventing cancer development in immunocompetent hosts [115, 116]. The evidences emerged in recent past stressed the dual host-protective and tumor-promoting actions of immunity on developing tumors, compelled the emergence of new hypothesis by Schreiber et al. called as "Cancer Immunoediting Hypothesis" [117]. During the dynamic process of immune cells – tumor cells interaction, the immune system not only protects against cancer development but also shapes the character of emerging tumours. The interaction is described in three phases — Elimination, Equilibrium and Escape.

In an elimination phase, the coordinated and balanced activation of innate and adaptive immune cells detect and destroy the tumor cells. The damage-associated molecular pattern molecules (DAMPs) released from dying tumor cells [such as high mobility group box 1 (HMGB1)] activates dendritic cells and secretion of interferons. Type I interferons act on $CD8\alpha^+/CD103^+$ DCs to enhance cross-presentation of tumour antigens to $CD8^+$ T cells [118]. The immune effector molecules, such as IFN-γ, perforin, Fas/FasL, and TRAIL (TNF-related apoptosis-inducing ligand); recognition molecules such as NKG2D; and an intact lymphocyte compartment altogether contribute in protective anti-tumour immunity and elimination of tumor cells [119].

The tumor cell variants which escape elimination phase exhibit long term state of dormancy with immune responses. The adaptive immunity prevents tumor cell outgrowth and simultaneously sculpts the immunogenicity of the tumor cells maintaining equilibrium. It is observed that adaptive immunity specifically, IL12, IFNγ, $CD4^+$, and $CD8^+$ T was responsible for maintaining the occult tumor cells in equilibrium [117]. The balance of IL12 promoting elimination, and IL23 promoting persistence, maintains tumor in equilibrium

[120]. In equilibrium phase, the immune system maintains residual tumor cells in a functional state of dormancy which may extend throughout the life of the host. The close interaction of immune cells with tumor cells facilitate the sculpting of tumor cell variants which are resistant to immune recognition and emerge as progressively growing, visible tumor.

The tumor cells deploy various mechanisms to escape from immune recognition. Tumor cells reduce expression of tumor antigen and inhibit cytotoxic effects of immunity ensuing decreased immunogenicity. Alternatively, tumor cells promote secretion of immunosuppressive cytokines such as VEGF, transforming growth factor β (TGFβ), galectin, indoleamine 2,3-dioxygenase (IDO), etc. thereby create immunosuppressive state within tumor environment. Recruitment of Treg cells (Regulatory T cells) and MDSCs (Myeloid-derived Suppressor Cells) into the tumor bed inhibits the function of tumor specific T lymphocytes by expressing the negative co-stimulatory molecules CTLA-4 (cytotoxic T-lymphocyte-associated ligand-4), PD-1 (Programmed cell death receptor-1), and PD-L1; and by consuming IL2, a cytokine that is critical for maintaining CTL function [117]. Eventually tumor environment mediates immune system deterioration, circumvents the tumor suppressor mechanisms of immunity and helps to develop into a progressively growing malignant disease. The advances in understanding the molecular pathogenesis of cancer regulated by tumor infiltrating immune cells have provided a scope to intervene the cancer progression using immunotherapeutic drugs. However, the progression of cancer at cellular and molecular level varies among cancer types and patient with different ethnic background. Thus further research is needed exploring the interaction between cancer and immunity specific to cancer type.

2.9 Inflammation and cancer

In recent years the emerging evidences have revealed that the tumor progression is not only confined to the cellular proliferation of epithelial cells but significantly regulated by

surrounding microenvironment. Tumor associated inflammation is one of the important hallmark of cancer [121]. Enormous efforts have elucidated that chronic inflammation plays decisive role in initiation, promotion, malignant conversion, invasion, and metastasis of cancer [54]. Initiation of tumorigenesis requires a genetic mutational hit. Activated inflammatory cells serve as sources of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) that are capable of inducing DNA damage and genomic instability [122, 123]. Inflammatory cytokines are reported to activate AID (activation-induced cytidine deaminase) enzyme in tumor cells which induces genomic instability and increases mutations in critical cancer genes, including Tp53, c-Myc, and Bcl-6 [124]. TNFα produced by tumor cells or infiltrating lymphocytes stimulates ROS accumulation, DNA damage, oncogene activation and epithelial mesenchymal transition in epithelial cells [125]. Dysregulation of IL6 contributes to tumor promoting effects on cancer cells and stromal cells in tumor environment. IL6 signals through STAT3 that promote cell proliferation, survival, angiogenesis and metastasis [126]. IL6 is reported to be involved in the pathogenesis of colorectal, gastric, liver, pancreatic, esophageal, breast, kidney, prostate and gynaecological cancers [126]. Various murine tumor models have shown that the inflammatory cytokines (IL6, TNFα, IL23 and IL1) activate transcription factors, such as NF-kB, STAT3, and AP-1, in premalignant cells to induce genes that stimulate cell proliferation and tumor promotion [54]. Growth of large tumors requires an increased intra-tumoral blood supply which is triggered by tumor hypoxia. Under hypoxic conditions HIF1α (hypoxia-inducible factor-1α) stimulated recruitment of Tumor associated macrophages (TAMs) and MDSCs induces local VEGF production [127]. Important proangiogenic genes, such as IL8, CXCL1, CXCL8, VEGF, and HIF1α, are directly regulated by NF-κB, STAT3, and AP-1 in TAMs, MDSCs, and other cell types [127, 128]. During metastasis of cancer, tumor cells undergo epithelial mesenchymal transition. Signalling through TGFβ activates SMAD transcription factors and

MAPKs, which control expression of other regulators of the epithelial-mesenchymal transition, such as Slug [129]. Signalling through TNFα induces Snail and Twist through activation of AKT/GSK-3β (Protein kinse B/ Glycogen synthase kinase-3β) or NF-κB pathway respectively [130, 131]. Survival of circulating metastatic cells is regulated by inflammatory mediators (TNFα, IL6, VEGF and epiregulin) released by immune cells [132]. The interaction of circulating cancer cells with platelets or macrophages may protect them from NK cell-mediated killing, thereby overcoming immunosurveillance [133]. Overall it is now appreciated that chronic inflammation orchestrates cancer initiation by generating genotoxic stress, cancer promotion by inducing cellular proliferation, and cancer progression by enhancing angiogenesis and tissue invasion. Given the crucial role of inflammation in cancer progression, understanding the immunological mediators of inflammations including cytokines and immune cells may provide platform for future therapeutic interventions.

2.10 Interleukin 17

Interleukin 17 (IL17) is a potent pro-inflammatory cytokine that contributes to the pathogenesis of several inflammatory diseases. The *Il17* gene and IL17 protein were first discovered as a product of T cells in rodents, where they were initially known as cytotoxic T lymphocyte-associated antigen 8 (CTLA8) [134]. IL17 was recognized to have homology to an open reading frame encoded within a T cell-tropic γ-herpesvirus, *Herpesvirus Saimiri* [134]. There are five related cytokines that were identified through database searches and degenerative RT-PCR, that share 20–50% homology to IL17 constitute an IL17-family [135]. The six members are designated as IL17A (commonly refer to IL17), IL17B, IL17C, IL17D, IL17E (also called IL25) and IL17F. IL17A is the founding member of the IL17 cytokine family [135]. These cytokines incorporate four conserved C-terminal cysteine residues [136] IL17 is most homologous to IL17F (~ 60%), and the genes encoding them are proximally located on longer arm of chromosome 6. The distance between these genes is 46 kbp. IL17A

is located on the forward strand and has a length of 4,252 bp, and IL17F is located on the reverse strand and has a length of 7,815 bp. Both genes are comprised of three exons [25]. IL25 has the least homology with IL17A. Little is known about the regulation and function of IL17B and IL17C; however, there is some evidence that these cytokines also regulate inflammatory response [137]. IL17 is a unique cytokine that bears no resemblance to other known interleukins. Furthermore, IL17 bears no resemblance to any other known proteins or structural domains.

2.10.1 IL17 structure

IL17A is a 155 amino acid homodimeric, disulfide linked protein that is secreted as glycoprotein with a molecular mass of 35 kDa. After splitting off the signal peptides (23 amino acids for IL17A and 30 amino acids for IL17F), the secreted polypeptides are 132 amino acids (IL17A) and 133 amino acids (IL17F) long [25]. Each subunit of the homodimer is approximately 15-20 KDa. The structure of IL17 consists of a signal peptide of 23 amino acids followed by a 123 amino acids chain region characteristic of the IL17 family [138]. Comparison of different members of the IL17 family revealed four conserved cysteines that form two disulfide bonds [138].

2.10.2 IL17 receptor

Similar to their cognate cytokines, IL17 receptor complexes are multimeric (Figure 2.2). The IL17 receptor family consists of 5 members (IL17RA, RB, RC, RD and RE), all of which, like their ligands, share sequence homology. IL17RA is ubiquitously expressed on a wide range of tissues and cell types [139]. Upon stimulation with IL17, IL17RA initiates the activation of downstream signalling pathways to induce the production of pro-inflammatory molecules. However, IL17RA alone is insufficient to mediated IL17 signalling. IL17 signals through a heterodimeric receptor complex composed of IL17RA and IL17RC (Figure 2.2). IL17RA subunits are pre-assembled in the plasma membrane prior to ligand binding [140].

The preassembly enables the receptor to respond rapidly and specifically to ligand, while preventing unproductive interactions with other receptors [139]. IL17F also signals through the same receptor complex with ~100 to1000 times lower affinity than does IL17A [141].

2.10.3 IL17 receptor signalling

At the C-terminus of the IL17 receptors is a conserved region known as the SEFIR (Similar Expression of Fibroblast growth factor genes and IL17Rs) domain which is closely related to the TIR (Toll/Interleukin-1 receptor) domain in Toll-like receptors (TLRs) and IL1 receptors [139]. SEFIR domain is also expressed by a cytosolic protein Act1(NF-κB activator 1) which is recruited to the IL17 receptor complex through homotypic interactions of the SEFIR domains upon IL17 stimulation [139]. Following binding to IL17R, Act1 mediates Lys-63 linked TRAF6 (TNF receptor associated factor-6) ubiquitination through its U-box domain [142]. Poly-ubiquitinated TRAF6 further activates downstream TRAF6 dependent TGFβ activated kinase 1 (TAK1) for NFκB activation (Figure 2.2). TRAF6 is essential for IL17A dependent activation of NFκB and MAPK cascades whereas TRAF2 and TRAF5 are involved in IL17A dependent chemokine production. Upon IL17A stimulation IKKi (I kappa B kinase I; regulator protein of ACT1) is recruited to the IL17R–Act1 complex, where it specifically phosphorylates Act1 at Ser-311. This generates a docking site that recruits TRAF2 and TRAF5, to form an Act1/TRAF2/TRAF5/arginine - and serine - rich splicing factor SRSF1 (SF2 (ASF)) complex. This complex prevents ASF from binding to the 3’ UTR of CXCL1 mRNA for cleavage and thereby enhances CXCL1 mRNA stability [143, 144]. TRAF3 and TRAF4 are the negative regulators of IL17 signalling. They bind directly to the IL17R to interfere with the formation of the IL17R–Act1–TRAF6 complex [145, 146]. IL17 stimulation also triggers the dual phosphorylation of C/EBPβ (CCAAT-enhancer-binding protein β) at Thr-188 and Thr-179 by ERK and GSK3β, respectively. These phosphorylation

events on C/EBPβ lead to inhibition of IL17 dependent pro-inflammatory gene induction [147].

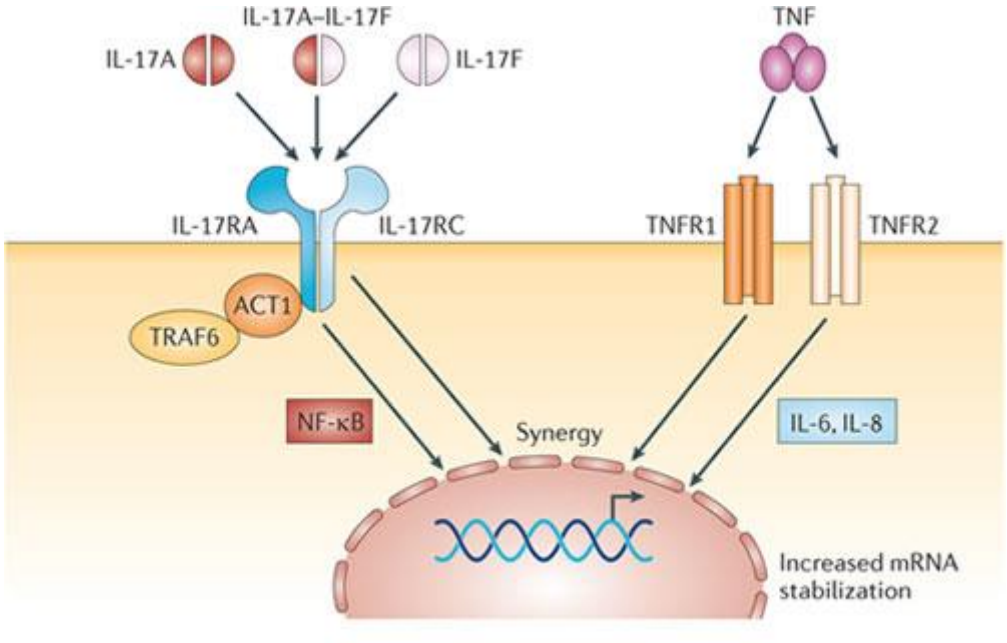


Figure 2.2 : IL17 receptor signalling. *Heterodimers of IL17A/IL17F interacts with heterodimer of IL17RA. Receptor signalling induces the activation of ACT1, NF-κB and TRAF6 which leads to increased transcription of the target genes. Signalling through TNG-TNFR synergizes the effect of IL17. The figure is adapted from* Miossec *et al, Nature reviews drug discovery,11, 2012*

2.10.4 Functions of IL17

IL17RA is expressed in nearly every cell type of the body, including epithelial cells, endothelial cells, fibroblasts and myeloid cells. The contribution of IL17 to disease is thus linked to the role of these cells in disease pathology. IL17 activity contributes to various aspects of acute and chronic inflammation. IL17 induces the production of many neutrophilic granulocyte attracting chemokines, such as CXCL1, CXCL2, CXCL5, CXCL8 and T cell and myeloid cell attracting chemokines CCL20, CCL2, and CCL7 in fibroblasts, epithelial cells, endothelial cells, and keratinocytes [25]. IL17 enhances the expression of G-CSF, GM-CSF, and stem cell factor in tissue cells, macrophages, and T cells and thereby led to strengthened granulopoiesis [148, 149]. This leads to a significant increase in the immigration of

neutrophilic granulocytes to the site of inflammation. The treatment of tissue cells with IL17F induces the expression of IL1β, TNFα, IL6, and a variety of metalloproteinases (MMP1, MMP2, MMP3, MMP9, and MMP13) [150, 151]. IL17 levels have been found increased in colon, ovarian, hepatocellular, esophageal, lung cancer, and glioblastoma [30]. The polymorphism G-197A in the IL17 promoter region is associated with increased gastric cancer risk independently of *Helicobacter pylori* presence [152]. IL17 induces AKT dependent IL6/JAK2/STAT3 activation and tumor progression in hepatocellular carcinoma by enhancing expression of downstream targets IL8, MMP2, and VEGF [153]. In carcinogen induced skin cancer models IL17 is shown to augment myeloid cell recruitment and STAT3 associated local inflammation [154]. IL17 acts synergistically with TNFα to stimulate glycolysis by increasing expression of the glucose transporter SLC2A1 and hexokinase-2 thereby supporting growth of colorectal cancer cells [155]. The major function of IL17 is to promote angiogenesis and invasiveness by stimulating VEGF production in cancer cells [29, 156]. Thus increasing levels of IL17 are reported to associate with poor prognosis of colorectal, Non-small-cell lung carcinoma (NSCLC) cancer patients [28, 29]. Contradictory reports demonstrated that higher levels of IL17 were correlated with progression free survival (PFS), and death rate in glioblastoma patients. The IL17 and PFS were also observed to be independent factors affecting the overall survival. Therefore, in glioblastoma, the IL17 may be modulating the tumor microenvironment as a defence mechanism [157]. Similarly in ovarian cancer, increased levels of IL17 associated with increased PFS and survival [158]. The increased levels of IL17 also associated with chemo-sensitivity of ovarian cancer cells to platinum-based chemotherapy [159].

2.11 Th17 cells

2.11.1 Differentiation and development of Th17

Th17 is a subset of $CD4^+$ T cells producing IL17, a lineage distinct from Th1, Th2, and Treg cells and are characterized by a unique molecular and functional signature (Figure 2.3) [160]. Th17 cells are characterized by their capacity to secrete IL17A, IL17F, IL21, IL22, and CCL20 [161, 162]. Th17 generation is controlled by the master transcription factors retinoic acid-related orphan receptor (ROR)γt, RORα, aryl hydrocarbon receptor (AHR), and interferon regulatory factor4 (IRF4) [163-166]. Differentiation of Th17 cells takes place from naïve $CD4^+$ T cells. Activation in the presence of TGFβ and IL6 primes the initial differentiation of naive $CD4^+$ T cells to IL17 producing Th17 cells [167, 168]. IL6 plays an indispensable role in initiating this process by activating STAT3, which directly regulate transcription of Rorc (RORγt), Il17, and Il23r [169, 170]. Recently it was shown that the ratio of activated STAT3 /STAT1 determines the Th17 differentiation program. IL6 and IL21 induce p-STAT3/p-STAT1 ratios > 1, leading to the promotion of Th17 differentiation, whereas IL27 or IL6+IL27 induce p-STAT3/p-STAT1 ratios < 1, resulting in inhibition of Th17 differentiation [171]. Expression of IL23 receptor is essential for the maturation of Th17 cells [172]. Activated $ROR\gamma t^+$ Th17 cells, but not naive $CD4^+$ T cells, express IL23R [173]. Addition of IL23 markedly enhances Il23r gene expression by $IL23R^+ROR\gamma t^+$ Th17 cells. Subsequent exposure to IL23 is required for the functional maturation and pathogenic function of Th17 cells and IL23R deficient Th17 cells fail to maintain their IL17 expression [172]. IL23 induces GM-CSF to regulate pathogenic functions of Th17 cells since GM-CSF deficient Th17 cells are unable to induce experimental autoimmune encephalomyelitis (EAE) upon adoptive transfer [174]. TGFβ1 plays an indirect role in the initial differentiation of Th17 cells by suppressing T-bet and GATA-3, thus inhibiting $CD4^+$ T cells from adopting alternate Th1 or Th2 cell fates [175]. Autocrine signalling by TGFβ1 also promotes Th17 cell

differentiation [176]. The effect of TGFβ1 may be dependent on its concentration in the cytokine milieu where low levels promote Th17 differentiation by inhibiting T-bet while high levels obstruct Th17 differentiation by inhibiting IL23R and inducing IL10 and Foxp3 [177]. However, TGFβ3 is shown to upregulate IL23R expression leading to highly pathogenic Th17 cells [178]. IL2 inhibits early Th17 differentiation by inducing STAT5 that displaces STAT3 binding to Il17 promoter and repress transcription [179]. IL23 directly suppresses IL2 signalling suggesting IL23 can prevent destabilization of the Th17 program [172].

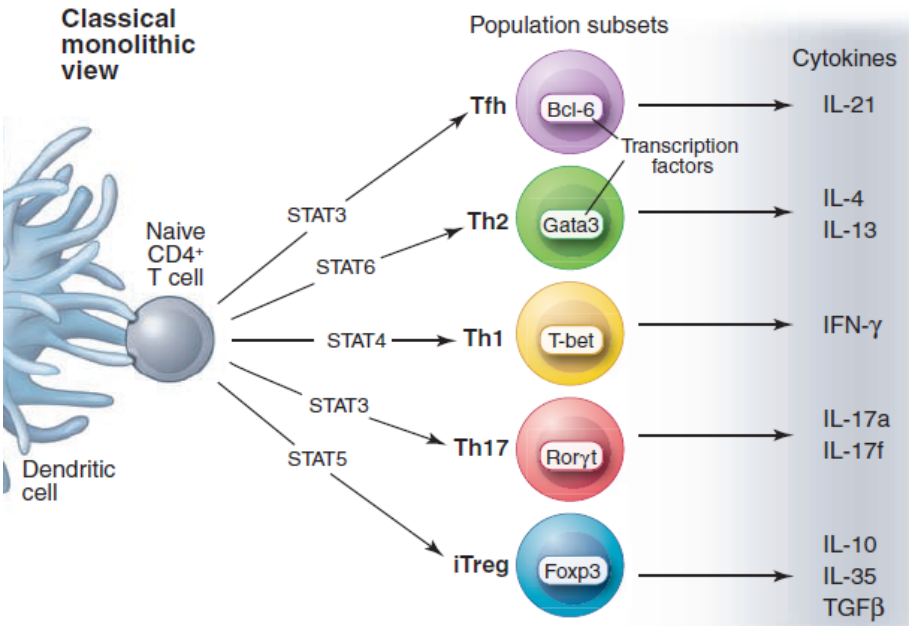


Figure 2.3 : Distinct lineages of CD4+ T cells. *The figure depicts the differentiation pathways of naïve CD4+ T cells to different subsets. The subset specific transcription factors determine the effector cytokines secreted by each subtype. The figure adapted from O'shea and Paul, Science, 327, 2010.*

2.11.2 Natural Th17 cells

In mice, Th17 cells developed in the thymus, express IL23R and RORγt and belong to innate immune cell compartment are natural Th17 cells (nTh17) [177, 180]. nTh17 cells develop in the thymus in the presence of strong interaction of TCR and MHC suggesting nTh17 cells may survive negative selection despite bearing TCRs with high affinity to MHC in the

thymus [181]. It is reported that thymic stromal MHC class II expression and RelB-dependent medullary thymic epithelial cells (mTEC), including Aire+ mTEC, are an essential requirement for nTh17 development suggesting critical role of the thymic medulla in the differential regulation of in nTh17 development [182]. Similar to inducible Th17 (iTh17) cells, development of nTh17 cells also require TGFβ as the deficiency of TGFβ receptor showed significant reduction in the thymic Th17 cells [181]. However, intracellular developmental pathways vary between two. Although Akt and the downstream mTORC1–ARNT–HIFα axis required for generation of iTH17 cells, nTh17 cells developed independently of mTORC1. In contrast, mTORC2 and inhibition of Foxo proteins are critical for development of nTh17 cells. Moreover, AKT2 is critical in development of iTh17 cells whereas AKT1 is required for nTh17 [180]. It is reported that IL17 expressing thymocytes are present in the DN1 T cell precursor compartment of $Rag1^{-/-}$ mice, indicating naturally occurring IL17 expressing T cell precursors develop in the absence of TCR rearrangement [183]. These data suggest that nTh17 commitment may occur before the process of thymic selection [183].

2.11.3 Classical and alternative Th17 cells

During differentiation of Th17, the cytokine milieu determines the functional characteristics as ‘classical’ or ‘alternative’ [184]. Although T-bet and IFNγ are classical features of Th1 lineage, *in vitro* or *in vivo* generated Th17 cells also express IFNγ in disease condition [184]. These $IL17^{+}IFN\gamma^{+}$ double producing cells are derived from Th17 cells and show reduced expression of RORγt as T-bet sequester the transactivation factor Runx-1 required for RORγt activation [185, 186]. Expression of T-bet and IFNγ in Th17 cells is dependent on IL23, but inhibited by TGFβ and thus is a characteristic of alternative rather than classical Th17 cells [185, 186]. Classical Th17 cell producing both IL17 and IFNγ are more plastic than alternative Th17 cells which are more terminally differentiated and therefore maintain a

stable IL17 production [184]. However, it remains to be determined whether the switch to IFNγ is actually beneficial or detrimental for the development of tissue inflammation and autoimmunity. In humans, the Th17 cells that convert to Th1 lineage (gain an ability to secrete IFNγ and lose their capacity to secrete IL17) express CD161 [187]. When Th17 cells encounter IL12, they convert to a Th17/Th1 phenotype that co-expresses RORγt, T-bet, CXCR3, CCR6, CD161, and IL23R. The transcription factors Runx1 or Runx3, in combination with T-bet, are crucial for the generation of IFNγ producing Th17 cells [188].

Alternative Th17 cells are also reported to produce IL10 [184, 189]. The exposure of Th17 cells to IL23 diminishes IL10 production, whereas TGFβ promotes the production of IL10 [190, 191]. In addition, IL1β inhibited IL10 production in differentiating and memory Th17 cells, whereas blockade of IL1β *in vivo* lead to increased IL10 production by memory Th17 cells [192]. IL10 may be produced by Th17, suppress Th17 induced inflammation [193]. $IL17^{+}IFN\gamma^{+}$ T cells express higher levels of IL10Rα compared to $IFN\gamma^{+}$ T cells and that their proliferation is preferentially suppressed by IL10 signalling [194]. Thus, in the absence of IL23 and IL1β, Th17 cells may eventually start producing IL10 in order to restrain themselves and prevent unnecessary tissue destruction. Another factor that is differentially expressed in 'alternative' vs. classical Th17 cells is GM-CSF [174]. TGFβ suppresses GM-CSF production in Th17 cells, while IL1β and especially IL23 drive the production of GM-CSF, which stimulates antigen presenting cells to produce more IL23 in a positive feedback loop [195]. In contrast, IL17 and GM-CSF expression are antagonistically regulated by human T cells [196]. Induction of GM-CSF expression by human $CD4^{+}$ T cells is constrained by the IL23/RORγt/Th17 cell axis but promoted by the IL12/T-bet/Th1 cell axis. IL2 mediated STAT5 signalling induce GM-CSF expression in naïve and memory $CD4^{+}$ T cells, whereas STAT3 signalling blocks it [196]. The study highlights the difference in the regulation of Th17 cells in mouse and human. GM-CSF not only induces antigen presenting

cells to produce pro-inflammatory cytokines including IL6 and IL23, it also attracts a wave of secondary infiltrating cells primarily macrophages, which then amplify the inflammatory process and promote tissue inflammation [184].

2.11.4 Recruitment of Th17 to the tumor

The role of Th17 cells in cancer displays complexity in various types of tumor immunity. Although it seems that the pathogenic role of IL23 induced Th17 cells has been consistently documented in autoimmunity, Th17 cells in cancer display both anti-tumorigenic and pro-tumorigenic functions [50]. The emerging evidences reported that the development of Th17 cells in tumor infiltrating lymphocytes is a general feature of cancers [197]. Th17 cells have been found in many different types of human tumors, including lymphoma, myeloma, breast cancer, colon cancer, gastric cancer, hepatocellular cancer, melanoma, ovarian cancer, pancreatic cancer, and prostate cancer [197]. In addition to universal expression of CCR6, Th17 cells can express Th1-associated (CCR2, CXCR3, CCR5, and CXCR6), Th2-associated (CCR4), and nonlymphoid tissue trafficking receptors (CCR4, CCR5, CCR6, CXCR3, and CXCR6), as well as homeostatic chemokine receptors (CD62L, CCR6, CCR7, CXCR4, and CXCR5) that are implicated in T-cell migration to and within lymphoid tissues [198]. The tumor cells, as well as tumor-derived fibroblasts, secrete MCP-1 (the ligand for CCR2 or CCR4) and RANTES (the ligand for CCR1, CCR3, or CCR5) and specifically recruit Th17 cells to the tumor environment [39]. In colorectal cancer and cervical cancer it is reported that Th17 cells are recruited into tumor tissues preferentially through CCR6-CCL20 pathway [199, 200]. Moreover, Th17 cells isolated from melanoma, colon, hepatocellular, ovarian, pancreatic, and renal cell carcinomas express high levels of CXCR4 and CCR6, several CD49 integrins, and CD161 which are involved in the Th17 cell trafficking and migration into the tumor sites [198]. Beside migration of Th17 cells from circulation to the tumor bed, Tumor cells and tumor environment stromal cells produce the proinflammatory cytokines

IL1β, IL6, IL23, and TGFβ, which can form an optimal proinflammatory cytokine milieu suitable for human Th17 cell differentiation and expansion [39, 42, 45].

2.11.5 Contrasting functions of Th17 cells in cancer

The potential mechanisms responsible for the pro-tumor activity of IL17 or Th17 cells mainly involve angiogenesis and cytokine induction in the tumor microenvironment resulting in the promotion of tumor growth. The accumulation of intra-tumoral Th17 cells enhances human hepatocellular carcinoma, head and neck squamous cell carcinoma, gastric cancer progression by fostering angiogenesis. [27, 42, 201]. In addition to its involvement in angiogenesis IL17 can induce IL6 production, which in turn activates the oncogenic signal STAT3, resulting in up-regulated pro-survival and proangiogenic genes [153]. A comparative study of emerging reports showing correlation of intra-tumoral Th17 cells and clinical outcome of patients showed association of increased Th17 cells with poor survival of patients [197]. However, Th17 cells are also reported to show antitumor immunity. In the murine models deletion of IL17 resulted faster tumor growth and metastasis than wild type mice [202]. Adoptive transfer of Th17 cells also showed elimination of tumor cells [203, 204]. Improved anti-tumor immunity has also resulted from immunotherapies directed towards increasing Th17 activity – these include injection of IL6 [205], blocking indoleamine 2,3-dioxygenase (IDO) [206] or use of IL7 as an adjuvant [207]. Th17 cells are also shown to promote dendritic cell recruitment into the tumor tissues and in draining lymph nodes containing tumor material which activate tumor-specific $CD8^+$ T cells [204]. In human intra-tumoral Th17 cells are associated with survival of cancer patients. Increased Th17 and IL17 in ovarian tumor predict longer survival of patients [45]. Th17 cells stimulate CXCL9 and CXCL10 production to recruit IFNγ producing effector T cells to the tumor microenvironment [45]. Elevated infiltration of Th17 in squamous cell carcinoma in also reported as independent prognostic factor for improved survival [208]. Another study showed that accumulation of Th17 cells in

lung cancer pleural effusion predicted improved patient survival [38, 209]. In patients with acute and chronic lymphocytic leukemia elevated levels of Th17 were also associated with longer survival [210, 211]. The contrasting functions of Th17 observed in cancer may be because of the source of the Th17 cells (arising naturally via tumor growth or adoptively transferred following *ex vivo* manipulation), the functional phenotype of the cells and/or exposure to therapeutic interventions such as chemotherapy. Understanding how Th17 cells cause inflammation in the context of these factors, as well as how these elements impact patient survival, is of considerable interest in the field of oncology.

2.12 γδT cells

2.12.1 Features of γδT cells

T cells expressing γδTCR is a separate lineage involved in early immune response in infections, inflammatory diseases, and cancer. Human γδ TCR expressing cells constitute 1–5% of total T cells in the peripheral blood but make up a major lymphoid subset (20–50%) in tissues such as the intestine and the dermis [212]. 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 [213]. Although activated by innate cytokines γδT cells undergo clonal propagation enhancing the immune response against invading pathogen or danger signal posed by "self" cells. Thus γδT cells are considered to link innate and adaptive immunity [214]. Antigen recognition by murine or human γδT cells does not require antigen presentation by major histocompatibility complex (MHC) class I or class II [215]. γδT cells recognise special class of antigens which are small mono- and pyrophosphates of linear C5 isoprenoids called as phosphoantigens [215]. In humans, during cholesterol biosynthesis, phosphorylated precursors such as isopentenyl pyrophosphate (IPP) and DMAPP

(dimethylallyl pyrophosphate) are synthesized by the mevalonate pathway. However, microbial pathogens use non-mevalonate pathway to produce these phosphorylated precursors [216, 217]. 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 [218]. Aminobisphosphonates is a class of drugs which indirectly activate γδT cells by inducing bioaccumulation of mammalian phosphoantigens [219]. The aminobisphosphonates (Zoledronic acid, pamidronate, ibandronate,alendronate ,etc) are selective inhibitors of the farnesyl-pyrophosphate synthase enzyme (FPPS) from the mevalonate pathway of cholesterol biosynthesis. bioaccumulation of the FPPS substrate, IPP and DMAPP, which activate γδT cells [219]. In addition to non-protein antigens, γδT cells recognize small peptides such as heat shock proteins (HSPs) [220, 221]. The exclusive response of γδT cells to these phosphoantigens has a potential therapeutic significance and can be used to harness the cytotoxic potential of γδT cells.

2.12.2 Development of γδT cells

γδT cells develop in the thymus from $CD4^{-}CD8^{-}$ (double negative, DN) thymocytes [222]. In humans, sustained notch signalling is required for the development of γδT cells [223]. Differential requirement of notch signalling for γδT cells development in thymus, leads to a scenario of DN, DP, and SP $TCR\gamma\delta^{+}$ population, which highlights heterogeneity in human γδT cell development [224]. Notch signalling also regulates extra thymic functions of γδT cells [225] thus validates the requirement of notch signalling in both thymic development and functions of human γδT cells.

2.12.3 Functions of γδT cells

γδT cells perform diverse effector functions determined by the TCR expressed, tissue localization, and activation status. γδT cells are potential cytotoxic cells based on MHC-independent recognition of antigens, production of IFNγ, and expression of cytotoxic

granules [212]. Human γδT cells recognise heat shock proteins (HSP60/70) expressed on tumor cells and enhance its cytolytic activity against the tumors [220, 221, 226] γδT cells support the maturation and activation of other lymphocytes, NK cells and macrophages with the help of secreted cheomkines (CCL3, CCL4, CXCL10) [212]. Another chemokine CXC - chemokine ligand 13 (CXCL13) produced by γδT cells can regulate B cell organization within lymphoid tissues and help B cells to produce antibodies [227] γδT cells secrete IL12 and IFNγ and influence maturation and antigen presentation of dendritic cells [213, 228]. Activated γδT 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 [229, 230]. Moreover, activated γδT cells are licenced to act as antigen presenting cells and activate CD4 and CD8 T cells [231]. Collectively, these observations highlight the multi-faceted role of γδT cells, having both Th and Tc like properties along with their APC like function. These properties of γδT cells aid in generation of an effective immune response in appropriate condition. Thus γδT cells 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 granzymes [232, 233]. These antitumor properties of γδT cells compelled investigators to undertake clinical trials in cancer patients. Adoptive transfer or *in vivo* expansion of γδT cells were implicated as treatment approaches. Results from Phase I and II clinical trials indicate that the efficacy of γδ T cell-based immunotherapy is comparable to that of conventional second-line therapies[234].

In contrast, γδT cells are reported to show pro-tumor activities. Recently, it is shown that γδT cells infiltrating pancreatic ductal adenocarcinoma express elevated levels of T cell exhaustion ligands (PD-L1) and suppress the activity of CD4 and CD8 T cells [235].

Tumour-infiltrating γδT cells are also reported to be the most significant predictor of relapse and poor survival in patients with breast cancer [236]. The pro-tumorigenic role of γδT cells is dependent on their regulatory function in the tumor microenvironment and secondary lymphoid tissues. Foxp3 expressing γδT cells are present in human PBMCs and tumor infiltrating lymphocytes and are reported to suppress T cell activation and proliferation [237, 238]. The suppressive activity of γδT cells did not correlate with Foxp3 but with CD86-CTLA-4 interaction and expression of CD39 [239].

2.13 Tγδ17: a subtype of γδ T cells

In recent past, the IL17 producing subtype of γδT cell is emerging as important contributor to cancer progression [240]. In humans, upon activation with different cytokines, γδT cells can be polarised towards different effector subtypes like γδ1, γδ2 [241], γδ17 [242, 243], γδTreg [237, 244]. 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. Similar to Th17 cells Tγδ17 cell express RORγt as a lineage determination transcriptional factor [245]. Like γδT cells human Tγδ17 cells present in non-lymphoid environment belong to $CD27^-$ $CD45RA^{+/-}$ effector [246] or terminally differentiated (T_{EMRA}) [243] memory phenotype.

2.13.1 Development of Tγδ17 cells

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 [247]. 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 [248]. Moreover, $CD27^+$ γδT cells differentiate into IFNγ producing cells whereas IL17 production was restricted to $CD27^-$ T

cells [249]. Another signalling pathway through lymphotoxin-β receptor (LTβR) controls Tγδ17 development by regulating transcription factors RORγt and RORα4, required for IL17 expression in γδ thymocytes [250]. Thymic development of human Tγδ17 cells is poorly investigated. Around 80% circulating human γδT cells are IFN-γ producers and express CD27 whereas CD27 negative cells are IL17 producing γδ T cells are less than 5% [242]. Human, γδ T cells can be polarised to Tγδ17 cells in periphery upon IPP activation and in 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 [242, 243]. The kinetics of IL17 production by human γδT cells showed that mRNA expression of IL17 and RORγt peaks by day 3-6 and decrease by day9 onwards, 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 [243].

2.13.2 Functions of Tγδ17 cells

Tγδ17 cells are reported to exacerbate inflammatory diseases (Figure 2.4) [251]. With the notion that IL17 is a proangiogenic cytokine [252], 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 in tumor cells [253]. Tγδ17 cells induce mobilization of pro-tumor 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 tumour cell proliferation [254]. In hepatocellular carcinoma mouse model, it was reported that IL17, majorly produced by $V\gamma4^{+}\gamma\delta T$ cells, induced CXCL5 production by tumor cells which enhance migration of MDSCs expressing CXCR2 to the tumor site. In addition, IL17 also enhanced suppressive functions of MDSCs by inhibition of T cells proliferation and IFNγ and TNFα production [255]. The importance

of Tγδ17 cells in human cancer is recently appreciated [251]. Tγδ17 cells are reported to secrete IL8, TNF and GM-CSF, which recruit immunosuppressive MDSCs into the malignant microenvironment, further driving progression of colorectal cancer [246]. Tγδ17 cells induce angiogenesis related factors (VEGF, uPA, MMP9, MCP-1, GM-CSF, CXCL16, Coagulation factor III, Angiogenin, etc.) in gallbladder tumor cells through IL17[256].

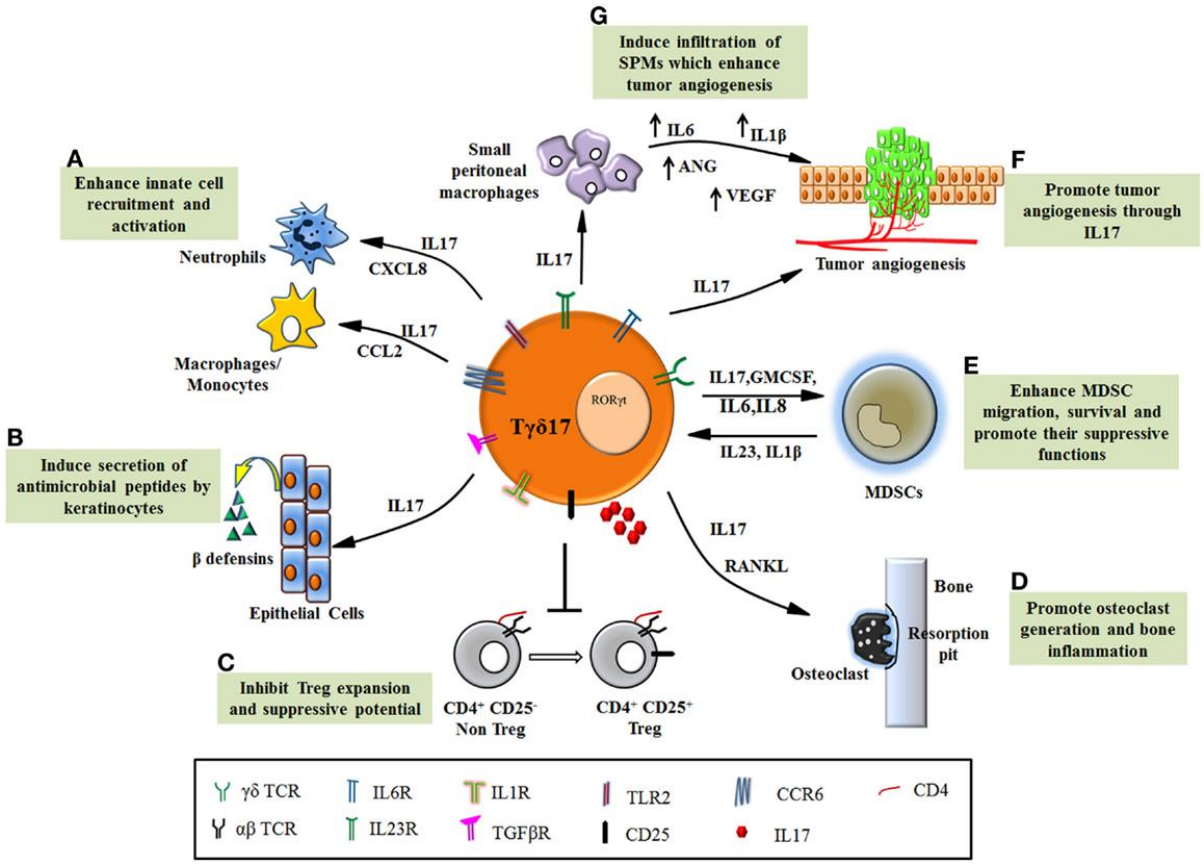


Figure 2.4 : Effector functions of Tγδ17 cells . *The figure illustrates the functions of Tγδ17 cells in innate cell activation, induction of antimicrobial peptides, inhibition of Treg expansion, promote osteoclast generation, enhance MDSC migration, promote tumor angiogenesis and recruitment of macrophages. The figure is adapted from Patil et. al, Frontiers in Immunology,6, 2015.*

In contrast, antitumor activity of Tγδ17 cells was also reported [251]. In a chemotherapeutic approach, in several transplantable tumor models, Tγδ17 cells are 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 [257].

Collectively, the apparent opposite roles of Tγδ17 cells in cancer immunity need to be understood in detail to consider it as potential target in immunotherapeutic interventions.

2.14 Inflammation and immunosuppression

Immune response in cancer patients is characterised by impairment of homeostatic levels of immune cells. Given that inflammation is one of the important hallmarks of cancer, the impairment of levels of inflammatory and immunosuppressive immune cells in cancer patients is obvious. The alteration in the Th17 and Treg cells and related cytokines is reported in various cancers. In patients with non-small cell lung cancer the levels of Treg cells were increased and that of Th17 cells decreased with cancer progression [258]. Similar trend was observed in salivary gland tumors [259]. In breast cancer patients analysis of tumor infiltrating lymphocytes showed that Th17 and Treg cell accumulation in the tumour microenvironment occurred in early disease; Th17 cell infiltration gradually decreased and Treg cells accumulated with disease progression [260]. Altered Th17 Treg balance was also reported in lung cancer, prostate cancer, and cervical cancer [58-60]. Thus the reports suggest that the balance of proinflammatory and immunosuppressive cells is crucial in cancer patients and investigation of the dynamics of proinflammatory and immunosuppressive cells is necessary for better understanding the pathogenesis of cancer.

2.15 Regulatory T cells

2.15.1 Features of Treg cells

Regulatory T cells (Tregs) is a subtype of CD4+ T cell that functionally suppresses an immune response by manipulating the activity of other immune cells [51]. It was first reported by Gershon et. al. that the subset of T cells exhibit suppressive function [261, 262]. Sakaguchi and his colleagues showed that Tregs are a small group of T cells co-expressing CD4 and CD25 (IL2 receptor-α chain) involved in maintaining self-tolerance since depletion of Treg cells lead to autoimmunity [263, 264]. Foxp3, a key transcription factor is crucial for

the development and functioning of $CD4^+CD25^+$ regulatory T cells [52, 265, 266]. In humans, the low expression of CD127, the α-chain of the interleukin-7 receptor, was used in combination with $CD25^+$ and $Foxp3^+$ 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 [267, 268]. 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 IL2 [269, 270]. Treg cells can be dissected into three sub-populations by the expression levels of Foxp3 and the cell surface molecules CD45RA and CD25 : (i) $Foxp3^{lo}CD45RA^+CD25^{lo}$ cells, designated naive or resting Treg cells, which differentiate into $Foxp3^{hi}CD45RA^-CD25^{hi}$ cells upon antigenic stimulation; (ii) $Foxp3^{hi}CD45RA^-CD25^{hi}$ cells, designated eTreg (effector Treg) cells, which are terminally differentiated and highly suppressive; and (iii) $Foxp3^{lo}CD45RA^-CD25^{lo}$ non-Treg cells, which do not possess suppressive activity but can secrete pro-inflammatory cytokines [51].

2.15.2 Treg cells in cancer

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 [271, 272]. 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 [273], gastric cancer [274], head and neck cancer [275] and ovarian cancer patients [276]. The accumulation of Tregs in the tumor takes place by three different, but not mutually exclusive, modes: (i) increased trafficking, (ii) preferential Treg expansion and (iii) de-novo differentiation, where the latter two can occur either locally within the tumor

microenvironment. 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 [277, 278]. An inflammatory condition in tumors also recruits Tregs in CCL20-CCR6 dependent fashion [279]. Other combinations of chemokines and chemokine receptors, such as CCR10-CCL28 and CXC chemokine receptor (CXCR) 3-CXCR3 ligands (such as CXCL9, 10, and 11), also reportedly contribute to Treg cell infiltration [280, 281]. A second mechanism could be through expansion of Tregs in the presence of IL2 and TGFβ within the tumor mass or in the tumor draining lymph nodes (TDLNs). Presence of IL2 and TGFβ within the tumor mass could promote Treg proliferation, development and homeostasis [282-284]. A third mechanism involving *de-novo* conversion of $Foxp3^-$ T cells into Tregs may play an important role in Treg accumulation in tumors [285]. The role of TGFβ in the induction of Tregs is well established and tumor cell derived TGFβ can contribute to the induction of Tregs [286, 287]. Compared with tumor-reactive $CD4^+$ T cells, natural Treg cells may be better at recognizing tumor-associated self-antigens because of their TCR repertoires are more self-reactive than those of conventional T cells. Moreover, the higher level expression of T cell accessory molecules including adhesion molecules (such as LFA-1) indicate their 'antigen- primed' states [51].

2.15.3 Mechanism of Treg function

Accumulating evidences have shown that $Foxp3^+CD25^+CD4^+$ natural Tregs suppress the activation and/or expansion of multiple types of immunocompetent cells. Based on these studies, multiple mechanisms have been proposed for Treg-mediated suppression, including soluble factor mediated as well as cell surface molecule dependent inhibition of T cells and antigen presenting cells [52]. Tregs majorly utilize the inhibitory cytokines like IL10, TGFβ, and IL35 to directly induce immune suppression [288]. Tregs can also use

perforin/granzyme-mediated cytotoxicity as a mechanism to suppress the function of effector T cells [289]. Tregs isolated from cancer patients were found to express Fas ligand (FasL) and mediate killing of $CD8^+$ T cells on activation via TCR ligation and high-dose IL2 [290]. 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 dependent fashion [291].

In addition to directly inhibiting effector lymphocytes through inhibitory cytokines or cytotoxic molecules, Tregs may also suppress immune responses by modulating APCs. Foxp3 regulated CTLA-4 (cytotoxic T lymphocyte antigen 4) expression on Tregs can downregulate the costimulatory molecules (CD80, CD86) on antigen-presenting cells to suppress T cell activation [292, 293]. 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 [294]. In addition, IDO-mediated degradation of tryptophan can generate toxic metabolites that induce apoptosis in lymphocytes [295, 296]. 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 [297]. Adenosine inhibits effector T cell function through activation of the adenosine receptor 2A [298]. Tregs harbour high levels of cyclic adenosine monophosphate (cAMP) which can potently inhibit IL-2 synthesis and T cell proliferation [299].

Chapter 3

Materials and Methods

Materials and methods:

3.1 Cell culture medium

RPMI-1640 medium (Invitrogen Life-Technologies, Grand Island, N.Y.) or Williams' E medium (Sigma-Aldrich, St. Louis, USA) powder was dissolved in deionized water and supplemented with sodium bicarbonate (Sarabhai Chemicals, India) as per manufacturer's instructions. The medium was sterilized by membrane filtration (0.45µm, Millipore Co, Bedford, MA). Sterility testing of the filtered media was carried out by adding 1-2ml of medium to the tube containing the sterility medium (Thioglycolate medium) (Annexure) and incubated at 37^0C. Sterility was monitored for 6 days and stored at -20^0C until use.

To prepare complete medium, RPMI/ Williams' E plain medium was supplemented with 10% heat inactivated Fetal calf serum, (FCS; Invitrogen Life Technologies, Grand Island, N.Y), Penicillin (100 IU/ml; Alembic Chemicals India), Streptomycin (100 mg/ml; Alembic chemicals India), Mycostatin (5 mg/ml; Sigma, USA), Gentamycin (40 mg/ml; Schering Corpn, India) and L-Glutamine (2 mM, Hi Media, India). Williams' E medium was supplemented with L-Glutamine thus it was not added while preparing complete medium.

3.2 Maintenance of cell lines

Gallbladder cancer cell lines (OCUG-1; JCRB-0191[300] and NOZ; JCRB-1033[301]) were purchased from Japan health science foundation, Health science research resources bank, Osaka, Japan.

Table 3.1 Characteristics of OCUG-1 cell line

OCUG-1	
Cell No	JCRB0191
Characteristics	Poorly differentiated adenocarcinoma. Cells are tumorigenic in nude mice.

Medium	Williams' E supplemented with 10% FCS
Growth rate	~ 31 h
Growth Temperature	37^0C
Lifespan	Infinite
Morphology	Clusters with monolayer cells
Source	Male (age 43)
Metastasis	Yes
Chromosome Number	Chromosome number distribute in a broad range from 52 to 139.

Table 3.2 Characteristics of NOZ cell line

NOZ	
Cell No	JCRB1033
Characteristics	Moderately differentiated adenocarcinoma.
Medium	Williams' E supplemented with 10% FCS
Growth rate	~ 24 h
Growth Temperature	37^0C
Life span	Infinite
Morphology	Epithelial like
Source	Female (age 48)
Metastasis	Cells are transplantable to nude mice and the morphology of the transplanted tumor was similar to the original one
Chromosome Number	46

Cells were cultured in Williams' E medium (Sigma Aldrich, USA) supplemented with 10% heat inactivated FBS under standard culture conditions (37^0C, 5% CO_2).The adherent cell lines were maintained in $25cm^2$ or $75cm^2$ flasks and split when confluent. The cells were trypsinized from the flask with PBS-trypsin (Sigma, USA; 0.3% trypsin in 0.01M PBS pH 7.5, containing 0.02% EDTA, sterilized by Millipore filtration), and washed with plain medium before use. For continuous maintenance of cultures, 0.2 to 0.3 $x10^6$ cells were reseeded into the flask with 5 ml of complete medium (containing 10% FCS).

GBC cell lines were cryopreserved as main stock and working stock. For cryopreservation of the cultures, cells were pelleted by centrifugation at 1000 rpm. Chilled freezing mixture (10% Dimethyl sulphoxide [DMSO] + 90% FCS) was added drop-wise to the pellet with constant mixing. 2-3$x10^6$ cells/ml of freezing mixture were transferred to cryotube (Nunc, Denmark) and frozen in liquid nitrogen with gradual decrease in temperature.

GBC cell lines were revived from working stock frozen vials. The vials were thawed rapidly by placing in a beaker containing lukewarm water (~37^0C). The cells were transferred to a centrifuge tube containing warm plain medium drop-wise with constant mixing to dilute the DMSO. Cells were washed twice with plain medium and checked for the viability using the trypan blue stain (0.4% trypan blue prepared in normal saline)

3.3 Study Group

Newly diagnosed GBC patients (n=52) were recruited from Tata Memorial Hospital, Mumbai. 22 males (mean age = 54±2 years) and 30 females (mean age = 51±2 years) were enrolled in the study group. Peripheral blood was collected prior to chemotherapy/radiotherapy or surgery after obtaining informed consent. The study protocol was approved by ACTREC-TMC Institutional review board for human studies. The patients were grouped according to the TNM classification as stage II (n=5), stage III (n=20) and

stage IV (n=27). Tumor tissue (n=17) were obtained from GBC patients undergoing cholecystectomy without radio or chemotherapy received. Peripheral blood was obtained from age and sex matched healthy individuals (n=30) who participated voluntarily and written informed consent was obtained.

Inclusion criteria for GBC patient accrual

- ✓ Patients presenting gallbladder as primary site of tumor origin
- ✓ Patient diagnosed with GBC with any clinical stage
- ✓ Treatment naïve patients

Exclusion criteria for GBC patient accrual

- ✓ Patients treated elsewhere with chemo/radiotherapy
- ✓ Patients refusing consent of the study
- ✓ Patients treated for cholecystectomy elsewhere
- ✓ Patients diagnosed with HIV/ hepatitis infection

3.4 Cell isolation and culture

3.4.1 Isolation of Peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from heparinized blood of GBC patients and healthy individuals using Ficoll Hypaque (FH) (Sigma-Aldrich, St. Louis, MO) by density gradient centrifugation. Briefly, peripheral blood collected in heparin (Sigma, USA; 100 IU/ml) was diluted with equal volume of normal saline (0.82% NaCl in double distilled water). 8ml of diluted blood was loaded on 2.5ml of FH [24 parts of 9% Ficoll 400 (Sigma, USA) + 10 parts 33.3% sodium diatrizoate (Sigma, USA), specific gravity adjusted to 1.077 ± 0.001] and centrifuged at 1,500 rpm for 20 min at room temperature (RT) using a swing-out rotor in Beckman centrifuge. PBMCs were collected from the interface between FH and plasma and washed

thrice with sterile normal saline. Viability was checked using Erythrosine B or Trypan blue vital dye (0.4%).

3.4.2 Preparation of single cell suspension of tumor tissue

Surgically resected gallbladder tumors were collected in a collection medium (sterile plain RPMI 1640 supplemented with double strength of antibiotics). The freshly collected tumor tissue was processed for preparation of single cell suspension. Briefly, necrotic, hemorrhagic and fatty tissues were removed and tumor tissues were thoroughly washed with RPMI medium containing double strength of antibiotics. Tumor tissues were minced finely and were incubated in RPMI medium containing double strength of antibiotics and enzyme mixture [0.05% collagenase, 0.02% DNase and 5U/ml hyaluronidase (Sigma, USA)] at 37^0C for 2 h with intermittent shaking. The minced tumor tissues were then passed through a 200 # wire mesh to obtain single cell suspension of lymphocytes. The cells were washed with plain RPMI medium containing antibiotics. The collected cells were washed twice and the cell viability was checked using Erythrosine B or trypan blue vital dye.

3.4.3 Collection of tumor supernatants

The single cells suspension obtained from freshly collected gallbladder tumor tissue were cultured in 4 well plate as $1x10^6$ cells/ml in a serum free medium for 24 h. The cell-free culture supernatant were collected, centrifuged and stored at – 80^0C until used.

3.4.4 Isolation of γδT cells from peripheral blood of HI

PBMCs were isolated from HI by Ficoll Hypaque density gradient centrifugation. γδT cells were purified by immunomagnetic purification using γδ-TCR microbeads (Miltenyi Biotech, Germany). PBMCs were washed with a degassed buffer containing PBS with 0.5% BSA and 2 mM EDTA. Supernatant was removed and cells were suspended in 40µl of buffer per total 10^7 cells. 10 µl of γδ-TCR antibody labelled with hapten was added per 10^7 total cells. Cells

were mixed and incubated for 10 min at 4^0C. 30µl of buffer and 20µl of anti-hapten antibody labelled with micro-beads and FITC were added per 10^7 cells, mixed well and incubated for 15 min at 4^0C. Cells were then washed by adding buffer 10-20 times of labeling volume and centrifuged at 1000 rpm for 10 min. Supernatant was removed and cells were suspended in 500µl buffer per 10^8 cells.

For isolation of magnetically labelled γδT cells, MACS column (MS column for 10^7 cells or LS MACS column for 10^7 to $5x10^7$of target cells) was placed in magnetic field of MACS separator. Column was washed thrice with 500µl of wash buffer. The cell suspension was applied onto the column. Unlabelled cells were washed out from the column as negative selection and collected separately. Column was removed from the separator and magnetically labelled cells (positively selected) cells were collected by flushing the cells from the column into 1 ml of buffer. The purity of positively selected cells was checked by flow cytometry. More than 90-95% pure γδT cells were re-suspended in the culture medium for further assay. 5-8 $x10^6$ γδT cells were isolated from starting PBMC population of 60–70 $x10^6$ cells.

3.4.5 Isolation of Tγδ17 cells from peripheral blood of HI

Tγδ17 cells were isolated using IL17 secretion assay-cell enrichment and detection kit (Miltenyi Biotec, Germany) according to manufacturer's instructions. Briefly, γδT cells were purified by positive selection from PBMCs of healthy individuals using anti TCR-γδ microbeads (Mltenyi Biotec, Germany). Purified γδT cells ($7-8x10^6$) were stimulated with 50ng/ml of PMA (phorbol 12-myristate 13-acetate) and 1µg/ml of Ionomycin for 5 h in RPMI medium supplemented with 10% FCS. Cells were washed at 4^0C, and labelled with IL17 catch reagent (5µl) for 5 min on ice. Cells were maintained at cold temperature. Cells were suspended in RPMI containing human AB serum (10ml) and incubated at 37^0C for 45 min with constant mixing for secretion phase. Cells were again maintained at 4^0C and after

washing labelled with anti-IL17 detection antibody (5µl) labelled with phycoerythrin. The cells were sorted for IL17-PE positive cells. Purity was determined by flow cytometry. Around $5x10^4$ Tγδ17 cells were isolated from $7\text{-}8x10^6$ γδT cells medium with > 90% purity.

3.4.6 Isolation of regulatory T cells

Regulatory T cells were isolated from PBMCs of healthy individuals and GBC patients using BD Imag immunomagnetic separation kit. PBMCs ($50\text{-}60x10^6$) isolated by ficoll density gradient were first labelled with cocktail of biotinylated antibodies (5µl per$1x10^6$ cells). The cocktail of antibodies consist of antibodies that recognize antigens on erythrocytes, platelets and peripheral leukocytes that are not $CD4^+$ T lymphocytes (CD8, CD11b, CD16, CD19, CD36, CD41a, CD56, CD123, TCR γδ and Glycophorin A) and APC-labelled mouse anti-human CD25 antibody. After peripheral blood lymphocytes were labelled with the cocktail, the $CD4^+$ $CD25^+$Treg cells were isolated in two immunomagnetic separation steps. First, the $CD4^+$T lymphocytes were enriched by negative selection (depletion of the non-$CD4^+$cells) using the Streptavidin Particles (7.5µl per$1x10^6$ cells). In the second immunomagnetic separation step, the Anti-APC Particles (5µl per$1x10^6$ cells) were used to select the $CD25^+$ cells, from the enriched $CD4^+$ cells, which were already labelled with the APC anti-CD25 mAb. The phenotype and purity of regulatory T cells was confirmed using flow cytometry. $1\text{-}2x10^5$ purified Treg cells were isolated with > 80% purity.

3.5 Flow cytometry

3.5.1 Multi-colour flow cytometry staining

Flow cytometric analysis was performed using FACS Aria flow cytometer (Becton Dickinson, CA, USA) and data was analysed using FlowJo software (Tree Star, Ashland, OR). Fluorescence minus one control was used in all experiments to determine background fluorescence. For intracellular staining, cells were washed with PBS and cold fixed with 500µl 1% Paraformaldehyde (Sigma Aldrich, USA) for 15 min at 4^0C, followed by

permeabilization for 5 min with 0.1% saponin (50µl) at room temperature. Cells were incubated with antibodies for 30 min at room temperature. For intracellular cytokines staining, PBMCs ($1x10^6$ cells per well) were first stimulated with PMA (50ng/ml) and ionomycin (1µg/ml) for 5 h in presence of Brefeldin A (5µg/ml) (All from Sigma Aldrich, USA). Stimulated cells were washed with PBS, fixed with paraformaldehyde, permeabilized with saponin and stained with antibodies. After staining cells were resuspended in FACS buffer (PBS+1% FCS+ sodium azide; Sigma Aldrich, USA) and acquired using FACS Aria flow cytometer (Becton Dickinson). 50,000-100,000 live cells (events) were collected to obtain reliable data.

Frequencies of T helper, cytotoxic T, γδT, B cells, NK cells, NKT cells, Th17, Tc17, Tγδ17, regulatory T and myeloid derived suppressor cells in PBMCs were compared between healthy individuals and GBC patients. The data was expressed as percent positive cells or MFI (median fluorescence intensity). Range of antibodies was used in multi-color flowcytometry to characterize these cell types in peripheral blood and tumor tissue of gallbladder cancer patients. Following are the antibodies used to characterize different cell subsets.

3.5.2 IL17 receptor expression on GBC cell line

To analyse the IL17 receptor expression on the OCUG-1 and NOZ cells, cells were cultured in the 60 mm petri dish (Nunc, Germany) to attain 70% confluency. The spent medium was removed and cells were washed with PBS. Cells were fixed with 1% paraformaldehyde for 15 min at 4^0C. After washing, cells were scraped from the plate and stained with IL17R-PE antibody for 30 min at RT. Unbound antibody was removed by washing the cells with FACS buffer and cells were re-suspended in 300µl buffer. Cells were acquired on FACS Aria.

3.5.3 Antibodies used

Table 3.3 List of Fluorochrome conjugated antibodies

Antibody	*Fluorochrome*	*Clone*	*Isotype*	*Company*
CD3ε	APCCy7	SK7	*Mouse IgG1κ*	BD Biosciences
CD3ε	Pacific Blue	SP34-2	*Mouse IgG1λ*	BD Biosciences
CD4	PECF594	RPA-T4	*Mouse IgG1κ*	BD Biosciences
CD4	APC	RPA-T4	*Mouse IgG1κ*	BD Biosciences
CD8	Pacific blue	RPA-T8	*Mouse IgG1κ*	BD Biosciences
Vδ2TCR	PE	B6	*Mouse IgG1κ*	BD Biosciences
Vδ2TCR	FITC	B6	*Mouse IgG1κ*	BD Biosciences
CD19	PE	HIB19	*Mouse IgG1κ*	BD Biosciences
CD56	PE	B159	*Mouse IgG1κ*	BD Biosciences
CD45RA	PECY5	HI100	*Mouse IgG2bκ*	BD Biosciences
CD27	APC	L128	*Mouse IgG1κ*	BD Biosciences
CD3ζ	PE	6B10.2	*Mouse IgG1κ*	Santacruz Biotech
IL17	Alexa fluor488	N49-653	*Mouse IgG1κ*	BD Biosciences
IL17	Alexa fluor 647	SCPL1362	*Mouse IgG1κ*	BD Biosciences
IFNγ	PECY7	B27	*Mouse IgG1κ*	BD Biosciences
CD33	PECF594	WM53	*Mouse IgG1κ*	BD Biosciences
HLADR	APCH7	G46-6	*Mouse IgG2aκ*	BD Biosciences
CD11b	Alexa fluor 488	ICRF-44	*Mouse IgG1κ*	BD Biosciences
CD25	PE	M-A251	*Mouse IgG1κ*	BD Biosciences

CD127	V450	HIL-7R-M21	*Mouse IgG1κ*	BD Biosciences
Foxp3	Alexa fluor 488	259D/C7	*Mouse IgG1*	BD Biosciences
IL17R	PE	FAB177P	*Mouse IgG1*	BD Biosciences
CD183 (CXCR3)	PE	1C6	*Mouse IgG1κ*	BD Biosciences
CD184 (CXCR4)	PECy7	12G5	*Mouse IgG2aκ*	BD Biosciences
CD196 (CCR6)	APC	11A9	*Mouse IgG1κ*	BD Biosciences
CD197 (CCR7)	Alexa fluor 647	3D12	*Mouse IgG2a*	BD Biosciences
CD194 (CCR4)	PerCP-Cy™5.5	1G1	*Mouse IgG1κ*	BD Biosciences

Table 3.4 List of purified antibodies

Antibody	*Clone*	*Isotype*	*Company*
CD3	UCHT1	*Mouse IgG1κ*	BD Biosciences
IL17	--	*Polyclonal Goat IgG*	R&D Systems
CXCR3	49801	*Mouse IgG1κ*	R&D Systems

3.5.4 Staining panel for immune cells

Table 3.5 List of staining panel of immune subtypes

Immune cell type	*Antibody panel*
T cell subsets	CD3-Pacific Blue, CD4-APC, CD8-FITC, Vδ2TCR-PE
B Cells	CD19 -PE
NK and NKT cells	CD3-FITC, CD56-PE

IL17 and IFNγ producing cells	CD3-APC-Cy7, CD4-PECF594, CD8-FITC, Vδ2TCR-PE, IL17-APC, IFNγ-PECY7
Regulatory T cells	CD3-APC-Cy7, CD4-PECF594, CD25-PE, CD127-APC, Foxp3-AF488
Myeloid derived suppressor cells	HLADR-APCH7, CD33-PECF594,CD11b-AF488,

Abbrevations: APC: allophycocyanin; FITC: fluorescein isothiocyanate; PE: phycoerythrin; Foxp3: forkhead box P3.

3.6 Proliferation assay

3.6.1 Proliferative response of PBMCs to mitogen and TCR agonist

Proliferative response of PBMCs was analysed by tritiated thymidine (^{3}H-Thymidine) incorporation assay. PBMCs were isolated from GBC patients and HI using ficoll-hypaque gradient centrifugation. Anti-CD3 mAb (1µg/well; 100µl in normal saline) was pre-coated overnight at 4^{0}C. Excess antibody was removed and PBMCs ($1x10^{5}$) were cultured with pre-coated anti-CD3mAb or phytohaemagglutinin (PHA; Sigma Aldrich, USA) (1%) for 72 h at 37^{0}C in round bottom 96 well plates (Nunc, Denmark). Only lymphocytes in complete medium (RPMI 1640 + 10% FCS) were used as control. 0.5µCi/10µl/well ^{3}H-Thymidine (specific activity 240GBq/mmol; Radiation and Isotype Technology, India) was added during last 18 h of the assay. The cells were harvested on glass-fibre filter paper (Titertek, Norwey) using cell harvester (Titertek, Norwey). The filter paper was dried at 60^{0}C and each disc corresponding to single well was placed in 3 ml liquid scintillation fluid [0.7% 2, 5 diphenyloxazole + 0.05% 1, 4 bis (5-phenyloxazole)]. Radioactivity was measured on a liquid β-scintillation counter (Model 1900 Packard, USA) as counts per minute (CPM). Stimulation index was calculated by following formula. Stimulation index = (Test CPM /

Control CPM). Proliferation of PBMCs in presence of stimulation was compared with cells cultured with medium alone. Data are presented as mean ± SEM.

3.6.2. Effect of rhIL17 on proliferation of GBC cell lines

Proliferative response of gallbladder cancer cell lines (OCUG-1 and NOZ) was analysed by tritiated thymidine (^{3}H-Thymidine) incorporation assay. To study the effect of IL17 on proliferation of GBC cells, OCUG-1 and NOZ cell lines were cultured in a 96 well flat bottom plate (Nunc, Denmark). Cells were seeded in triplicates with density $1x10^4$ per well in presence of complete Williams' E medium for total 72 h. After 24 h, cells were washed with serum-free medium and rhIL17 (R&D systems, MN, USA) was added at different concentrations (100, 10, 1, and 0.1ng/ml) to the wells. Cells cultured with medium alone were used as control. 0.5µCi/10µl/well ^{3}H-Thymidine (specific activity 240 GBq/mmol; Radiation and Isotype Technology, India) was added during last 18 h of the assay. After 72 h, cells were harvested on glass-fibre filter paper (Titertek, Norway) using cell harvester (Titertek, Norway). The filter paper was dried at 60^0C and each disc corresponding to single well was placed in 3 ml liquid scintillation fluid [0.7% 2, 5 diphenyloxazole + 0.05% 1, 4 bis (5-phenyloxazole)]. Radioactivity was measured on liquid β-scintillation counter (Packard USA) as counts per minute (CPM). Proliferation of OCUG-1 and NOZ cells in the presence of rhIL17 was compared with control cells cultured with medium alone by measuring the radioactivity in CPM. Data was preseted as mean ± SEM.

3.7 Estimation of cytokines

3.7.1 Cytokine secretion by stimulated PBMCs

PBMCs ($1x10^5$) from HI and GBC patients were stimulated with pre-coated anti-CD3 (1µg/well; 100µl in normal saline) or PHA (1%) for 24 h at 37^0C in round bottomed 96 well plates (Nunc, Denmark). The cell free supernatants were collected and stored at -80^0C until used. Cytokines and chemokines in culture supernatants were measured by Th1/Th2/Th17

cytometric bead array kit (BD Biosciences) and flex sets for IL8, IL1β, IL12p70, CXCL9, CCL5, CXCL10, CCL2 (BD Biosciences) as per manufacturer's instructions. Samples were acquired on FACS Aria and analysed using BD FCAP Array (BD Biosciences). TGFβ (BD Biosciences) and IL23 (eBiosciences, CA, USA) were determined by ELISA as per manufacturer instructions.

3.7.2 Cytokine estimation in serum samples of GBC patients and HI

Peripheral blood was collected from GBC patients and HI in vaccutainers (BD biosciences). Blood was allowed to clot at 4^0C overnight. Serum was collected in eppendorff tubes after centrifuging the clotted blood. Serum samples were stored at -80^0C until used. Cytokines and chemokines in sera samples were measured by Th1/Th2/Th17 cytometric bead array, flex sets (BD Biosciences) and ELISA.

Cytometric bead array

The CBA technique utilizes microparticles or beads labelled with discrete fluorescence intensity. The maximum emission of capture beads is at 650 nm on RED parameter. Cytokine specific capture antibody is covalently attached to beads. The captured cytokines are detected using specific antibodies with phycoerythrin (PE) fluorochrome which emits at 585 nm on yellow parameter. The intensity of fluorescence of yellow parameter is proportional to the amount of cytokines present in test samples. Cytokines were determined in the test samples according to the manufacturer instructions. Briefly test samples (50µl) and PE detection antibody were incubated with capture bead reagent for 3 h in the dark at room temperature. All unbound antibodies were washed (1.0 ml wash buffer), re-suspended in 300µl before acquisition on BD FACS Aria cytometer (BD Bioscience, San Jose, CA, USA). All cytokines exhibited single, well separated peaks. The individual cytokine standard curves (range 20–5000 pg/ml) were run in each assay. The data was presented as pg/ml.

Enzyme Linked Immunosorbent Assay

ELISA was performed as per the instructions in commercially available kits. Briefly 100µl capture antibody (with required dilution in coating buffer) specific for cytokine (TGBβ / IL23 / VEGF) was coated in flat bottom 96 well plates (Maxisorp, Nunc, Germany) for overnight at 4^0C. After three times washing with wash buffer (0.01M phosphate buffered saline + 0.05% Tween 20; 300µl) the wells were coated with assay diluent (PBS + 10% bovine serum albumin or FCS; 200µl) for 1 h at RT. After washing with wash buffer, serially diluted recombinant cytokine (100µl) was added to the wells. The standard and serum/supernatant samples (100µl) were incubated for 2 h at RT. Unbound proteins were removed by washing (3-5 times) with wash buffer. The bound cytokines were determined by detection antibody (100µl with required dilution) labelled with biotin. The wells were washed (5-7 times). The ELISA was developed using HRP tagged streptavidin and chromogenic substrate for 30 min in dark (100µl). The reaction was terminated using 2N H_2SO_4 (50µl). Optical density was measured at 450nm on ELISA plate reader. The concentrations of cytokines in the supernatants were calculated by extrapolating the OD values of unknown samples on calibration curve of standards. The data was expressed as pg/ml.

3.8 Real time Polymerase chain reaction (RT-PCR)

3.8.1 Extraction of RNA

PBMCs isolated from GBC patients and HI were stored at -80^0 C in TRIzol (Invitrogen Life-Technologies, N.Y.) in the ratio of $1x10^6$ cells per 100µl TRIzol solution until further use. During RNA extraction, chloroform was added in 1: 5 ratio of chloroform : TRIzol (eg. 20µl choloroform in 100 µlTRIzol). The mixture was vigorously vortexed and centrifuged at 10,000 rpm for 15 min at 4^0C. The aqueous phase was collected in separate tube and treated with chilled Isopropyl alcohol (Qualigens, India) equivalent to half the volume of TRIzol. After mixing gently, the precipitated RNA was centrifuged for 10 min at 10,000 rpm at 4^0C.

The pelleted RNA was washed with 75% ethanol (10,000 rpm for 5 min at 4^0C). The pellet was air dried and dissolved in appropriate volume of DEPC (Diethyl Pyrocarbonate; Sigma, USA) 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.

3.8.2 Complementary DNA (cDNA) synthesis by reverse transcription

Total RNA isolated from PBMCs was used for first strand cDNA synthesis using oligo dT primers (Invitrogen Life-Technologies, N.Y.). 1- 5µg of RNA (10µl volume with DEPC treated water) was reverse transcribed using 1µl of oligo dT and 1µl (10 mM) of dNTP. The mixture was heated at 65^0C for 5 min and then chilled on ice for 10 min. The mixture for reverse transcriptase containing the components given below was prepared and was added to the previously made RNA-primer mixture.

Table 3.6 Composition of cDNA synthesis PCR reaction

Component	*Volume (µl)*
5 X first strand buffer	4
0.1M DTT	2
RNAse out inhibitor (40U/µl)	1
MMLV reverse transcriptase enzyme (200U/µl)	1

This total mixture was heated at 37^0C for 52 min followed by 70^0C for 15 min in PTC-100TM Programmable Thermal Controller (MJ Research Inc.). The final cDNA volume was 20µl. The reverse transcriptase enzyme used was Murine Moloney Leukemia Virus (MMLV) reverse transcriptase enzyme (Invitrogen Life-Technologies, N.Y.; 200U/µl).

3.8.3 Real Time PCR

Quantitative RT-PCR for transcription factors (Foxp3 and RORc) and cytokines (IL6 and TGFβ) was performed using Assays-on-Demand Gene Expression probes (Applied Biosystems). RT-PCR reaction mixture was prepared as follows:

Table 3.7 Composition of real time PCR reaction

Component	*Volume (µl)*
DEPC water	2.5
Primer probe mixture	0.25
PCR Master-mix	0.25
cDNA (10ng)	2.0

Samples were analysed using QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific). Gene Expression probes for RORC (Hs01076112_m1), Foxp3 (Hs01085834_m1), IL6 (Hs00985639_m1) and TGFβ (Hs00234244_m1) were purchased from Applied Biosystems. Gene expression was normalized using housekeeping gene β actin (Hs99999903_m1) (Applied Biosystems). Relative mRNA expression of target genes was calculated as $2^{(Ct\ control\ RNA-\ Ct\ test\ RNA)}$. The relative mRNA expression of target genes (IL6, IL23, RORc and Foxp3) in PBMCs of GBC patients was compared with HI.

3.9 Regulatory T cells suppression assay

Tregs ($CD4^+CD25^+$) were isolated from PBMCs using BD IMag regulatory T lymphocyte separation set-DM (BD Biosciences). Briefly $CD4^+$ T cells were negatively selected from PBMCs followed by positive selection of $CD25^+$ T cells. $CD4^+CD25^-$ T cells were used as responder T cells (Tres) and labelled with carboxyfluorescein succinimidyl ester (CFSE; 5µM) (CellTrace proliferation kit, Life technologies). Briefly, 1-5 x 10^6 cells suspended in PBS + 5% FCS were incubated with 5µM CFSE for 10 min at RT in dark. After incubation

cells were washed thrice with PBS + 5% FCS. Labelled Tres cells ($1x10^4$) were co-cultured with Tregs for 5 days at different ratios (Tres: Treg = 1:2, 1:1, 1:0.5, 1:0) in round bottom 96 well plate. Co-cultures were stimulated with anti-CD3/anti-CD28 coated beads (1bead: 1cell) (Treg suppression inspector, Miltenyi biotech). CFSE labelled Tres cells cultured without Treg cells in the presence of anti-CD3/anti-CD28 coated beads were kept as control. Cells were acquired on FACS Aria and analysed by FlowJo software. The stimulation of Tres cells was analysed by calculating the number of peaks representing generation of divided cells. The undivided cells having peak with high CFSE intensity were considered as mother population. The suppressive effect of Treg cells on Tres cells was determined by decrease in the number of peaks. The data was presented as division index calculated by the software using percentage of cells in each generation and number of peaks.

3.10 Cell migration assay

3.10.1 Migration of Tγδ17 cells

Migration of Tγδ17cells was studied by trans-well assay using Millicell cell-culture inserts with pore size 8.0µm (Merck Millipore, MA, USA). OCUG-1 cells ($5x10^4$), cultured in 24 well plate were washed with plain medium and 600µl serum-free Williams' E medium was added to the lower chamber. In some experiments rhCXCL9 (100ng/ml; PeproTech, NJ, USA) or tumor supernatants of gallbladder tumor tissue were added to the lower chamber. Isolated Tγδ17 cells ($5x10^4$/100µl medium) were added onto the trans-well inserts. Tγδ17 cells cultured with medium alone were used as control. Migrated cells from lower chamber were counted using hemocytometer after 7 h. For blocking experiments, Tγδ17 cells were incubated with anti-CXCR3 antibody (10µg/ml; R&D Systems, MN, USA), 30 min before trans-well co-culture. Migration of Tγδ17 cells was compared with control wells where Tγδ17 cells were cultured with medium alone.

3.10.2 Migration of γδT cells

γδT cells were isolated from peripheral blood of HI. Purified γδT cells ($1x10^5$) were cultured to the upper chamber of Millicell cell-culture inserts with pore size 8.0µm (Merck Millipore, MA, USA). OCUG-1 cells ($1x10^5$) were cultured in 24 well plates (lower chamber of trans-well). Cells were washed and 600µl serum-free Williams' E medium was added to the lower chamber. In some experiments tumor supernatants were added to the lower chamber. Migrated γδT cells to the lower chamber were collected and counted using hemocytometer after 7 h. For blocking experiments, γδT cells were incubated with anti-CXCR3 antibody (10µg/ml; R&D Systems, MN, USA), 30 min before trans-well co-culture. Migration of γδT cells was compared with control wells where Tγδ17 cells were cultured with medium alone

3.11 Estimation of angiogenesis factors

Tγδ17 cells ($5x10^4$) were cultured in serum-free RPMI medium for 24 h in the presence of anti-CD3/anti-CD28 coated beads. Cell-free supernatant of Tγδ17 was collected and added to OCUG-1 cells ($2x10^4$ cells/well) cultured in 96 well plate. Neutralizing anti-IL17 antibody (10µg/ml; R&D Systems, MN, USA) was added to some wells. OCUG-1 cells cultured with medium alone were used as control. After 48 h, supernatants were collected and analyzed for VEGF by ELISA (R&D systems, MN, USA) or for presence of angiogenesis related proteins by human proteome profiler angiogenesis antibody array (R&D systems, MN, USA) as per manufacturer's instructions.

3.11.1 Angiogenesis Array

The cell free supernatants collected from above experiment were diluted (1ml supernatant + 500µl array buffer provided in the kit) and mixed with a 15µl of reconstituted cocktail of biotinylated detection antibodies specific for angiogenesis related proteins. The nitrocellulose membranes coated with array of antibodies (spotted in duplicate with capture antibodies

specific for angiogenesis related proteins) were dipped in 2ml blocking buffer for 1 h on rocking platform. The mixture of supernatant and diluted antibodies was incubated with the nitrocellulose membranes overnight at 4^0C with intermittent shaking. Membranes were washed three times on rocking platform shaker to remove unbound material followed by incubation with 2ml HRP-conjugated streptavidin for 30min at room temperature. After washing the membranes, chemiluminescence was used for signal detection using x-ray film with exposure of 10min. The signal produced was proportional to the amount of bound analyte. The data was evaluated using Image J 1.48V software (NIH, USA) and expressed as mean pixel density. The mean pixel density of angiogenesis related factors produced by OCUG-1 cells in presence of Tγδ17 supernatant was compared with OCUG-1 cells cultured with medium alone.

3.11.2 VEGF ELISA

The cell free supernatants collected from above experiment were analyzed for the presence of VEGF using VEGF ELISA as per the manufacturer's instructions. Briefly, the capture antibody was diluted to working concentration in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4). 100µl capture antibody specific for VEGF was coated in flat bottom 96 well plates (Maxisorp, Nunc, Germany) and incubated overnight at room temperature. The wells were washed three times with 300µl wash buffer (PBS + 0.05% Tween 20). The wells were coated with reagent diluent (1% BSA in PBS) for 1 h at room temperature. After washing with wash buffer, serially diluted recombinant VEGF or cell-free supernatants (100µl) was added to the wells. The standard and supernatant samples were incubated for 2 h at room temperature. Unbound proteins were removed by washing (3-5 times) with wash buffer. The VEGF present in the wells was determined by detection antibody labelled with biotin (100µl with required dilution prepared in reagent diluent). The wells were washed to remove unbound detection antibody (3 times). HRP tagged streptavidin

(100µl prepared in reagent diluent) was added to well and incubated for 20min at room temperature. The ELISA was developed using chromogenic substrate (100µl per well) for 30 min in dark. The reaction was terminated using 2N H_2SO_4 (50µl). Optical density was measured at 450nm on ELISA plate reader. The concentrations of cytokines in the supernatants were calculated by extrapolating the OD values of unknown samples on calibration curve of standards. The data was expressed as pg/ml.

3.12 Chorioallantoic membrane (CAM) assay

Fertilized chicken eggs (Central Poultry Development Organization, Goregaon, Mumbai) were incubated in humidified incubator at 37^0C. On embryonic day 5, the eggs were screened for presence of embryo by exposing against torch. A small window was made in the shell and 200µl medium/ Tγδ17 supernatant/ rhIL17 (100ng/ml, R&D systems, MN, USA) was added onto the CAM of growing embryo. In some eggs, Tγδ17 supernatant pre-incubated with neutralizing anti-IL17 antibody was added. The window was sealed using parafilm (Sigma Aldrich, USA) and the eggs were incubated in humidified incubator at 37^0C. After 48 h, eggs were cracked open and embryos were carefully transferred to 100mm petri dish and images were captured. CAM was cut and transferred to a glass slide to observe under the microscope. Angiogenesis was quantitatively evaluated by scoring number of branching points in control and treated CAMs.

3.13 Immunofluorescence staining

To study the expression of IL17R on OCUG-1 and NOZ cells, OCUG-1 and NOZ cells were cultured in glass bottom plates for 48 h with seeding density of $2x10^4$. Cells were washed with PBS and fixed with 4% paraformaldehyde at 4^0C for 15-20 min. Bovine serum albumin (5% prepared in PBS; Sigma Aldrich, USA) was used for blocking at room temperature for 1 h. Primary antibody specific for IL17R (1µg/ml, R&D Biosystems) was incubated overnight at 4^0C in humid chamber. Cells were washed three times with PBS. Secondary antibody

labelled with FITC (1: 200 dilution; Sigma, USA) was incubated for 2 h in humid chamber at room temperature. Cells were washed for three times with PBS and counterstained with DAPI (Sigma, USA) for 2min. Cells were maintained in PBS and images were acquired using Leica STED super resolution microscope (Leica Microsystems, Germany). The images were analysed by Image J1.48V software (NIH, USA).

3.14 Effect of IL17 on VEGF production in gallbladder cancer cell lines

To study the proangiogenic effect of rhIL17 on gallbladder tumor cells, OCUG-1 and NOZ cells were cultured in presence or absence of rhIL17 and levels of VEGF were measured in the supernatant by ELISA. Briefly, OCUG-1 and NOZ cells (2-3x10^5) were cultured in 6 well plate in Williams' E medium supplemented with 10% FCS for 48 h. The cells were seeded to attain 80% confluency in 48 h. The cells were washed gently with plain Williams' E to remove FCS. Plain medium with or without rhIL17 (50ng/ml or 100ng/ml) was added to the wells. Cells cultured with plain medium were considered as control. After 48 h, supernatant was collected and levels of VEGF were measured by ELISA (R&D systems, USA) as described in 3.11.2. The effect of rhIL17 on VEGF production in cell lines was analysed by comparing with the control wells where cells were cultured with medium alone.

3.15 Wound healing assay

GBC cell lines were cultured in 6 well plate in complete Williams' E medium. When OCUG-1 and NOZ cell lines formed monolayer with 90% confluency the spent medium was removed and cells were washed twice with plain Williams' E medium. The cells were treated with Mitomycin C (10µg/ml; Sigma Aldrich, USA) in plain medium for 2 h at 37^{o}C with 5% CO_2. Cells were gently washed two times with plain medium to remove traces of Mitomycin C. Three scratches were made precisely with the help of T-10 micro tips. The scratched cells were removed further by rinsing the wells with plain Williams' E medium. rhIL17 (50ng/ml, 100ng/ml) was added in plain Williams' E medium to the respective wells. Cells with plain

Williams' E medium were used as control. The cell migration was monitored for 21 h using time lapse inverted microscope (Carl Zeiss, Germany). The images were acquired with interval of every 1 h. Images were analysed using Image J1.48V software (NIH, USA). The effect of IL17 on migration of GBC cells was measured by comparing the percent wound closure of cells with the control. The percent wound closure was calculated by the formula

$$\text{Percent (\%) wound closure} = \frac{\text{Area of wound at 0 h X 100}}{\text{Area of wound at 21 h}}$$

3.16 Matrigel invasion assay

To study the effect of rhIL17 on invasion potential of gallbladder tumor cells, OCUG-1 and NOZ cells were treated with rhIL17 and analysed by matrigel invasion assay. In a 24 well trans-well assay the cell culture inserts (8.0µm pore size, BD Falcon, USA), were coated with the matrigel (20µl of Matrigel [1mg/ml stock] diluted in 140µl of plain Williams' E; BD Biosciences, San Jose, CA) for 2 h at 37^0C. The matrigel was maintained at 4^0C during coating process. The inserts were washed with plain medium to remove unpolymerised matrigel. OCUG-1 ($5x10^4$) and NOZ ($3x10^4$) cells were suspended in 200µl plain Williams' E medium containing IL17 (50ng/ml or 100ng/ml). The untreated cells were considered as control. The cells were added to the inserts of trans-well and 600µl of Williams' E medium with 10% FCS was added to the lower chamber. OCUG-1 cells were incubated for 48 h and NOZ cells were incubated for 24 h. The incubation periods were decided by prior standardization experiments. After incubation, the inserts were lifted from the plate and washed by gentle dipping in PBS. The cells were fixed in absolute methanol for 5-10sec followed by washing with PBS for 30sec. The cells were stained by 1% crystal violet (prepared in 70% methanol) for 5min and excess stain was removed by dipping the trans-well in PBS. Cells remained in the upper chamber (unmigrated cells) were removed using cotton swabs. The membrane was cut from inserts and carefully transferred to the glass slide with

cells adhered to the membrane facing upward. The cells were observed using 10X objectives of microscope (Carl Zeiss, Germany). Minimum 10 microscopic fields were photographed and the migrated cells were counted. The fold increase in invasion upon rhIL17 treatment was calculated by normalizing the migrated number of cells with the control.

3.17 Survival analysis

The survival analysis was performed using GraphPad Prism software (Prism Software, Lake Forest, CA). The clinical significance of Tγδ17, Th17, Tc17, and Treg, $\gamma\delta^{+}IFN\gamma^{+}$ cells and serum IL17 and serum IFNγ levels was analysed by comparing with survival time of GBC patients. The survival time of patients was determined from date of diagnosis and date of death. The data was censored for patients alive or lost to follow up at the time of analysis. As there was no clinically defined cut off points for Th17, Tc17, Tγδ17, Treg cells, the high-expressing or low-expressing groups of GBC patients were defined based on mean values of expression of these lymphocytes (4.7 for Tγδ17, 1.8 for Th17, 1.8 for Tc17 and 3.2 for Treg). Overall patient survival was calculated by Kaplan-Meier curve and compared by Log-rank test.

3.18 Statistical analysis

Statistical analysis of data was performed using GraphPad Prism software (Prism Software, Lake Forest, CA). Statistical significances were calculated by two-tailed student's t-test or Mann-Whitney test. $P < 0.05$ was considered statistically significant. The data was presented as mean ± standard error.

Chapter 4

Analysis of immune scenario in peripheral blood of GBC patients

4.1 Introduction

Tumor infiltrating immune cells and tumor cells are in continuous crosstalk with each other and significantly contribute in shaping malignant progression of tumor [54, 55]. The studies in cancer patients have shown that modulation of immune response in tumor environment is also reflected in peripheral blood [258, 302-304]. Systemic levels of T cells are reported as a potential prognostic marker and are associated with survival of patients with melanoma, breast cancer, gastric cancer, ovarian cancer, etc. [305-307]. Circulating frequencies of lymphocytes can also serve as a measure of effectiveness of therapeutic treatment of patients [302, 308]. Therefore, understanding the immune scenario in peripheral blood of cancer patients is important for successful therapeutic interventions. The significance of immune response regulating development of gallbladder cancer is not well understood. Knowledge of prognostic or predictive markers could be of great importance for the choice of appropriate individual treatment for GBC patients. The earlier studies in GBC patients have shown the prevalence of CD4 and CD8 T cells in tumor tissue [309, 310]. However, the comprehensive analysis of immune cell types, their response to antigenic stimulus and secretion of effector cytokines by adaptive and innate immune cells in GBC patients is not investigated.

In the present prospective study we used flowcytometry based immunophenotyping of GBC patients to delineate the importance of adaptive and innate lymphocytes. The data highlights functional parameters of peripheral blood lymphocytes of GBC patients in comparison to healthy individuals.

4.2 Results

4.2.1 Cells contributing to adaptive immunity are decreased in peripheral blood of GBC patients

In order to study the innate and adaptive immune cells in GBC patients, peripheral blood mononuclear cells (PBMCs) were isolated from GBC patients (n=52) and healthy individuals (HI; n=30). For multicolour flow cytometry, PBMCs were surface stained for $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells. Figure 4.1 describes gating strategy used to analyse T cells ($CD3^+$), T-helper cells ($CD3^+CD4^+$), T-cytotoxic cells ($CD3^+CD8^+$), B cells ($CD19^+$), natural killer cells ($CD3^-CD56^+$), natural killer T ($CD3^+CD56^+$) cells and γδT cells ($CD3^+V\delta_2TCR^+$) cells.

The data showed that $CD3^+$ T lymphocytes were decreased in peripheral blood of GBC patients compared to HI (Figure 4.1 A). The subtype analysis of T cells revealed that $CD3^+CD4^+$ helper T cells and $CD3^+CD8^+$ cytotoxic T cells were significantly low in peripheral blood of GBC patients compared to HI (Figure 4.1 B and C). However, the ratio of CD4 : CD8 T cells was comparable in GBC patients and HI (Figure 4.1 D). $CD19^+$ B cells and NKT cells showed marked decrease in percentage compared to HI (Figure 4.1 E and F). In contrast, $CD3^-CD56^+$ NK cells were elevated and γδT cells were unaltered in GBC patients compared to HI (Figure 4.1 G and H). The data revealed that decrease in the percentages of lymphocytes contributing to adaptive immunity suggesting inadequate immune response in GBC patients to maintain the immune-surveillance.

4.2.2 PBMCs from GBC patients show poor lymphocyte proliferative response

Antigen recognition leads to activation and clonal propagation of lymphocytes. To understand the proliferative response of lymphocytes, the PBMCs from GBC patients (n=26) and HI (n=19) were stimulated in separate sets with anti CD3 mAb (1μg/well) and

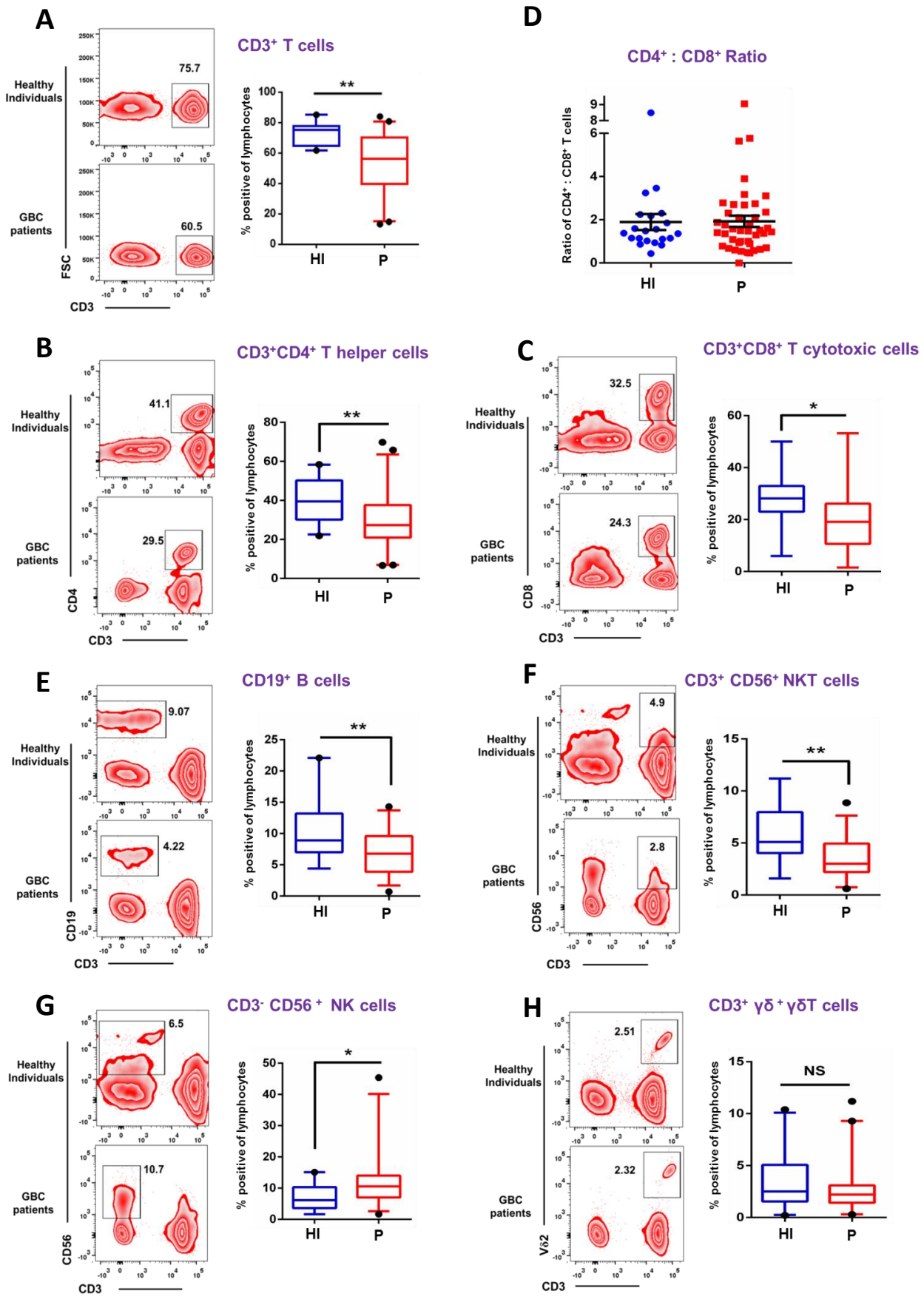

A
Healthy Individuals
GBC patients
75.7
60.5
FSC
CD3
CD3⁺ T cells
% positive of lymphocytes
**
HI
P
D
CD4⁺ : CD8⁺ Ratio
Ratio of CD4⁺ : CD8⁺ T cells
HI
P
B
CD3⁺CD4⁺ T helper cells
41.1
29.5
CD4
CD3
% positive of lymphocytes
**
HI
P
C
CD3⁺CD8⁺ T cytotoxic cells
32.5
24.3
CD8
CD3
% positive of lymphocytes
*
HI
P
E
CD19⁺ B cells
9.07
4.22
CD19
CD3
% positive of lymphocytes
**
HI
P
F
CD3⁺ CD56⁺ NKT cells
4.9
2.8
CD56
CD3
% positive of lymphocytes
**
HI
P
G
CD3⁻ CD56⁺ NK cells
6.5
10.7
CD56
CD3
% positive of lymphocytes
*
HI
P
H
CD3⁺ γδ⁺ γδT cells
2.51
2.32
Vδ2
CD3
% positive of lymphocytes
NS
HI
P

Figure 4.1 : Immunophenotyping of peripheral blood lymphocytes of GBC patients. *PBMCs from GBC patients (n=52) and HI (n=30) were analysed by flowcytometry for percent levels of lymphocytes in peripheral blood of GBC patients compared to HI.* ***(A-C)*** *A summarized data for the percent levels of $CD3^+$ (A), $CD3^+CD4^+$ (B), $CD3^+CD8^+$ (C) is presented as box whisker plots. Representative figures of flowcytometry analysis are shown as zebra plots.* ***(D)*** *Ratio of $CD3^+CD4^+$: $CD3^+CD8^+$ T cells in GBC patients and HI is presented as dot plot analysis.* ***(E-H)*** *Representative flowcytometry figures and box whisker plots showing expression of B cells (E), NKT cells (F), NK cells (G) and γδT cells (H) in peripheral blood of GBC patients in comparison to HI. The box plots show the median (middle line), 5^{th} and 95^{th} percentiles (box), the extreme values (whiskers) and the outliers (dark circles). Data are shown as mean ± SEM with *p<0.05 or **p<0.01.*

Phytohaemagglutinin (PHA; 1%) for 72 h. The proliferative responses were monitored by ^3H Thymidine incorporation assay.

PBMCs from GBC patients showed decreased basal level proliferative potential in comparison to HI (Figure 4.2). Further, stimulation of PBMCs with anti-CD3 mAb, showed decreased proliferative response of lymphocytes from GBC patients compared to HI. Similar results were obtained when PHA was used to stimulate the PBMCs (Figure 4.2). The results indicate that PBMCs from GBC patients exhibit decreased proliferative responses to PHA (mitogen) and anti-CD3 mAb (TCR agonist) as compared to HI.

4.2.3 T lymphocytes in peripheral blood of GBC patients exhibit effector memory phenotype

Activated T cells differentiate into memory phenotype and attain variety of effector functions. To study the memory phenotype of T cells, PBMCs from GBC patients (n=27) and HI (n=14) were analysed by flow cytometry for the surface expression of CD27 and CD45RA. Naïve T cells were defined as $CD27^+CD45RA^+$ (T_{Naive}), central memory T cells as $CD27^+CD45RA^-$ (T_{CM}), effector memory T cells as $CD27^-CD45RA^-$ (T_{EM}) and terminally differentiated effector memory T cells as $CD27^-CD45RA^+$ (T_{EMRA}) (Figure 4.3 A). The data revealed that the percentages of naïve cells in total T lymphocytes were

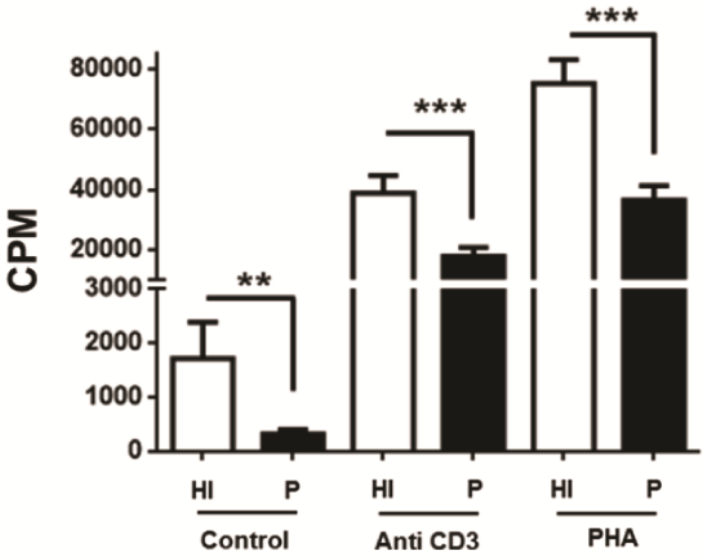


Figure 4.2 : Proliferative response of PBMCs from GBC patients to anti-CD3 and PHA. *PBMCs from GBC patients (n=26) and HI (n=19) were stimulated with anti-CD3 or PHA and proliferation was monitored by ^{3}H-Thymidine incorporation assay. The data is shown as bar diagram presenting radiations counted as counts per minutes.*
*Data are shown as mean ± SEM with * p<0.05; ** p<0.01; *** P<0.001.*

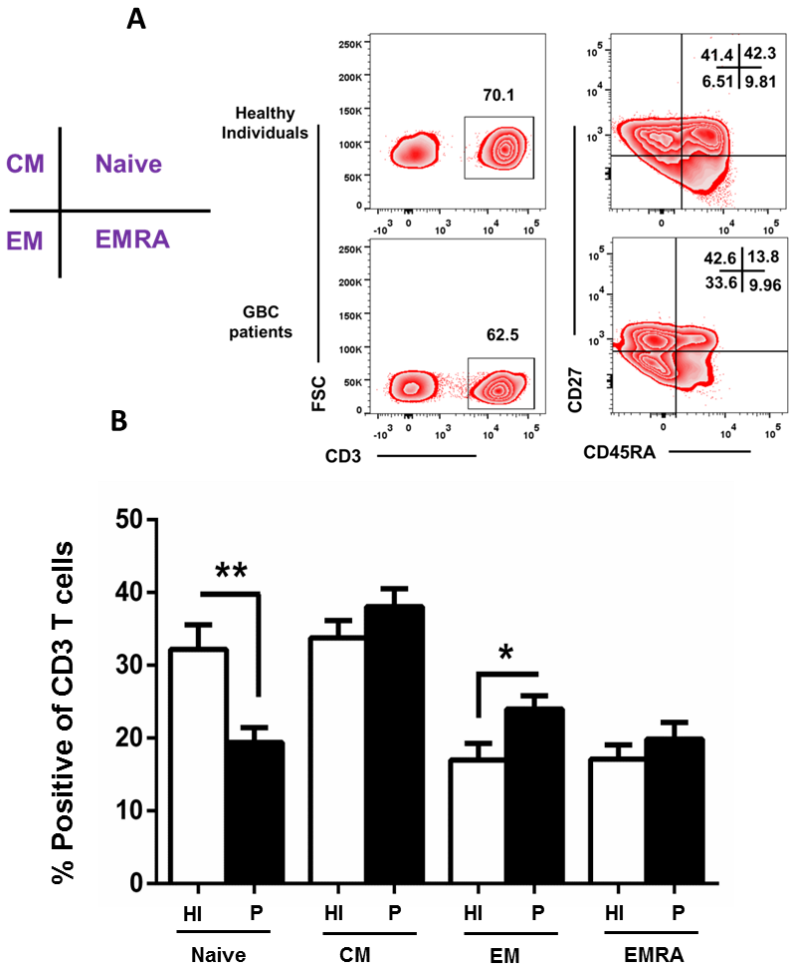


Figure 4.3 : Memory phenotypes of T lymphocytes in GBC patients. (A) *Representative zebra plot showing expression of CD27 and CD45RA on T lymphocytes of GBC patients and HI.* ***(B)*** *Proportion of naïve and memory T cells in peripheral blood of GBC patients compared to HI are shown as bar diagram.*
*Data are shown as mean ± SEM with * $p<0.05$; ** $p<0.01$.*

significantly decreased in peripheral blood of GBC patients. However, T cells exhibiting effector memory phenotype were increased in GBC patients. The percentage of central memory and terminally differentiated effector memory (T_{EMRA}) cells were unaltered in GBC patients compared to HI (Figure 4.3 B). The analysis of memory phenotypes indicates that the T lymphocytes may be functionally active in GBC patients.

4.2.4 PBMCs of GBC patients secrete low levels of effector cytokines compared to HI

Activated lymphocytes secrete effector cytokines in response to the antigenic stimulation. To determine the cytokine profile of activated lymphocytes, PBMCs from GBC patients (n=42) and HI (n=21) were stimulated with anti-CD3 mAb and PHA. Cell free supernatants were collected after 24 h and analysed by cytometric bead array.

The PBMCs from GBC patients secreted decreased levels of IFNγ, IL6, TNFα, IL8 and IL10 at baseline i.e. without any stimulation compared to HI (Figure 4.4). Upon stimulation with anti-CD3 mAb, PBMCs from GBC patients produced significantly low levels of IFNγ, IL6, IL17, IL8, IL12p70, IL1β, IL23, IL10 and TGFβ whereas levels of IL2, IL4 and TNFα did not alter in comparison to HI. Similar results were observed in GBC patients upon stimulation with PHA that the levels of IFNγ, IL6, IL17, IL8, IL1β, IL10, IL2p70, IL4 and TGFβ were decreased compared to HI (Figure 4.4). Overall, the poor production of effector cytokines by activated PBMCs from GBC patients, suggests that the immune response in GBC patients is defective.

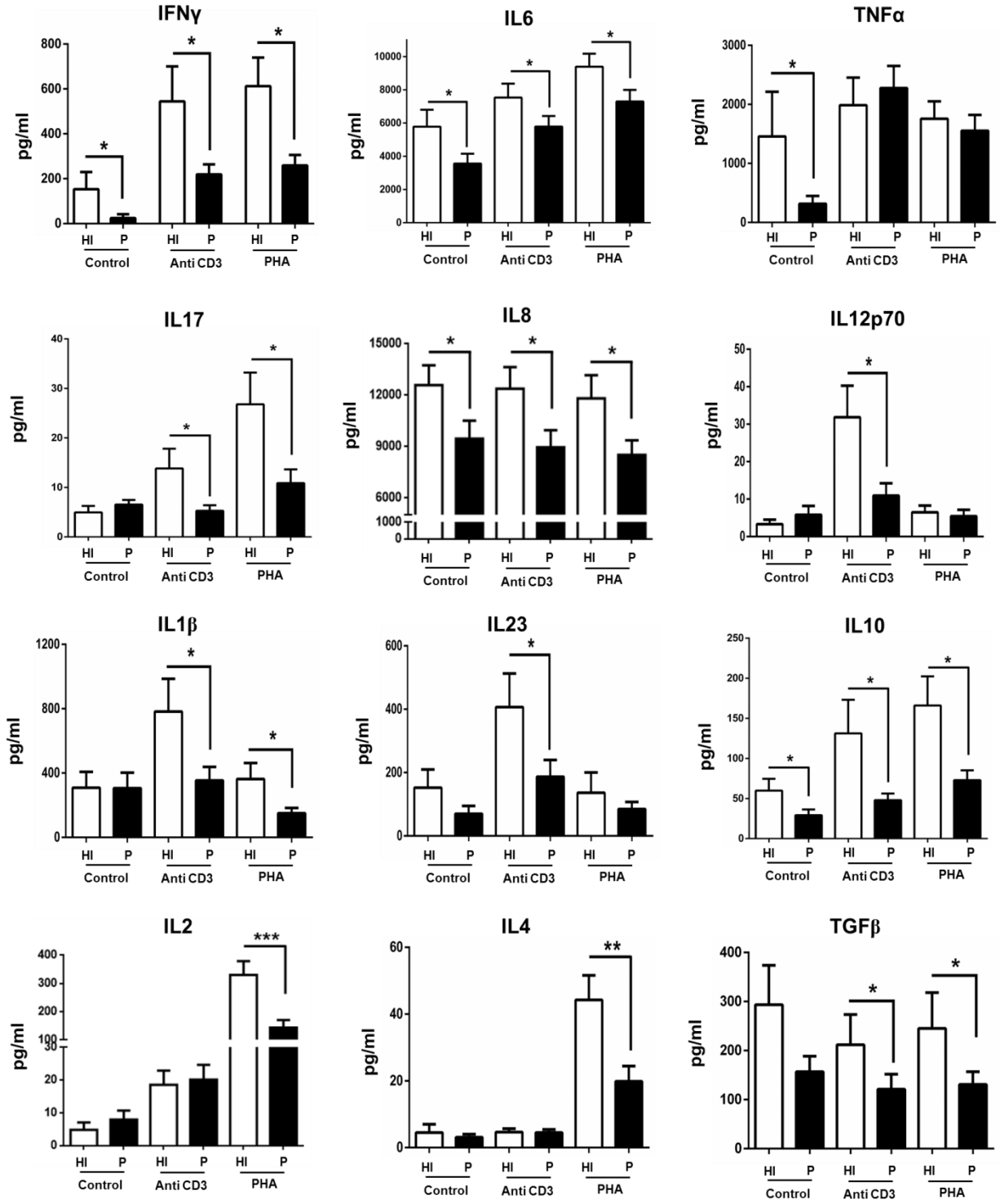


Figure 4.4 : Cytokines secreted by activated PBMCs of GBC patients. *PBMCs from GBC patients and HI were stimulated with anti-CD3 or PHA and cell-free culture supernatants were collected after 24 hrs. The cytokines were estimated by cytometric bead array. The levels of secreted cytokines are presented as pg/ml. Data are shown as mean ± SEM with * $p<0.05$; ** $p<0.01$.*

4.2.5 TCR ζ chain is downregulated in T lymphocytes of GBC patients

To understand the mechanism of poor effector functions of T lymphocytes, the defects in the T cell receptor (TCR) signalling were analysed. PBMCs from GBC patients (n=50) and HI (n=24) were analysed for expression of TCR ζ chain by flow cytometry. It was observed that more than 90% of T lymphocytes from GBC patients expressed TCR ζ chain as shown by percent positive expression. However, the median fluorescence intensity of TCR ζ chain expression in T cells was significantly low in peripheral blood of GBC patients compared to HI (Figure 4.5 A and B). Investigation of TCR ζ chain in tumor infiltrating lymphocytes (n=13) showed further decrease in the expression compared to peripheral blood of GBC patients and HI (Figure 4.5 B). In addition, the classification of GBC patients with clinical stage II (n=5), stage III (n=18) and stage IV (n=27) showed that the TCR ζ chain expression in T lymphocytes inversely correlated with clinical stage of the patients (Figure 4.5 C). This suggests that the expression of TCR ζ chain is downregulated in T lymphocytes of GBC patients.

Further, correlation analysis revealed that TCR ζ chain expression positively correlated with proliferation of T lymphocytes (stimulation index) stimulated with anti-CD3 mAb or PHA (Figure 4.5 D and E). The data suggests that defective TCR mediated signalling may be responsible for poor activation and effector functions in T lymphocytes of GBC patients.

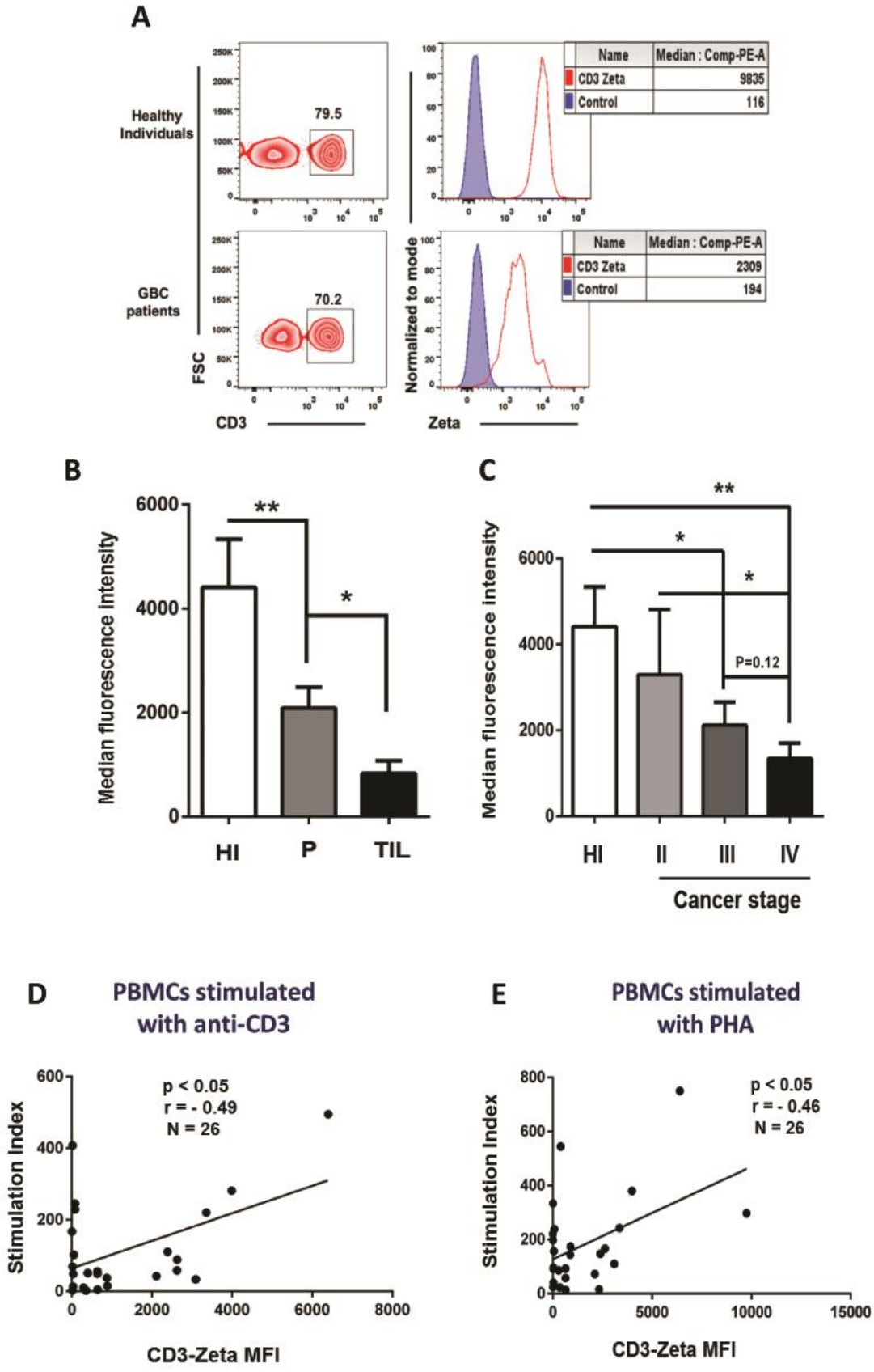

A
Healthy Individuals
GBC patients
79.5
70.2
FSC
CD3
Zeta
Normalized to mode
Name
Median : Comp-PE-A
CD3 Zeta
Control
9835
116
2309
194
B
Median fluorescence intensity
HI
P
TIL
C
HI
II
III
IV
Cancer stage
P=0.12
D
PBMCs stimulated with anti-CD3
p < 0.05
r = - 0.49
N = 26
Stimulation Index
CD3-Zeta MFI
E
PBMCs stimulated with PHA
p < 0.05
r = - 0.46
N = 26
Stimulation Index
CD3-Zeta MFI

Figure 4.5 : Expression of TCR ζ chain in T lymphocytes of GBC patients. ***(A)*** *A representative zebra plot and histogram of expression of TCR ζ chain in $CD3^+$ T lymphocytes of GBC patients and HI. Figures in the histogram indicate median fluorescence intensity (MFI) of TCR ζ chain expression (empty histogram) and isocontrol (filled histogram).* ***(B)*** *Bar diagram shows summarised data of expression of TCR ζ chain presented as MFI in GBC patients (n=50) and HI (n=24).* ***(C)*** *Expression of TCR ζ chain in T lymphocytes of peripheral blood was analysed with clinical stages of GBC patients.* ***(D)*** *Correlation graph of expression of TCR ζ chain in T lymphocytes with stimulation index of PBMCs stimulated with anti-CD3 mAb.* ***(E)*** *Correlation graph of Expression of TCR ζ chain in T lymphocytes with stimulation index of PBMCs stimulated with PHA. "r" indicate coefficient of correlation and "N" indicate number of pairs in correlation.* Data are shown as mean ± SEM with * $p<0.05$; ** $p<0.01$.

4.3 Summary

The results described in this chapter revealed dysfunctions in immune response in peripheral blood of GBC patients. The lymphocytes contributing to adaptive immune response were decreased in PBMCs of GBC patients compared to HI. Although T lymphocytes belonging to effector memory phenotype were elevated in GBC patients, the proliferative response of T cells stimulated with mitogen or TCR agonist was decreased compared to HI. Moreover, the PBMCs from GBC patients produced decreased levels of effector cytokines upon stimulation in comparison to HI. The reason behind poor immunological response by PBMCs of GBC patients may be rooted in the downregulated expression of TCR ζ chain involved in the signal transduction and T cell activation. Overall, the present data showed that the peripheral blood lymphocytes in GBC patients are dysfunctional.

Chapter 5

Understanding the functional dynamics of pro-inflammatory and anti-inflammatory T cells in peripheral blood and tumor microenvironment of GBC patients

5.1 Introduction

Chronic inflammation is one of the important hallmark of cancer progression [121]. The tumor microenvironment selects the type of inflammatory reactions most favourable to tumor growth and progression [311]. It also subverts tumor antigen specific adaptive immune responses, and alters responses to chemotherapeutic agents [31]. Infiltration of lymphocytes is an integral part of tumor associated inflammation. Chronic inflammation activates tumor associated macrophages (TAMs), neutrophils (TANs), immunosuppressive cells (regulatory T cells, myeloid derived suppressor cells) which promote cancer progression. In contrast, signals that trigger acute inflammatory reactions often stimulate dendritic cells maturation and antigen presentation thereby trigger antitumor immunity [55]. The expression of various immune modulators and the abundance and activation state of different cell types in the tumor microenvironment dictate the balance of tumor-promoting inflammation and antitumor immunity. Thus it is necessary to study the type, functional orientation and dynamics of lymphocytes, cytokines and chemokines in inflammatory tumor environment.

GBC is an interesting model to understand such nexus of immune infiltrates and inflammation in tumor. GBC is predisposed with chronic inflammatory condition of cholelithiasis but the impact of immune infiltrates on inflammation and tumorigenic events remains largely unknown. Therefore, the efforts are needed to understand the tumor solicited inflammation present in GBC tumor microenvironment regulating the immune response.

The antitumor immune response is dysfunctional in GBC patients as demonstrated in chapter 4. In the present chapter, the dynamics of inflammatory (IL17 producing CD4, CD8 and γδT cells) and immunosuppressive (Treg, MDSCs) lymphocytes in gallbladder tumor environment and peripheral blood of GBC patients were investigated.

5.2 Results

5.2.1 IL17 producing CD4, CD8 and γδ T cells are elevated in tumor environment and peripheral blood of GBC patients

In order to study the prevalence of circulating IL17 producing cells, peripheral blood mononuclear cells (PBMCs) were isolated from GBC patients (n=52) and healthy individuals (HI; n=30). To investigate the tumor infiltrating lymphocytes (TILs) the single cell suspension of tumor tissue (n=17) was prepared by enzyme digestion as discussed in materials and methods. Cells were stimulated with PMA and Ionomycin in the presence of Brefeldin A. T lymphocytes in the peripheral blood and TILs were analysed by staining PBMCs and single cell suspension of tumor tissue using fluorochrome labelled subset specific antibodies.

Figure 5.1 describes representative gating strategy to define Th17, Tc17, Tγδ17 cells as $CD4^+IL17^+$, $CD8^+IL17^+$ and $\gamma\delta^+IL17^+$ cells. IL17 producing cells were gated on $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+V\delta2\text{-}TCR^+$ T cells respectively.

Th17 and Tc17 cells were significantly increased in PBMCs of GBC patients compared to HI (Figure 5.2 A and B). Interestingly, it was observed that IL17 producing γδT cells (Tγδ17) were also increased in PBMCs of GBC patients (Figure 5.2 C). A marked increase in the levels of these cells (Th17, Tc17 and Tγδ17) was observed in tumor compartment compared to the peripheral blood of GBC patients (Figure 5.2 A, B and C). It was Interesting to note that the relative percentages of Tγδ17 were higher compared to Th17 and Tc17 in TILs of GBC patients. Collectively, the data indicates that Tγδ17 cells are emerging as an important phenotype in GBC patients.

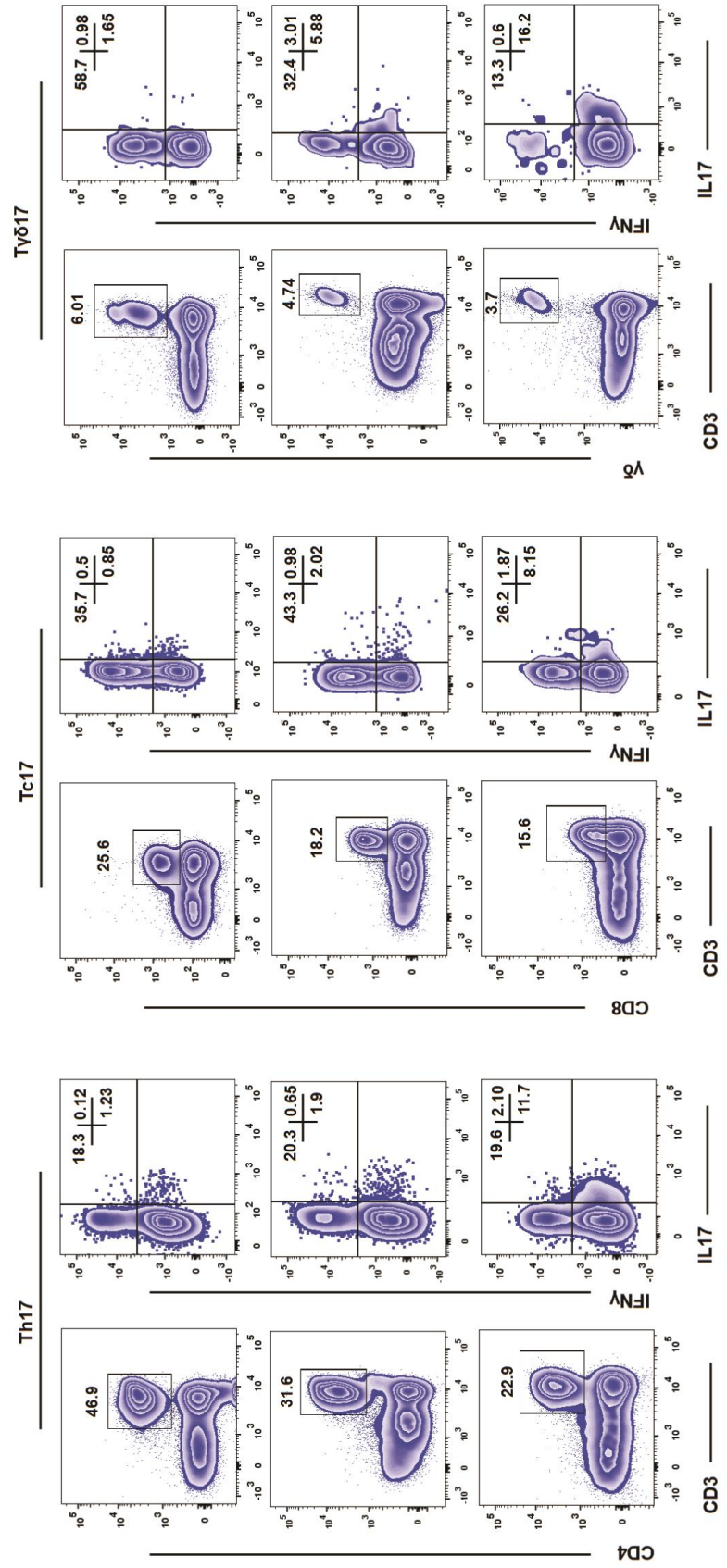


Figure 5.1. Gating strategy to define IL17 producing cells in GBC patients and HI. *Representative zebra plot analysis of Th17 (left), Tc17 (middle) and Tγδ17 (right) cells from PBMCs (n=52) and TILs (n=17) of GBC patient and HI (n=30). PBMCs and TILs were stimulated with PMA and ionomycin in presence of brefeldin A followed by intracellular staining for IL17 and IFNγ. IL17 and IFNγ producing cells were gated on $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+V\delta2TCR^+$ population respectively. Numbers in the plot indicate percent positive cells.*

To examine which T cell subset have higher propensity to produce IL17 in GBC patients, the data was analysed by another gating strategy. $CD4^+$, $CD8^+$ and $V\delta2TCR^+$ T cells from peripheral blood (n=24) and TILs (n=13) of GBC patients were analysed by gating on $CD3^+IL17^+$ T cells (total IL17 producing cells) (Figure 5.3 A). It was observed that percentage of $CD4^+$ cells within $CD3^+IL17^+$ T cells were significantly decreased in peripheral blood and TILs of GBC patients compared to HI (Figure 5.3 B). In contrast, $CD8^+$ T cells within $CD3^+IL17^+$ T cells were increased in TILs of GBC patients (Figure 5.3 C). Next, it was observed that among total $CD3^+IL17^+$ T cells, the levels of γδT cells were significantly increased in peripheral blood and further elevated in TILs of GBC patients compared to HI (Figure 5.3 D). Thus, the data revealed that the potential of IL17 production by T cell subsets was different at different anatomical locations (peripheral blood and tumor environment) in GBC patients. The γδT cells have higher propensity to produce IL17 compared to $CD4^+$ T cells in tumor environment of GBC patients.

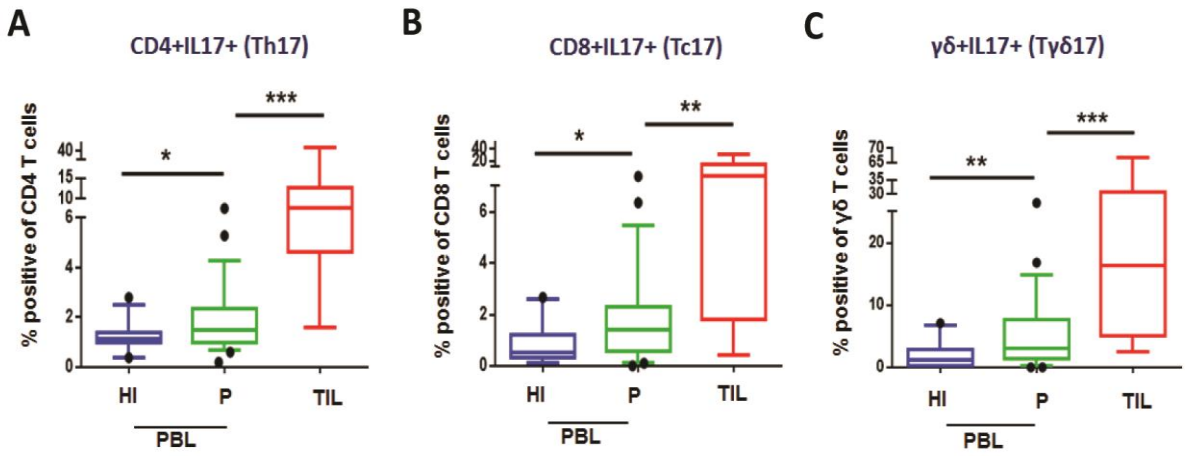


Figure 5.2 : Prevalence of Th17, Tc17 and Tγδ17 cells in GBC patients. (A-C) *The levels of Th17, Tc17 and Tγδ17 cells were analysed in PBMCs (n=52) and TILs (n=17) of GBC patients and compared with HI (n=30). Summarised data in box whisker plots show percentages of Th17 (A), Tc17 (B) and Tγδ17 (C) in PBMCs and TILs of GBC patients compared with HI.*
*HI, healthy individuals; P, GBC patients; TIL, Tumor infiltrating lymphocytes. The box plots show median (middle line), 5th and 95th percentiles (box), extreme values (whiskers) and outliers (dark circles). Results are analysed by Mann-Whitney test and student's t test with *(p < 0.05); **(p < 0.01); ***(p < 0.001).*

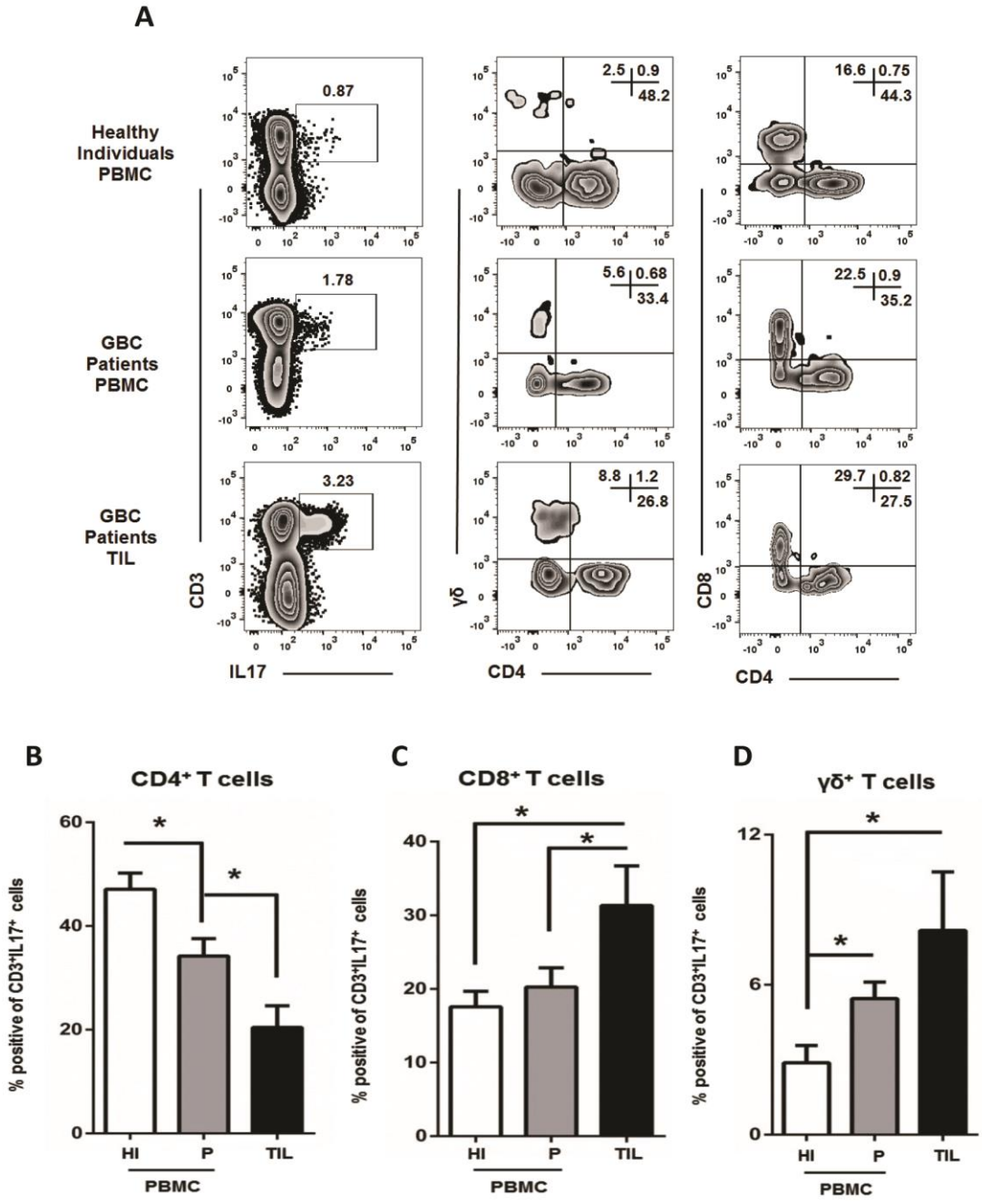


Figure 5.3 : Propensity of IL17 production in subsets of CD3+ T cells in GBC patients. *To study the proportion of IL17 produced by T cells subsets, $CD4^+$, $CD8^+$ and γδT cells were gated on $CD3^+IL17^+$ cells in peripheral blood (n=24) and TILs (n=13) of GBC patients and compared with HI (n=11).* ***(A)*** *A representative zebra plot analysis show gating strategy of $CD4,^+$ $CD8^+$ and γδT cells within $CD3^+IL17^+$ population in GBC patients and HI. Numbers in the plot indicate percent positive cells.* ***(B-D)*** *A summarized data of $CD4^+$ T cells (B), $CD8^+$ T cells (C) and γδT cells (D) within $CD3^+IL17^+$ population in GBC patients and HI. HI, healthy individuals; P, GBC patients; TIL, Tumor infiltrating lymphocytes; PBMCs, peripheral blood mononuclear cells. Data was analysed by Mann Whitney test and student's t test and presented as mean ± SEM with * $p<0.05$.*

5.2.2 IFNγ producing γδ and CD8$^+$ T cells are decreased in tumor environment of GBC patients

IFNγ producing T cells are reported to be tumoricidal and are associated with better prognosis of cancer patients [312]. To study the role of IFNγ producing cells in GBC patients, PBMCs and single cells suspension of tumor tissue were stained for intracellular expression of IFNγ after stimulation with PMA and Ionomycin in presence of Brefeldin A. IFNγ producing CD4, CD8 and γδT cells were defined as CD4$^+$IFNγ$^+$, CD8$^+$IFNγ$^+$ and γδ$^+$IFNγ$^+$ respectively where IFNγ$^+$ cells were gated on CD3$^+$CD4$^+$, CD3$^+$CD8$^+$ and CD3$^+$Vδ2-TCR$^+$ T cells respectively (as shown in Figure 5.1).

The data revealed that γδ$^+$IFNγ$^+$ cells were significantly decreased in TILs compared to PBMCs of GBC patients and HI (Figure 5.4 A). A significant decrease was also observed in

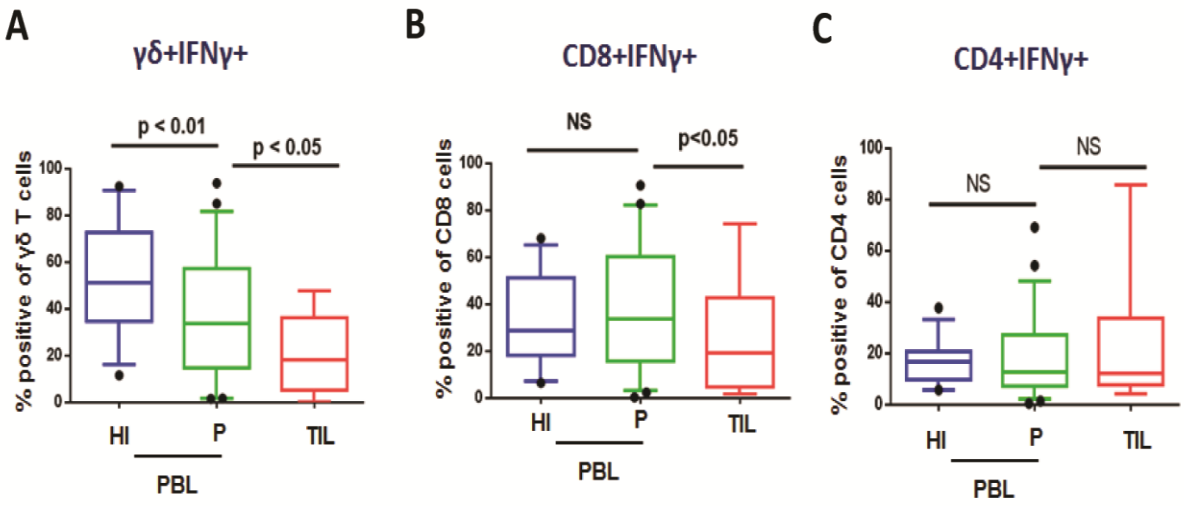


Figure 5.4 : Frequency of IFNγ producing cells in GBC patients*. IFNγ producing cells were studied in PBMCs (n=52) and TILs (n=17) of GBC patients and compared with HI (n=30). The cumulative data presented in box whisker plots show γδ$^+$IFNγ$^+$ (A), CD8$^+$IFNγ$^+$ (B) and CD4$^+$IFNγ$^+$ (C) cells in PBMCs and TILs of GBC patients compared with HI.*
*HI, healthy individuals; P, GBC patients; TIL, Tumor infiltrating lymphocytes. The box plots show median (middle line), 5th and 95th percentiles (box), extreme values (whiskers) and outliers (dark circles). Results are analysed by Mann-Whitney test and student's t test with *(p < 0.05); **(p < 0.01).*

$CD8^+IFN\gamma^+$ cells in TILs compared to PBMCs of GBC patients (Figure 5.4 B). However, the levels of $CD4^+IFN\gamma^+$ cells were comparable in GBC patients and HI (Figure 5.4 C). The data suggests that the anti-tumor immunity contributed by $\gamma\delta^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ cells may be dysfunctional in GBC patients.

5.2.3 Cytokine profile of γδ T cells is skewed towards IL17 production in GBC patients

The immune-phenotype analysis of γδT cells showed that the levels Tγδ17 cell were increased and that of $\gamma\delta^+IFN\gamma^+$ cells were decreased in GBC patients in comparison to HI. The correlation analysis of Tγδ17 and $\gamma\delta^+IFN\gamma^+$ cells in peripheral blood of GBC patients further confirmed the significant negative correlation among these cells (Figure 5.5 A). Th17 and Tc17 cells also negatively correlated with $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ cells respectively (Figure 5.5 A).

It is reported that the expression of CD27 on surface of γδT cells, determine the cytokine profile of γδT cells towards IFNγ production. γδT cells lacking the expression of CD27 develop to produce IL17 [249]. In GBC patients, the levels of γδT cells deficient in CD27 expression were significantly elevated in peripheral blood (n=27) and TILs (n=10) compared to $CD27^+\gamma\delta T$ cells (Figure 5.5 B). Next, the analysis of memory phenotype of γδT cells in peripheral blood revealed that more than 90% of γδT cells were memory T cells. However, the categorization of γδT cells from GBC patients (n=27) into different memory phenotypes (central memory, effector memory and terminally differentiated memory phenotype) did not show significant difference in comparison to HI (n=14) (Figure 5.5 C). In addition, as shown in Figure 4.1 H, the levels of total γδT cells in GBC patients were comparable to HI. Thus the data suggests that although the frequency of total γδT cells and their memory phenotype in

GBC patients did not alter, the cytokine secretion profile of γδT cells was skewed towards IL17 production.

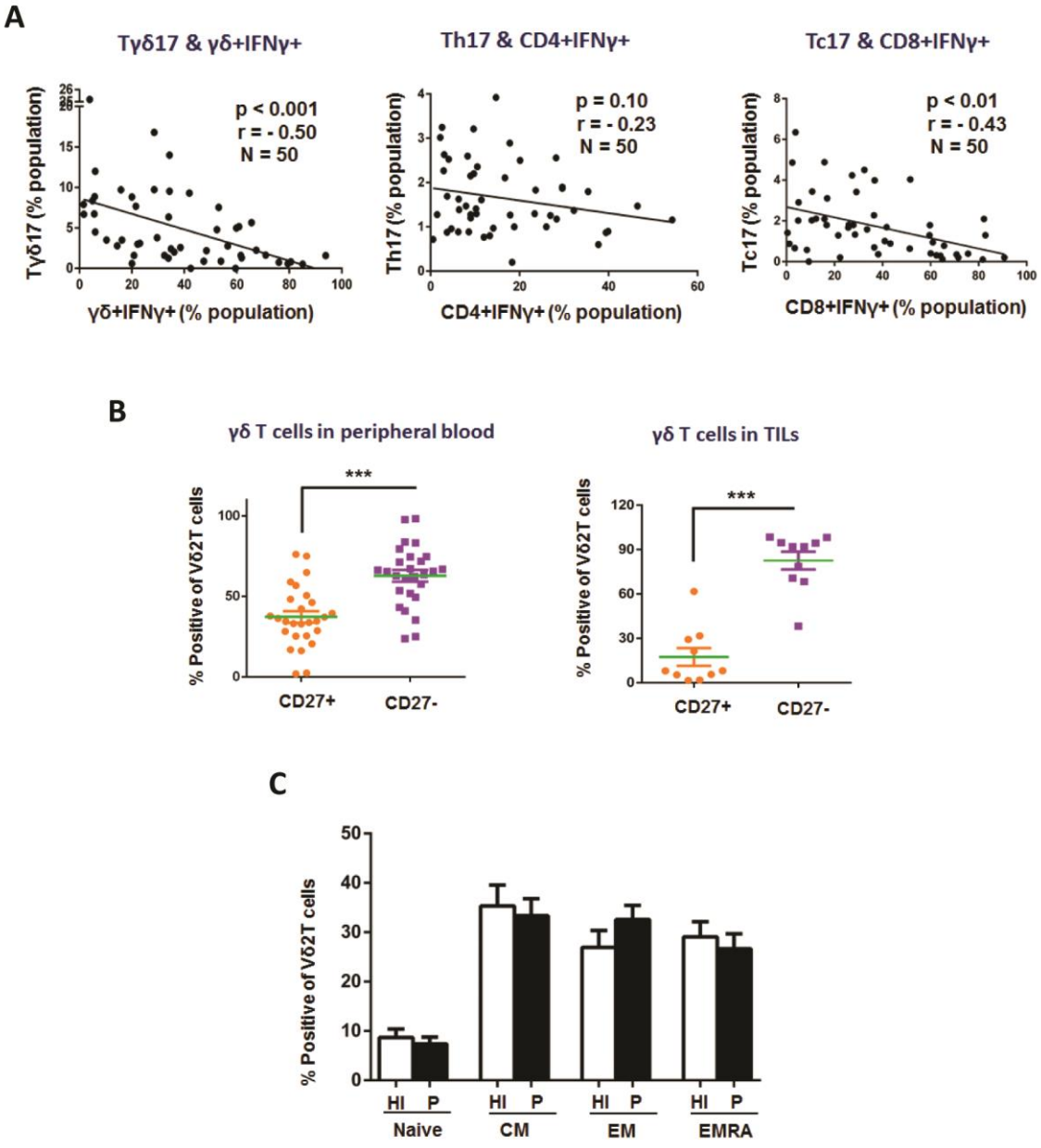


Figure 5.5 : Cytokine profile of γδ T cells in GBC patients. ***(A)*** *The correlation of IL17 producing and IFNγ producing cells was analysed in peripheral blood of GBC patients. Pearson coefficient of correlation was calculated between Tγδ17 and $\gamma\delta^{+}IFN\gamma^{+}$ (left), Th17 and $CD4^{+}IFN\gamma^{+}$ (centre) and Tc17 and $CD8^{+}IFN\gamma^{+}$ (right). "r" indicates Pearson coefficient of correlation and N indicates number of pairs in correlation.* ***(B)*** *Expression of CD27 was analysed on γδ T cells in PBMCs (n=27) and TILs (n=10) of GBC patients. The levels of*

$CD27^{+}$γδT cells and $CD27^{-}$γδT cells in peripheral blood (left) and TILs (right) are presented as scatter plots. ***(C)*** *The proportion of memory phenotypes of γδT cells were analysed by expression of CD27 and CD45RA on γδT cells. The levels of naïve and memory (central memory, effector memory and terminally differentiated effector memory) γδT cells in peripheral blood of GBC patients (n=27) and HI (n=14) are presented as bar diagram.*
*HI, healthy individuals; P, GBC patients; TIL, Tumor infiltrating lymphocytes. Data are shown as mean ± SEM with * $p<0.05$; ** $p<0.01$; *** $P<0.001$.*

5.2.4 Cytokines involved in differentiation of Tγδ17 cells are elevated in sera and tumor environment of GBC patients

Studies have shown that the differentiation of Tγδ17 and Th17 from naive T cells is facilitated by combinations of IL6, TGFβ, IL1β and IL23 [243, 313]. The serum levels of these and related cytokines and chemokines were evaluated in GBC patients (n=49) and HI (n=25) by cytometric bead array (CBA). The data was analysed by flow cytometry. As shown in Figure 5.6 A, the levels of IL6, IL1β, and IL23 were high in GBC patients whereas TGFβ was low compared to HI. IL17 was significantly elevated in sera of GBC patients. However, IFNγ was decreased and IL12p70 remained unaltered compared to HI. Levels of IL10 also did not alter among HI and GBC patients.

The chemokines in the sera of GBC patients and HI were evaluated by combining individual flex sets of CBA. Analysis of chemokines revealed that monokine induced by gamma interferon (MIG; CXCL9), interferon induced protein 10 (IP-10; CXCL10) and IL8 were increased in sera of GBC patients. Levels of monocyte chemoattractant protein-1 (MCP-1; CCL2) and RANTES (CCL5) in GBC patients were comparable to HI (Figure 5.6 B).

Further to evaluate the cytokine profile in tumor environment, single cell suspension of surgically resected tumor tissue was prepared and cytokines were measured in cell-free culture supernatant collected after 24 h. It was observed that cytokines involved in

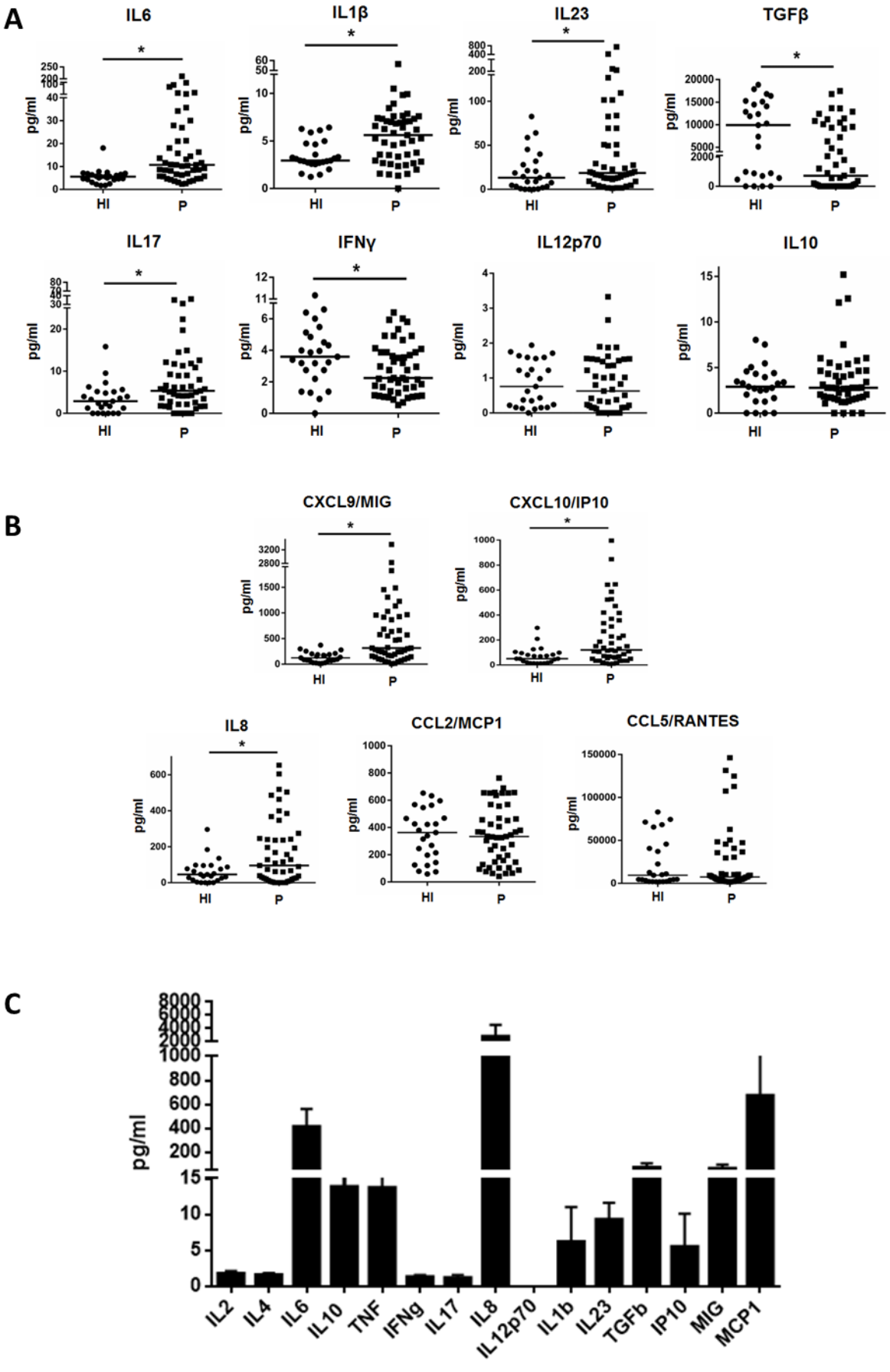

A
IL6
IL1β
IL23
TGFβ
IL17
IFNγ
IL12p70
IL10
pg/ml
HI
P
B
CXCL9/MIG
CXCL10/IP10
IL8
CCL2/MCP1
CCL5/RANTES
C
pg/ml
IL2
IL4
IL6
IL10
TNF
IFNg
IL17
IL8
IL12p70
IL1b
IL23
TGFb
IP10
MIG
MCP1

Figure 5.6 : Cytokine profile in serum and tumor environment of GBC patients. ***(A-B)*** *Scatter plots depict concentration of various cytokines (A) and chemokines (B) in sera of GBC patients (n=49) and HI (n=25) analysed by cytometric bead array. Horizontal lines indicate median values. Data was analysed by Mann Whitney test with *($p < 0.05$)* ***(C)*** *Cytokines and chemokines were measured in cell-free tumor supernatants (n=15) by cytometric bead array. Data presented as concentration in pg/ml.*

polarization of IL17 producing cells (IL6, TGFβ, IL1β and IL23) were present in the tumor environment. Levels of IL12, IFNγ, IL2 and IL4 were low whereas chemokines such as IL8, CXCL9, CXCL10 and CCL2 were remarkably high (Figure 5.6 C). Taken together, these results suggest that cytokines involved in the polarization of Tγδ17 are elevated in sera and tumor environment of GBC patients.

5.2.5 Regulatory T cells are decreased in peripheral blood of GBC patients

The cytokine profile of sera and tumor environment of GBC patients showed that the levels of TGFβ were decreased and IL10 were comparable to HI. TGFβ is required by Treg cells for differentiation from naïve $CD4^+$ T cells [314], and IL10 is secreted by Treg cells [315]. The frequency of Tregs in PBMCs of GBC patients (n=52) and HI (n=30) were analysed by surface staining for CD4, CD25 and CD127 followed by intracellular staining for Foxp3. Figure 5.7 A describes the gating strategy used for Tregs where Tregs were defined as $CD25^+CD127^{low/-}$ cells within $CD4^+$ T cells with Foxp3 expression of ≥80%.

It was observed that Tregs were significantly decreased in PBMCs of GBC patients compared to HI. However, the percentages of Tregs in TILs were higher than PBMCs of GBC patients but comparable to HI (Figure 5.7 B). The median fluorescence intensity (MFI) of Foxp3 expression on Tregs was significantly increased in TILs than PBMCs of GBC patients (Figure 5.7 C).

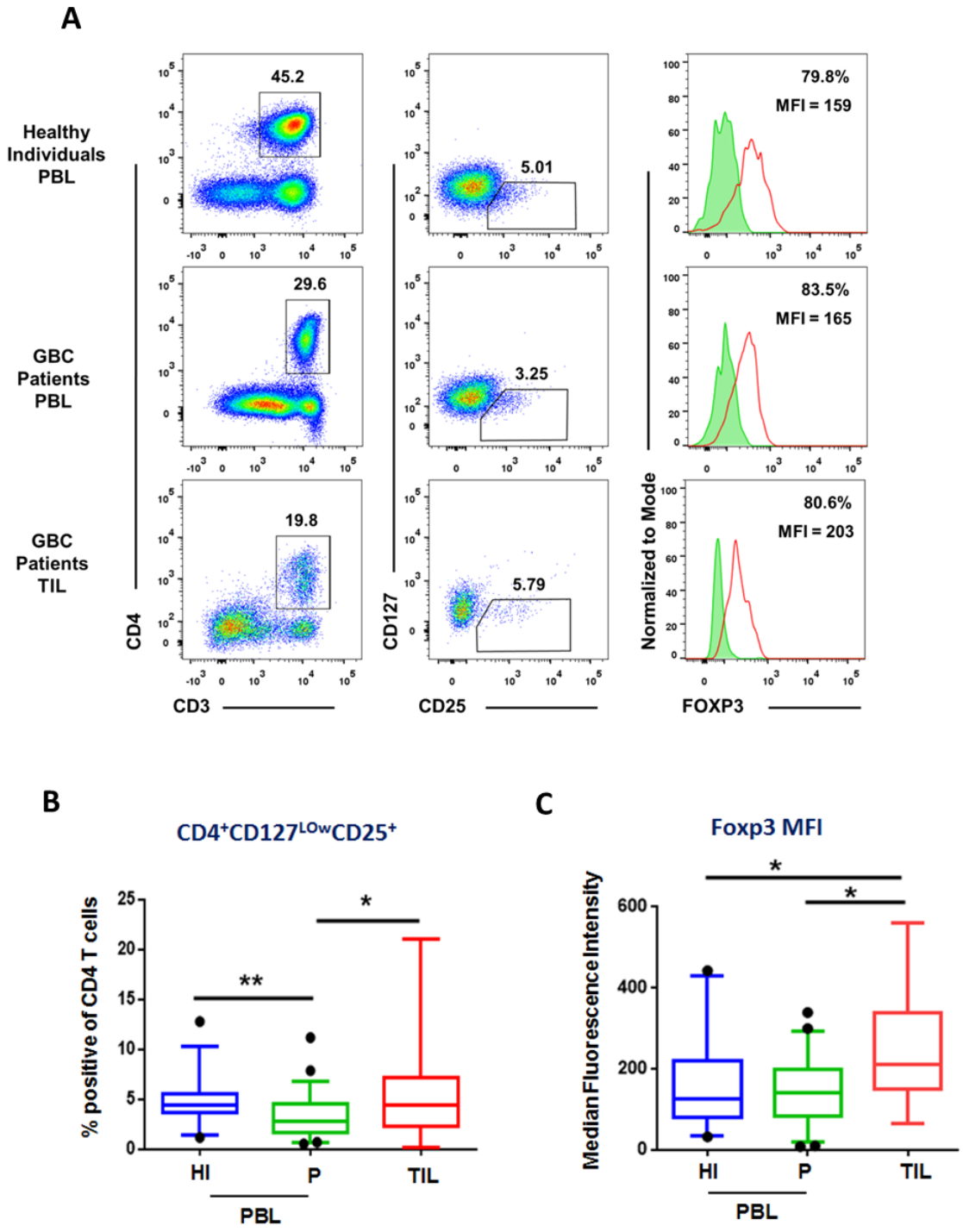


Figure 5.7 : Frequency of regulatory T cells in GBC patients. ***(A)*** *A representative dot plot describing Tregs, characterized as $CD25^{+}CD127^{low/-}$ within $CD4^{+}$ T cells. Histograms indicate expression of Foxp3 within $CD25^{+}CD127^{low/-}$ population. Figures in the histogram indicate median fluorescence intensity of Foxp3 expression (blank histogram) corrected with isocontrol (shaded histogram).* ***(B)*** *Box whisker plots showing composite results of Tregs in PBMCs (n=52) and TILs (n=17) of GBC patients compared with HI (n=30).* ***(C)*** *Comparison of median fluorescence intensity of Foxp3 expression within Tregs in GBC*

*patients and HI. HI, healthy individuals; P, GBC patients; TIL, Tumor infiltrating lymphocytes; PBMCs, peripheral blood mononuclear cells. The box plots show median (middle line), 5th and 95th percentiles (box), extreme values (whiskers) and outliers (dark circles). Data was analysed by Mann Whitney test and student's t test and presented as mean ± SEM with * p<0.05; ** p<0.01.*

5.2.6 Suppressive potential of Tregs is comparable in GBC patients and HI

In order to investigate functional potential of Tregs in GBC patients, Tregs were isolated from peripheral blood of GBC patients and HI with more than 80% purity (Figure 5.8 A). The purified Treg cells were co-cultured with autologous responder T cells (Tres; $CD4^+CD25^-$) labelled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with anti-CD3/anti-CD28. It was observed that percent dividing Tres cells were decreased with increase in Treg cells in culture. The co-culture of Tres with Treg in 1:2 ratio significantly inhibited the proliferation of Tres. However, the suppressive potential of Tregs in GBC patients was comparable to HI (Figure 5.8 B and C). Thus, the results indicate that although Tregs were decreased in PBMCs of GBC patients, their suppressive potential was not compromised.

5.2.7 Dynamics of Tγδ17, Th17, Tc17 and Treg cells in GBC patients

We further analyzed the relationship between Tγδ17, Th17, and Tc17 cells with Tregs in GBC patients. The ratios of Tγδ17/Treg, Th17/Treg and Tc17/Treg were significantly increased in peripheral blood as well as in tumor environment of GBC patients indicating an inverse correlation of IL17 producing cells and Tregs (Figure 5.9 A). Next, we found that peripheral blood Tγδ17 cells positively correlated with serum IL17 levels (Figure 5.9 B). However, the levels of Th17 and Tc17 cells in peripheral blood of GBC patients did not correlate with serum IL17 levels. Thus the data highlighted the importance of Tγδ17 cells in inflammation associated with GBC (Figure 5.9 B).

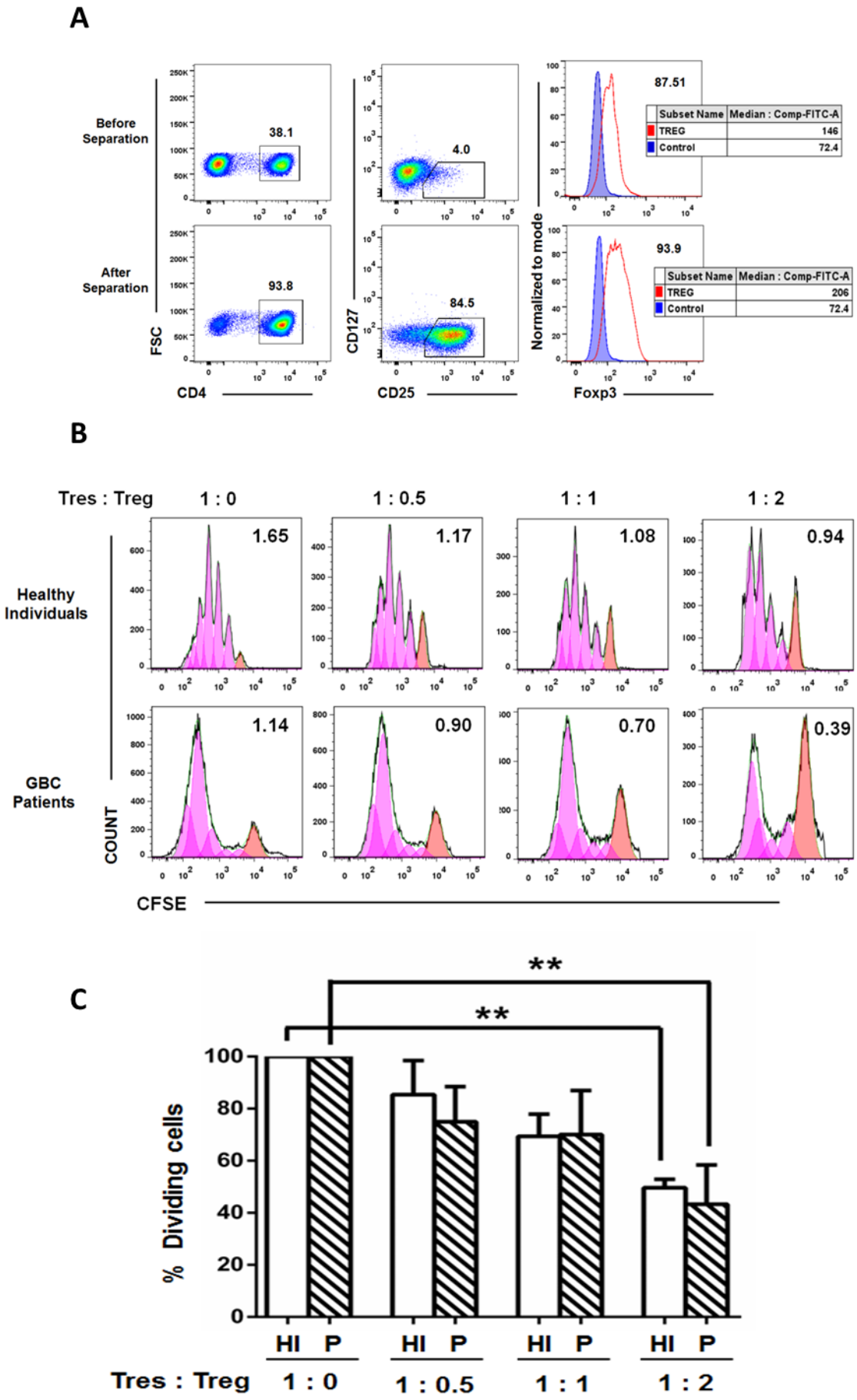

A
Before Separation
After Separation
38.1
93.8
4.0
84.5
87.51
93.9
Subset Name | Median : Comp-FITC-A
TREG 146
Control 72.4
Subset Name | Median : Comp-FITC-A
TREG 206
Control 72.4
FSC
CD4
CD127
CD25
Normalized to mode
Foxp3
B
Tres : Treg
1 : 0
1 : 0.5
1 : 1
1 : 2
Healthy Individuals
GBC Patients
1.65
1.17
1.08
0.94
1.14
0.90
0.70
0.39
COUNT
CFSE
C
**
**
% Dividing cells
HI P
Tres : Treg
1 : 0
1 : 0.5
1 : 1
1 : 2

Figure 5.8 : Suppressive potential of Treg cells in GBC patients. ***(A)*** *The representative dot plot analysis show purity of Treg cells before and after immunomagnetic separation from peripheral blood of GBC patients and HI. The histograms show percent expression of Foxp3 by $CD4^+CD25^+CD127^{low/-}$ cells presented as numbers. The horizontal boxes in the histograms indicate median fluorescence intensity of Foxp3 expression (blank histogram) in compared with isocontrol (shaded histogram).* ***(B)*** *Representative figure of three independent experiments depicting suppressive potential of Tregs ($CD4^+CD25^+$) on CFSE labelled autologous responder cells ($CD4^+CD25^-$) from GBC patients (n=3) and HI (n=3). First peak from right indicates mother population. Figures in the plot indicate division index at respective ratio.* ***(C)*** *Bar diagram summarizes the percent dividing responder T cells at respective Tres:Treg ratio.*
*HI, healthy individuals; P, GBC patients; CFSE, carboxyfluorescein succinimidyl ester. Data was analysed by student's t test and presented as mean ± SEM with ** $p<0.01$.*

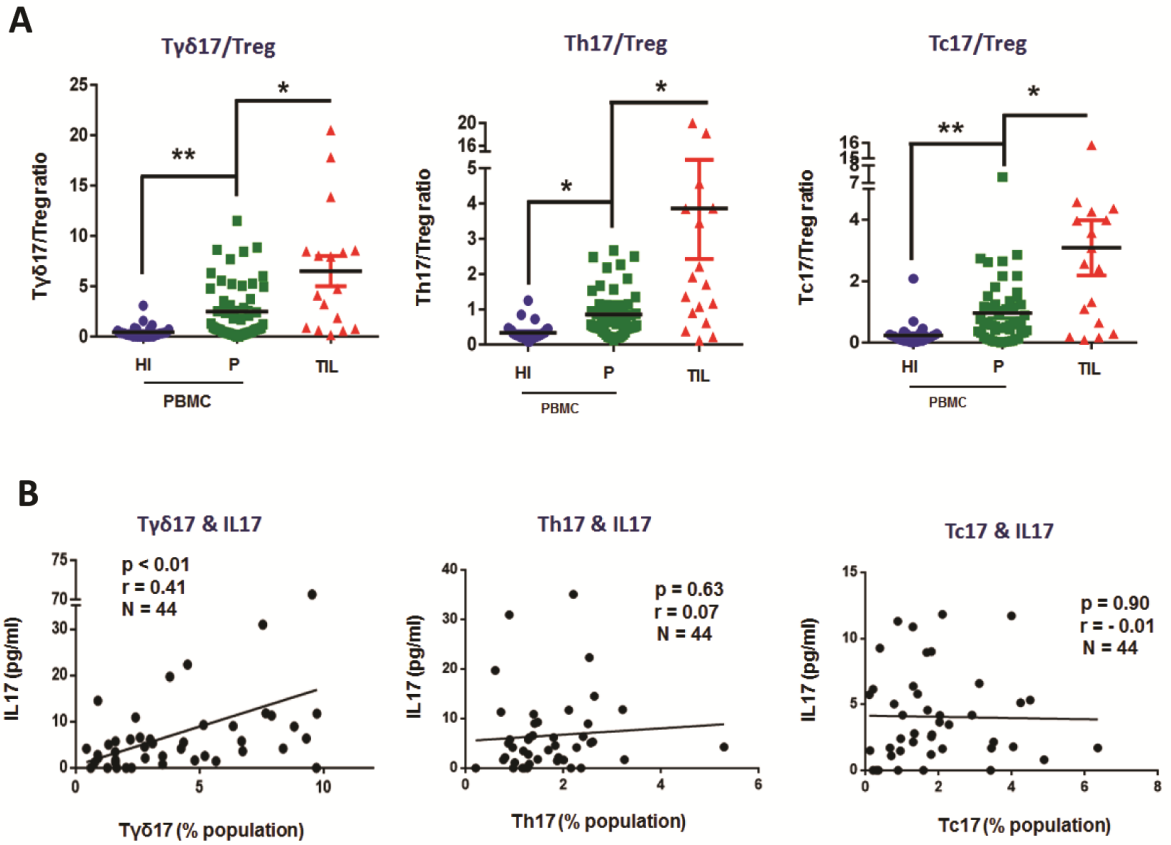


Figure 5.9 : Dynamics of Tγδ17, Th17, Tc17 and Treg in peripheral blood of GBC patients. ***(A)*** *Scatter plot showing the ratios of Tγδ17/Treg (left), Th17/Treg (middle) and Tc17/Treg (right) in PBMCs (n=52) and TIL (n=17) of GBC patients and HI (n=30).* ***(B)*** *The correlations of Tγδ17 (left), Th17 (middle) and Tc17 (right) with serum IL17 levels*

were evaluated respectively. "r" indicates Pearson coefficient of correlation and N indicates number of pairs in correlation.
*HI, healthy individuals; P, GBC patients TIL, Tumor infiltrating lymphocytes; PBMCs, peripheral blood mononuclear cells. Data was analysed by student's t test and presented as mean ± SEM with * p<0.05; ** p<0.01.*

5.2.8 Tγδ17, Th17 and Tc17 cells do not correlate with clinical stage of GBC patients

To investigate the implication of IL17 producing cells on GBC progression, we analyzed the peripheral blood levels of Tγδ17, Tc17, Th17 and Treg cells with different stages of GBC patients. The patients were grouped according to the TNM classification as stage II (n=5), stage III (n=20) and stage IV (n=27). Tγδ17, Th17 and Tc17 cells showed no correlation with clinical stage (II to IV) of GBC patients. However, their levels remained high in all stages of GBC patients compared to HI (Figure 5.10 A, B and C). In contrast, the levels of Tregs in GBC patients of all stages (II to IV) remained lower than HI (Figure 5.10 D). This clearly indicates that the immune response was skewed towards IL17 producing cells in GBC patients.

5.2.9 mRNA expressions of cytokines and transcription factors regulating Tγδ17 and Treg in PBMCs of GBC patients

It is reported that IL6 regulates the differentiation of $CD4^+$ T cells towards Th17 or Treg cell lineage. Higher expression of IL6 induces Th17 differentiation and inhibits Treg whereas TGFβ promotes differentiation and maturation of Tregs [57]. Thus to understand the dynamics of IL6 and TGFβ, PBMCs from GBC patients (n=18) and HI (n=18) were analyzed for expression of IL6 and TGFβ at mRNA level using real time PCR. The expression of IL6 was significantly higher in PBMCs from GBC patients than HI (Figure 5.11 A). However, expression of TGFβ was decreased in GBC patients compared to HI (Figure 5.11 B). These results corroborate our observation of IL6 and TGFβ expression at protein level in sera of GBC patients. Moreover, the mRNA expression of RORc, a lineage determining transcription

factor of IL17 producing T cells, was elevated in PBMCs of GBC patients compared to HI (Figure 5.11 C) whereas the mRNA expression of Foxp3 was comparable in GBC patients and HI (Figure 5.11 D). Overall the data showed that the immune response in GBC patients was tilted towards IL17 producing inflammatory T cells.

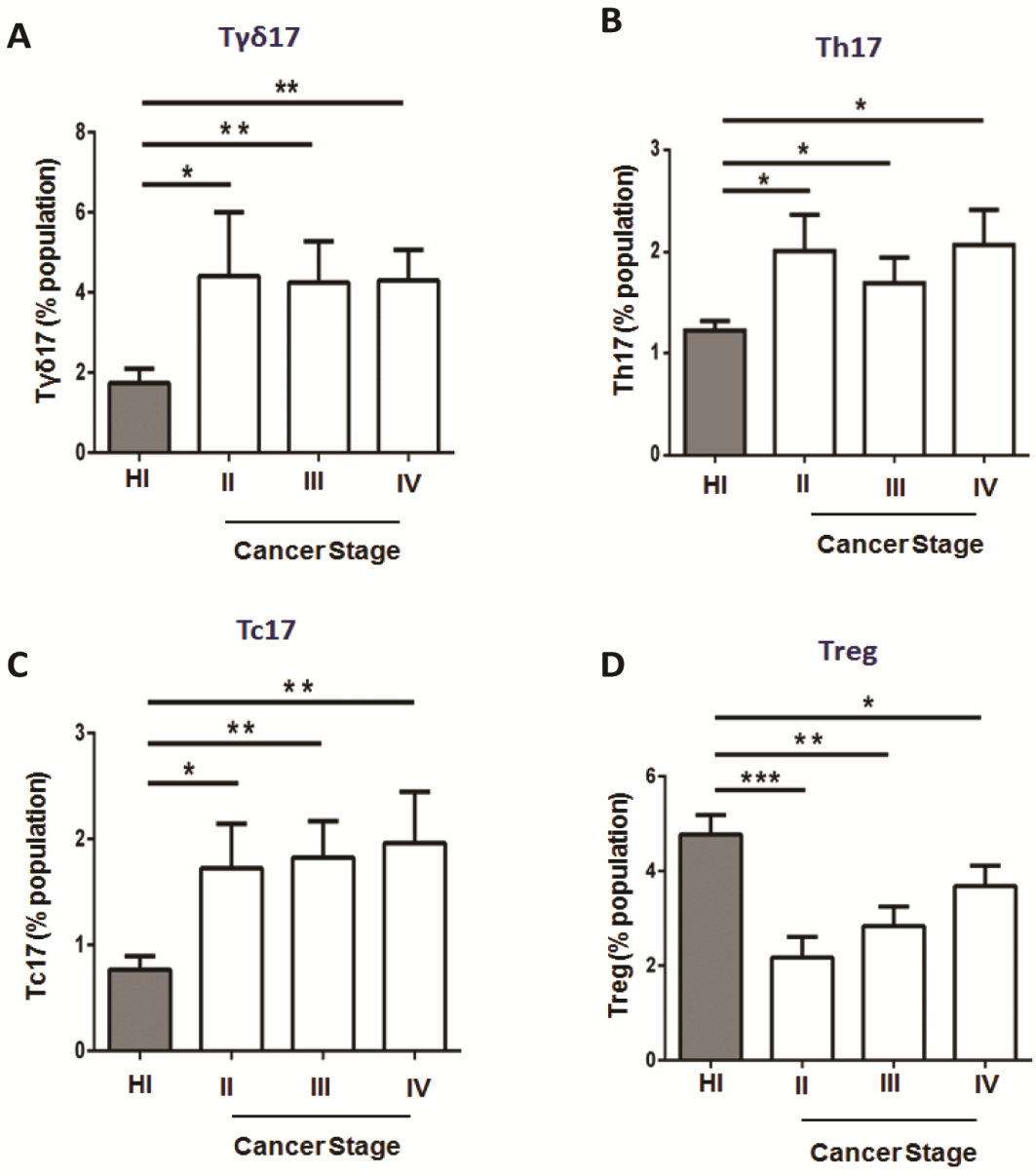


Figure 5.10 : Association of Tγδ17, Th17, Tc17 and Treg cells with clinical stage of GBC patients. *The bar diagram depicts frequencies of Tγδ17 (A), Th17 (B), Tc17 (C) and Treg (D) were compared with clinical stages of GBC patients. Results are shown as mean ± SEM with *($p < 0.05$); **($p < 0.01$).*

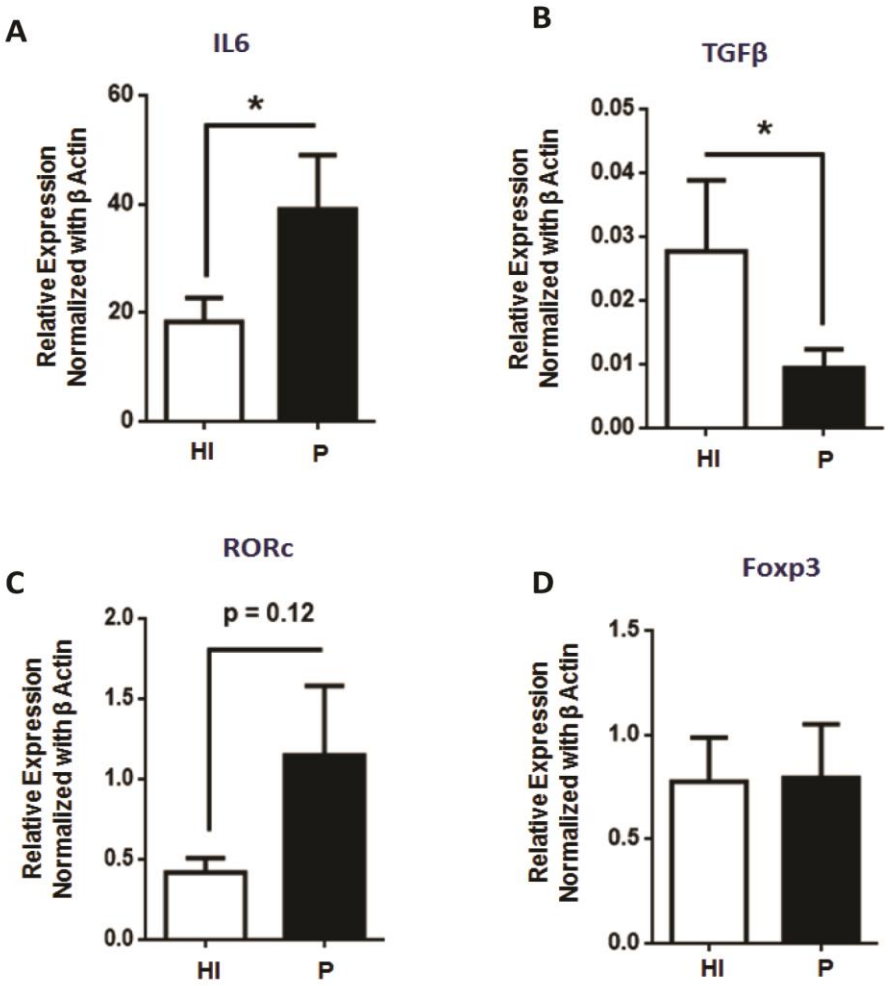


Figure 5.11 : Expression of Tγδ17 associated genes in PBMCs of GBC patients*. Total RNA extracted from PBMCs was reversed transcribed to cDNA and expression of IL6 (A), TGFβ (B), RORc (C) and Foxp3 (D) was evaluated in PBMCs of GBC patients (n=18) and HI (n=16). The expression of genes was normalized to β-actin expression. Results are shown as mean ± SEM with *(p < 0.05).*

5.2.10 Myeloid derived suppressor cells are increased in peripheral blood of GBC patients

Recent studies have shown that IL17 is involved in the accumulation of tumor-infiltrating myeloid derived suppressor cells (MDSCs) in mice [316]. Since, IL17 and IL17 producing cells were elevated in GBC; it was hypothesized that MDSCs may be increased in GBC patients. To phenotype MDSCs, PBMCs from GBC patients (n=5) and HI (n=5) and single cells suspension of tumor tissue (n=5) were stained for surface expression of HLADR, CD33

and CD11b. MDSCs were defined as $CD33^{+}CD11b^{+}$ cells gated on $HLADR^{-}$ cells [317] (Figure 5.12 A). It was observed that $CD33^{+}CD11b^{+}$ MDSCs were significantly increased in peripheral blood of GBC patients compared to HI (Figure 5.12 B). Interestingly, $CD33^{+}CD11b^{+}HLADR^{-}$ MDSCs were absent in TILs whereas $CD11b^{+}HLADR^{-}$ phenotype of MDSCs was present in tumor environment of GBC patients (Figure 5.12 B).

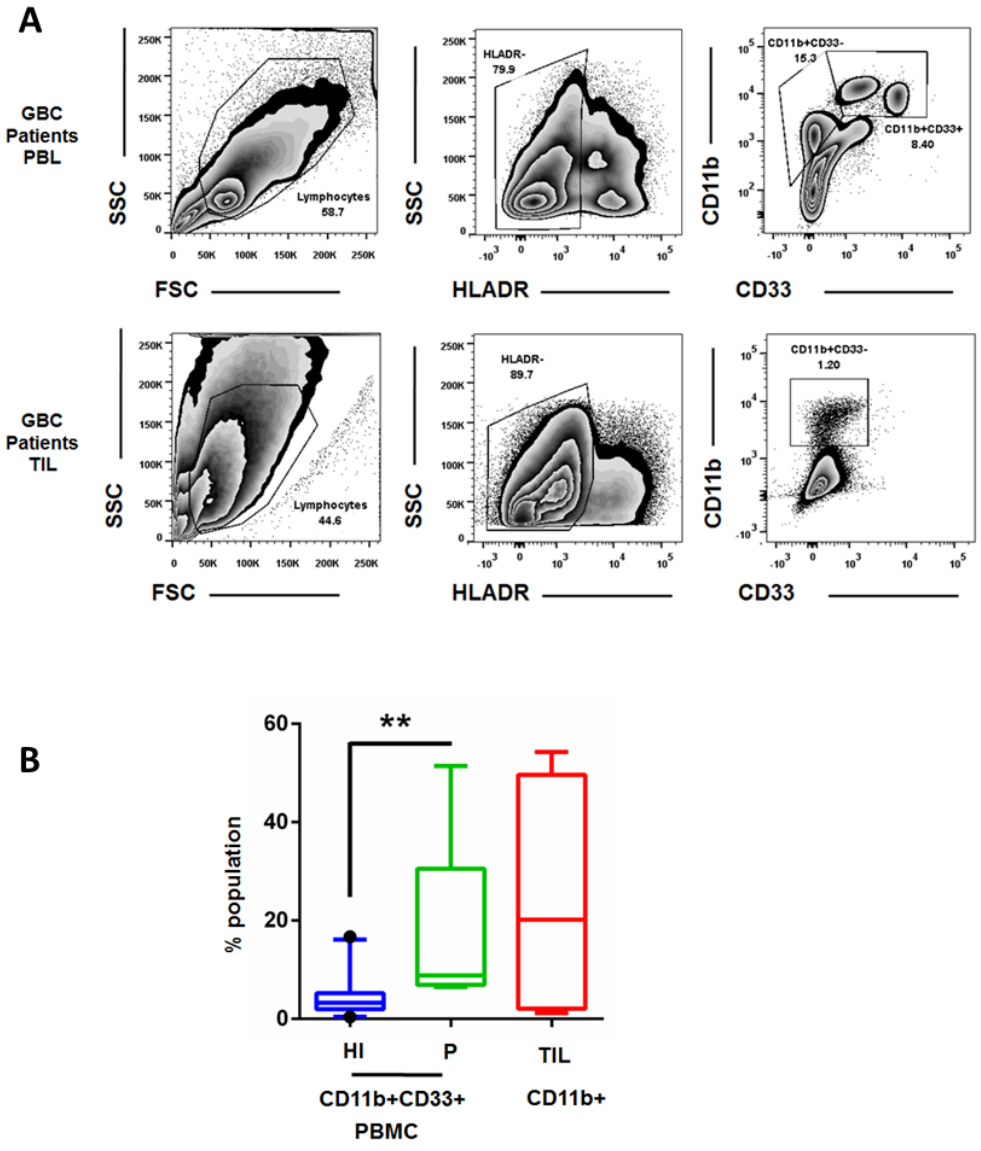


Figure 5.12 : Prevalence of MDSCs in peripheral blood and TILs of GBC patients. ***(A)*** *Representative zebra plot analysis of MDSCs from peripheral blood (n=5) and TILs (n=5) of GBC patients. $CD33^{+}CD11b^{+}$ cells were gated on $HLADR^{-}$ population.* ***(B)*** *The levels of MDSCs in PBMCs and TILs of GBC patients compared with HI are*

*presented as box whisker plot. HI, healthy individuals; P, GBC patients; TIL, Tumor infiltrating lymphocytes. The box plots show median (middle line), 5^{th} and 95^{th} percentiles (box), extreme values (whiskers) and outliers (dark circles). Results are analysed by Mann-Whitney test with **($p < 0.01$).*

5.2.11 Tγδ17, Th17 and Treg cells are associated with poor overall survival of GBC patients

To investigate the clinical significance of Tγδ17, Th17, Tc17 and Tregs, the survival time of patients was analyzed with frequency of these cells in peripheral blood of GBC patients (n=40). As there was no clinically defined cut off points for Th17, Tc17, Tγδ17, Treg cells, the high-expressing or low-expressing groups of GBC patients were defined based on mean values of expression of these lymphocytes (4.7 for Tγδ17, 1.8 for Th17, 1.8 for Tc17 and 3.2 for Treg). As shown in Figure 5.13 A, cox proportional regression analysis revealed that patients with high levels of Tγδ17 cells showed poor overall survival (median survival: 8.95 months) than patients with low levels of Tγδ17 (median survival: 15.97 months). The individuals with high Tγδ17 levels were at higher risk compared to those with low Tγδ17 levels (Hazard ratio (HR): 2.4). In contrast, the patients with high levels of $\gamma\delta^{+}IFN\gamma^{+}$ had longer overall survival than patients with low levels of $\gamma\delta^{+}IFN\gamma^{+}$ cells (HR: 0.4; Figure 5.13 B).

Similarly, GBC patients with high levels of Th17 cells had shorter overall survival compared to patients with low levels (HR: 2.32; Figure 5.13 C). Tc17 were not associated with survival of patients (Figure 5.13 D). However, patients with increased Treg cells had poor overall survival (HR: 2.07; Figure 5.13 G). Next, it was observed that the high levels of serum IL17 were associated with poor survival of patients (HR: 2.12; Figure 5.13 E). In contrast, the patients with high levels of IFNγ had survival benefit over patients with low serum levels of

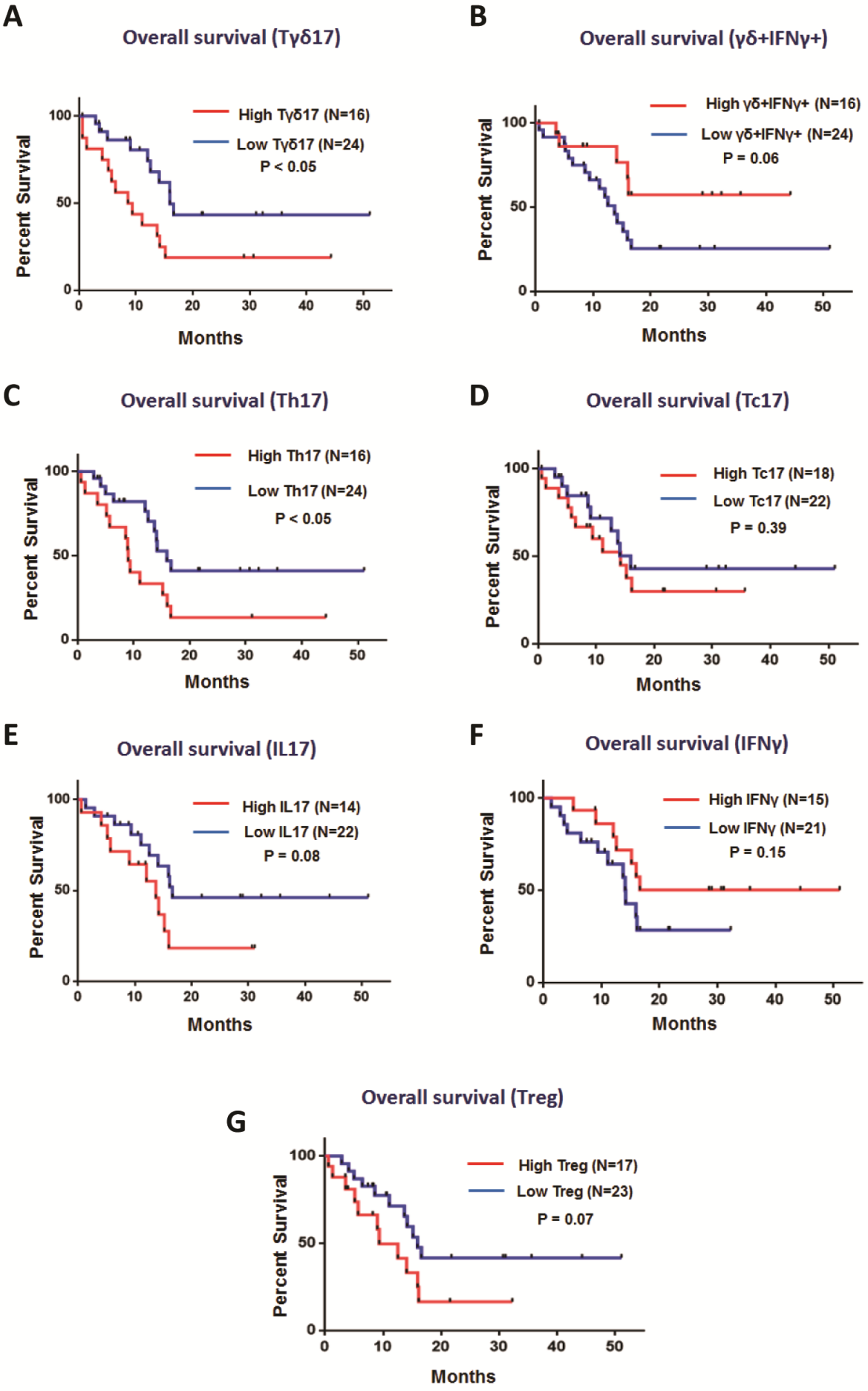

A
Overall survival (Tγδ17)
High Tγδ17 (N=16)
Low Tγδ17 (N=24)
P < 0.05
Percent Survival
Months
B
Overall survival (γδ+IFNγ+)
High γδ+IFNγ+ (N=16)
Low γδ+IFNγ+ (N=24)
P = 0.06
Percent Survival
Months
C
Overall survival (Th17)
High Th17 (N=16)
Low Th17 (N=24)
P < 0.05
Percent Survival
Months
D
Overall survival (Tc17)
High Tc17 (N=18)
Low Tc17 (N=22)
P = 0.39
Percent Survival
Months
E
Overall survival (IL17)
High IL17 (N=14)
Low IL17 (N=22)
P = 0.08
Percent Survival
Months
F
Overall survival (IFNγ)
High IFNγ (N=15)
Low IFNγ (N=21)
P = 0.15
Percent Survival
Months
G
Overall survival (Treg)
High Treg (N=17)
Low Treg (N=23)
P = 0.07
Percent Survival
Months

Figure 5.13 : Increased Tγδ17 cells in GBC patients associate with poor survival. *Overall survival of GBC patients (n=40) was analysed by Kaplan-Meier method for low or high levels of Tγδ17 cells (A), γδ+IFNγ+ cells (B) Th17 cells (C), Tc17 cells (D), Serum IL17 levels (E), Serum IFNγ levels (F) and Treg cells (G). The curve statistics were compared by log-rank test with $p < 0.05$.*

IFNγ (HR: 0.51; Figure 5.13 F). Altogether, the data suggests that Tγδ17, Th17 and Treg cells may serve as predictive biomarkers for prognosis of GBC.

5.3 Summary

The immune-phenotyping of peripheral blood lymphocytes and tumor infiltrating lymphocytes revealed elevated levels of IL17 producing T cells in GBC patients irrespective of their clinical stage. Interestingly Tγδ17 cells emerged as significant contributor of IL17 in tumor tissue and in circulation compared to Th17 and Tc17 cells. In contrast $\gamma\delta^{+}IFN\gamma^{+}$ and $CD8^{+}IFN\gamma^{+}$ cells were decreased in tumor compartment of GBC patients. The cytokine profile of sera and tumor environment highlighted the abundance of inflammatory cytokines involved in differentiation and maturation of Tγδ17 and Th17 cells. Analysis of Treg cells showed that although the peripheral blood levels of Tregs were decreased, their suppressive potential was not compromised. Moreover, Foxp3 expressing Treg cells were elevated in tumor tissue compared to peripheral blood of GBC patients but remained comparable to HI. Study of dynamics of IL17 producing cells and Treg cells showed that immune response was biased towards Tγδ17, Th17 and Tc17 cell types in tumor environment. High levels of MDSCs observed in peripheral blood and tumor environment suggest that Tγδ17 are involved in recruitment of MDSCs towards tumor environment which may further support tumor progression by inducing immunosuppression of antitumor immune response. The survival analysis of GBC patients showed significant association of Tγδ17 cells with poor prognosis. Thus, the results presented in this chapter unravelled Tγδ17 cells to be the central player in the pathogenesis of gallbladder cancer.

Chapter 6

Exploring the pro-tumor role of *IL*17 producing γδ*T* (*T*γδ17) cells in GBC

6.1 Introduction

IL17 is a potent pro-inflammatory cytokine. It acts through IL17 receptor expressed by epithelial cells and activates MAPK (mitogen-activated protein kinases) and NF-kB (nuclear factor-kB) by TRAF6 (tumour necrosis factor receptor-associated factor-6) and has also been found to physically associate with the NF-kB activator protein (Act1) [135]. IL17A expression has been detected in several human tumours, and is shown to associate with poor prognosis of patients [318]. However, a consensus on the specific role of IL17 in cancer promotion is not achieved which may be specific to cancer type [318]. Our data demonstrated that the IL17 was present in the tumor environment and the levels were elevated in sera of GBC patients. The elevated IL17 in GBC patients was also correlated with poor survival of patients. Thus it was imperative to investigate the mechanism of GBC progression mediated by IL17. The results presented in current chapter highlights the contribution of IL17 in proliferation, migration and invasion of GBC cells.

It was demonstrated in previous chapter that Tγδ17 is major contributor of IL17 and was associated with poor prognosis of GBC patients. It is reported that healthy adult human peripheral blood γδT cells distinctively express Th1 signature and 50–80% produce IFNγ but < 5% produce IL17 [319]. However, Tγδ17 cells have been demonstrated to be involved in the pathogenesis of transplantation rejection [320], autoimmune disease [321], allergy [322], and cancer [246] in humans, suggesting exclusive role of Tγδ17 cells in modulating immune response in disease condition and has significant influence on the outcome of the disease. Various infection and autoimmunity models have shown that Tγδ17 are distinctly involved in early immune response in the tissue and can modulate the functions of other immune and epithelial cells [251]. However, most of the studies carried out to understand the functions of Tγδ17 cells are based on the murine models. Scanty literature is available on functions of Tγδ17 cells in human cancers.

Tγδ17 function influencing the clinical outcome in GBC patients has not been reported. The present chapter is focused on studying the factors involved in the recruitment of Tγδ17 cells to the tumor bed in GBC patients and understanding the role of Tγδ17cells in GBC progression.

6.2 Results

6.2.1 rhIL17 induces proliferation of gallbladder tumor cells

To study whether IL17 has direct role in GBC promotion, two GBC cell lines - OCUG-1 (poorly differentiated) and NOZ (moderately differentiated) were cultured with rhIL17. The proliferative response of GBC cells was monitored by ^{3}H Thymidine incorporation assay. OCUG-1 and NOZ cells were cultured in flat bottom in 96 well plate for 24 h in William's E medium supplemented with 10% FBS. rhIL17 in different concentration (0.1, 1, 10 and 100 ng/ml) was added to the cells and monitored for another 48 h. It was observed that addition of rhIL17 enhanced the proliferation of OCUG-1 cells in a concentration dependent manner (Figure 6.1 A). Interestingly, rhIL17 did not affect the proliferation of NOZ cells (Figure 6.1 B). Overall the results demonstrated that poorly differentiated GBC cells were more responsive to the tumor promoting effects of IL17.

6.2.2 Gallbladder tumor cells produce increased VEGF in presence of rhIL17

VEGF is a key inducer of tumor angiogenesis [323]. To investigate the role of IL17 in VEGF induction in GBC, OCUG-1 and NOZ cells were cultured in presence or absence of rhIL17. Production of VEGF was monitored for 48 h in presence of different concentrations of IL17 (0.1, 1, 10 and 100 ng/ml). It was observed that stimulation of OCUG-1 with rhIL17 resulted into significant increase in the production of VEGF in dose-dependent fashion (Figure 6.1 C). However, secretion of VEGF by NOZ cells did not alter upon stimulation with rhIL17

(Figure 6.1 D). Thus the data indicates that IL17 present in the tumor environment induce GBC cells to secrete VEGF which may enhance the GBC angiogenesis.

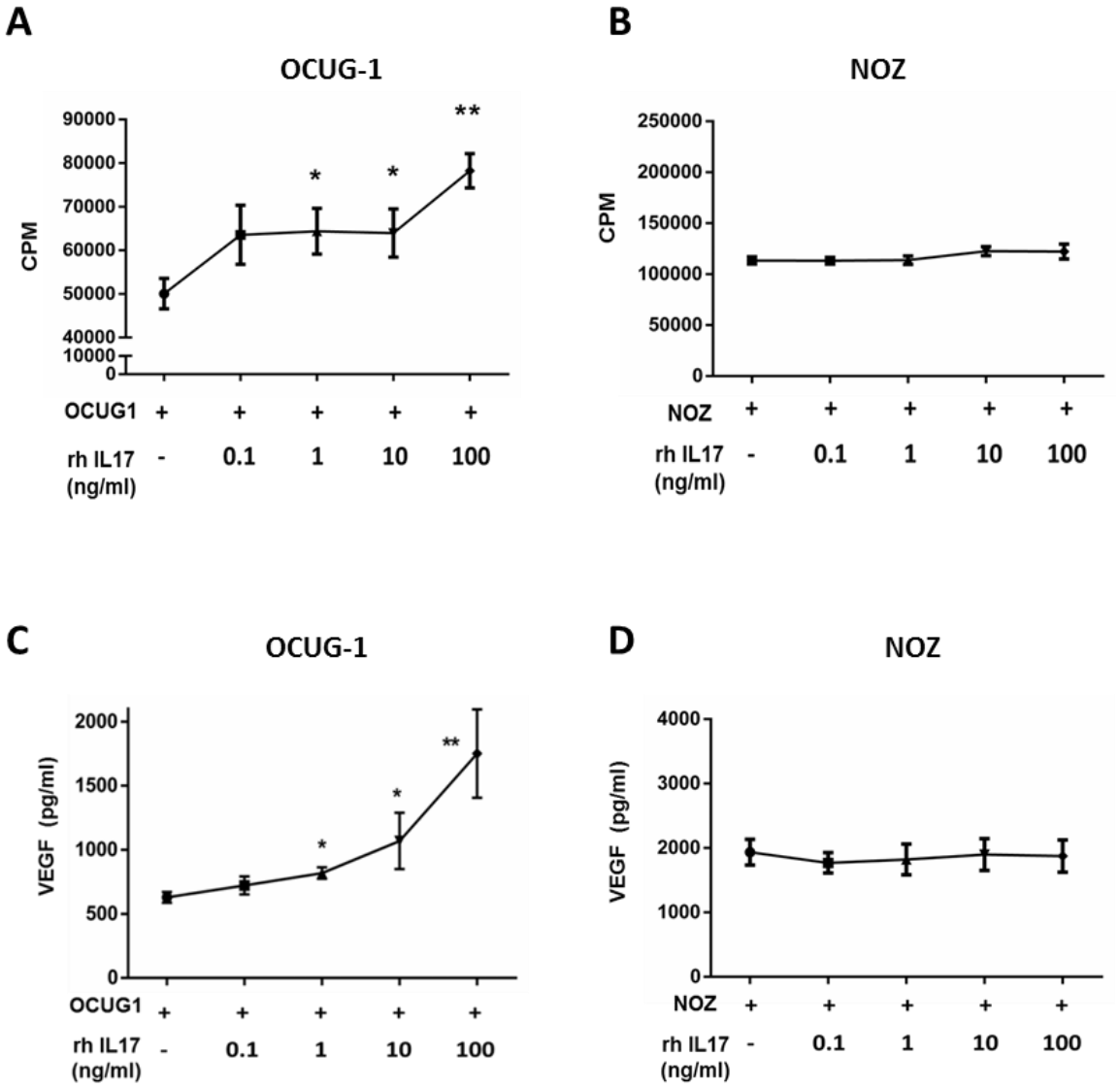


Figure 6.1 : Effect of IL17 on proliferation and VEGF production in GBC cell line. *OCUG-1 cells (A) and NOZ cells (B) were cultured in serum free medium and were treated with different concentrations of rhIL17. Proliferation was measured by ^{3}H-Thymidine incorporation and represented as counts per minute (n=6). OCUG-I (C) and NOZ (D) cell lines were cultured with different concentrations of rhIL17 in a serum-free medium for 48 h. VEGF was estimated in supernatants by ELISA (n=6). Results were statistically analysed using Student's t test and shown as mean ± SEM with * p<0.05; ** p<0.01.*

6.2.3 rhIL17 enhances migration of gallbladder tumor cells

The influence of rhIL17 on migratory potential of gallbladder tumor cells was analysed by wound healing assay. OCUG-1 and NOZ cells were cultured in 6 well plates for 24 h and treated with Mitomycin C (10 μg/ml) for 2 h to inhibit the cell proliferation. The scratches were made in monolayers and treated with rhIL17 (50, 100 ng/ml). The closure of the wounds was monitored using time lapse microscope for 21 h. It was observed that the rate of wound closure was high with increase in concentration of rhIL17 compared to GBC cells treated with medium alone (Figure 6.2 A and B). Interestingly, the data revealed that fold increase in wound closure compared to medium alone was higher in OCUG-1 cells (Figure 6.2 A) treated with rhIL17 than that observed with NOZ cells (Figure 6.2 B). Thus the data suggests that pro-migratory effect of rhIL17 was more pronounced in poorly differentiated (OCUG-1) than moderately differentiated GBC cells (NOZ).

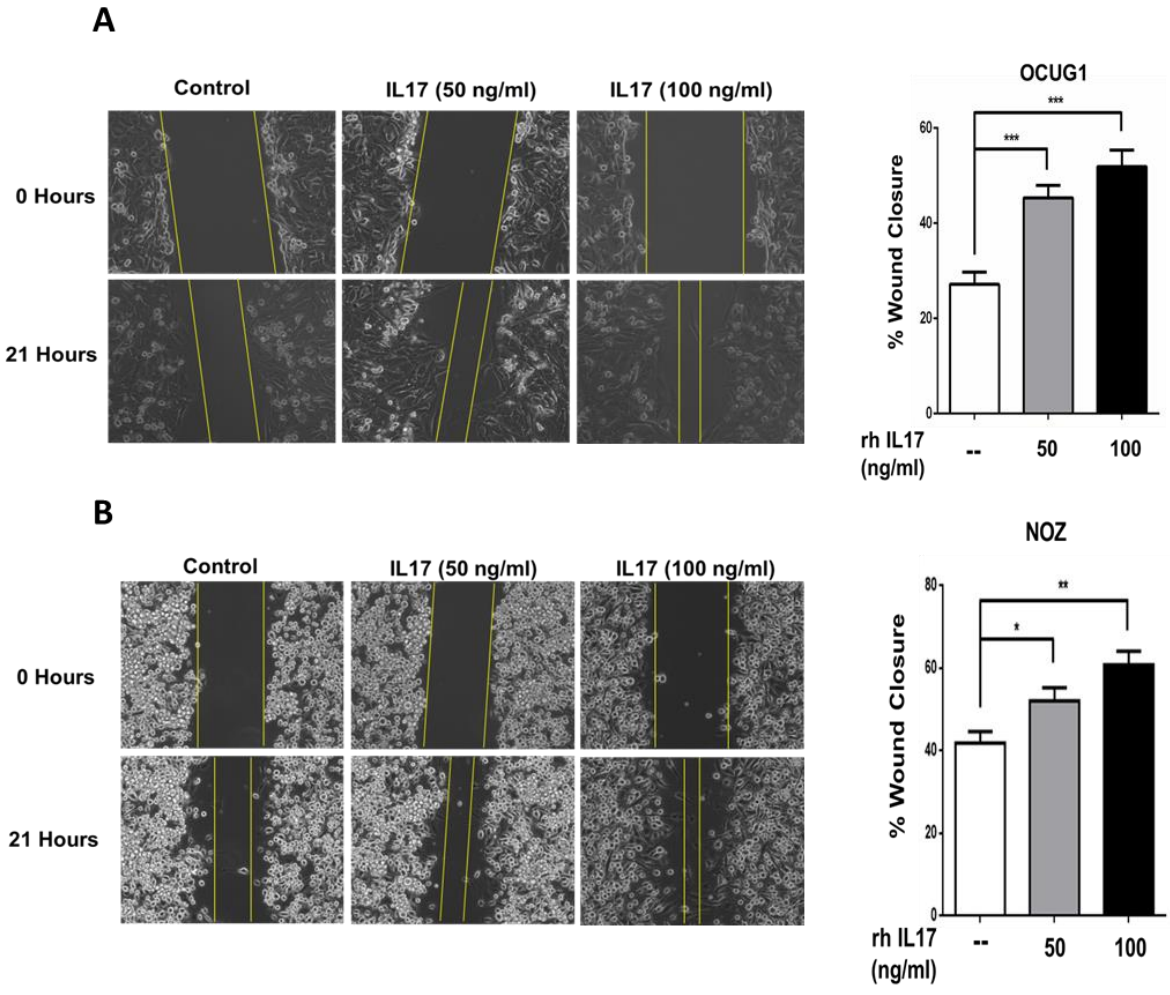


Figure 6.2 : Effect of IL17 on migratory potential of GBC cells. *OCUG-1 (A) and NOZ (B) cell lines were analysed for migratory potential by wound healing assay in presence of different concentrations of rhIL17 for 21 h. Representative figures of three independent experiments are depicted. Summarized data is presented as percent wound closure and shown as bar diagram. Data was analysed using Student's t test and shown as mean ± SEM with * $p<0.05$; ** $p<0.01$.*

6.2.4 rhIL17 promotes invasive potential of gallbladder tumor cells

To investigate whether rhIL17 influences the invasive potential of GBC, trans-well migration of GBC cells through matrigel (mimicking basement membrane) was analysed upon treatment with rhIL17. OCUG-1 or NOZ cells treated with rhIL17 (50, 100 ng/ml) were added to the upper chamber (trans-well insert) of 24-well trans-well system pre-coated with matrigel. William's E medium supplemented with 10% FBS was used as chemoattractant in the lower chamber. It was observed that the numbers of OCUG-1 and NOZ cells invaded to the lower side of the trans-well were significantly increased upon treatment of rhIL17 compared to treatment with medium alone (Figure 6.3 A and B). However, the fold increase in invasive potential of OCUG-1 cells (Figure 6.3 A) was higher than NOZ cells (Figure 6.3 B). Thus the data demonstrated that the rhIL17 aids in invasiveness of gallbladder tumor cells and has profound effect on poorly differentiated cells of GBC.

6.2.5 Gallbladder tumor cells express IL17 receptor on the surface

The proinflammatory functions of IL17 are mediated through IL17 receptor. Expression of IL17R on GBC cells was determined on poorly differentiated (OCUG-1) and moderately differentiated (NOZ) GBC cell lines by flow cytometry. It was observed that more than 80% of OCUG-1 and NOZ cells express IL17R (Figure 6.4 A and B). The localization of IL17 receptor was further confirmed by immunofluorescence staining using confocal imaging. As shown in figure 6.4 C and D, IL17 receptor is expressed by both the cell lines (OCUG-1 and NOZ) on the surface. This study clearly indicates that the IL17 receptor present extracellularly on poorly differentiated as well as on moderately differentiated GBC cells.

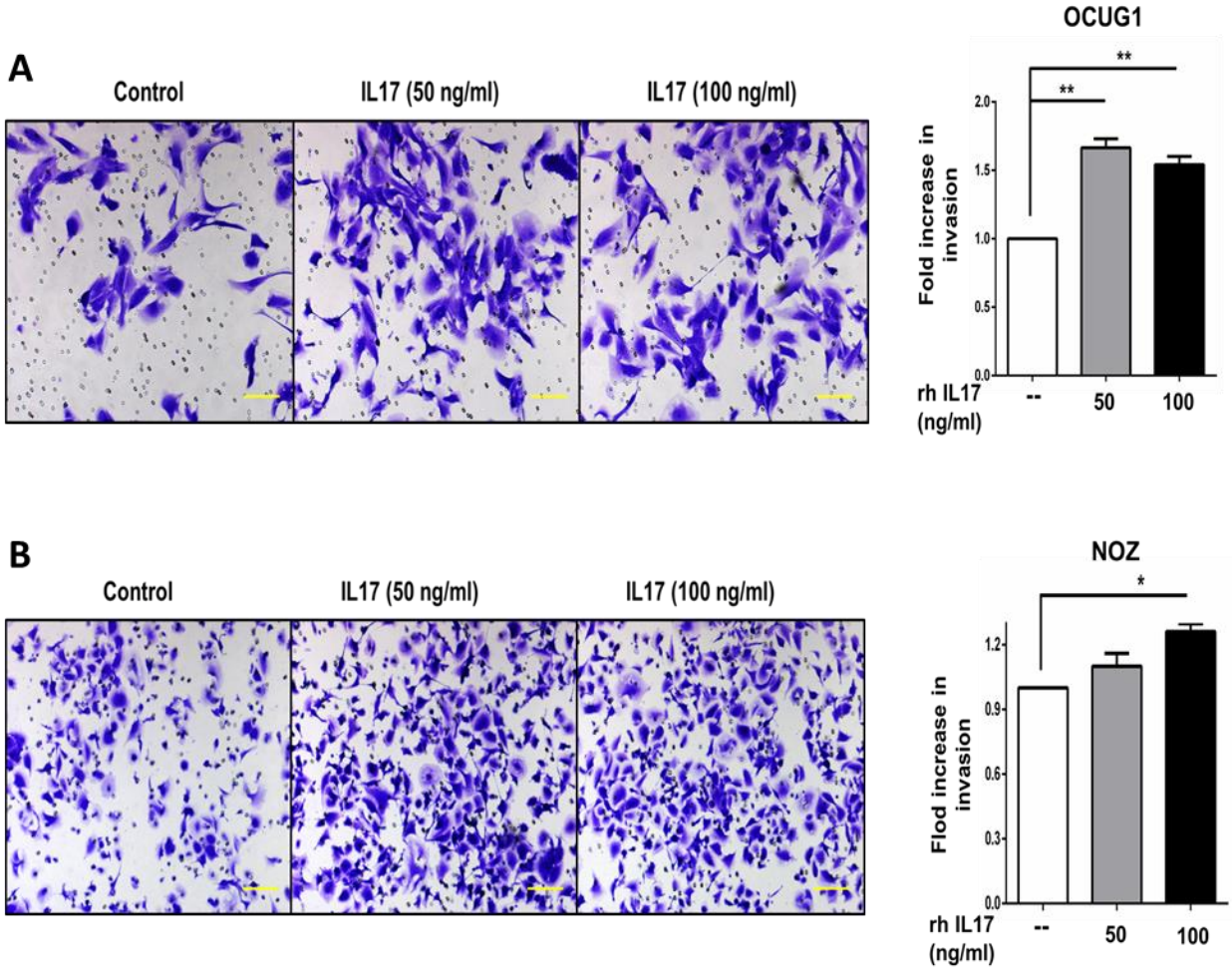


Figure 6.3 : Effect of IL17 on invasion potential of gallbladder tumor cells. *The figure depicts invasion potential OCUG-1 (A) and NOZ (B) cell lines depicting in presence of different concentration of rhIL17 using matrigel invasion assay. The Bar graph represents fold increase in invasion. Data was analysed using student's t test and shown as mean ± SEM with * p<0.05; ** p<0.01.*

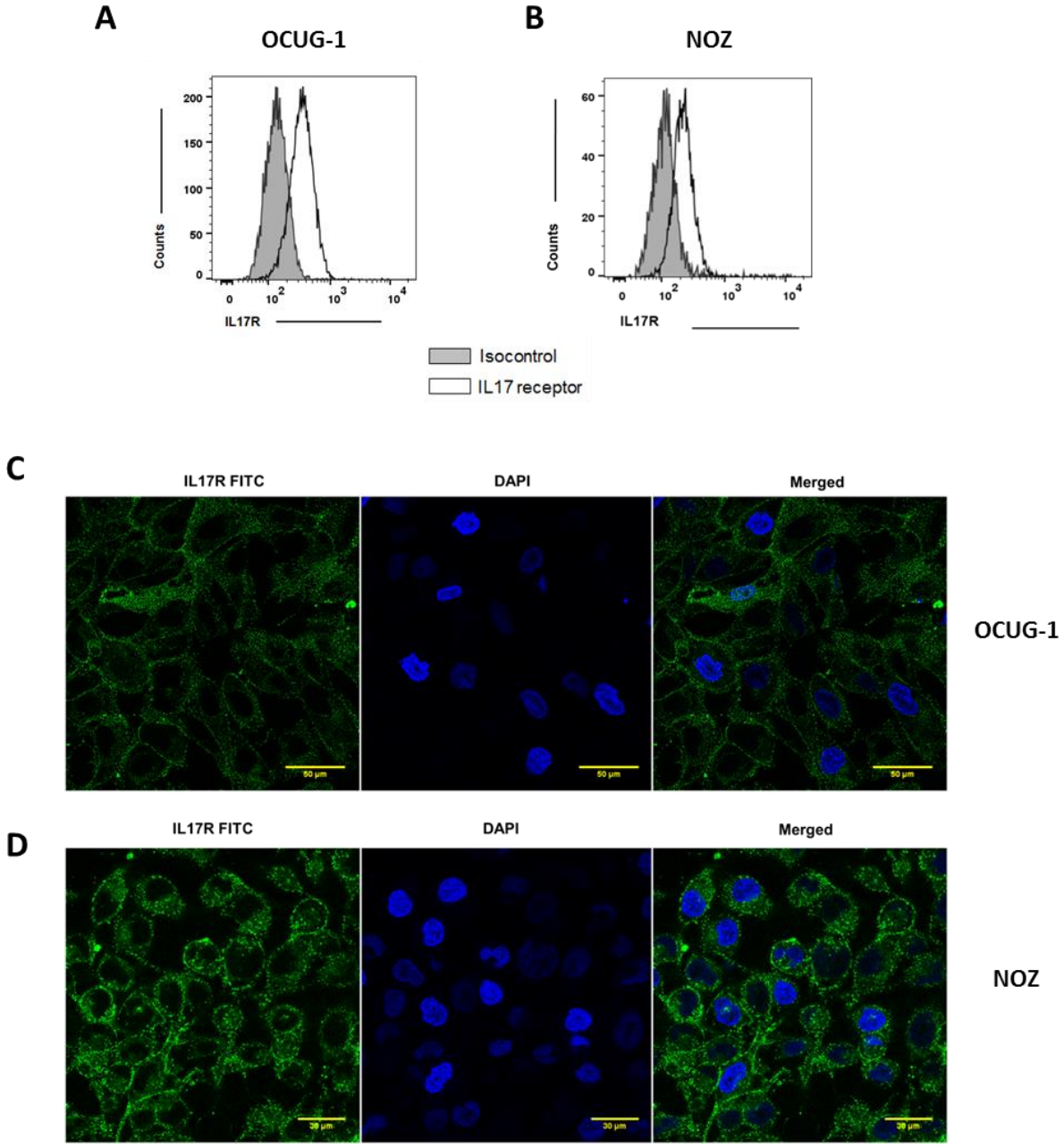


Figure 6.4 : GBC cells express IL17 receptor. (A-B) *A histogram indicates expression of IL17 receptor on OCUG-1 cells (A) and NOZ cells (B) analysed by flow cytometry.* ***(D-E)*** *Expression of IL17 receptor on OCUG-1 cells (C) and NOZ cells (D) analysed by immunofluorescence staining.*

6.2.6 Tγδ17 cells express CXCR3 chemokine receptor

Immunophenotyping of IL17 producing cells in GBC patients revealed that Tγδ17 cells were elevated in tumor environment. We proposed that Tγδ17 cells would be migrating to the

tumor tissue from peripheral blood. To test this, the expression of chemokine receptors (CCR6, CCR7, CCR4, CXCR4 and CXCR3) were analysed on Th17, Tc17 and Tγδ17 cells in peripheral blood of GBC patients (n=35) and HI (n=15) by flow cytometry. It was observed that the expression of these chemokine receptors on Tγδ17, Th17 and Tc17 cells (measured as median fluorescence intensity) were comparable in GBC patients and HI (Figure 6.5).

Next, the expression of chemokine receptors was compared among Th17, Tc17 and Tγδ17 cells in peripheral blood of GBC patients (n=35). The data showed that Tγδ17 expressed elevated levels of CCR4 and CXCR3 expression compared to Th17 or Tc17 (Figure 6.6 C and E). However, CCR6, CCR7 and CXCR4 were expressed at comparable levels by Tγδ17, Th17 and Tc17 (Figure 6.6 A, B and D). The data suggests that Tγδ17 cells may be using chemokine axis through CXCR3 and CCR4 receptors.

6.2.7 Tγδ17 cells migrate towards tumor environment through CXCR3/CXCL9 chemokine axis

It is reported that γδT cells express CCR4 as homing receptor [324-326]. Thus to investigate the contribution by CXCR3 in migration of Tγδ17 cells to the tumor, Tγδ17 cells were first purified to > 90% purity using immunomagnetic separation from peripheral blood of HI (Figure 6.7 A). Purified Tγδ17 cells were cultured with GBC cell line (OCUG-1) or in presence of rhCXCL9 (ligand of CXCR3) or cell-free tumor supernatants of surgically resected gall bladder tumors in trans-well assay. Tγδ17 cells were pre-incubated in presence or absence of neutralizing anti-CXCR3 antibody. Enhanced migration of Tγδ17 cells was observed towards OCUG-1 or rhCXCL9 or tumor supernatants compared to medium alone. The migration of Tγδ17 was significantly curtailed in presence of neutralizing anti-CXCR3 antibody (Figure 6.7 B).

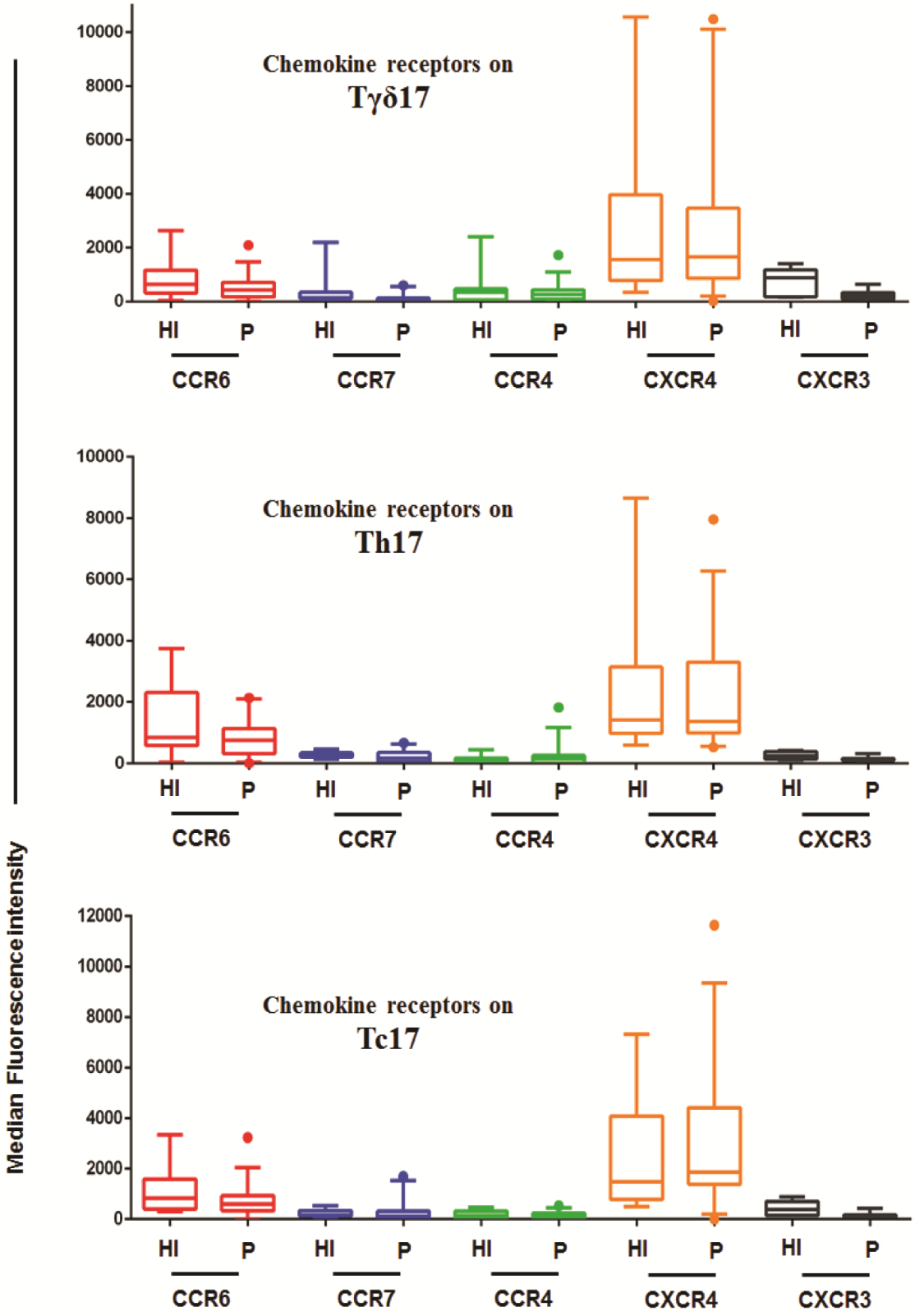


Figure 6.5 : Expression of chemokine receptors on Tγδ17, Th17 and Tc17 in GBC patients and HI. *Summarized data presented as box whisker plots shows expression of chemokine receptors (CCR6, CCR7, CCR4, CXCR4 and CXCR3) on Tγδ17 (A),*

Th17 (B) and Tc17 (C) in peripheral blood of GBC patients (n=35) and HI (n=15). The data is presented as median fluorescence intensity. HI, healthy individuals; P, GBC patients. The box plots show median (middle line), 5th and 95th percentiles (box), extreme values (whiskers) and outliers (dark circles).

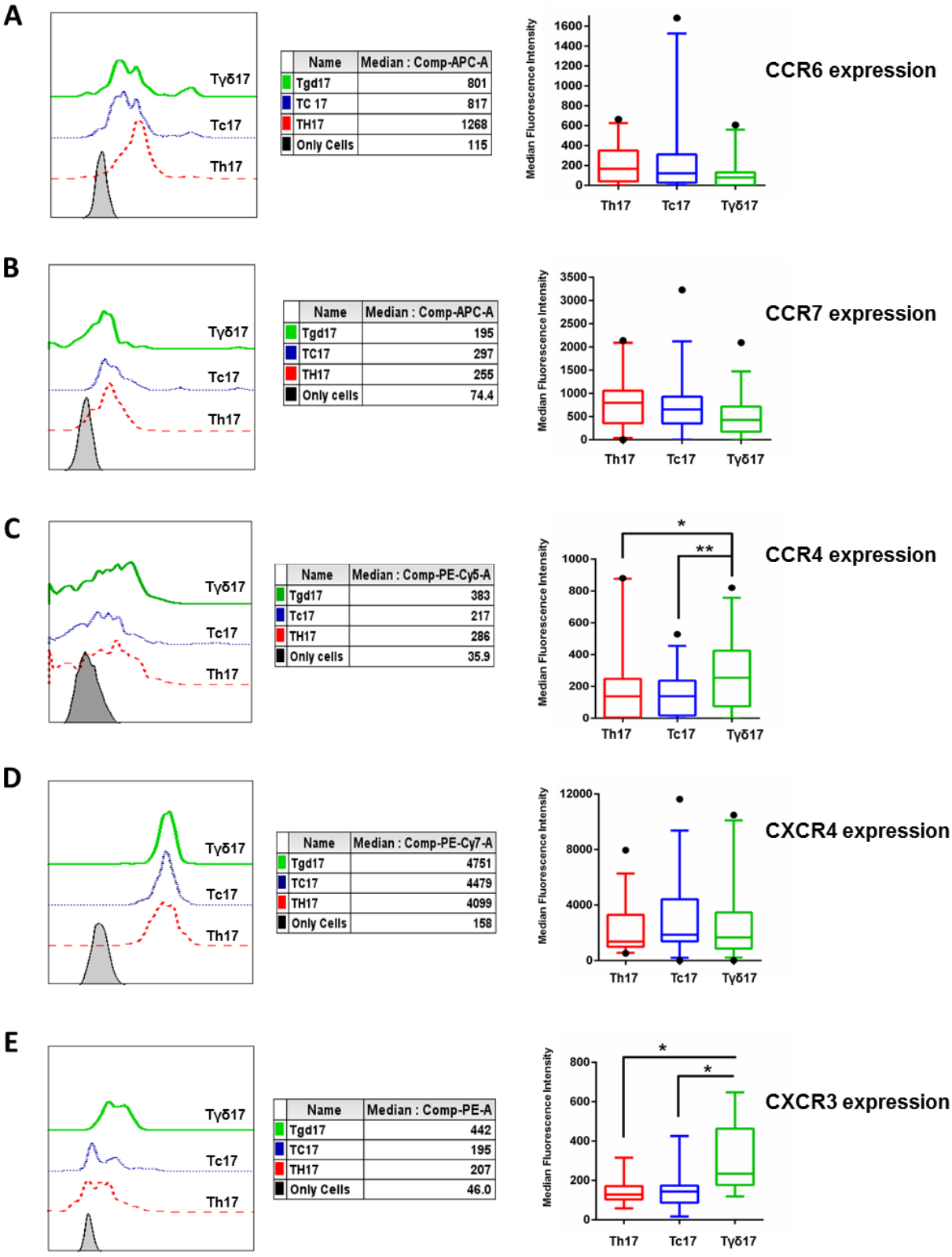


Figure 6.6 : Comparative analysis of chemokine receptor expression on Tγδ17, Th17 and Tc17 in GBC patients. *Expression of chemokine receptors on Tγδ17 (Green), Th17 (Blue) and Tc17 (Red) in peripheral blood of GBC patients (n=35) were analysed by flow cytometry and depicted as representative overlaid histograms. Shaded histogram represents isocontrol of respective fluorochrome. Median expression of respective chemokine receptors on Tγδ17, Th17 and Tc17 is shown as representative table. A collective data of expression of CCR6 (A), CCR7 (B), CCR4 (C), CXCR4 (D) and CXCR3 (E) is presented as box whisker plots. The box plots show median (middle line), 5^{th} and 95^{th} percentiles (box), extreme values (whiskers) and outliers (dark circles). Results are analysed by Mann-Whitney test and student's t test with *($p < 0.05$); **($p < 0.01$).*

Further to study if total γδT cells can also migrate towards GBC tumor environment, purified γδT cells (Figure 6.8 A) were co-cultured with OCUG1 or cell free tumor supernatant in a trans-well assay in presence or absence of neutralizing anti-CXCR3 antibody. We observed an increased migration of γδT cells towards OCUG-1 cells as well as tumor supernatant compared to medium alone which was significantly abrogated in presence of anti-CXCR3 antibody (Figure 6.8 B). The data suggests that the tumor environment induces recruitment of Tγδ17 and γδT cells towards tumor bed through CXCL9-CXCR3 axis.

6.2.8 GBC tumor environment promotes Tγδ17 phenotype

As shown in figure 5.6 C, the cytokines (IL6, IL1β, IL23, TGFβ) required for differentiation of γδT cells towards Tγδ17 phenotype were present in GBC tumor environment. Thus to study the immunomodulatory effect of GBC tumor environment, isolated γδT cells were cultured in presence or absence of tumor supernatant of freshly resected gallbladder tumor tissue in the presence of anti-CD3/anti-CD28 for 48 h. The levels of Tγδ17 cells were analysed in the cultures by flowcytometry. γδT cells cultured in the presence of tumor supernatant showed increased levels of Tγδ17 cells compared to cells cultured with medium alone (Figure 6.8 C). Thus the data suggests that the GBC tumor environment support the differentiation of γδT cells towards IL17 producing phenotype.

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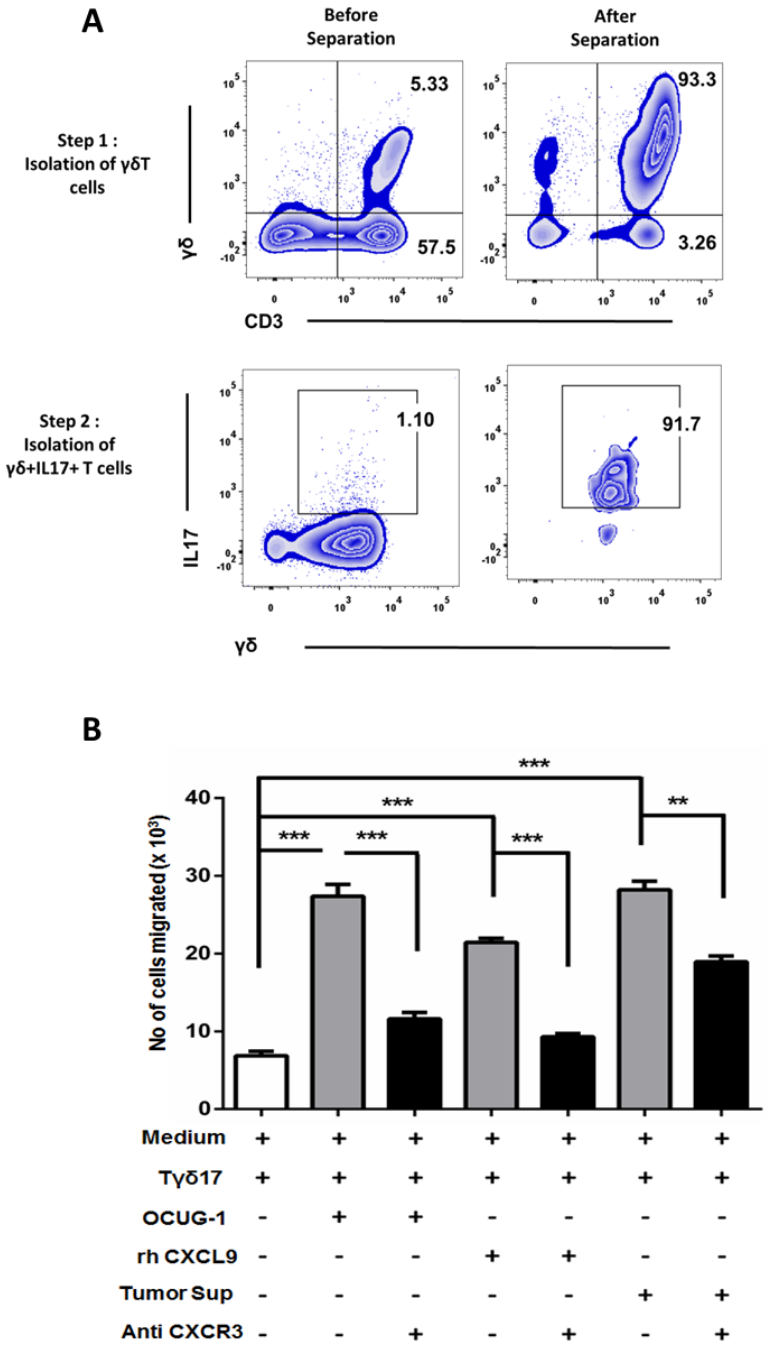


Figure 6.7 : Migration of Purified Tγδ17 cells towards GBC tumor tissue. (A) *Representative figure of purity of sorted Tγδ17 cells from peripheral blood of HI. Upper panel shows purity of γδT cells. IL17 producing γδT cells were sorted to more than 90% purity as shown in lower panel.* ***(B)*** *Isolated Tγδ17 cells were cultured with OCUG-1 cells or rhCXCL9 or tumor supernatants in a trans-well assay in presence or absence of anti-CXCR3 mAb. Data presented as total number of migrated cells (n=3). Data was analysed by student's t test and shown as mean ± SEM with * p<0.05; ** p<0.01; *** P<0.001.*

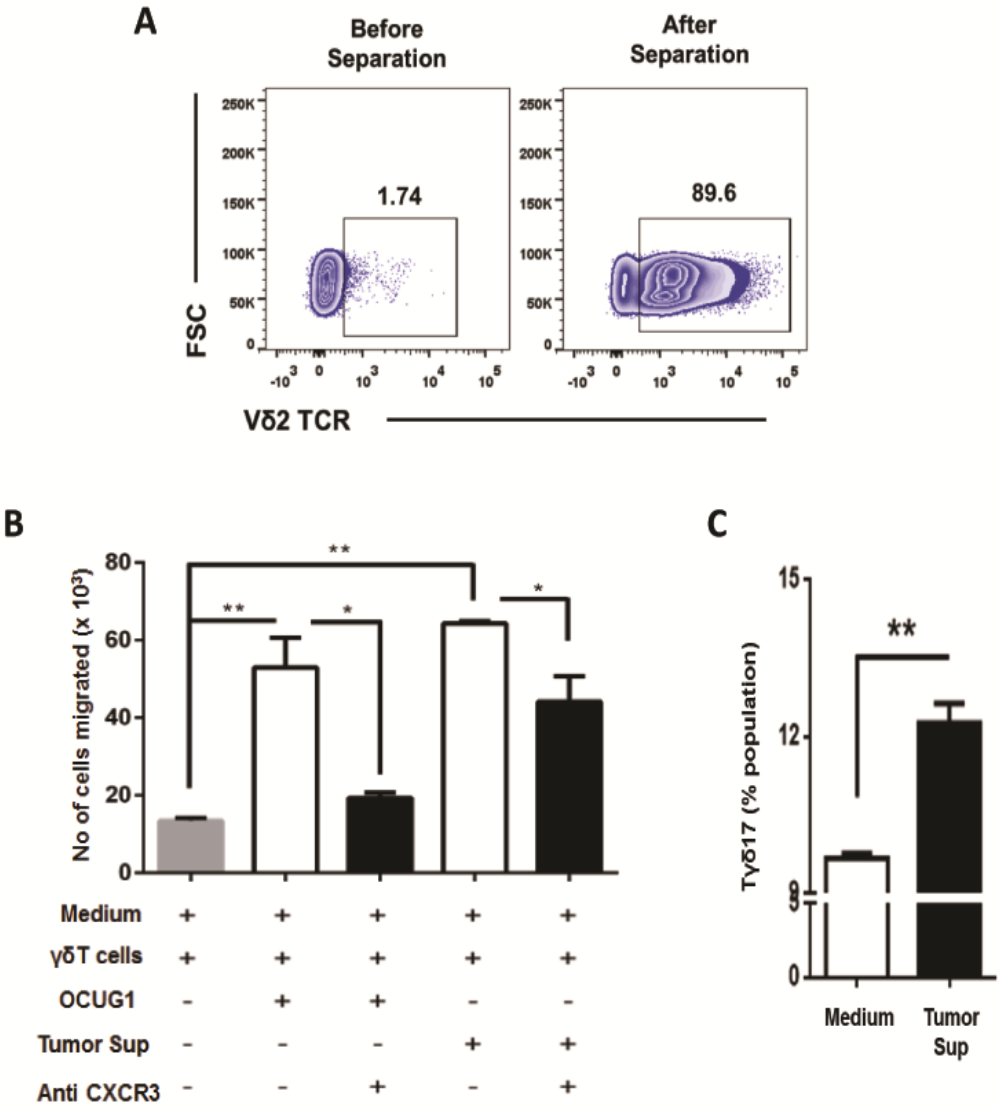


Figure 6.8 : Recruitment of γδT cells to GBC tumor environment. (A) *Representative zebra plot of purity of γδT cells after immune-magnetic separation from peripheral blood of HI.* ***(B)*** *In a trans-well assay, isolated γδT cells were cultured with OCUG-1 cells or tumor supernatants in presence or absence of anti-CXCR3 mAb. Migration was monitored by counting total number of migrated cells to the lower chamber (n=3).* ***(C)*** *Purified γδT cells were cultured with tumor supernatant or medium alone in presence of anti-CD3/anti-CD28 stimulus. IL17 producing γδT cells were determined by flow cytometry (n=3). Data was analysed by student's t test and shown as mean ± SEM with * p<0.05; ** p<0.01*

6.2.9 Tγδ17 cells predominantly secrete IL17

In order to characterise Tγδ17 cells, the cytokine profile of Tγδ17 was analysed. Purified Tγδ17 cells were stimulated with anti-CD3/anti-CD28 for 24 h in serum free medium. Cytokine estimation in cell free supernatant showed that Tγδ17 primarily secrete high levels

of IL17 and low levels of IL2 and TNFα but did not produce other cytokines (IL4, IL6, IL10 and IFNγ) (Figure 6.9 A). Thus the data confirms the purity of Tγδ17 cells and suggests that IL17 is the signature cytokine of Tγδ17 cells.

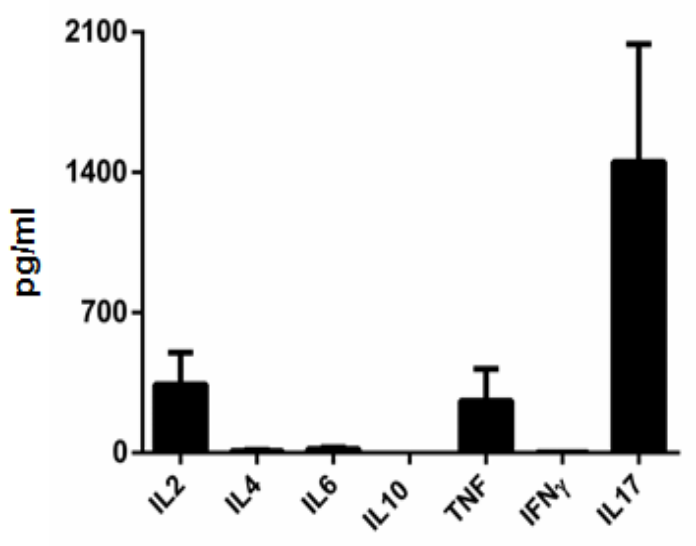


Figure 6.9 : Characterization of cytokine profile of Tγδ17 cells. *Isolated Tγδ17 cell were stimulated with anti-CD3/anti-CD28 for 24 h (n=5). The cytokines were measured in cell-free culture supernatants by cytometric bead array. The data are presented as pg/ml.*

6.2.10 Tγδ17 cells induce angiogenesis related factors in GBC cells

VEGF is a key mediator of tumor angiogenesis and metastasis. As shown in figure 6.10 A, the serum levels of VEGF were significantly increased in GBC patients compared to HI. Thus to investigate whether the elevated levels of Tγδ17 in GBC patients contribute to VEGF production and tumor progression, the proangiogenic activity of Tγδ17 cells was studied *in vitro*. The cell-free supernatant of Tγδ17 cells was cultured with GBC cells (OCUG-1 cell line) for 48 h and the levels of VEGF were estimated in culture supernatants. The data demonstrated that Tγδ17 cells alone secreted marginal amount of VEGF. However, culture of Tγδ17 cells with OCUG-1 cells upregulated the secretion of VEGF by OCUG-1 cells compared to OCUG-1 cells cultured with medium alone. Addition of neutralizing anti-IL17

antibody to the Tγδ17 – supernatant, significantly abrogated VEGF production by OCUG-1 cells (Figure 6.10 B). The data suggests that the VEGF production by the GBC cells is regulated by Tγδ17 through IL17 secretion.

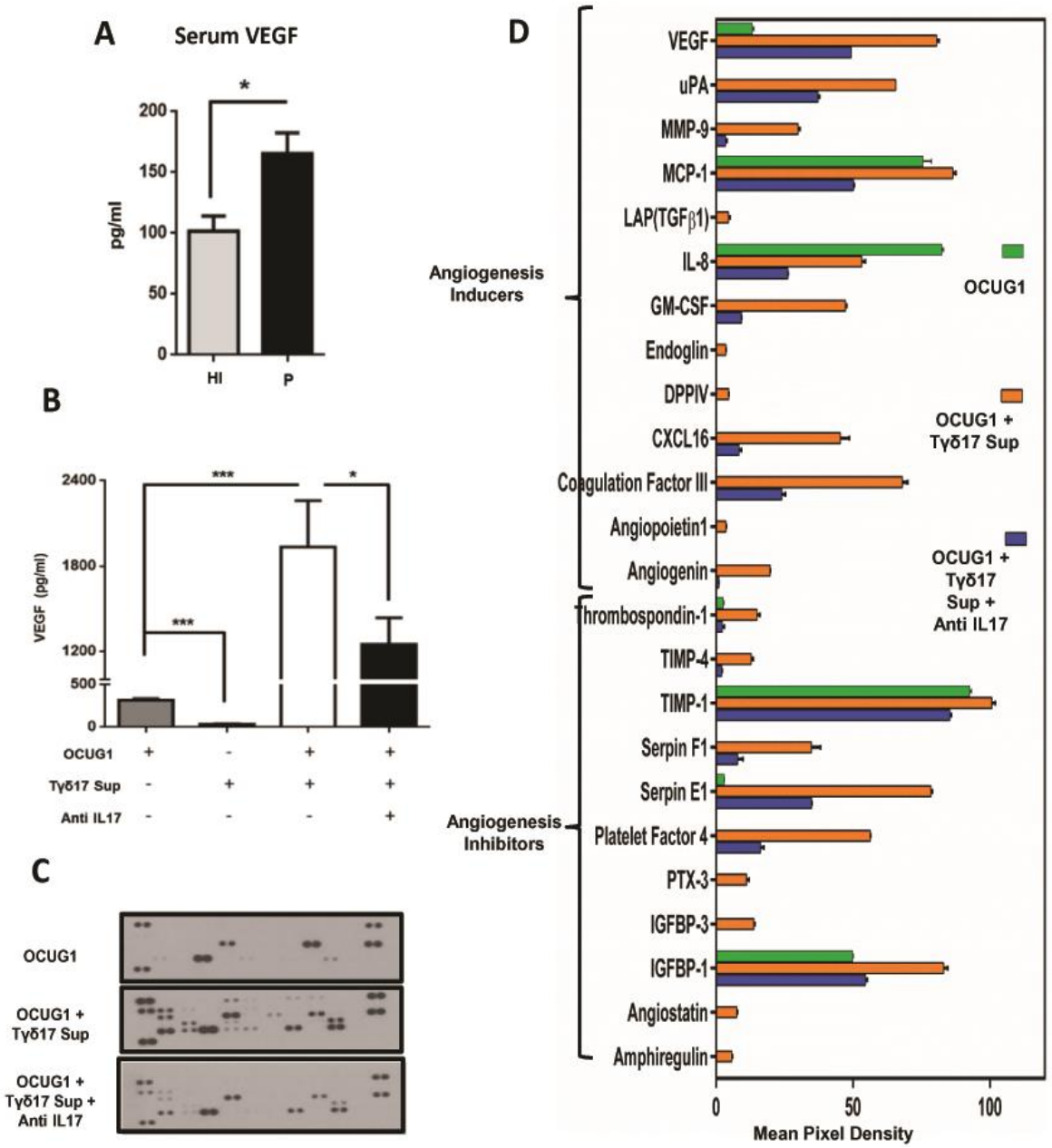


Figure 6.10 : Tγδ17 cells induce angiogenesis related proteins in gallbladder cancer cells. (A) *Serum VEGF levels in GBC patients (n=11) and HI (n=7) were measured by ELISA. VEGF levels are presented as pg/ml.* ***(B)*** *OCUG-1 cells were cultured with cell-*

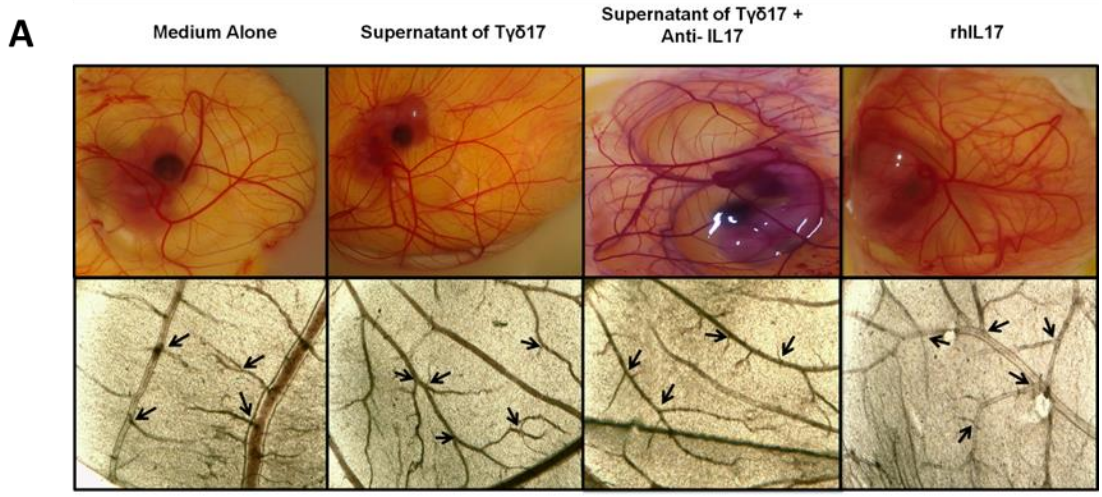
free supernatants of Tγδ17 in presence or absence of neutralizing anti-IL17 antibody. VEGF levels in the supernatants were estimated using ELISA (n=6). ***(C-D)*** *Supernatant of Tγδ17 was incubated with OCUG-1 cells in presence or absence of anti-IL17. Angiogenesis related proteins in culture supernatants were analysed by human angiogenesis proteome profiler array.* ***(C)*** *Representative membranes showing array blot developed using chemi-luminescence.* ***(D)*** *The bar diagram showing densitometric analysis of angiogenesis array blots represented as mean pixel density (n=2). Data was analysed by student's t test and shown as mean ± SEM with * p<0.05; ** p<0.01*

IL17 is reported to regulate many downstream target genes associated with angiogenesis. Thus the effects of Tγδ17 cells on induction of angiogenesis related genes in GBC cells were studied by human protein profiler angiogenesis array. Cell-free supernatants from Tγδ17 cells significantly upregulated secretion of angiogenesis promoting factors from OCUG-1 cells such as VEGF, uPA, MMP9, MCP1, GM-CSF, CXCL16, coagulation factor III, angiogenin, etc. compared to OCUG-1 cells cultured with medium alone (Figure 6.10 C and D). This effect was abrogated by neutralizing IL17 in Tγδ17-supernatant using anti-IL17 mAb. OCUG-1 cells secreted high IL8 and addition of Tγδ17 supernatant did not further increase its levels. Along with angiogenesis inducers, certain angiogenesis inhibitors like thrombospondin-1, TIMP-1, serpine-1, platelet factor 4, IGFBP-1, etc. were also secreted by OCUG-1 cells in presence of Tγδ17 supernatant. The levels of angiogenesis inhibitors also decreased in presence of anti-IL17 mAb (Figure 6.6 C and D). Thus the data demonstrated that Tγδ17 cells induce proangiogenic proteins in GBC cells.

6.2.11 Tγδ17 cells promote blood vessel formation in chorioallantoic membrane (CAM) of chick embryo

The proangiogenic effects of Tγδ17 were further validated by CAM assay. Cell-free supernatants of Tγδ17 were *ex vivo* inoculated in CAM of 5 days old chick embryos. The number of blood vessels and branching pattern were monitored for 48 h and analysed individually by capturing images. It was observed that treatment of CAM with of Tγδ17

supernatant significantly enhanced the vascularization (measured as number of branching points emerging from secondary blood vessels) compared to treatment of CAM with medium alone (Figure 6.11 A). Similar pronounced effect of vasculogenesis was observed in presence of rhIL17. The neutralization of IL17 in Tγδ17 supernatant showed decreased blood vessel formation in the CAM treated with Tγδ17 supernatant (Figure 6.11 A and B). Further, the blood vessels formed in presence of Tγδ17 supernatant and rhIL17 showed tree-like branching with increased number of branching points from secondary blood vessels compared to parallel branching observed in presence of medium alone (Figure 6.11 A lower panel). Thus the data suggests that Tγδ17 cells can modulate the blood vessel formation through IL17.



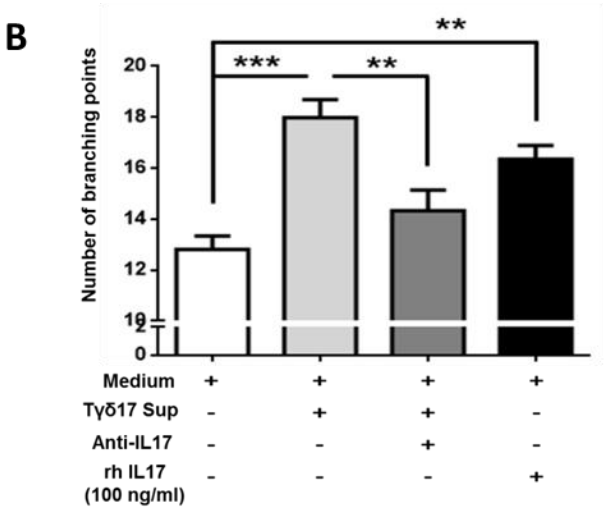
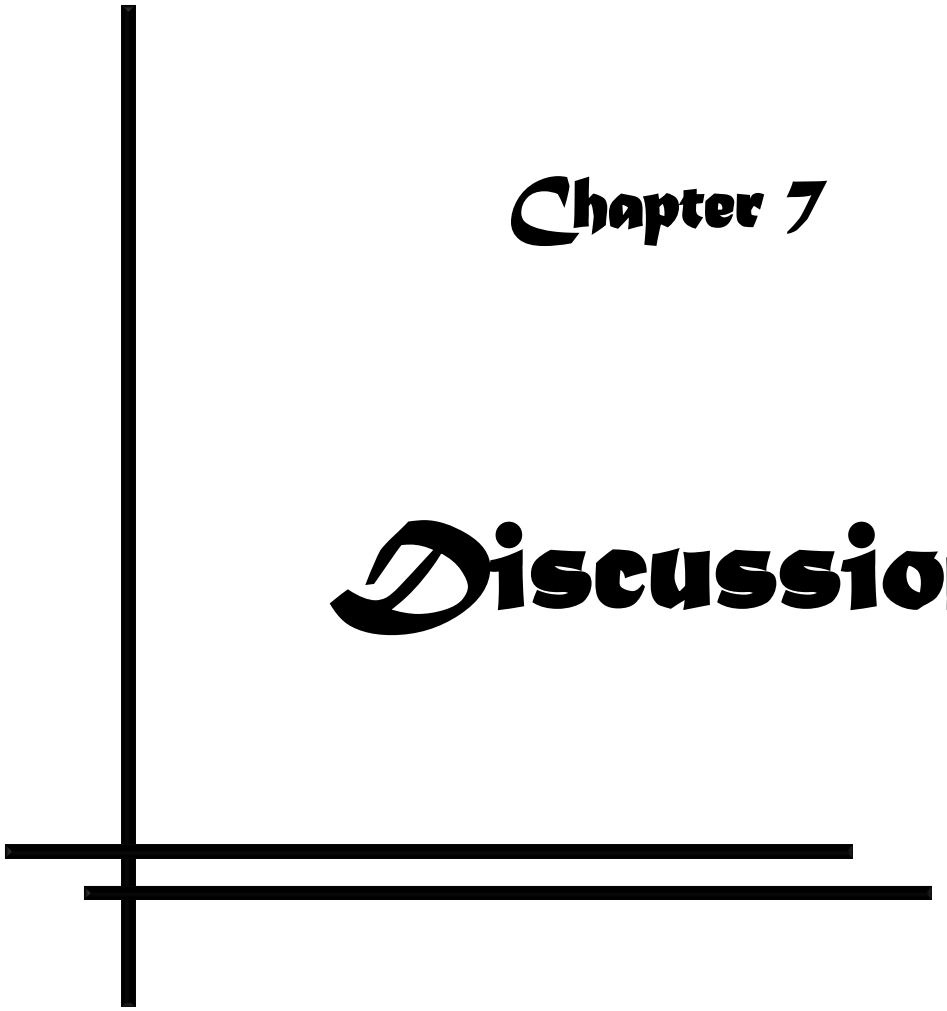


Figure 6.11 : Pro-angiogenic effect of Tγδ17 cells on chorioallantoic membrane of chick embryo (A) *Cell free culture supernatant of Tγδ17 cells was inoculated to 5 days old CAM of chick embryo in presence or absence of anti-IL17 antibody. Representative images of CAM assay depicting enhanced vasculogenesis in presence of supernatant of Tγδ17 or rhIL17 compared to medium alone. Addition of anti-IL17 showed decreased blood vessels. The lower panel shows images of CAM recorded using 4X objective. Arrows indicate branching points.* ***(B)*** *Bar diagram showing summarized data of number of branching points in CAM assay (n=3). Data was analysed by student's t test and shown as mean ± SEM with * p<0.05; ** p<0.01*

6.3 Summary

The results presented in current chapter revealed that IL17 induces proliferation, migration and invasion of GBC cells in concentration dependent manner. The pro-tumor functions of IL17 may be mediated through IL17 receptor expressed by GBC cells. It was observed that Tγδ17 cells expressed elevated levels of CXCR3 receptor than Th17 and Tc17 cells. Tγδ17 utilize novel CXCR3-CXCL9 chemokine axis to migrate towards GBC cells. Culture of γδT cells with cell free tumor supernatants of gallbladder tumor showed increased Tγδ17 phenotype. The Tγδ17 cells were successfully isolated from peripheral blood of HI and the cytokine characterization showed that Tγδ17 cells predominantly secretes IL17. Culture of Tγδ17 cells with GBC cells induced VEGF and other angiogenesis related proteins. It was further confirmed by CAM assay that Tγδ17 cells aid in blood vessel formation through IL17 production. Overall the data demonstrated that Tγδ17 is a pro-tumorigenic subtype of γδT cells.



Chapter 7

Discussion

Discussion

GBC is highly malignant cancer known for its aggressive biological nature and poor clinical presentation. The poor prognosis is due to lack of sensitive screening tests for early detection resulting in delayed diagnosis [11]. Although surgical resection of gallbladder is a curative treatment, less than 10% of GBC patients present with early stage disease and can be considered for surgery [12]. As of today, prognostic biomarkers and effective adjuvant immunotherapy for GBC are unavailable. Use of monoclonal antibodies targeting antigens expressed on tumor cells have shown therapeutic efficacy in cancer patients [327]. Antibodies such as rituximab targeting CD20 in non-Hodgkin B cell lymphoma, trastuzumab targeting HER2 in breast cancer, and cetuximab targeting EGFR in colorectal cancer are introduced in clinical practice [327]. These antibodies induce tumor cell death by blocking ligand-receptor growth and survival pathways [327]. The clinical trials using antibodies targeting tumor antigens in biliary tract cancer patients have shown unfavourable outcome [113]. Erlotinib, a small-molecule inhibiting the EGFR tyrosine kinase domain was evaluated in biliary tract cancer patients in phase II study. The study showed that overall survival of patients was 7.5 months with 52% of patients alive after 6 months [328]. Similarly Lapatinib, a dual EGFR1 and ErbB2 inhibitor, when tested in a phase II trial in biliary tract cancer patients showed progression free survival of 1.8 months and overall survival of 5.2 months [329]. The combination therapies using antibodies specific for tumor antigens and chemotherapeutic drugs showed dismal long term survival of patients with biliary tract [113]. These reports suggest that it is necessary to distinguish biliary tract cancer from other hepato-pancreatic malignancies in view of treatment modalities [113]. Therefore, there is a need for developing novel approaches for successful management of GBC.

The emerging evidences in recent past have highlighted the importance of antibody-based strategies focussing on enhancing antitumor immune responses by targeting immune cells,

irrespective of tumor antigens [330, 331]. Promising results are obtained in patients with advanced melanoma, non-small cell lung cancer, prostate cancer, renal cell cancer, colorectal cancer, etc. [332, 333]. The antibodies used in these studies were targeted against immune cells expressing inhibitory molecules CTLA-4, PD-1 and PD-L1 (immune checkpoints) [333]. Cytotoxic T-lymphocyte antigen-4, CTLA-4 (CD152), is a type I transmembrane glycoprotein that presents homology to CD28. It down-regulates T cell activation by inhibiting co-stimulation by CD28, playing a key role in the regulation of immune homeostasis [332]. CTLA-4 is predominantly expressed on activated $CD4^+$ helper T cells and not on $CD8^+$ cytotoxic T cells. Therefore, heightened CD8 responses is observed in anti-CTLA-4-treated patients [334].

Similarly PD-1 (CD279) is another inhibitory receptor expressed by activated T cells. TCR engagement with MHC-antigen complex induces PD-1 expression, and PD-1/ PD-L1 binding leads to the inhibition of T cell activation and effector functions [335]. PD-L1 is highly expressed on human tumors and it has been suggested that PD-L1 has a role in attenuating anti-tumor immune responses of $CD4^+$ and $CD8^+$ T cells expressing PD-1 [336]. The anti-CTLA-4 monoclonal antibody ipilimumab, a fully human IgG1, and anti-PD-1 antibody nivolumab were the first immune checkpoint-blocking drugs to enter clinical practice for treatment of melanoma, colorectal and prostate cancer patients [334]. Another approach in the treatment of cancer is the use of engineered T cells expressing chimeric antigen receptor (CAR-T cell therapy). T cells are engineered to express TCR derived from the variable region of an antigen-specific antibody and is linked to signalling components of costimulatory signals leading to T cell activation. The treatment protocols for this therapy are under development [337]. Monoclonal antibodies specific for regulatory T cells have also showed improved patient outcome. Treg cells suppress antitumor immune response through CTLA-4 mediated downregulation of costimulatory molecules on APCs [51]. Thus targeting CTLA-4

or CD25 on Treg cells leads to decrease in Treg cells and improved antitumor immune response [51].

The current available modalities of cancer treatment have transformed from non-specific chemotherapeutic drugs to more specific targeted therapy providing long-term clinical benefit in some advanced cancer patients [337]. The understanding of the immune evasion strategies of tumor at molecular level has led to the emergence of novel molecular targets. To extrapolate the advent of targeted therapies to gallbladder cancer treatment, it is necessary to understand the role of immune response which is not investigated in GBC patients.

The GBC patients are predisposed with chronic inflammatory condition of cholelithiasis. The studies in the murine model of cholesterol gallstone disease, presence of cholesterol crystals led to increased mucus layer thickness, interleukin-1 and myeloperoxidase activity in the wall of the gallbladder [91]. These changes were accompanied by inflammatory infiltrate composed of eosinophils, macrophages, neutrophils and lymphocytes within the lamina propia [92]. Another study reported that $Rag2^{-/-}$ mice, (deficient for B- and T-cells), were resistant to cholesterol gallstone formation suggesting that functional T cells are crucial in the development of gallstones [93]. Although these studies indicate the significance of inflammatory immune response in gallstone disease, the role of inflammatory factors contributed by immune cells is not investigated in GBC patients.

Thus the present study aimed at understanding the immune scenario in GBC patients and identify the proinflammatory (IL17 producing T cells) and immunosuppressive (Treg) subsets that may contribute to pathogenesis of GBC.

The important hallmark of malignant progression of cancer is an escape from immunosurveillance [121]. The immune escape of cancer is accompanied by profound immune destruction [338]. Immunophenotyping of PBMCs in GBC patients revealed that the

cells contributing to adaptive immunity ($CD4^+$ T cells, $CD8^+$ T cells, B cells and NKT cells) were significantly decreased in peripheral blood of GBC patients. However, the ratio of $CD4^+/CD8^+$ T cells did not alter in GBC patients compared to HI, suggesting that the decline in the percentage of T lymphocytes observed in GBC patients is not subset specific. The PBMCs of GBC patients stimulated with anti-CD3 (TCR agonist) or PHA (mitogen) showed poor proliferative response and secreted decreased levels of effector cytokines (IFNγ, IL6, IL10, IL17, IL8, IL12p70 and IL1β). The results suggest that the PBMCs in GBC patients are unable to show optimum response to TCR or mitogenic stimulatory signal. The activation of T cells is determined by the engagement of TCR with MHC-antigen complex and efficient signal transduction. The intracellular expression of CD3-ζ chain associated with TCR is involved in transduction of stimulatory signal and thus plays an important role in T cell activation [339]. Investigation of CD3-ζ chain in T cells of GBC patients showed that the expression of CD3-ζ chain was downregulated in T lymphocytes present in peripheral blood and tumor tissue. Moreover, the decrease in CD3-ζ chain expression was correlated with clinical stage of GBC patients. Our earlier studies in oral cancer patients have demonstrated that CD3-ζ chain in T lymphocytes of cancer patients undergoes ubiquitination and is subsequently targeted for degradation in the lysosome [340]. However, in tumor compartment, the reduced expression of ELF1, a transcription factor for CD3-ζ chain expression, resulted into decreased transcription of TCR ζ gene [339, 340].

The decreased CD3-ζ chain expression, poor proliferative response and reduced production of effector cytokines by PBMCs suggest that the immune response in GBC patients is dysfunctional. The alterations observed in immune response in GBC patients and the predisposition of GBC to chronic inflammatory condition of cholelithiasis suggests the significant role of coexistence of poor immunosurveillance and chronic inflammation in GBC. Immunosuppressive cells such as regulatory T cells (Treg) also play significant role in

cancer progression by suppressing antitumor $CD4^+$ and $CD8^+$ T cells [51]. The balance of proinflammatory Th17 and immunosuppressive Treg cells is crucial in determining immune response in cancer patients. It is reported that in squamous cell carcinoma the levels of Treg cells were increased and that of Th17 cells decreased with cancer progression [258]. Similar trend was observed in salivary gland tumors [259]. The impaired balance of the Th17 and Treg was also reported in lung cancer, prostate cancer, and cervical cancer [58-60].

Immunophenotyping of GBC patients in the current study revealed that the levels of IL17 producing CD4, CD8 and γδT cells were significantly elevated in peripheral blood and tumor tissue compared to the levels observed in HI. Interestingly, the data highlighted the emergence of recently discovered IL17 producing phenotype of γδT cells. Conventionally γδT cells secrete high levels of IFNγ and contribute to antiviral, anti-bacterial, and anti-tumor immunity in humans [212, 234, 341, 342]. γδT cells do not require APCs and recognition of antigen is MHC unrestricted, resembling B cells [343]. They can kill infected, activated, 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 [232, 233]. Human γδT cells recognize HSP (HSP60/70) expressed on tumor cells and enhance its cytolytic activity against the tumors [220, 226]. Similar to Natural killer (NK) cells, human γδT cells also recognise the stress-induced MHC class I-related molecules MICA, MICB and the UL16-binding proteins that are upregulated on malignant or stressed cells [218, 344]. The stress related molecules are ligands for NKG2D expressed by γδT cells and this engagement also enhances γδT cells' response to non-peptide antigens [345].

Studies in recent years have shown the importance of cytotoxic behavior of γδT cells in cell based therapies against cancer [214, 234]. Studies from our lab and others have reported that

γδT cells exhibit potent cytotoxicity against tumors [233, 346]. Various clinical trials have been launched in breast, prostate, hepatocellular and renal cell carcinoma patients using *in vivo* activation of γδT cells or by adoptive transfer of activated γδT cells [234]. In contrast, γδT cells isolated from breast tumor biopsies were shown to mediate immunosuppression by inducing senescence in dendritic cells and $CD4^+$ T cells to suppress their antitumor response [347]. In a murine model of hepatocellular carcinoma, γδT cells were shown as major source of IL17. These cells inhibit $CD8^+$ T cell response and recruit myeloid derived suppressor cells (MDSCs) thereby promoting development of tumor [255]. Nitric oxide synthase (NOS2) has been shown to polarize γδT cells to produce IL17 and promote metastasis formation in transgenic model of melanoma [348]. In a breast cancer metastasis model, Tγδ17 cells were shown to induce expansion and polarization of neutrophils which suppress cytotoxic antitumor response [349]. Recently, it is shown that γδT cells infiltrating pancreatic ductal adenocarcinoma express elevated levels of T cells exhaustion ligand (PD-L1) and suppress the activity of CD4 and CD8 T cells [235]. Thus, for successful application of γδT cell based immunotherapies in clinics, it is necessary to have a deeper insight into pro-tumor role of γδT cells where data in humans is lacking.

In the present study, we report that Tγδ17 cells are increased in tumor environment and peripheral blood of GBC patients irrespective of clinical stage. Th17 and Tc17 cells were also elevated but $CD8^+IFN\gamma^+$ and $\gamma\delta^+IFN\gamma^+$ cells were decreased in GB tumor environment. This supports the existence of chronic inflammation in GBC contributed by immune cells. A murine study in hepatocellular carcinoma has shown that the depletion of $V\gamma4^+$ γδ T cells (major source of IL17) resulted in significant reduction in tumor volumes in comparison with wild type mice [255]. Moreover, the infiltrations of effector $CD8^+IFN\gamma^+$ cells in tumors were significantly increased in $V\gamma4^+$ γδT cell–depleted mice [255]. This suggests that Tγδ17 cells

exhibit pro-tumor functions by dampening anti-tumor immune response. In GBC patients, we observed that Tγδ17 and Th17 cells were associated with poor survival. On the contrary, GBC patients with increased $\gamma\delta^{+}IFN\gamma^{+}$ experienced longer survival. These results strongly suggest that IL17 producing cells play a critical role in immune pathogenesis of human GBC. Moreover, it appears that GBC tumor environment selectively promotes IL17 producing cells as $CD8^{+}IFN\gamma^{+}$ and $\gamma\delta^{+}IFN\gamma^{+}$ cells were decreased in TILs. Collectively, these results highlight the pathogenic role of Tγδ17 cells in GBC.

Previously, it was reported that Th17 cells infiltrate the tumor environment and significantly influence the tumor growth through secretion of IL17 [350]. However, the emerging evidences in murine models of hepatocellular carcinoma, breast cancer, skin cancer have shown that major source of IL17 are γδT cells [253, 255, 349]. In GBC patients, it was observed that the propensity of IL17 secretion by γδT cells was increased in tumor compartment than peripheral blood and it was higher compared to Th17 cells. In human colorectal cancer, tumor infiltrating γδT cells were the major contributors of IL17 production and were associated with poor prognosis of patients [246]. It is reported that γδT cells produce IL17 in response to the stimulation by innate cytokines (IL23 and IL1β) in MHC unrestricted manner [351]. IL17 and IL21 derived from γδT cells promote IL17 production from Th17 cells [351]. Thus the inherent property of γδT cells to respond to early inflammatory signals through innate cytokines may lead to the activation and polarization of γδT cells towards IL17 producing phenotype which can modulate the immune response in the tumor environment.

As increased levels of Tγδ17 were observed in GBC patients, we reasoned that the cytokine milieu promoting Tγδ17 differentiation should be present in sera and tumor environment. It was documented that like Th17 cells, Tγδ17 also differentiate from naïve γδT cells in the

presence of IL6, IL1β, IL23 and TGFβ upon antigenic stimulation [243]. Serum cytokine profile of GBC patients revealed that the levels of IL6, IL1β and IL23 were elevated in GBC patients than HI. The estimation of cytokines in tumor supernatants of freshly resected tumor tissue showed that these cytokines were also present in tumor compartment and may be responsible for intra-tumoral differentiation and maintenance of Tγδ17 cells.

In GBC patients, the total percentage and memory phenotype of γδT cells were comparable to that observed in HI. However, the levels of Tγδ17 were increased and that of $\gamma\delta^{+}IFN\gamma^{+}$ were decreased in peripheral blood and tumor environment. Tγδ17 cells negatively correlated with $\gamma\delta^{+}IFN\gamma^{+}$ cells. Moreover, incubation of isolated γδT cells in presence of cell free supernatant of cultured gallbladder tumor cells showed increased percentages of Tγδ17 cells after 48 h of culture compared to incubation with medium alone. Thus the data suggests that the cytokine milieu present in the GBC tumor environment is conducive for differentiation of γδT cells towards Tγδ17 phenotype. It is reported that γδT cells producing IFNγ, express TNFR superfamily member CD27 and IL17 producing γδT cells are restricted to the $CD27^{-}$ subset [249]. In GBC patients, it was observed that the $CD27^{-}$ γδT cells were elevated in peripheral blood and tumor tissue than $CD27^{+}$ γδT cells.

The analysis of TGFβ showed decreased mRNA expression in PBMCs and low levels in sera of GBC patients. TGFβ is required for Treg differentiation and maturation [314]. We observed that compared to HI, Tregs were decreased in peripheral blood of GBC patients at all clinical stages of disease. However, as observed by CFSE dye dilution assay, the suppressive potential of Tregs in GBC patients was not compromised as compared to HI. In addition, the levels of Foxp3 expression (at mRNA and protein level) were comparable to HI, suggesting that the Tregs in GBC patients are functionally normal. Low levels of TGFβ observed in serum may be responsible for reduced levels of Tregs in peripheral blood of these

patients. Similar observations were reported in patients with multiple myeloma and pancreatic ductal adenocarcinoma [352, 353]. It is reported that the Th17 cells differentiate from Foxp3 expressing $CD4^+$ T cells [354] and there exists plasticity in the $CD4^+$ T cells in inflammatory environment [355]. Low concentration of TGFβ and presence of proinflammatory cytokines (IL23, IL21, and IL6) upregulate RORγt expression and inhibit Foxp3 in $CD4^+$ T cells [356]. Thus the decreased levels of Treg cells in peripheral blood of GBC patients would be because of $CD4^+$ T cell polarization towards Th17 phenotype in response to the presence of an inflammatory cytokine milieu.

After categorizing the GBC patients having high or low levels of Tregs, we observed that patients with high peripheral blood Treg cells have decreased survival compared to those with low levels (hazard ratio: 2.07). We also noted that Tregs were increased in tumor compartment and express elevated levels of Foxp3 compared to peripheral blood of GBC patients. Given that the suppressive activity of Tregs is determined by Foxp3 expression [357], the Tregs in TILs of GBC patients appear to be more immunosuppressive. A recent study in colorectal cancer patients demonstrated that Tγδ17 cells promote migration and survival of MDSCs which enhanced immunosuppression in these patients [246]. In GBC patients it was observed that the $HLADR^-CD33^+CD11b^+$ MDSCs were elevated in the peripheral blood and tumor environment. MDSCs are known to induce Treg cells in cancer patients [358] Thus, the increased levels of Tregs in tumor environment of GBC may be attributed to the Tγδ17 driven inflammation leading to accumulation of MDSCs and subsequent upregulation of Tregs. However, the mechanism regulating accumulation and suppressive functions of MDSCs mediated by Tγδ17 cells in GBC warrants further investigation. It is reported that Th17 and Treg frequently co-localize at the same anatomic compartments and mutually promote each other's generation and function [359]. We observed the infiltration of Tregs and IL17 producing cells in the tumor environment of GBC

patients which corroborate the earlier observations in GBC patients reported by Zhang et al [360]. This suggests that although the ratios of Tγδ17/Treg, Th17/Treg and Tc17/Treg were increased in GBC patients, IL17 producing cells and Tregs may act cooperatively and eventually contribute to the poor survival observed in GBC patients.

Next we addressed the functional role of Tγδ17 cells on GBC tumor progression. The migration of Tγδ17 cells mediated by chemokines was studied in GBC patients. Murine Tγδ17 cells have been reported to express various chemokine receptors including CCR6, CCR1, CCR2, CCR4, CCR5, CCR7, CCR9, CXCR1, CXCR3, CXCR4, CXCR5 and CXCR6 [251]. Murine Tγδ17 cells expressing CCR9 show selective migration towards allergic inflamed tissue in response to CCL25 [361]. The recruitment of Tγδ17 cells towards inflammatory environment is poorly investigated in human. In GBC patients, it was observed that Tγδ17 cells showed elevated expression of CXCR3 than Th17 and Tc17. Expression of CCR6, CCR7 and CXCR4 was comparable in Tγδ17, Th17 and Tc17 cells. We demonstrated that Tγδ17 cells migrate towards tumor milieu through CXCL9-CXCR3 axis. The increased levels of CXCL9 and CXCL10 observed in sera of GBC patients, further supports the elevated levels of Tγδ17 cells observed in the tumor environment. Earlier it was reported that Th17 utilized this axis to migrate towards inflamed liver [362]. This is the first report demonstrating migration of Tγδ17 cells to the tumor environment using CXCL9-CXCR3 axis.

Angiogenesis is a critical step in the progression of solid tumors providing nutrients, growth factors and oxygen for growth of malignant cells. Th17 cells are shown to be proangiogenic in human head and neck squamous cell carcinoma [42]. Proangiogenic functions of Tγδ17 in human cancer are not yet reported. Our data showed that human Tγδ17 cells induce blood vessel formation through secretion of IL17 as observed in *ex vivo* chick embryo CAM assay.

Tγδ17 induced GBC cells to produce proangiogenic factors such as VEGF, uPA, MMP9, MCP1, GM-CSF, CXCL16, coagulation factor III, angiogenin, etc. through secretion of IL17. Interestingly, we observed that Tγδ17 also induced anti-angiogenic factors (Thrombospondin-1, TIMP1, Serpine1, Platelet factor-4, IGFBP1, etc.). However, TIMP-1 and serpine-1 are also reported as markers of poor prognosis in cancer [363, 364]. A recent study in glioblastoma showed that IGFBP1 secretion by microglial cells induced by MCSF is essential for angiogenesis [365]. A study in $IL17^{-/-}$ CMS-G4 fibrosarcoma murine model has shown that γδT cells were the major source of IL17 and depletion of IL17 resulted in decreased vascular density and tumor growth [253]. In the ID8 ovarian cancer model, tumors grown in $IL17^{-/-}$ and $TCR\delta^{-/-}$ mice express lower levels of *ang-2* and *vegf* compared with tumors grown in wild-type animals [254]. Tγδ17 also mobilizes the proangiogenic *Tie2*-expressing macrophages into the peritoneal cavity [254]. Thus Tγδ17 is a major contributor to the tumor angiogenesis. To the best of our knowledge, our data for the first time provides evidence that Tγδ17 promote angiogenesis in gallbladder cancer and is a pro-tumor subtype of γδT cells in human.

IL17 is a key cytokine in the proangiogenic function of Tγδ17 cells in GBC. Inhibition of IL17 showed reduced production of angiogenic factors and decreased blood vessel formation in CAM assay. The levels of IL17 were increased in sera of GBC patients than HI. GBC patients with elevated levels of IL17 in sera were associated with poor survival whereas GBC patients with higher levels of IFNγ showed better clinical outcome. The data from study in murine model of melanoma showed that the growth of tumor was reduced in double knockout $IL17^{-/-}IFN\gamma^{-/-}$ mice as observed in $IL17^{-/-}$ mice. However, the fast tumor growth was observed in $IFN\gamma^{-/-}$ mice [366]. These results suggest importance of IL17 in tumor progression.

IL17 is reported to contribute to neoangiogenesis, activation of matrix metalloproteinases, carcinogenesis, tumor metastasis and resistance to chemotherapy in diverse solid tumors [30, 31, 318]. IL17 induces IL6 production which activates STAT3 upregulating pro-survival and proangiogenic genes [154, 366]. In a 4T-1 metastatic breast cancer model IL17 participates in tumor progression by recruiting neutrophils to the tumor which produce CXCL1, MMP9, VEGF and TNFα. IL17 also induces production of IL6 and CCL20 favouring the migration and differentiation of IL17 producing cells and correlate with poor prognosis of patients with invasive ductal carcinoma [367]. In the present study, it was observed that IL17 enhanced the neoplastic transformation of GBC cells. Addition of IL17 enhanced the proliferation of poorly differentiated GBC cells in a dose dependent manner. IL17 acts through IL17 receptor and activates Tumor progression locus 2 (TPL2) which induces protein kinase/extracellular signal-regulated kinase kinases, c-jun N-terminal kinases and STAT3 signaling pathways [368]. Knockdown of IL17 and/or inhibition of TPL2 attenuated tumorigenecity of human breast cancer MCF7 cells [368]. It was observed that the proliferative effect of IL17 was absent in moderately differentiated NOZ cells although, both cell lines expressed IL17 receptor on the surface. It is reported that IL17 induce phosphorylation of ERK 1/2 and stimulated proliferation of breast cancer cell line T47D. MCF-7, another breast cancer cell line was less sensitive to ERK recruitment and failed to respond to IL17 [31]. Thus it needs to be further investigated whether the differential activation of signalling molecules in two GBC cell lines is responsible for non-responsiveness of NOZ cells to IL17 treatment.

Since, angiogenesis plays a crucial role in local invasion and metastasis of tumor cells, the role of IL17 in angiogenesis of GBC was explored. VEGF is a key mediator of tumor angiogenesis and metastasis [323]. VEGF is reported as an independent prognostic marker and associates with poor survival of GBC patients [369, 370]. Treatment of gallbladder tumor cells (OCUG-1 cells line) with rhIL17 showed dose dependent increase in VEGF production.

The serum levels of VEGF were elevated in GBC patients compared to HI. Moreover, IL17 induced migration and invasion potential of GBC cells in a dose dependent manner. In colorectal cancer, the high expression of IL17 in tumor tissue was correlated with high expression of VEGF, increased micro-vessel density and poor survival of patients [29]. IL17 directly promotes the invasion of non-small cell lung cancer (NSCLC) cells *in vitro* and *in vivo* and the metastasis of NSCLC was impaired in $IL17^{-/-}$ mice [371]. IL17 activates JAK2/STAT3 signalling through AKT mediated IL6 production. This signalling subsequently upregulates the downstream targets IL8, MMP2, and VEGF and promote migration and invasion in hepatocellular carcinoma [153]. High expression of molecules involved in JAK2/STAT3 signalling pathway are reported to be associated with high expression of VEGF, increased micro-vessel density and poor survival of patients with NSCLC [372]. The accumulating evidences have suggested the pathogenic role of IL17 in cancer scenario. Thus the role of IL17 to promote angiogenesis and invasiveness of gallbladder tumor cells may explain the pro-tumor effect of γδT cells when IL17 is produced as primary cytokine.

The present study has identified a key role of IL17 and its producer cells (Tγδ17, Th17 and Tc17) in pathogenesis of GBC. Therapeutic interventions to the IL17 – inflammatory axis exhibits great clinical potential. Recently, Secukinumab, a fully human IL17A specific monoclonal antibody and Ixekizumab a humanized IL17A specific antibody that neutralizes human IL17A have been approved for treatment of psoriasis [373, 374]. Both these antibodies showed superior results than etanercept (fusion protein inhibiting TNFα) [373, 374]. There are more drug candidates specific for IL17 that include CNTO 6785, ABT-122, COVA322 are under clinical trials [375]. Drugs targeting IL17F (CJM112, ALX-0761 and bimekizumab) are also under clinical trial for patients with inflammatory disease [375]. Although IL17 and TNFα work synergistically to induce a pro-inflammatory signaling cascade [155], patients who are unresponsive to anti-TNF agents may respond to therapies

that target IL17. Other approaches to target IL17 inflammatory axis including monoclonal antibody targeting IL17 receptor (brodalumab), IL23p40 subunit (ustekinumab), IL23p19 subunit and small molecules with inverse agonist activity against RORγt (Digoxin, SR 1001, Ursolic acid) are under phase II/III clinical trials for inflammatory diseases [26, 376, 377]. However, inhibitors of IL17 may offer superior and more specific response as IL12 and IL23 sit upstream of both T_h1 and T_h17 pathways and targeting IL17 do not affect functions of Th1 lineage [375].

Chemotherapeutic drugs like oxaliplatin, doxorubicin, gemcitabine, 5-fluorouracil trigger cancer cell death which activate antitumor immune response. Gemcitabine (gem) and 5-fluorouracil (5-fu) were shown to induce apoptosis in MDSCs and release of IL1β which activates Th17 cells. Release of IL17 further compromised the antitumor effect of gem and 5-fu [378]. However, in a murine study, Tγδ17 cells improved the anti-tumor efficacy of anthracycline doxorubicin [257]. The studies suggest that the chemotherapeutic drugs should be combined with immunomodulatory agents for increasing efficacy of anticancer therapy. Bevacizumab which target VEGF, when combined with chemotherapy has shown direct antitumor effect and improved patient survival [379]. The present study provides insights into pathogenic role of Tγδ17 in GBC through IL17 production and inducing angiogenesis. Targeting IL17 - Tγδ17 axis could be a promising approach for the management of GBC.

The Tregs observed in GBC tumor could also be targeted using various immunotherapeutic strategies. Use of antibodies specific for CD25 (daclizumab), CTLA4 (ipilimumab), GITR, OX-40, PD-L1 or PD-1 (nivolumab) subvert the immunosuppression mediated by Treg cells and have demonstrated efficacy in clinical trials [51, 380]. Implication of combination immunotherapy, targeting both co-inhibitory and co-stimulatory molecules, is advantageous over monotherapy and capable of overcoming tumor immune tolerance ultimately leading to

tumor regression. Combination therapy using anti-OX40/anti-CTLA-4 mAb, Anti-PD1/anti PDL-1 mAb, anti-OX40/anti-PD-1 have shown improved survival and effector functions of CD4 and CD8 T cells along with depletion of Treg cells [381]. Disrupting tumor homing of Tregs by blocking CCR4 mediated migration is advantageous as it transiently inhibit Treg cells only during priming phase and avoid potential autoimmune complications caused by long term depletion of Treg cells by mAbs [53, 380].

The present study has explored the relationship of IL17 pathway, specifically Tγδ17 and GBC development and progression. We report for the first time Tγδ17 and Th17 as predictive markers in GBC and provide evidence for the proangiogenic role of human Tγδ17. The coexistence of Tγδ17, Th17 and Treg as well as cytokines other than IL17 present in the tumor microenvironment may have modulated the immune response and tilted the balance towards GBC immune evasion. Our data strongly suggests that Tγδ17 mediated angiogenesis and Treg cells mediated immunosuppression may contribute to the negative clinical outcome of GBC patients. It may be possible to manipulate γδT cell polarization *in situ* by targeting cytokines such as IL1β, IL6, IL23, and TGFβ. Thus, future immunotherapeutic treatment modality for GBC may use a combined approach to block the trafficking of Tγδ17 cells to the tumor, inhibit functions of IL17 and reverse the immunosuppression mediated by Treg cells.

Chapter 8

Summary and Conclusion

Summary and Conclusion

Gallbladder Cancer (GBC) is a relatively uncommon but lethal biliary tract related cancer. The highly aggressive nature of tumor renders dismal prognosis of patients diagnosed with GBC and show median survival of 3 to 6 months irrespective of treatment. Anatomical location of gallbladder in the abdomen and elusive early symptoms, leads to diagnosis of these patients at an advanced stage. As of today, prognostic biomarkers and effective adjuvant immunotherapy for GBC are unavailable. Targeting tumor associated antigens has not shown promising outcome in GBC. Thus novel approach is necessary for successful management of GBC. The application of immunotherapies targeting immune cells requires deeper insights of the crosstalk of immune cells with tumor cells which is lacking in GBC.

Cholelithiasis is a major risk factor of GBC which predisposes gall bladder wall to persistent inflammation. Chronic inflammation leads to dysplastic changes in gallbladder resulting into high grade premalignant carcinoma *in situ*. The murine models of gallstone disease have reported the role of eosinophils, macrophages, neutrophils and lymphocytes in gallbladder associated inflammation. The inflammatory markers such as COX-2, iNOS, inflammatory cytokines (IL1, TNF) are elevated in the gallbladder wall. In cancer associated inflammation the immune repose by innate and adaptive immunity in response to injury is subverted by tumor cells for their advantage. Thus the investigation of immune scenario (inflammatory and immunosuppressive) in GBC patients is essential to understand the pathogenesis of GBC.

With this background, the present prospective study aimed at investigating how the dynamics of proinflammatory (IL17 producing T cells) and immunosuppressive (Treg) cells contributes to inflammation and thereby progression of GBC. The key questions we asked were:

1. What is the frequency of proinflammatory (Th17, Tc17 and Tγδ17) and immunosuppressive (Treg) cells in peripheral blood and tumor tissue of GBC patients?

2. Which cytokines are involved in the regulation of these phenotypes?

3. How pro- and anti-inflammatory subtypes contribute to pathogenesis of GBC?

The immunophenotyping of peripheral blood lymphocytes in GBC patients and HI showed that the percentages of cells contributing to adaptive immunity were decreased in peripheral blood of GBC patients. PBMCs of GBC patients also showed poor proliferative response to TCR agonist and mitogen with reduced production of effector cytokines. Investigations of TCR signalling pathway in GBC patients showed downregulated expression of CD3-ξ chain in T cells compared to HI. The compromised signalling through TCR resulted in low lymphocyte proliferative response and decreased secretion of effector cytokines upon stimulation with anti-CD3 mAb and mitogen. Overall the data suggests that the immune response in GBC patients is dysfunctional.

The dysfunctional immune response and predisposition to chronic cholecystitis suggest significant role of impaired immunity and inflammation in GBC. The multicolor flowcytometry analysis of inflammatory T cells subtypes in peripheral blood and tumor tissue of GBC patients, showed that IL17 producing TCRγδ^{+} (Tγδ17), CD4^{+} (Th17), CD8^{+} (Tc17) cells were significantly increased in PBMCs of GBC patients compared to HI. The increase in the levels of Tγδ17, Th17 and Tc17 cells were irrespective of clinical stages of GBC patients. Levels of these cells were further elevated in tumor compartment than the peripheral blood of GBC patients. Thus the data highlights the significance of IL17 producing T cells in GBC patients. Interestingly Tγδ17 cells emerged as an important phenotype in GBC patients. Further it was noted that, the relative percentages and propensity to secrete IL17 were higher in Tγδ17 cells compared to Th17 and Tc17 in TILs. In contrast, γδ^{+}IFNγ^{+} cells were significantly decreased in TILs compared to PBMCs of GBC patients and HI. Survival analysis of GBC patients showed that Tγδ17 and Th17 cells in peripheral blood were

associated with poor survival of GBC patients. In contrast, the patients with high levels of $\gamma\delta^{+}IFN\gamma^{+}$ had longer overall survival than patients with low levels of $\gamma\delta^{+}IFN\gamma^{+}$ cells. Thus the data showed that IL17 is majorly contributed by γδT cells in GBC patients. It also underscored the clinical significance of Tγδ17 cells in GBC patients.

Since, the levels of Tγδ17 cells were increased in GBC patients, we investigated the cytokine milieu promoting Tγδ17 differentiation in sera and tumor environment. The cytokine profile of GBC patients showed that the cytokines (IL6, IL23, IL1β) involved in the polarization and/or stabilization of Tγδ17 cells were elevated in sera and were present in the tumor environment. We observed that, the levels of TGFβ were decreased in sera of GBC patients. Since, TGFβ is required for differentiation of Treg cells, the levels of Treg cells were investigated in peripheral blood and tumor tissue of GBC patients. The analysis of $CD4^{+}CD25^{+}CD127^{low/-}$ regulatory T cells revealed that the levels of Tregs were decreased in peripheral blood but increased in TILs of GBC patients from all clinical stages (II to IV). The expression of Foxp3 was elevated in Tregs present in tumor environment. However, the suppressive potential of Tregs in peripheral blood was comparable to HI. This indicated that although Tregs were decreased in PBMCs of GBC patients, their suppressive potential was not compromised. Moreover, the GBC patients with high peripheral blood levels of Treg were associated with poor survival. The data suggests that although the ratios of Th17/Treg, Tγδ17/Treg and Tc17/Treg were increased in TILs, IL17 producing cells and Treg cells coexist in GBC tumor environment and may contribute to poor clinical outcome of GBC patients.

The association of Tγδ17 cells with poor prognosis of GBC patients highlighted their importance in pathogenesis of GBC. To further address the functional role of Tγδ17, the expression of chemokine receptors (CCR6, CCR7, CXCR4 and CXCR3) on Th17, Tc17 and

Tγδ17 cells was analysed in peripheral blood of GBC patients. It was observed that Tγδ17 expressed elevated levels of CXCR3 than Th17 or Tc17. However, CCR6, CCR7 and CXCR4 were expressed at comparable levels by Tγδ17, Th17 and Tc17. Using neutralizing antibody for CXCR3 in a trans-well assay, it was shown that Tγδ17 cells use novel chemokine axis (CXCL9/CXCL10-CXCR3) to migrate towards GBC tumor bed.

In order to investigate the mechanism of Tγδ17 cells contributing to tumor progression in GBC patients, the cytokine profile of Tγδ17 was analysed. It was observed that Tγδ17 primarily secretes high levels of IL17 and low levels of IL2 and TNFα but did not produce other cytokines like IL4, IL6, IL10 and IFNγ. In addition, the inherent property of γδT cells to respond to innate cytokines like IL23 and IL1β may explain the current observation that Tγδ17 are major producers of IL17 than Th17. The analysis of gallbladder tumor cells (OCUG-1 and NOZ cell lines) by flow cytometry and immunofluorescence staining showed that more than 80% of gallbladder tumor cells (OCUG-1 and NOZ cell lines) expressed IL17 receptor on the surface. Thus IL17 secreted by Tγδ17 cells may act through IL17 receptor to show its effects on gallbladder tumor cells.

Next, the proangiogenic action of Tγδ17 cells on GBC was studied by human protein profiler angiogenesis array. Cell-free supernatants from Tγδ17 cells significantly upregulated secretion of angiogenesis promoting factors from OCUG-1 cells such as VEGF, uPA, MMP9, MCP1, GM-CSF, CXCL16, coagulation factor III, angiogenin, etc. This effect was abrogated by addition of anti-IL17 mAb. The proangiogenic effects of Tγδ17 were validated by chorioallantoic membrane (CAM) assay. Cell-free supernatants of Tγδ17 enhanced vascularization of CAM and the effect was mediated through secretion of IL17. Next to study whether IL17 has direct role in GBC promotion, GBC cells were treated with rhIL17 and analysed for their proliferation, migration and invasion potential. It was observed that

addition of rhIL17 to gallbladder tumor cells (OCUG-1) induced proliferation, migration, matrigel invasion and VEGF production in concentration dependent manner. Thus the results highlighted that Tγδ17 is a proangiogenic subtype of γδT cells and explains the correlation of this subset with poor prognosis of GBC patients.

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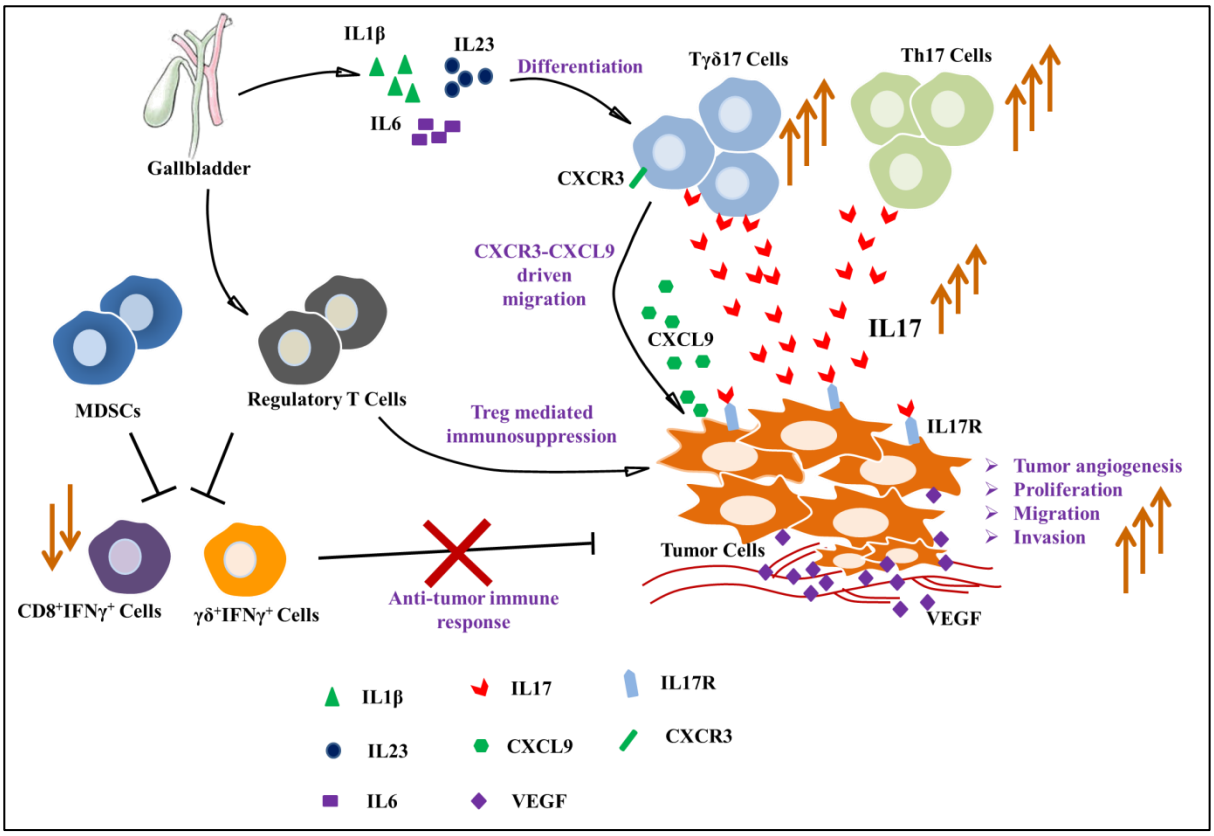

Figure 8.1 : Role of pro-inflammatory (Tγδ17, Th17 and Tc17) cells and immunosuppressive (Treg) cells in progression of GBC. *The proinflammatory cytokines (IL1β, IL6, IL23) released by gallbladder tissue promotes differentiation of Tγδ17 cells.CXCL9 secreted by gallbladder tumor cells induces migration of Tγδ17 cells to the tumor bed through CXCR3-CXCL9 chemokine axis.. The IL17 secreted by Tγδ17 cells induce proangiogenic factor such as VEGF in gallbladder tumor cells and promotes angiogenesis, migration and invasion of tumour cells. The Treg cells and MDSCs present in the tumor environment suppress the IFNγ producing CD8 and γδT cells thereby inhibit antitumor immune responses. Overall proinflammatory and immunosuppressive cells contribute to gallbladder tumor progression*

In conclusion, the inflammatory tumor environment fosters the development and recruitment of Tγδ17 and Th17 cells to the tumor bed (Figure 8.1). Increased levels of Treg cells and reduced levels of $CD8^{+}IFN\gamma^{+}$ and $\gamma\delta^{+}IFN\gamma^{+}$ cells in the tumor environment suggests the immunosuppression of antitumor immune response. IL17 secreted by Tγδ17 cells induce proangiogenic factors in GBC tumor cells through IL17 receptor and may promote the migration and invasion of GBC. Thus our data unravelled Tγδ17 as a "druggable immune target" that plays an important role in GBC progression.

γδT cells have been conventionally shown to exhibit broad anti-tumor effects thus it is important to expand our understanding to exploit them for successful immunotherapy of cancer. γδT cells based immunotherapy, although has shown safety and efficacy, IL17 producing γδ cells associate with poor prognosis of patients. Our studies suggest that Tγδ17 can be viewed as opposite of γδT cells. The pro-tumor functions of Tγδ17 cells suggest that it will be important to evaluate the stable functional polarization of effector γδT cells during adoptive transfer of γδT cells. Targeting Tγδ17 cells and IL17 inflammatory axis holds significance in future immunotherapeutic interventions. For favourable results of γδT cells based immunotherapy, clinical protocols should maximize IFNγ production and minimize IL17 secretion. Combination of γδT cells with immunomodulatory antibodies targeting inflammatory cytokines may also be a promising approach. In this context the data presented in the thesis adds a new dimension to the understanding of GBC pathogenesis and effectively using γδT cells for cancer immunotherapy.

Chapter 9

Bibliography

1. D'Souza, N.D., N.S. Murthy, and R.Y. Aras, *Projection of cancer incident cases for India -till 2026.* Asian Pac J Cancer Prev, 2013. **14**(7): p. 4379-86.
2. Mallath, M.K., et al., *The growing burden of cancer in India: epidemiology and social context.* Lancet Oncol, 2014. **15**(6): p. e205-12.
3. Torre, L.A., et al., *Global cancer statistics, 2012.* CA Cancer J Clin, 2015. **65**(2): p. 87-108.
4. Hundal, R. and E.A. Shaffer, *Gallbladder cancer: epidemiology and outcome.* Clin Epidemiol, 2014. **6**: p. 99-109.
5. Boutros, C., et al., *Gallbladder cancer: past, present and an uncertain future.* Surg Oncol, 2012. **21**(4): p. e183-91.
6. Randi, G., S. Franceschi, and C. La Vecchia, *Gallbladder cancer worldwide: geographical distribution and risk factors.* Int J Cancer, 2006. **118**(7): p. 1591-602.
7. Murthy1, N.S., et al., *Trends in incidence of gallbladder cancer – Indian scenario.* Gastrointestinal Cancer: Targets and Therapy, 2011. **1**: p. 1-9.
8. Stinton, L.M. and E.A. Shaffer, *Epidemiology of gallbladder disease: cholelithiasis and cancer.* Gut Liver, 2012. **6**(2): p. 172-87.
9. Wistuba, II and A.F. Gazdar, *Gallbladder cancer: lessons from a rare tumour.* Nat Rev Cancer, 2004. **4**(9): p. 695-706.
10. Li, Y., J. Zhang, and H. Ma, *Chronic inflammation and gallbladder cancer.* Cancer Lett, 2014. **345**(2): p. 242-8.
11. Dutta, U., *Gallbladder cancer: can newer insights improve the outcome?* J Gastroenterol Hepatol, 2012. **27**(4): p. 642-53.
12. Zhu, A.X., et al., *Current management of gallbladder carcinoma.* Oncologist, 2010. **15**(2): p. 168-81.
13. Shukla, H.S., et al., *Indian Council of Medical Research consensus document for the management of gall bladder cancer.* Indian J Med Paediatr Oncol, 2015. **36**(2): p. 79-84.
14. Sirohi, B., et al., *Neoadjuvant chemotherapy in patients with locally advanced gallbladder cancer.* Future Oncol, 2015. **11**(10): p. 1501-9.
15. Han, H.S., et al., *Preoperative inflammation is a prognostic factor for gallbladder carcinoma.* Br J Surg, 2011. **98**(1): p. 111-6.
16. van Erpecum, K.J., et al., *Gallbladder histopathology during murine gallstone formation: relation to motility and concentrating function.* J Lipid Res, 2006. **47**(1): p. 32-41.
17. Wu, T., et al., *Gut microbiota dysbiosis and bacterial community assembly associated with cholesterol gallstones in large-scale study.* BMC Genomics, 2013. **14**: p. 669.
18. Sithithaworn, P., et al., *Roles of liver fluke infection as risk factor for cholangiocarcinoma.* J Hepatobiliary Pancreat Sci, 2014. **21**(5): p. 301-8.
19. Xia, J., S.C. Jiang, and H.J. Peng, *Association between Liver Fluke Infection and Hepatobiliary Pathological Changes: A Systematic Review and Meta-Analysis.* PLoS One, 2015. **10**(7): p. e0132673.
20. Espinoza, J.A., et al., *The inflammatory inception of gallbladder cancer.* Biochim Biophys Acta, 2016. **1865**(2): p. 245-54.
21. Zhi, Y.H., et al., *Cyclooxygenase-2 promotes angiogenesis by increasing vascular endothelial growth factor and predicts prognosis in gallbladder carcinoma.* World J Gastroenterol, 2005. **11**(24): p. 3724-8.
22. Chen, Y., et al., *Vascular endothelial growth factor-C promotes the growth and invasion of gallbladder cancer via an autocrine mechanism.* Mol Cell Biochem, 2010. **345**(1-2): p. 77-89.

23. Oguro, S., et al., *Clinical significance of tumor-infiltrating immune cells focusing on BTLA and Cbl-b in patients with gallbladder cancer.* Cancer Sci, 2015. **106**(12): p. 1750-60.
24. Iwakura, Y., et al., *Functional specialization of interleukin-17 family members.* Immunity, 2011. **34**(2): p. 149-62.
25. Sabat, R., et al., *IL-22 and IL-17: An Overview*, in *IL-17, IL-22 and Their Producing Cells: Role in Inflammation and Autoimmunity*, V. Quesniaux, B. Ryffel, and F. Padova, Editors. 2013, Springer Basel. p. 11-35.
26. Miossec, P. and J.K. Kolls, *Targeting IL-17 and TH17 cells in chronic inflammation.* Nat Rev Drug Discov, 2012. **11**(10): p. 763-76.
27. 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.
28. Chen, X., et al., *Increased IL-17-producing cells correlate with poor survival and lymphangiogenesis in NSCLC patients.* Lung Cancer, 2010. **69**(3): p. 348-54.
29. Liu, J., et al., *IL-17 is associated with poor prognosis and promotes angiogenesis via stimulating VEGF production of cancer cells in colorectal carcinoma.* Biochem Biophys Res Commun, 2011. **407**(2): p. 348-54.
30. Zarogoulidis, P., et al., *Interleukin-8 and interleukin-17 for cancer.* Cancer Invest, 2014. **32**(5): p. 197-205.
31. Cochaud, S., et al., *IL-17A is produced by breast cancer TILs and promotes chemoresistance and proliferation through ERK1/2.* Sci Rep, 2013. **3**: p. 3456.
32. Fossiez, F., et al., *T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines.* J Exp Med, 1996. **183**(6): p. 2593-603.
33. Zhou, L., et al., *IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways.* Nat Immunol, 2007. **8**(9): p. 967-74.
34. Dong, C., *Genetic controls of Th17 cell differentiation and plasticity.* Exp Mol Med, 2011. **43**(1): p. 1-6.
35. He, Z., et al., *Ubiquitination of RORgammat at Lysine 446 Limits Th17 Differentiation by Controlling Coactivator Recruitment.* J Immunol, 2016. **197**(4): p. 1148-58.
36. Kane, A., et al., *STAT3 is a central regulator of lymphocyte differentiation and function.* Curr Opin Immunol, 2014. **28**: p. 49-57.
37. Wei, L., et al., *IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner.* J Biol Chem, 2007. **282**(48): p. 34605-10.
38. Ye, Z.J., et al., *Generation and differentiation of IL-17-producing CD4+ T cells in malignant pleural effusion.* J Immunol, 2010. **185**(10): p. 6348-54.
39. Su, X., et al., *Tumor microenvironments direct the recruitment and expansion of human Th17 cells.* J Immunol, 2010. **184**(3): p. 1630-41.
40. Wilke, C. and W. Zou, *TH17 Cells in the Tumor Micro-environment*, in *TH17 Cells in Health and Disease*, S. Jiang, Editor 2011, Springer New York. p. 481-496.
41. Martin, F., L. Apetoh, and F. Ghiringhelli, *Controversies on the role of Th17 in cancer: a TGF-beta-dependent immunosuppressive activity?* Trends Mol Med, 2012. **18**(12): p. 742-9.
42. Kesselring, R., et al., *Human Th17 cells can be induced through head and neck cancer and have a functional impact on HNSCC development.* Br J Cancer, 2010. **103**(8): p. 1245-54.
43. Zhao, F., et al., *Human CCR4+ CCR6+ Th17 cells suppress autologous CD8+ T cell responses.* J Immunol, 2012. **188**(12): p. 6055-62.
44. Chauhan, S.K., et al., *A novel pro-lymphangiogenic function for Th17/IL-17.* Blood, 2011. **118**(17): p. 4630-4.

45. Kryczek, I., et al., *Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments.* Blood, 2009. **114**(6): p. 1141-9.
46. Lv, L., et al., *The accumulation and prognosis value of tumor infiltrating IL-17 producing cells in esophageal squamous cell carcinoma.* PLoS One, 2011. **6**(3): p. e18219.
47. Du, J., et al., *Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3.* J Immunol, 2008. **180**(7): p. 4785-92.
48. Muranski, P. and N.P. Restifo, *Essentials of Th17 cell commitment and plasticity.* Blood, 2013. **121**(13): p. 2402-14.
49. Chalmin, F., et al., *Stat3 and Gfi-1 transcription factors control Th17 cell immunosuppressive activity via the regulation of ectonucleotidase expression.* Immunity, 2012. **36**(3): p. 362-73.
50. Bailey, S.R., et al., *Th17 cells in cancer: the ultimate identity crisis.* Front Immunol, 2014. **5**: p. 276.
51. Nishikawa, H. and S. Sakaguchi, *Regulatory T cells in cancer immunotherapy.* Curr Opin Immunol, 2014. **27**: p. 1-7.
52. Shevach, E.M., *Mechanisms of foxp3+ T regulatory cell-mediated suppression.* Immunity, 2009. **30**(5): p. 636-45.
53. Bayry, J., E. Tartour, and D.F. Tough, *Targeting CCR4 as an emerging strategy for cancer therapy and vaccines.* Trends Pharmacol Sci, 2014. **35**(4): p. 163-5.
54. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-99.
55. Shalapour, S. and M. Karin, *Immunity, inflammation, and cancer: an eternal fight between good and evil.* J Clin Invest, 2015. **125**(9): p. 3347-55.
56. Fridman, W.H., et al., *The immune contexture in human tumours: impact on clinical outcome.* Nat Rev Cancer, 2012. **12**(4): p. 298-306.
57. Kimura, A. and T. Kishimoto, *IL-6: regulator of Treg/Th17 balance.* Eur J Immunol, 2010. **40**(7): p. 1830-5.
58. Sfanos, K.S., et al., *Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing.* Clin Cancer Res, 2008. **14**(11): p. 3254-61.
59. Chen, Z., et al., *The Th17/Treg balance and the expression of related cytokines in Uygur cervical cancer patients.* Diagn Pathol, 2013. **8**: p. 61.
60. Duan, M.C., et al., *The Treg/Th17 paradigm in lung cancer.* J Immunol Res, 2014. **2014**: p. 730380.
61. Pilgrim, C.H., V. Usatoff, and P. Evans, *Consideration of anatomical structures relevant to the surgical strategy for managing gallbladder carcinoma.* Eur J Surg Oncol, 2009. **35**(11): p. 1131-6.
62. Lai, C.H. and W.Y. Lau, *Gallbladder cancer--a comprehensive review.* Surgeon, 2008. **6**(2): p. 101-10.
63. Yamaguchi, K., et al., *Retrospective analysis of 70 operations for gallbladder carcinoma.* Br J Surg, 1997. **84**(2): p. 200-4.
64. Duffy, A., et al., *Gallbladder cancer (GBC): 10-year experience at Memorial Sloan-Kettering Cancer Centre (MSKCC).* J Surg Oncol, 2008. **98**(7): p. 485-9.
65. Nevin, J.E., et al., *Carcinoma of the gallbladder: staging, treatment, and prognosis.* Cancer, 1976. **37**(1): p. 141-8.
66. Onoyama, H., et al., *Extended cholecystectomy for carcinoma of the gallbladder.* World J Surg, 1995. **19**(5): p. 758-63.
67. Jayaraman, S. and W.R. Jarnagin, *Management of gallbladder cancer.* Gastroenterol Clin North Am, 2010. **39**(2): p. 331-42, x.

68. Henson, D.E., J. Albores-Saavedra, and D. Corle, *Carcinoma of the gallbladder. Histologic types, stage of disease, grade, and survival rates.* Cancer, 1992. **70**(6): p. 1493-7.
69. Andia, M.E., et al., *Geographic variation of gallbladder cancer mortality and risk factors in Chile: a population-based ecologic study.* Int J Cancer, 2008. **123**(6): p. 1411-6.
70. Sachidananda, S., et al., *Characteristics of gallbladder cancer in South India.* Indian J Surg Oncol, 2012. **3**(3): p. 228-30.
71. Jain, K., et al., *Risk factors for gallbladder cancer: a case-control study.* Int J Cancer, 2013. **132**(7): p. 1660-6.
72. Basu, S., et al., *Heavy and trace metals in carcinoma of the gallbladder.* World J Surg, 2013. **37**(11): p. 2641-6.
73. Mustafa Ahmed Barbhuiya, T.D.S., Sanjeev Gupta,Braj Raj Shrivastav,Pramod Kumar Tiwari, *Incidence of gall bladder cancer in rural and semi-urban population of north central India: A first insight.* The Internet Journal of Epidemiology, 2009. **7**(2): p. 1-14.
74. Unisa, S., et al., *Population-based study to estimate prevalence and determine risk factors of gallbladder diseases in the rural Gangetic basin of North India.* HPB (Oxford), 2011. **13**(2): p. 117-25.
75. Barbhuiya, M.A., et al., *Gallbladder cancer incidence in Gwalior district of India: Five-year trend based on the registry of a regional cancer center.* Indian J Cancer, 2015. **52**(3): p. 430-7.
76. Takiar, R., D. Nadayil, and A. Nandakumar, *Projections of number of cancer cases in India (2010-2020) by cancer groups.* Asian Pac J Cancer Prev, 2010. **11**(4): p. 1045-9.
77. Kanthan, R., et al., *Gallbladder Cancer in the 21st Century.* J Oncol, 2015. **2015**: p. 967472.
78. Shipra Dwivedi, A.M., Devendra Singh, Shraddha Singh, Akhilesh Krishna, *Gall Bladder Cancer and some epidemiological factors: A cross sectional study.* Biomedical Research, 2013. **24**(1): p. 83-87.
79. Barreto, S.G., H. Haga, and P.J. Shukla, *Hormones and gallbladder cancer in women.* Indian J Gastroenterol, 2009. **28**(4): p. 126-30.
80. Shrikhande, S.V., et al., *Cholelithiasis in gallbladder cancer: coincidence, cofactor, or cause!* Eur J Surg Oncol, 2010. **36**(6): p. 514-9.
81. Roa, I., et al., *Gallstones and gallbladder cancer-volume and weight of gallstones are associated with gallbladder cancer: a case-control study.* J Surg Oncol, 2006. **93**(8): p. 624-8.
82. Diehl, A.K., *Gallstone size and the risk of gallbladder cancer.* JAMA, 1983. **250**(17): p. 2323-6.
83. Portincasa, P., et al., *Therapy of gallstone disease: What it was, what it is, what it will be.* World J Gastrointest Pharmacol Ther, 2012. **3**(2): p. 7-20.
84. Krawczyk, M., et al., *Dissecting the genetic heterogeneity of gallbladder stone formation.* Semin Liver Dis, 2011. **31**(2): p. 157-72.
85. von Kampen, O., et al., *Genetic and functional identification of the likely causative variant for cholesterol gallstone disease at the ABCG5/8 lithogenic locus.* Hepatology, 2013. **57**(6): p. 2407-17.
86. Albores-Saavedra, J., et al., *The precursor lesions of invasive gallbladder carcinoma. Hyperplasia, atypical hyperplasia and carcinoma in situ.* Cancer, 1980. **45**(5): p. 919-27.

87. Slack, J.M., *Metaplasia and somatic cell reprogramming.* J Pathol, 2009. **217**(2): p. 161-8.
88. Seretis, C., et al., *Metaplastic changes in chronic cholecystitis: implications for early diagnosis and surgical intervention to prevent the gallbladder metaplasia-dysplasia-carcinoma sequence.* J Clin Med Res, 2014. **6**(1): p. 26-9.
89. Kim, H., et al., *Strong cytoplasmic expression of COX2 at the invasive fronts of gallbladder cancer is associated with a poor prognosis.* J Clin Pathol, 2010. **63**(12): p. 1048-53.
90. Liu, E., et al., *Aspirin use and risk of biliary tract cancer: a population-based study in Shanghai, China.* Cancer Epidemiol Biomarkers Prev, 2005. **14**(5): p. 1315-8.
91. Rege, R.V. and J.B. Prystowsky, *Inflammation and a thickened mucus layer in mice with cholesterol gallstones.* J Surg Res, 1998. **74**(1): p. 81-5.
92. Lavoie, B., et al., *Disruption of gallbladder smooth muscle function is an early feature in the development of cholesterol gallstone disease.* Neurogastroenterol Motil, 2012. **24**(7): p. e313-24.
93. Maurer, K.J., et al., *T-cell function is critical for murine cholesterol gallstone formation.* Gastroenterology, 2007. **133**(4): p. 1304-15.
94. Carotti, S., et al., *Effect of ursodeoxycholic acid on inflammatory infiltrate in gallbladder muscle of cholesterol gallstone patients.* Neurogastroenterol Motil, 2010. **22**(8): p. 866-73, e232.
95. Miquel, J.F., et al., *Increased activity in the biliary Con A-binding fraction accounts for the difference in crystallization behavior in bile from Chilean gallstone patients compared with Dutch gallstone patients.* Hepatology, 2001. **33**(2): p. 328-32.
96. Elinav, E., et al., *Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms.* Nat Rev Cancer, 2013. **13**(11): p. 759-71.
97. Kumar, S., *Infection as a risk factor for gallbladder cancer.* J Surg Oncol, 2006. **93**(8): p. 633-9.
98. Dutta, U., et al., *Typhoid carriers among patients with gallstones are at increased risk for carcinoma of the gallbladder.* Am J Gastroenterol, 2000. **95**(3): p. 784-7.
99. Shukla, V.K., et al., *Carcinoma of the gallbladder--is it a sequel of typhoid?* Dig Dis Sci, 2000. **45**(5): p. 900-3.
100. Nath, G., H. Singh, and V.K. Shukla, *Chronic typhoid carriage and carcinoma of the gallbladder.* Eur J Cancer Prev, 1997. **6**(6): p. 557-9.
101. Nath, G., et al., *Association of carcinoma of the gallbladder with typhoid carriage in a typhoid endemic area using nested PCR.* J Infect Dev Ctries, 2008. **2**(4): p. 302-7.
102. Scanu, T., et al., *Salmonella Manipulation of Host Signaling Pathways Provokes Cellular Transformation Associated with Gallbladder Carcinoma.* Cell Host Microbe, 2015. **17**(6): p. 763-74.
103. Lee, J.W., et al., *Identification of Helicobacter pylori in Gallstone, Bile, and Other Hepatobiliary Tissues of Patients with Cholecystitis.* Gut Liver, 2010. **4**(1): p. 60-7.
104. Sabbaghian, M.S., et al., *Identification of Helicobacter spp. in bile and gallbladder tissue of patients with symptomatic gallbladder disease.* HPB (Oxford), 2010. **12**(2): p. 129-33.
105. Bansal, V.K., et al., *Helicobacter pylori in gallbladder mucosa in patients with gallbladder disease.* Indian J Gastroenterol, 2012. **31**(2): p. 57-60.
106. Takayama, S., et al., *Effect of Helicobacter bilis infection on human bile duct cancer cells.* Dig Dis Sci, 2010. **55**(7): p. 1905-10.
107. Mishra, R.R., M. Tewari, and H.S. Shukla, *Association of Helicobacter pylori infection with inflammatory cytokine expression in patients with gallbladder cancer.* Indian J Gastroenterol, 2013. **32**(4): p. 232-5.

108. Pandey, M., et al., *Helicobacter bilis in human gallbladder cancer: results of a case-control study and a meta-analysis.* Asian Pac J Cancer Prev, 2010. **11**(2): p. 343-7.
109. Mishra, R.R., M. Tewari, and H.S. Shukla, *Helicobacter pylori and pathogenesis of gallbladder cancer.* J Gastroenterol Hepatol, 2011. **26**(2): p. 260-6.
110. Misra, S., et al., *Carcinoma of the gallbladder.* Lancet Oncol, 2003. **4**(3): p. 167-76.
111. Lee, S.E., et al., *Systematic review on the surgical treatment for T1 gallbladder cancer.* World J Gastroenterol, 2011. **17**(2): p. 174-80.
112. Valle, J.W., et al., *Cisplatin and gemcitabine for advanced biliary tract cancer: a meta-analysis of two randomised trials.* Ann Oncol, 2014. **25**(2): p. 391-8.
113. Marino, D., et al., *Biliary tract carcinomas: from chemotherapy to targeted therapy.* Crit Rev Oncol Hematol, 2013. **85**(2): p. 136-48.
114. Kerkar, S.P. and N.P. Restifo, *Cellular constituents of immune escape within the tumor microenvironment.* Cancer Res, 2012. **72**(13): p. 3125-30.
115. Burnet, M., *Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications.* Br Med J, 1957. **1**(5023): p. 841-7.
116. Dunn, G.P., L.J. Old, and R.D. Schreiber, *The three Es of cancer immunoediting.* Annu Rev Immunol, 2004. **22**: p. 329-60.
117. 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.
118. Fuertes, M.B., et al., *Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells.* J Exp Med, 2011. **208**(10): p. 2005-16.
119. Mittal, D., et al., *New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape.* Curr Opin Immunol, 2014. **27**: p. 16-25.
120. 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.
121. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
122. Schumacker, P.T., *Reactive oxygen species in cancer: a dance with the devil.* Cancer Cell, 2015. **27**(2): p. 156-7.
123. Gupta, S.C., et al., *Upsides and downsides of reactive oxygen species for cancer: the roles of reactive oxygen species in tumorigenesis, prevention, and therapy.* Antioxid Redox Signal, 2012. **16**(11): p. 1295-322.
124. Colotta, F., et al., *Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability.* Carcinogenesis, 2009. **30**(7): p. 1073-81.
125. Balkwill, F.R. and A. Mantovani, *Cancer-related inflammation: common themes and therapeutic opportunities.* Semin Cancer Biol, 2012. **22**(1): p. 33-40.
126. Taniguchi, K. and M. Karin, *IL-6 and related cytokines as the critical lynchpins between inflammation and cancer.* Semin Immunol, 2014. **26**(1): p. 54-74.
127. Palazon, A., et al., *HIF transcription factors, inflammation, and immunity.* Immunity, 2014. **41**(4): p. 518-28.
128. Semenza, G.L., *Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy.* Trends Pharmacol Sci, 2012. **33**(4): p. 207-14.
129. Katsuno, Y., S. Lamouille, and R. Derynck, *TGF-beta signaling and epithelial-mesenchymal transition in cancer progression.* Curr Opin Oncol, 2013. **25**(1): p. 76-84.
130. Li, C.W., et al., *Epithelial-mesenchymal transition induced by TNF-alpha requires NF-kappaB-mediated transcriptional upregulation of Twist1.* Cancer Res, 2012. **72**(5): p. 1290-300.

131. Wang, H., et al., *Epithelial-mesenchymal transition (EMT) induced by TNF-alpha requires AKT/GSK-3beta-mediated stabilization of snail in colorectal cancer.* PLoS One, 2013. **8**(2): p. e56664.
132. Wan, L., K. Pantel, and Y. Kang, *Tumor metastasis: moving new biological insights into the clinic.* Nat Med, 2013. **19**(11): p. 1450-64.
133. Palumbo, J.S., et al., *Tumor cell-associated tissue factor and circulating hemostatic factors cooperate to increase metastatic potential through natural killer cell-dependent and-independent mechanisms.* Blood, 2007. **110**(1): p. 133-41.
134. Rouvier, E., et al., *CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene.* J Immunol, 1993. **150**(12): p. 5445-56.
135. Chang, S.H. and C. Dong, *Signaling of interleukin-17 family cytokines in immunity and inflammation.* Cell Signal, 2011. **23**(7): p. 1069-75.
136. Moseley, T.A., et al., *Interleukin-17 family and IL-17 receptors.* Cytokine Growth Factor Rev, 2003. **14**(2): p. 155-74.
137. Yamaguchi, Y., et al., *IL-17B and IL-17C are associated with TNF-alpha production and contribute to the exacerbation of inflammatory arthritis.* J Immunol, 2007. **179**(10): p. 7128-36.
138. Zhang, X., et al., *Structure and function of interleukin-17 family cytokines.* Protein Cell, 2011. **2**(1): p. 26-40.
139. Gu, C., L. Wu, and X. Li, *IL-17 family: cytokines, receptors and signaling.* Cytokine, 2013. **64**(2): p. 477-85.
140. Toy, D., et al., *Cutting edge: interleukin 17 signals through a heteromeric receptor complex.* J Immunol, 2006. **177**(1): p. 36-9.
141. Wright, J.F., et al., *The human IL-17F/IL-17A heterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex.* J Immunol, 2008. **181**(4): p. 2799-805.
142. Liu, C., et al., *Act1, a U-box E3 ubiquitin ligase for IL-17 signaling.* Sci Signal, 2009. **2**(92): p. ra63.
143. Sun, D., et al., *Treatment with IL-17 prolongs the half-life of chemokine CXCL1 mRNA via the adaptor TRAF5 and the splicing-regulatory factor SF2 (ASF).* Nat Immunol, 2011. **12**(9): p. 853-60.
144. Herjan, T., et al., *HuR is required for IL-17-induced Act1-mediated CXCL1 and CXCL5 mRNA stabilization.* J Immunol, 2013. **191**(2): p. 640-9.
145. Zepp, J.A., et al., *Cutting edge: TNF receptor-associated factor 4 restricts IL-17-mediated pathology and signaling processes.* J Immunol, 2012. **189**(1): p. 33-7.
146. Zhu, S., et al., *Modulation of experimental autoimmune encephalomyelitis through TRAF3-mediated suppression of interleukin 17 receptor signaling.* J Exp Med, 2010. **207**(12): p. 2647-62.
147. Shen, F., et al., *IL-17 receptor signaling inhibits C/EBPbeta by sequential phosphorylation of the regulatory 2 domain.* Sci Signal, 2009. **2**(59): p. ra8.
148. Liu, B., et al., *IL-17 is a potent synergistic factor with GM-CSF in mice in stimulating myelopoiesis, dendritic cell expansion, proliferation, and functional enhancement.* Exp Hematol, 2010. **38**(10): p. 877-884 e1.
149. Parsa, R., et al., *BAFF-secreting neutrophils drive plasma cell responses during emergency granulopoiesis.* J Exp Med, 2016. **213**(8): p. 1537-53.
150. Shibata, M., et al., *The effect of IL-17 on the production of proinflammatory cytokines and matrix metalloproteinase-1 by human periodontal ligament fibroblasts.* Orthod Craniofac Res, 2014. **17**(1): p. 60-8.

151. Wang, Y., et al., *Interleukin 17A promotes gastric cancer invasiveness via NF-kappaB mediated matrix metalloproteinases 2 and 9 expression.* PLoS One, 2014. **9**(6): p. e96678.
152. Rafiei, A., et al., *Polymorphism in the interleukin-17A promoter contributes to gastric cancer.* World J Gastroenterol, 2013. **19**(34): p. 5693-9.
153. Gu, F.M., et al., *IL-17 induces AKT-dependent IL-6/JAK2/STAT3 activation and tumor progression in hepatocellular carcinoma.* Mol Cancer, 2011. **10**: p. 150.
154. Wang, L., et al., *IL-17 enhances tumor development in carcinogen-induced skin cancer.* Cancer Res, 2010. **70**(24): p. 10112-20.
155. Straus, D.S., *TNFalpha and IL-17 cooperatively stimulate glucose metabolism and growth factor production in human colorectal cancer cells.* Mol Cancer, 2013. **12**: p. 78.
156. Zhu, X., et al., *IL-17 expression by breast-cancer-associated macrophages: IL-17 promotes invasiveness of breast cancer cell lines.* Breast Cancer Res, 2008. **10**(6): p. R95.
157. Cui, X., et al., *Analysis of CD137L and IL-17 expression in tumor tissue as prognostic indicators for gliblastoma.* Int J Biol Sci, 2013. **9**(2): p. 134-41.
158. Lan, C., et al., *High density of IL-17-producing cells is associated with improved prognosis for advanced epithelial ovarian cancer.* Cell Tissue Res, 2013. **352**(2): p. 351-9.
159. Droeser, R.A., et al., *High IL-17-positive tumor immune cell infiltration is indicative for chemosensitivity of ovarian carcinoma.* J Cancer Res Clin Oncol, 2013. **139**(8): p. 1295-302.
160. Korn, T., et al., *IL-17 and Th17 Cells.* Annu Rev Immunol, 2009. **27**: p. 485-517.
161. Dong, C., *TH17 cells in development: an updated view of their molecular identity and genetic programming.* Nat Rev Immunol, 2008. **8**(5): p. 337-48.
162. Liang, S.C., et al., *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* J Exp Med, 2006. **203**(10): p. 2271-9.
163. Ivanov, II, et al., *The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell, 2006. **126**(6): p. 1121-33.
164. Ivanov, II, L. Zhou, and D.R. Littman, *Transcriptional regulation of Th17 cell differentiation.* Semin Immunol, 2007. **19**(6): p. 409-17.
165. Veldhoen, M., et al., *The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins.* Nature, 2008. **453**(7191): p. 106-9.
166. Ciofani, M., et al., *A validated regulatory network for Th17 cell specification.* Cell, 2012. **151**(2): p. 289-303.
167. Mangan, P.R., et al., *Transforming growth factor-beta induces development of the T(H)17 lineage.* Nature, 2006. **441**(7090): p. 231-4.
168. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
169. Yang, X.O., et al., *STAT3 regulates cytokine-mediated generation of inflammatory helper T cells.* J Biol Chem, 2007. **282**(13): p. 9358-63.
170. Durant, L., et al., *Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis.* Immunity, 2010. **32**(5): p. 605-15.
171. Peters, A., et al., *IL-27 Induces Th17 Differentiation in the Absence of STAT1 Signaling.* J Immunol, 2015. **195**(9): p. 4144-53.
172. McGeachy, M.J., 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): p. 314-24.

173. Ghoreschi, K., et al., *Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling.* Nature, 2010. **467**(7318): p. 967-71.
174. Codarri, L., et al., *RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation.* Nat Immunol, 2011. **12**(6): p. 560-7.
175. Das, J., et al., *Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation.* J Exp Med, 2009. **206**(11): p. 2407-16.
176. Gutcher, I., et al., *Autocrine transforming growth factor-beta1 promotes in vivo Th17 cell differentiation.* Immunity, 2011. **34**(3): p. 396-408.
177. Zuniga, L.A., et al., *Th17 cell development: from the cradle to the grave.* Immunol Rev, 2013. **252**(1): p. 78-88.
178. Lee, Y., et al., *Induction and molecular signature of pathogenic TH17 cells.* Nat Immunol, 2012. **13**(10): p. 991-9.
179. Laurence, A., et al., *Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation.* Immunity, 2007. **26**(3): p. 371-81.
180. Kim, J.S., et al., *Natural and inducible TH17 cells are regulated differently by Akt and mTOR pathways.* Nat Immunol, 2013. **14**(6): p. 611-8.
181. Marks, B.R., et al., *Thymic self-reactivity selects natural interleukin 17-producing T cells that can regulate peripheral inflammation.* Nat Immunol, 2009. **10**(10): p. 1125-32.
182. Jenkinson, W.E., et al., *Natural Th17 cells are critically regulated by functional medullary thymic microenvironments.* J Autoimmun, 2015. **63**: p. 13-22.
183. Smith, E., et al., *T-lineage cells require the thymus but not VDJ recombination to produce IL-17A and regulate granulopoiesis in vivo.* J Immunol, 2009. **183**(9): p. 5685-93.
184. Peters, A., Y. Lee, and V.K. Kuchroo, *The many faces of Th17 cells.* Curr Opin Immunol, 2011. **23**(6): p. 702-6.
185. Hirota, K., et al., *Fate mapping of IL-17-producing T cells in inflammatory responses.* Nat Immunol, 2011. **12**(3): p. 255-63.
186. Lazarevic, V., et al., *T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat.* Nat Immunol, 2011. **12**(1): p. 96-104.
187. Cosmi, L., et al., *Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor.* J Exp Med, 2008. **205**(8): p. 1903-16.
188. Wang, Y., et al., *The transcription factors T-bet and Runx are required for the ontogeny of pathogenic interferon-gamma-producing T helper 17 cells.* Immunity, 2014. **40**(3): p. 355-66.
189. Musuraca, G., et al., *IL-17/IL-10 double-producing T cells: new link between infections, immunosuppression and acute myeloid leukemia.* J Transl Med, 2015. **13**: p. 229.
190. Ahern, P.P., et al., *Interleukin-23 drives intestinal inflammation through direct activity on T cells.* Immunity, 2010. **33**(2): p. 279-88.
191. McGeachy, M.J., et al., *TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology.* Nat Immunol, 2007. **8**(12): p. 1390-7.
192. Zielinski, C.E., et al., *Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta.* Nature, 2012. **484**(7395): p. 514-8.
193. Stewart, C.A., et al., *Interferon-dependent IL-10 production by Tregs limits tumor Th17 inflammation.* J Clin Invest, 2013. **123**(11): p. 4859-74.

194. Huber, S., et al., *Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner.* Immunity, 2011. **34**(4): p. 554-65.
195. El-Behi, M., et al., *The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF.* Nat Immunol, 2011. **12**(6): p. 568-75.
196. Noster, R., et al., *IL-17 and GM-CSF expression are antagonistically regulated by human T helper cells.* Sci Transl Med, 2014. **6**(241): p. 241ra80.
197. Punt, S., et al., *The correlations between IL-17 vs. Th17 cells and cancer patient survival: a systematic review.* Oncoimmunology, 2015. **4**(2): p. e984547.
198. Ye, J., R.S. Livergood, and G. Peng, *The role and regulation of human Th17 cells in tumor immunity.* Am J Pathol, 2013. **182**(1): p. 10-20.
199. Yu, Q., X.M. Lou, and Y. He, *Preferential recruitment of Th17 cells to cervical cancer via CCR6-CCL20 pathway.* PLoS One, 2015. **10**(3): p. e0120855.
200. Walch-Ruckheim, B., et al., *Stromal Fibroblasts Induce CCL20 through IL6/C/EBPbeta to Support the Recruitment of Th17 Cells during Cervical Cancer Progression.* Cancer Res, 2015. **75**(24): p. 5248-59.
201. Iida, T., et al., *Tumor-infiltrating CD4+ Th17 cells produce IL-17 in tumor microenvironment and promote tumor progression in human gastric cancer.* Oncol Rep, 2011. **25**(5): p. 1271-7.
202. Kryczek, I., et al., *Endogenous IL-17 contributes to reduced tumor growth and metastasis.* Blood, 2009. **114**(2): p. 357-9.
203. Muranski, P., et al., *Tumor-specific Th17-polarized cells eradicate large established melanoma.* Blood, 2008. **112**(2): p. 362-73.
204. Martin-Orozco, N., et al., *T helper 17 cells promote cytotoxic T cell activation in tumor immunity.* Immunity, 2009. **31**(5): p. 787-98.
205. Gnerlich, J.L., et al., *Induction of Th17 cells in the tumor microenvironment improves survival in a murine model of pancreatic cancer.* J Immunol, 2010. **185**(7): p. 4063-71.
206. Sharma, M.D., et al., *Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes.* Blood, 2009. **113**(24): p. 6102-11.
207. Pellegrini, M., et al., *Adjuvant IL-7 antagonizes multiple cellular and molecular inhibitory networks to enhance immunotherapies.* Nat Med, 2009. **15**(5): p. 528-36.
208. Punt, S., et al., *Angels and demons: Th17 cells represent a beneficial response, while neutrophil IL-17 is associated with poor prognosis in squamous cervical cancer.* Oncoimmunology, 2015. **4**(1): p. e984539.
209. Gong, Y., et al., *Cell origins and significance of IL-17 in malignant pleural effusion.* Clin Transl Oncol, 2014. **16**(9): p. 807-13.
210. Jain, P., et al., *Th17 and non-Th17 interleukin-17-expressing cells in chronic lymphocytic leukemia: delineation, distribution, and clinical relevance.* Haematologica, 2012. **97**(4): p. 599-607.
211. Abousamra, N.K., M. Salah El-Din, and R. Helal, *Prognostic value of Th17 cells in acute leukemia.* Med Oncol, 2013. **30**(4): p. 732.
212. Vantourout, P. and A. Hayday, *Six-of-the-best: unique contributions of gammadelta T cells to immunology.* Nat Rev Immunol, 2013. **13**(2): p. 88-100.
213. 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.

214. Silva-Santos, B., K. Serre, and H. Norell, *gammadelta T cells in cancer.* Nat Rev Immunol, 2015. **15**(11): p. 683-91.
215. Born, W.K., M. Kemal Aydintug, and R.L. O'Brien, *Diversity of gammadelta T-cell antigens.* Cell Mol Immunol, 2013. **10**(1): p. 13-20.
216. Buhaescu, I. and H. Izzedine, *Mevalonate pathway: a review of clinical and therapeutical implications.* Clin Biochem, 2007. **40**(9-10): p. 575-84.
217. Hunter, W.N., *The non-mevalonate pathway of isoprenoid precursor biosynthesis.* J Biol Chem, 2007. **282**(30): p. 21573-7.
218. Groh, V., et al., *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): p. 6879-84.
219. Christian Belmant, D.D., Jean-Jacques Fournié, *Phosphoantigens and aminobisphosphonates: New leads targeting γδ T lymphocytes for cancer immunotherapy.* Drug Discovery Today: Therapeutic Strategies, 2006. **3**(1): p. 17–23.
220. Thomas, M.L., et al., *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): p. 653-9.
221. Laad, A.D., et al., *Human gamma delta T cells recognize heat shock protein-60 on oral tumor cells.* Int J Cancer, 1999. **80**(5): p. 709-14.
222. Prinz, I., et al., *Visualization of the earliest steps of gammadelta T cell development in the adult thymus.* Nat Immunol, 2006. **7**(9): p. 995-1003.
223. Garcia-Peydro, M., V.G. de Yebenes, and M.L. Toribio, *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): p. 2444-51.
224. Van Coppernolle, S., 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): p. 127-38.
225. 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.
226. Zhang, H., et al., *Membrane HSP70: the molecule triggering gammadelta T cells in the early stage of tumorigenesis.* Immunol Invest, 2005. **34**(4): p. 453-68.
227. Ansel, K.M., et al., *A chemokine-driven positive feedback loop organizes lymphoid follicles.* Nature, 2000. **406**(6793): p. 309-14.
228. Conti, L., 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): p. 252-60.
229. Brandes, 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): p. 2307-12.
230. Himoudi, N., et al., *Human gammadelta T lymphocytes are licensed for professional antigen presentation by interaction with opsonized target cells.* J Immunol, 2012. **188**(4): p. 1708-16.
231. Brandes, M., K. Willimann, and B. Moser, *Professional antigen-presentation function by human gammadelta T Cells.* Science, 2005. **309**(5732): p. 264-8.
232. Qin, G., 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): p. 858-65.
233. Dhar, S. and S.V. Chiplunkar, *Lysis of aminobisphosphonate-sensitized MCF-7 breast tumor cells by Vgamma9Vdelta2 T cells.* Cancer Immun, 2010. **10**: p. 10.
234. Fisher, J.P., et al., *gammadelta T cells for cancer immunotherapy: A systematic review of clinical trials.* Oncoimmunology, 2014. **3**(1): p. e27572.

235. Daley, D., et al., *gammadelta T Cells Support Pancreatic Oncogenesis by Restraining alphabeta T Cell Activation.* Cell, 2016. **166**(6): p. 1485-1499 e15.
236. Ma, C., et al., *Tumor-infiltrating gammadelta T lymphocytes predict clinical outcome in human breast cancer.* J Immunol, 2012. **189**(10): p. 5029-36.
237. Li, X., 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): p. 6693-700.
238. Kang, N., et al., *Identification and characterization of Foxp3(+) gammadelta T cells in mouse and human.* Immunol Lett, 2009. **125**(2): p. 105-13.
239. Peters, C., et al., *Phenotype and regulation of immunosuppressive Vdelta2-expressing gammadelta T cells.* Cell Mol Life Sci, 2014. **71**(10): p. 1943-60.
240. Rei, M., D.J. Pennington, and B. Silva-Santos, *The emerging Protumor role of gammadelta T lymphocytes: implications for cancer immunotherapy.* Cancer Res, 2015. **75**(5): p. 798-802.
241. Wesch, D., A. Glatzel, and D. Kabelitz, *Differentiation of resting human peripheral blood gamma delta T cells toward Th1- or Th2-phenotype.* Cell Immunol, 2001. **212**(2): p. 110-7.
242. Ness-Schwickerath, K.J., C. Jin, and C.T. Morita, *Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells.* J Immunol, 2010. **184**(12): p. 7268-80.
243. Caccamo, N., et al., *Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells.* Blood, 2011. **118**(1): p. 129-38.
244. Casetti, R., 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): p. 3574-7.
245. Martin, B., et al., *Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals.* Immunity, 2009. **31**(2): p. 321-30.
246. Wu, P., et al., *gammadeltaT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer.* Immunity, 2014. **40**(5): p. 785-800.
247. Price, A.E., et al., *Marking and quantifying IL-17A-producing cells in vivo.* PLoS One, 2012. **7**(6): p. e39750.
248. Jensen, K.D., 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): p. 90-100.
249. Ribot, J.C., 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): p. 427-36.
250. Powolny-Budnicka, I., et al., *RelA and RelB transcription factors in distinct thymocyte populations control lymphotoxin-dependent interleukin-17 production in gammadelta T cells.* Immunity, 2011. **34**(3): p. 364-74.
251. Patil, R.S., et al., *The Jekyll and Hyde story of IL17-Producing gammadeltaT Cells.* Front Immunol, 2015. **6**: p. 37.
252. Yang, B., et al., *The role of interleukin 17 in tumour proliferation, angiogenesis, and metastasis.* Mediators Inflamm, 2014. **2014**: p. 623759.
253. Wakita, D., et al., *Tumor-infiltrating IL-17-producing gammadelta T cells support the progression of tumor by promoting angiogenesis.* Eur J Immunol, 2010. **40**(7): p. 1927-37.

254. Rei, M., 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): p. E3562-70.
255. Ma, S., et al., *IL-17A produced by gammadelta T cells promotes tumor growth in hepatocellular carcinoma.* Cancer Res, 2014. **74**(7): p. 1969-82.
256. Sudam Patil, R., et al., *IL17 producing gammadeltaT cells induce angiogenesis and are associated with poor survival in gallbladder cancer patients.* Int J Cancer, 2016. **139**(4): p. 869-81.
257. Ma, Y., et al., *Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy.* J Exp Med, 2011. **208**(3): p. 491-503.
258. Zhao, L., et al., *Imbalance in the Th17/Treg and cytokine environment in peripheral blood of patients with adenocarcinoma and squamous cell carcinoma.* Med Oncol, 2013. **30**(1): p. 461.
259. Haghshenas, M.R., et al., *Immune regulatory cells and IL17-producing lymphocytes in patients with benign and malignant salivary gland tumors.* Immunol Lett, 2015. **164**(2): p. 109-16.
260. Wang, J., et al., *Skewing the balance of regulatory T-cells and T-helper 17 cells in breast cancer patients.* J Int Med Res, 2011. **39**(3): p. 691-701.
261. 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.
262. Gershon, R.K. and K. Kondo, *Infectious immunological tolerance.* Immunology, 1971. **21**(6): p. 903-14.
263. 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.
264. 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.
265. Ziegler, S.F., *FOXP3: of mice and men.* Annu Rev Immunol, 2006. **24**: p. 209-26.
266. 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.
267. 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.
268. 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.
269. 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.
270. Gavin, M.A., et al., *Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo.* Nat Immunol, 2002. **3**(1): p. 33-41.
271. Ha, T.Y., *The role of regulatory T cells in cancer.* Immune Netw, 2009. **9**(6): p. 209-35.
272. Mougiakakos, D., et al., *Regulatory T cells in cancer.* Adv Cancer Res, 2010. **107**: p. 57-117.
273. 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.

274. 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.
275. 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.
276. 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.
277. 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.
278. 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.
279. 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.
280. Facciabene, A., et al., *Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells.* Nature, 2011. **475**(7355): p. 226-30.
281. Redjimi, N., et al., *CXCR3+ T regulatory cells selectively accumulate in human ovarian carcinomas to limit type I immunity.* Cancer Res, 2012. **72**(17): p. 4351-60.
282. 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.
283. 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.
284. 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.
285. 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.
286. 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.
287. 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.
288. 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.
289. 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.
290. 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.
291. 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.

292. 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.
293. 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.
294. 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.
295. 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.
296. Munn, D.H. and A.L. Mellor, *Indoleamine 2,3-dioxygenase and tumor-induced tolerance.* J Clin Invest, 2007. **117**(5): p. 1147-54.
297. 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.
298. Vignali, D.A., L.W. Collison, and C.J. Workman, *How regulatory T cells work.* Nat Rev Immunol, 2008. **8**(7): p. 523-32.
299. 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.
300. Yamada, N., et al., *Establishment and characterization of a new human gallbladder carcinoma cell line (OCUG-1) producing TA-4.* Int J Oncol, 1997. **10**(6): p. 1251-5.
301. Homma, S., et al., *[Establishment and characterization of a human gall bladder carcinoma cell line NOZ].* Hum Cell, 1988. **1**(1): p. 95-7.
302. Yao, X., et al., *Levels of peripheral CD4(+)FoxP3(+) regulatory T cells are negatively associated with clinical response to adoptive immunotherapy of human cancer.* Blood, 2012. **119**(24): p. 5688-96.
303. Gros, A., et al., *Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients.* Nat Med, 2016. **22**(4): p. 433-8.
304. Germain, C., et al., *Presence of B cells in tertiary lymphoid structures is associated with a protective immunity in patients with lung cancer.* Am J Respir Crit Care Med, 2014. **189**(7): p. 832-44.
305. Song, G., et al., *Elevated level of peripheral CD8(+)CD28(-) T lymphocytes are an independent predictor of progression-free survival in patients with metastatic breast cancer during the course of chemotherapy.* Cancer Immunol Immunother, 2013. **62**(6): p. 1123-30.
306. Zhuang, Y., et al., *CD8(+) T cells that produce interleukin-17 regulate myeloid-derived suppressor cells and are associated with survival time of patients with gastric cancer.* Gastroenterology, 2012. **143**(4): p. 951-62 e8.
307. Thedrez, A., et al., *A quantitative deficiency in peripheral blood Vgamma9Vdelta2 cells is a negative prognostic biomarker in ovarian cancer patients.* PLoS One, 2013. **8**(5): p. e63322.
308. Homma, Y., et al., *Changes in the immune cell population and cell proliferation in peripheral blood after gemcitabine-based chemotherapy for pancreatic cancer.* Clin Transl Oncol, 2014. **16**(3): p. 330-5.
309. Liu, G., et al., *Distribution of natural killer cells and T-lymphocyte subsets in peripheral blood, gallbladder cancer and surrounding tissue.* Hepatobiliary Pancreat Dis Int, 2007. **6**(1): p. 81-6.
310. Nakakubo, Y., et al., *Clinical significance of immune cell infiltration within gallbladder cancer.* Br J Cancer, 2003. **89**(9): p. 1736-42.

311. Balkwill, F., K.A. Charles, and A. Mantovani, *Smoldering and polarized inflammation in the initiation and promotion of malignant disease.* Cancer Cell, 2005. **7**(3): p. 211-7.
312. Gajewski, T.F., H. Schreiber, and Y.X. Fu, *Innate and adaptive immune cells in the tumor microenvironment.* Nat Immunol, 2013. **14**(10): p. 1014-22.
313. Ganjalikhani Hakemi, M., et al., *Optimization of human Th17 cell differentiation in vitro: evaluating different polarizing factors.* In Vitro Cell Dev Biol Anim, 2011. **47**(8): p. 581-92.
314. Yamagiwa, S., et al., *A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood.* J Immunol, 2001. **166**(12): p. 7282-9.
315. Dennis, K.L., et al., *Current status of interleukin-10 and regulatory T-cells in cancer.* Curr Opin Oncol, 2013. **25**(6): p. 637-45.
316. He, D., et al., *IL-17 promotes tumor development through the induction of tumor promoting microenvironments at tumor sites and myeloid-derived suppressor cells.* J Immunol, 2010. **184**(5): p. 2281-8.
317. 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.
318. Fabre, J., et al., *Targeting the Tumor Microenvironment: The Protumor Effects of IL-17 Related to Cancer Type.* Int J Mol Sci, 2016. **17**(9).
319. Peng, M.Y., et al., *Interleukin 17-producing gamma delta T cells increased in patients with active pulmonary tuberculosis.* Cell Mol Immunol, 2008. **5**(3): p. 203-8.
320. Itoh, S., et al., *Interleukin-17 accelerates allograft rejection by suppressing regulatory T cell expansion.* Circulation, 2011. **124**(11 Suppl): p. S187-96.
321. Petermann, F., et al., *gammadelta T cells enhance autoimmunity by restraining regulatory T cell responses via an interleukin-23-dependent mechanism.* Immunity, 2010. **33**(3): p. 351-63.
322. Lo Re, S., et al., *IL-17A-producing gammadelta T and Th17 lymphocytes mediate lung inflammation but not fibrosis in experimental silicosis.* J Immunol, 2010. **184**(11): p. 6367-77.
323. Saharinen, P., et al., *VEGF and angiopoietin signaling in tumor angiogenesis and metastasis.* Trends Mol Med, 2011. **17**(7): p. 347-62.
324. Fenoglio, D., et al., *Vdelta1 T lymphocytes producing IFN-gamma and IL-17 are expanded in HIV-1-infected patients and respond to Candida albicans.* Blood, 2009. **113**(26): p. 6611-8.
325. Brandes, M., et al., *Flexible migration program regulates gamma delta T-cell involvement in humoral immunity.* Blood, 2003. **102**(10): p. 3693-701.
326. Vrieling, M., et al., *gammadelta T cell homing to skin and migration to skin-draining lymph nodes is CCR7 independent.* J Immunol, 2012. **188**(2): p. 578-84.
327. Weiner, L.M., J.C. Murray, and C.W. Shuptrine, *Antibody-based immunotherapy of cancer.* Cell, 2012. **148**(6): p. 1081-4.
328. Philip, P.A., et al., *Phase II study of erlotinib in patients with advanced biliary cancer.* J Clin Oncol, 2006. **24**(19): p. 3069-74.
329. Ramanathan, R.K., et al., *A phase II study of lapatinib in patients with advanced biliary tree and hepatocellular cancer.* Cancer Chemother Pharmacol, 2009. **64**(4): p. 777-83.
330. Ophir, E., et al., *Personalized approaches to active immunotherapy in cancer.* Biochim Biophys Acta, 2016. **1865**(1): p. 72-82.

331. Baumeister, S.H., et al., *Coinhibitory Pathways in Immunotherapy for Cancer.* Annu Rev Immunol, 2016. **34**: p. 539-73.
332. Madorsky Rowdo, F.P., et al., *Immunotherapy in Cancer: A Combat between Tumors and the Immune System; You Win Some, You Lose Some.* Front Immunol, 2015. **6**: p. 127.
333. Bordon, Y., *Immunotherapy: Checkpoint parley.* Nat Rev Cancer, 2015. **15**(1): p. 3.
334. Topalian, S.L., C.G. Drake, and D.M. Pardoll, *Immune checkpoint blockade: a common denominator approach to cancer therapy.* Cancer Cell, 2015. **27**(4): p. 450-61.
335. Honda, T., et al., *Tuning of antigen sensitivity by T cell receptor-dependent negative feedback controls T cell effector function in inflamed tissues.* Immunity, 2014. **40**(2): p. 235-47.
336. Sznol, M. and L. Chen, *Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer.* Clin Cancer Res, 2013. **19**(5): p. 1021-34.
337. Emens, L.A., et al., *Cancer immunotherapy trials: leading a paradigm shift in drug development.* J Immunother Cancer, 2016. **4**: p. 42.
338. Finn, O.J., *Immuno-oncology: understanding the function and dysfunction of the immune system in cancer.* Ann Oncol, 2012. **23 Suppl 8**: p. viii6-9.
339. Baniyash, M., *TCR zeta-chain downregulation: curtailing an excessive inflammatory immune response.* Nat Rev Immunol, 2004. **4**(9): p. 675-87.
340. 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.
341. Qin, G., et al., *Phenotypic and functional characterization of human gammadelta T-cell subsets in response to influenza A viruses.* J Infect Dis, 2012. **205**(11): p. 1646-53.
342. Zheng, J., et al., *gammadelta-T cells: an unpolished sword in human anti-infection immunity.* Cell Mol Immunol, 2013. **10**(1): p. 50-7.
343. Born, W.K., et al., *Peptide antigens for gamma/delta T cells.* Cell Mol Life Sci, 2011. **68**(14): p. 2335-43.
344. Groh, V., et al., *Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells.* Science, 1998. **279**(5357): p. 1737-40.
345. Das, H., et al., *MICA engagement by human Vgamma2Vdelta2 T cells enhances their antigen-dependent effector function.* Immunity, 2001. **15**(1): p. 83-93.
346. Hannani, D., et al., *Harnessing gammadelta T cells in anticancer immunotherapy.* Trends Immunol, 2012. **33**(5): p. 199-206.
347. Ye, J., et al., *Tumor-derived gammadelta regulatory T cells suppress innate and adaptive immunity through the induction of immunosenescence.* J Immunol, 2013. **190**(5): p. 2403-14.
348. Laetitia Douguet, L.B., Renée Lengagne, Laura Labarthe, Masashi Kato, Marie-Françoise Avril & Armelle Prévost-Blondel, *Nitric oxide synthase 2 is involved in the pro-tumorigenic potential of γδ17 T cells in melanoma.* OncoImmunology 2016. **5**(8).
349. Coffelt, S.B., et al., *IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis.* Nature, 2015. **522**(7556): p. 345-8.
350. Alizadeh, D., E. Katsanis, and N. Larmonier, *The multifaceted role of Th17 lymphocytes and their associated cytokines in cancer.* Clin Dev Immunol, 2013. **2013**: p. 957878.
351. Sutton, C.E., et al., *Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity.* Immunity, 2009. **31**(2): p. 331-41.

352. Prabhala, R.H., et al., *Dysfunctional T regulatory cells in multiple myeloma.* Blood, 2006. **107**(1): p. 301-4.
353. Shibuya, K.C., et al., *Pancreatic ductal adenocarcinoma contains an effector and regulatory immune cell infiltrate that is altered by multimodal neoadjuvant treatment.* PLoS One, 2014. **9**(5): p. e96565.
354. Valmori, D., et al., *Human RORgammat+ TH17 cells preferentially differentiate from naive FOXP3+Treg in the presence of lineage-specific polarizing factors.* Proc Natl Acad Sci U S A, 2010. **107**(45): p. 19402-7.
355. Nakayamada, S., et al., *Helper T cell diversity and plasticity.* Curr Opin Immunol, 2012. **24**(3): p. 297-302.
356. Zhou, L., et al., *TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function.* Nature, 2008. **453**(7192): p. 236-40.
357. Walker, M.R., et al., *Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells.* J Clin Invest, 2003. **112**(9): p. 1437-43.
358. 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.
359. Chen, X. and J.J. Oppenheim, *Th17 cells and Tregs: unlikely allies.* J Leukoc Biol, 2014.
360. Zhang, Y., Y. Huang, and M. Qin, *Tumour-infiltrating FoxP3+ and IL-17-producing T cells affect the progression and prognosis of gallbladder carcinoma after surgery.* Scand J Immunol, 2013. **78**(6): p. 516-22.
361. Costa, M.F., et al., *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): p. 1250-60.
362. Oo, Y.H., et al., *CXCR3-dependent recruitment and CCR6-mediated positioning of Th-17 cells in the inflamed liver.* J Hepatol, 2012. **57**(5): p. 1044-51.
363. Ma, J., et al., *Upregulated TIMP-1 correlates with poor prognosis of laryngeal squamous cell carcinoma.* Int J Clin Exp Pathol, 2014. **7**(1): p. 246-54.
364. Klein, R.M., et al., *SERPINE1 expression discriminates site-specific metastasis in human melanoma.* Exp Dermatol, 2012. **21**(7): p. 551-4.
365. Nijaguna, M.B., et al., *Glioblastoma-derived Macrophage Colony-stimulating Factor (MCSF) Induces Microglial Release of Insulin-like Growth Factor-binding Protein 1 (IGFBP1) to Promote Angiogenesis.* J Biol Chem, 2015. **290**(38): p. 23401-15.
366. Wang, L., et al., *IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway.* J Exp Med, 2009. **206**(7): p. 1457-64.
367. Benevides, L., et al., *IL17 Promotes Mammary Tumor Progression by Changing the Behavior of Tumor Cells and Eliciting Tumorigenic Neutrophils Recruitment.* Cancer Res, 2015. **75**(18): p. 3788-99.
368. Kim, G., et al., *Interleukin-17 induces AP-1 activity and cellular transformation via upregulation of tumor progression locus 2 activity.* Carcinogenesis, 2013. **34**(2): p. 341-50.
369. Sun, X.N., et al., *Prognostic impact of vascular endothelial growth factor-A expression in resected gallbladder carcinoma.* Tumour Biol, 2011. **32**(6): p. 1183-90.
370. Letelier, P., et al., *Immunohistochemical expression of vascular endothelial growth factor A in advanced gallbladder carcinoma.* Appl Immunohistochem Mol Morphol, 2014. **22**(7): p. 530-6.
371. Li, Q., et al., *IL-17 promoted metastasis of non-small-cell lung cancer cells.* Immunol Lett, 2012. **148**(2): p. 144-50.

372. Zhao, M., et al., *JAK2/STAT3 signaling pathway activation mediates tumor angiogenesis by upregulation of VEGF and bFGF in non-small-cell lung cancer.* Lung Cancer, 2011. **73**(3): p. 366-74.
373. Gordon, K.B., et al., *Phase 3 Trials of Ixekizumab in Moderate-to-Severe Plaque Psoriasis.* N Engl J Med, 2016. **375**(4): p. 345-56.
374. Langley, R.G., et al., *Secukinumab in plaque psoriasis--results of two phase 3 trials.* N Engl J Med, 2014. **371**(4): p. 326-38.
375. Bartlett, H.S. and R.P. Million, *Targeting the IL-17-T(H)17 pathway.* Nat Rev Drug Discov, 2015. **14**(1): p. 11-2.
376. Lebwohl, M., et al., *Phase 3 Studies Comparing Brodalumab with Ustekinumab in Psoriasis.* N Engl J Med, 2015. **373**(14): p. 1318-28.
377. Yang, J., et al., *Targeting Th17 cells in autoimmune diseases.* Trends Pharmacol Sci, 2014. **35**(10): p. 493-500.
378. Ghiringhelli, F. and L. Apetoh, *Enhancing the anticancer effects of 5-fluorouracil: current challenges and future perspectives.* Biomed J, 2015. **38**(2): p. 111-6.
379. Bizama, C., et al., *Targeting specific molecular pathways holds promise for advanced gallbladder cancer therapy.* Cancer Treat Rev, 2015. **41**(3): p. 222-34.
380. Pere, H., et al., *Comprehensive analysis of current approaches to inhibit regulatory T cells in cancer.* Oncoimmunology, 2012. **1**(3): p. 326-333.
381. Linch, S.N., M.J. McNamara, and W.L. Redmond, *OX40 Agonists and Combination Immunotherapy: Putting the Pedal to the Metal.* Front Oncol, 2015. **5**: p. 34.

Publications

IJC
International Journal of Cancer

IL17 producing γδT cells induce angiogenesis and are associated with poor survival in gallbladder cancer patients

Rushikesh Sudam Patil[1], Sagar Umesh Shah[1], Shailesh Vinayak Shrikhande[2], Mahesh Goel[2], Rajesh Prabhakar Dikshit[3] and Shubhada Vivek Chiplunkar[1]

[1] Chiplunkar Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai, Maharashtra, India
[2] Department of Surgical Oncology
[3] Department of Cancer Epidemiology, Tata Memorial Hospital, Parel, Mumbai, Maharashtra, India

Despite conventional treatment modalities, gallbladder cancer (GBC) remains a highly lethal malignancy. Prognostic biomarkers and effective adjuvant immunotherapy for GBC are not available. In the recent past, immunotherapeutic approaches targeting tumor associated inflammation have gained importance but the mediators of inflammatory circuit remain unexplored in GBC patients. In the current prospective study, we investigated the role of IL17 producing TCRγδ$^+$ (Tγδ17), CD4$^+$ (Th17), CD8$^+$ (Tc17) and regulatory T cells (Tregs) in pathogenesis of GBC. Analysis by multi-color flow cytometry revealed that compared to healthy individuals (HI), Tγδ17, Th17 and Tc17 cells were increased in peripheral blood mononuclear cells (PBMCs) and tumor infiltrating lymphocytes (TIL) of GBC patients. Tregs were decreased in PBMCs but increased in TILs of GBC patients. The suppressive potential of Tregs from GBC patients and HI were comparable. Serum cytokines profile of GBC patients showed elevated levels of cytokines (IL6, IL23 and IL1β) required for polarization and/or stabilization of IL17 producing cells. We demonstrated that Tγδ17 cells migrate toward tumor bed using CXCL9-CXCR3 axis. IL17 secreted by Tγδ17 induced productions of vascular endothelial growth factor and other angiogenesis related factors in GBC cells. Tγδ17 cells promote vasculogenesis as studied by chick chorioallantoic membrane assay. Survival analysis showed that Tγδ17, Th17 and Treg cells in peripheral blood were associated with poor survival of GBC patients. Our findings suggest that Tγδ17 is a protumorigenic subtype of γδT cells which induces angiogenesis. Tγδ17 may be considered as a predictive biomarker in GBC thus opening avenues for targeted therapies.

Key words: Tγδ17, Th17, Treg, vascular endothelial growth factor

Abbreviations: CFSE: carboxyfluorescein succinimidyl ester; Foxp3: forkhead box p3; GBC: gallbladder cancer; HI: healthy individuals; IL: interleukin; IFNγ: interferon γ; PBMC: peripheral blood mononuclear cells; PMA: phorbol 12-myristate 13-acetate; RANTES: regulated on activation normal T cell expressed and secreted; Treg: regulatory T cells; Tres: responder T cells; TIL: tumor infiltrating lymphocytes; TCR: T cell receptor; TGF: transforming growth factor; VEGF: vascular endothelial growth factor

Additional Supporting Information may be found in the online version of this article.

The authors have no conflicts of interest to declare

Study concept and design: SVC. Acquisition of data: RSP and SUS. Analysis and interpretation of data: RSP and SVC. Drafting of the manuscript: RSP and SVC. Critical revision of the manuscript for important intellectual content: SVC. Statistical analysis: RSP. Obtained funding: SVC and RD. Study supervision: SVC. Providing patient samples: SVS, MG and RD

DOI: 10.1002/ijc.30134

History: Received 2 Dec 2015; Accepted 31 Mar 2016; Online 8 Apr 2016

Correspondence to: S.V. Chiplunkar, Chiplunkar Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai, Maharashtra 410210, India Tel.: +91-22-27405075, Fax: +91-22-27405085, E-mail: schiplunkar@actrec.gov.in

Introduction

Gallbladder cancer (GBC) is a relatively uncommon but lethal biliary tract related cancer. Its occurrence shows ethnic and geographical variations and is prevalent in Peru, Ecuador, Poland, Chile, Pakistan, Japan and northern India.[1] GBC is two to three times common in women than men and highest incidences are reported in north Indian women.[2] Cholelithiasis is a major risk factor for GBC which induces chronic inflammation leading to dysplastic changes and high grade premalignant carcinoma *in situ*.[3,4] Anatomic location, elusive symptoms and diagnosis at advanced stage reduces 5-year survival of GBC patients to <5%.[1] Complete surgical resection is the only curative option available, but >90% GBC patients are with un-resectable or metastatic disease.[5] Despite improved results of chemotherapy and surgery, the long-term outcome remains disappointing.[6] Thus, the need is to identify other etiological factors which would provide better insights into the process of carcinogenesis in GBC patients.

The inflammatory microenvironment is an essential component of a tumor and plays a decisive role at different stages of tumor development. It modulates host immune response to facilitate tumor growth.[7] Interleukin-17 (IL17) is a potent proinflammatory cytokine and its elevated levels have been found to be detrimental in autoimmune diseases and cancers.[8] It increases the immigration of neutrophils, macrophages and

What's new?
Human T cells expressing γδ-TCR exhibit potent anti-tumor activity and are potential candidates for cell-based therapies. Evidence however also exists of the ability of γδ-TCR cells to suppress anti-tumor responses, making deeper insight necessary for the successful clinical application of γδT cell-based immunotherapies. This study identified IL17-producing γδT cells (Tγδ17) as a pro-tumorigenic subtype of γδT cells associated with poor survival in gallbladder cancer (GBC) patients. Tγδ17 cells infiltrate the tumor bed via CXCL9-CXCR3 axis and IL17 induces pro-angiogenic factors in GBC cells. Tγδ17 may be considered as a predictive biomarker in GBC, opening up new avenues for targeted therapies.

monocytes to inflamed tissues. IL17 induces tumor necrosis factor, IL-1, IL-6, colony stimulating factors, chemokines, matrix metalloproteinases, *etc.* which further augment tumor progression.[8] However, the protumor role of IL17 in the progression of GBC remains unexplored. Additionally, the cellular source of IL17 and its clinical relevance in GBC is not well investigated.

$CD4^+$ $IL17^+$ (Th17) cells and its related cytokines are reported to be present in tumor environment of various malignancies.[9] Recently, γδT cells have been shown as major innate source of IL17. IL17 producing γδT cells (Tγδ17) are protective in host defense against extracellular fungi and bacteria but exacerbate autoimmune and inflammatory diseases.[10] However, the role of Tγδ17 cells in pathogenesis of human malignancies is not well understood. In contrast, regulatory T cells (Treg), are anti-inflammatory and play a critical role in immune tolerance and autoimmunity.[11] They inhibit activation of CD4 and CD8 T cells and impair antitumor immune response.[12] Elevated proportions of Tregs have been identified in peripheral blood as well as in tumor environment and are associated with poor prognosis in several human cancers.[11]

The tumor infiltrating immune cells are engaged in extensive crosstalk with cancer cells, affecting immune surveillance and response to therapy.[7] An expanding body of literature has implicated the dynamics of Th17 and Tregs to play a major role in pathogenesis of several malignancies.[13] Thus, the present study aims at understanding how a specific subset of γδT cells, namely, Tγδ17, contributes to inflammation and thereby progression of GBC.

Material and Methods

Patient samples

Newly diagnosed GBC patients ($n = 52$) were recruited from Tata Memorial Hospital, Mumbai. There were 22 males (mean age = 54 ± 2 years) and 30 females (mean age = 51 ± 2 years) in the study group. Peripheral blood of GBC patients was collected prior to chemotherapy/radiotherapy or surgery after obtaining written informed consent. The study protocol was approved by ACTREC-TMC Institutional review board for human studies. The patients were grouped according to the TNM classification as stage II ($n = 5$), stage III ($n = 20$) and stage IV ($n = 27$). Tumor tissues ($n = 17$) were obtained from GBC patients undergoing cholecystectomy without radiotherapy or chemotherapy. Peripheral blood was obtained from age and sex matched healthy individuals ($n = 30$) who participated voluntarily and written informed consent was obtained.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of GBC patients and healthy individuals using Ficoll Hypaque (Sigma-Aldrich, St. Louis, MO). Surgically resected gallbladder tumors were minced finely and incubated in RPMI medium containing enzyme mixture (0.05% collagenase, 0.02% DNase and 5U/ml hyaluronidase [Sigma-Aldrich]), at 37°C for 2 hr with intermittent shaking. The tissue was passed through wire mesh and washed with saline to obtain single cell suspension. Cells were cultured in serum-free medium and tumor supernatants were collected after 24 hr.

Tγδ17 cells were isolated using IL17 secretion assay-cell enrichment and detection kit (Miltenyi Biotec, Germany) according to manufacturer's instructions. Briefly, γδT cells were purified by positive selection from PBMCs of healthy individuals using anti TCR-γδ microbeads (Mltenyi Biotec, Germany) and stimulated with PMA (phorbol 12-myristate 13-acetate) and Ionomycin for 5 hr. Cells were then labelled with IL17 catch reagent for 5 min on ice followed by secretion phase of 45 min at 37°C with constant mixing in RPMI containing human AB serum. After labelling with anti-IL17 detection antibody, cells were sorted for IL17-PE positive cells. Purity (>90%) was determined by flow cytometry.

GBC cell line (OCUG-1; JCRB-0191)[14] was purchased from Japan Health Science Research Resources Bank (Osaka, Japan). Cells were cultured in William's E medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS under standard culture conditions (37°C, 5%CO_2).

Flow cytometry

Flow cytometric analysis was performed using FACS Aria flow cytometer (Becton Dickinson, CA) and analyzed by FlowJo software (Tree Star, Ashland, OR). Fluorescence minus one control was used in all experiments to determine background fluorescence. For intracellular cytokines staining, PBMCs were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 5 hr in presence of Brefeldin A (5 μg/ml; all from Sigma-Aldrich). Cells were washed with PBS and cold fixed with 1% paraformaldehyde (Sigma-Aldrich) for 15 min at 4°C, followed by permeabilization for 5 min with 0.1% saponin. Cells were incubated with antibodies for 30 min at room temperature. Minimum 50,000 events were acquired on FACS Aria flow cytometer. Antibodies used for flow cytometry staining are provided as Supporting Information Table 1.

Regulatory T cells suppression assay

Tregs were isolated from PBMCs using BD IMag regulatory T lymphocyte separation set-DM (BD Biosciences). Briefly, $CD4^+$ T cells were negatively selected from PBMCs followed by positive selection of $CD25^+$ T cells. $CD4^+$ $CD25^-$ T cells were used as responder T cells (Tres) and labelled with carboxyfluorescein succinimidyl ester (CFSE; 5 μM; CellTrace proliferation kit, Life technologies) for 10 min at room temperature. Tres cells (1×10^4) were cocultured with Tregs for 5 days at different ratios (Tres:Treg = 1:2, 1:1, 1:0.5 and 1:0). Cocultures were stimulated with anti-CD3/anti-CD28 coated beads (1bead:1cell; Treg suppression inspector, Miltenyi biotech). Cells were acquired on FACS Aria and analyzed by FlowJo software.

Cytokines measurement

Serum samples were obtained from GBC patients and healthy individuals and stored at −80°C until used. Cytokines and chemokines in sera samples and culture supernatants were measured by Th1/Th2/Th17 cytometric bead array kit and flex sets for IL8, IL1β, IL12p70, CXCL9, CCL5, CXCL10 and CCL2 (BD Biosciences) as per manufacturer's instructions. Samples were acquired on FACS Aria and analyzed using BD FCAP Array (BD Biosciences). TGFβ (BD Biosciences) and IL23 (eBiosciences, CA) were determined by ELISA.

Cell migration assay

Migration of Tγδ17 cells was studied by trans-well assay using Millicell cell-culture inserts (Merck Millipore, MA) with pore size 8.0 μm. OCUG-1 cells (5×10^4), cultured in 24 well plate were washed and 600 μl serum-free William's E medium was added to the lower chamber. In some experiments, rhCXCL9 (100 ng/ml; PeproTech, NJ) or tumor supernatants were added to the lower chamber. Isolated Tγδ17 cells (5×10^4/100 μl medium) were added onto the trans-well filter. Migrated cells from lower chamber were counted using hemocytometer after 7 hr. For blocking experiments, Tγδ17 cells were incubated with anti-CXCR3 antibody (10 μg/ml; R&D Systems, MN), 30 min before transwell coculture.

Angiogenesis array

Tγδ17 cells were cultured in serum-free RPMI medium for 24 hr in the presence of anti-CD3/anti-CD28 coated beads. Cell-free supernatant of Tγδ17 was incubated with OCUG-1 cells (2×10^4/well) in presence or absence of neutralizing anti-IL17 antibody (10 μg/ml; R&D Systems). After 48 hr, supernatants were collected and analyzed for VEGF by ELISA (R&D Systems) or angiogenesis related proteins by human proteome profiler angiogenesis antibody array (R&D Systems). Briefly, equal amounts of cell-free supernatants were diluted (1: 3) and mixed with a cocktail of biotinylated detection antibodies specific for angiogenesis related proteins and incubated with the membranes coated with array of antibodies for overnight at 4°C. Membranes were washed to remove unbound material followed by incubation with HRP-conjugated streptavidin. Chemiluminescence was used for signal detection. The data were evaluated using Image J 1.48V software (NIH) and expressed as mean pixel density.

Chorioallantoic membrane assay

Fertilized chicken eggs were incubated in humidified incubator at 37°C. On embryonic Day 5, a small window was made in the shell and 200 μl medium/Tγδ17 supernatant/rhIL17 (100 ng/ml, R&D Systems) was added onto the chorioallantoic membrane (CAM) of growing embryo. After 48 hr, eggs were cracked open and embryos were carefully transferred to 100 mm petri dish and images were captured. CAM was cut and transferred to a glass slide to observe under the microscope. Angiogenesis was quantitatively evaluated by scoring number of branching points in control and treated CAMs.

OCUG-1 cell proliferation assay

Proliferative response of OCUG-1 cells was analyzed by tritiated thymidine (^{3}H-Thymidine) incorporation assay. OCUG-1 cells (1×10^4) were cultured in flat bottom 96 well plates (Nunc, Denmark) in medium containing serum. After 24 hr, cells were washed with serum-free medium and rhIL17 (R&D Systems) was added in different concentrations. 0.5μCi/10 μl/well ^{3}H-Thymidine (specific activity 240 GBq/mmol; Radiation and Isotype Technology, India) was added during last 18 hr of the assay. After 72 hr, cells were harvested on glass-fibre filter paper (Titertek, Norway) using cell harvester (Titertek, Norway). The filter paper was dried at 60°C and each disc corresponding to single well was placed in 3 ml liquid scintillation fluid [0.7% 2,5 diphenyloxazole + 0.05% 1,4 bis(5-phenyloxazole)]. Radioactivity was measured on liquid β-scintillation counter (Packard USA) as counts per minute (CPM).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (Prism Software, Lake Forest, CA). Statistical significances were calculated by two-tailed Student's *t* test or Mann–Whitney test. As there was no clinically defined cut off points for Th17, Tc17, Tγδ17, Treg cells, the high-expressing or low-expressing groups of GBC patients were defined based on mean values of expression of these lymphocytes (4.7 for Tγδ17, 1.8 for Th17, 1.8 for Tc17 and 3.2 for Treg). Overall patient survival was calculated by Kaplan-Meier curve and compared by Log-rank test. Survival time was defined as the interval between date of diagnosis and date of death or last follow-up, whichever occurred earlier. Data were censored for patients who were alive at the time of last follow-up. $p < 0.05$ was considered statistically significant.

Results

Tγδ17 cells are increased in peripheral blood and tumor infiltrating lymphocytes (TILs) of GBC patients

In order to study the prevalence of circulating IL17 producing cells, peripheral blood mononuclear cells (PBMCs) were isolated from GBC patients ($n = 52$) and healthy individuals (HI; $n = 30$). The single cell suspension of tumor tissue ($n = 17$) was prepared by enzyme digestion. Cells were stimulated with PMA and Ionomycin in the presence of Brefeldin A followed by

immunophenotyping. Figure 1*a* describes representative gating strategy to define Th17, Tc17 and Tγδ17 cells. IL17$^+$ cells were gated on CD3$^+$ CD4$^+$, CD3$^+$ CD8$^+$ and CD3$^+$ Vδ2TCR$^+$ cells. A similar strategy was used to define CD4$^+$ IFNγ$^+$, CD8$^+$ IFNγ$^+$ and γδ$^+$IFNγ$^+$ cells.

Th17, Tc17 and Tγδ17 cells were significantly increased in PBMCs of GBC patients compared to HI. Levels of these cells were further elevated in tumor compartment than the peripheral blood of GBC patients. Interestingly, the relative percentages of Tγδ17 were higher in TILs compared to Th17 and Tc17 (Fig. 1*b*). In contrast, γδ$^+$IFNγ$^+$ cells were significantly decreased in TILs compared to PBMCs of GBC patients and HI. A significant decrease was also observed in CD8$^+$ IFNγ$^+$ cells in TILs compared to PBMCs of GBC patients. However, no change was observed in CD4$^+$ IFNγ$^+$ in GBC patients compared to HI (Fig. 1*c*). Collectively, the data indicate that Tγδ17 cells are emerging as an important phenotype in GBC patients.

CD4$^+$ CD25$^+$ CD127$^{low/-}$Foxp3$^+$ Tregs are decreased in peripheral blood of GBC patients

The frequency of Tregs in PBMCs of GBC patients ($n = 52$) and HI ($n = 30$) were analyzed by surface staining for CD4, CD25 and CD127 followed by intracellular staining for Foxp3. Figure 2*a* describes the gating strategy of Tregs where Tregs were defined as CD25$^+$ CD127$^{low/-}$ cells within CD4$^+$ cells with Foxp3 expression of ≥80%.

Tregs were significantly decreased in PBMCs of GBC patients compared to HI. However, the percentages of Tregs in TILs were higher than PBMCs of GBC patients but comparable to HI (Fig. 2*b*). The median fluorescence intensity (MFI) of Foxp3 expression on Tregs was significantly increased in TILs than PBMCs of GBC patients (Fig. 2*c*). In order to investigate functional potential of Tregs in GBC patients, Tregs from peripheral blood were cocultured with autologous responder T cells (Tres; CD4$^+$ CD25$^-$) stimulated with anti-CD3/anti-CD28. It was observed that culture of Tres with Treg in 1:2 ratio significantly inhibited proliferation of Tres. However, the suppressive potential of Tregs in GBC patients was comparable to HI (Figs. 2*d* and 2*e*). Thus, the results indicate that although Tregs were decreased in PBMCs of GBC patients, their suppressive potential was not compromised.

Dynamics of Tγδ17, Th17, Tc17 and Tregs in GBC patients

The ratios of Tγδ17/Treg, Th17/Treg and Tc17/Treg were significantly increased in PBMCs as well as in TILs of GBC patients indicating an inverse correlation of IL17 producing cells and Tregs (Fig. 2*f*). Tγδ17, Th17 and Tc17 cells showed no correlation with clinical stage (II to IV) of GBC patients. However, their levels remained high in all stages of GBC patients compared to HI. In contrast, the levels of Tregs in GBC patients of all stages (II to IV) remained lower than HI (Fig. 2*g*). This clearly indicates that the immune response is skewed toward IL17 producing cells in GBC patients.

Tγδ17 polarizing cytokines are present in sera and tumor of GBC patients

Studies have shown that the differentiation of Tγδ17 and Th17 from naive T cells is facilitated by combinations of IL6, TGFβ, IL1β and IL23.[15,16] The serum levels of these and related cytokines/chemokines were evaluated in GBC patients ($n = 49$) and HI ($n = 25$). As shown in Figure 3*a*, levels of IL6, IL1β and IL23 were high in GBC patients whereas TGFβ was low compared to HI. IL17 was significantly elevated in GBC patients. However, IFNγ was decreased and IL12 remained unaltered compared to HI. Levels of IL10 also did not alter among HI and GBC patients. Analysis of chemokines revealed that monokine induced by gamma interferon (MIG; CXCL9), interferon induced protein 10 (IP-10; CXCL10) and IL8 were increased in sera of GBC patients. Levels of Monocyte chemoattractant protein-1 (MCP-1; CCL2) and RANTES (CCL5) in GBC patients were comparable to HI (Fig. 3*b*).

Further to evaluate the cytokine profile in tumor environment, single cell suspension of tumor tissue was prepared and cytokines were measured in cell-free culture supernatant collected after 24 hr. It was observed that cytokines involved in polarization of IL17 producing cells (IL6, TGFβ, IL1β and IL23) were present in the tumor environment. Levels of IL12, IFNγ, IL2 and IL4 were low whereas chemokines such as IL8, CXCL9, CXCL10 and CCL2 were remarkably high (Fig. 3*c*). Taken together, these results suggest that cytokines involved in the polarization of Tγδ17 are elevated in sera and tumor environment of GBC patients.

Tγδ17 cells are recruited to the tumor environment of GBC

The increased levels of Tγδ17 cells observed in tumor environment of GBC patients suggest that they would be migrating to the tumor. To test this hypothesis, expression of chemokine receptors (CCR6, CCR7, CXCR4 and CXCR3) on Th17, Tc17 and Tγδ17 cells was analyzed in peripheral blood of GBC patients ($n = 35$). It was observed that Tγδ17 expressed elevated levels of CXCR3 than Th17 or Tc17 (Fig. 4*a*). However, CCR6, CCR7 and CXCR4 were expressed at comparable levels by Tγδ17, Th17 and Tc17 (Supporting Information Fig. 1).

Next, to investigate migration of Tγδ17 cells to the tumor, purified Tγδ17 cells (Fig. 4*b*) were cultured with GBC cell line (OCUG-1) or in presence of rhCXCL9 or cell-free tumor supernatants of surgically resected tumors in trans-well assay. Enhanced migration of Tγδ17 cells was observed toward OCUG-1 or rhCXCL9 or tumor supernatants compared to medium alone. The migration of Tγδ17 was significantly curtailed in presence of neutralizing anti-CXCR3 antibody (Fig. 4*c*). The data suggest that tumor environment induces infiltration of Tγδ17 cells toward tumor bed through CXCL9-CXCR3 axis.

Tγδ17 cells promote angiogenesis in GBC

In order to investigate whether the elevated levels of Tγδ17 in GBC patients, contribute to tumor progression, the cytokine profile of Tγδ17 was analyzed. It was observed that

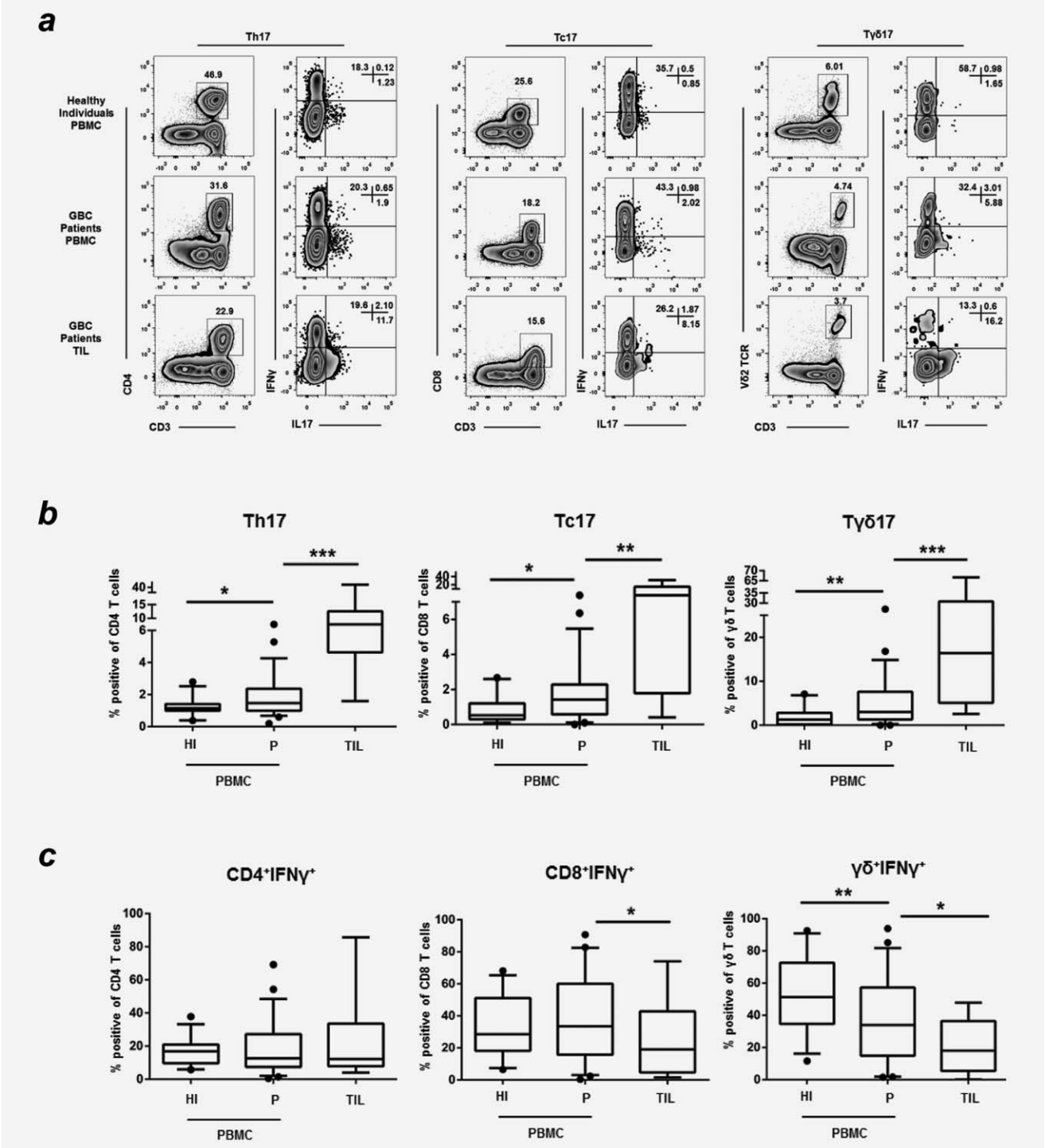

Figure 1. Prevalence of Th17, Tc17 and Tγδ17 cells in GBC patients. (*a*) Representative zebra plot analysis of Th17 (left), Tc17 (middle) and Tγδ17 (right) from PBMCs ($n = 52$) and TILs ($n = 17$) of GBC patients and HI ($n = 30$). Cells were gated as $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$ and $CD3^+$ $V\delta2TCR^+$ population, respectively. Numbers in the plot indicate percent positive cells. (*b*) A summarized data show percentages of Th17 (left), Tc17 (middle) and Tγδ17 (right) in PBMCs and TILs of GBC patients compared with HI. (*c*) Frequency of $CD4^+$ $IFN\gamma^+$ (left), $CD8^+$ $IFN\gamma^+$ (middle) and $\gamma\delta^+IFN\gamma^+$ (right) cells in PBMCs and TILs of GBC patients compared with HI. The box plots (*b*, *c*) show median (middle line), 5th and 95th percentiles (box), extreme values (whiskers) and outliers (dark circles). Results are analyzed by Mann-Whitney test with $^*p < 0.05$; $^{**}p < 0.01$.

Tγδ17 primarily secrete high levels of IL17 and low levels of IL2 and TNFα but did not produce other cytokines (IL4, IL6, IL10 and IFNγ; Fig. 4*d*). Interestingly, GBC cells (OCUG-1) express IL17 receptor as observed by flow cytometry (Fig. 4*e*). Next, to investigate the effect of IL17 on proliferation of GBC, OCUG-1 cells were cultured with rhIL17. It

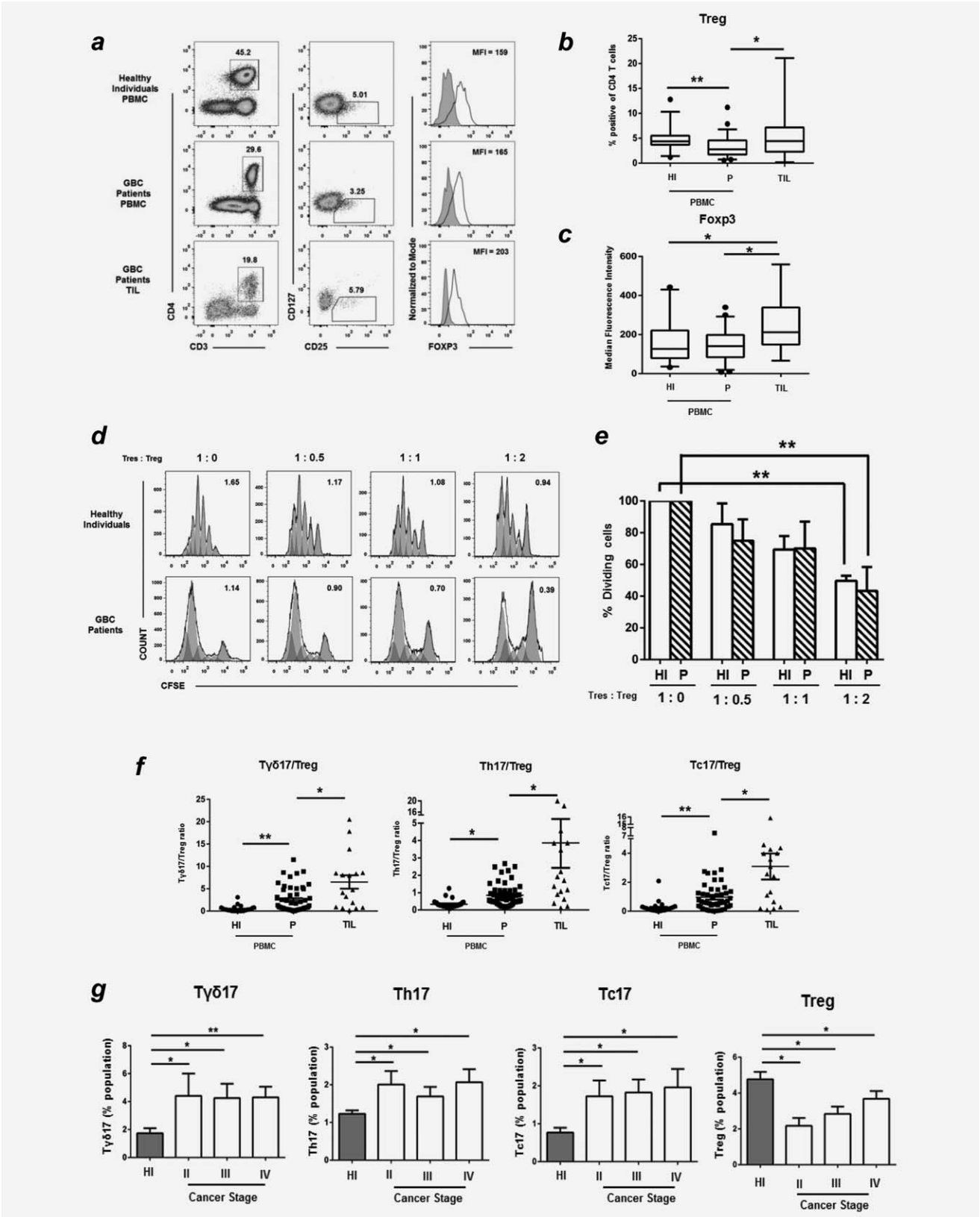

Figure 2. Frequency of regulatory T cells in GBC patients. (*a*) A representative dot plot describing Tregs, characterized as $CD25^{+}$ $CD127^{low/-}$ within $CD4^{+}$ T cells. Histograms indicate expression of Foxp3 within $CD25^{+}$ $CD127^{low/-}$ population. Figures in the histogram indicate median fluorescence intensity of Foxp3 expression (blank histogram) corrected with isocontrol (shaded histogram). (*b*) Box plots showing composite results of Tregs in PBMCs ($n = 52$) and TILs ($n = 17$) of GBC patients compared with HI ($n = 30$). (*c*) Comparison of median fluorescence intensity of Foxp3 expression within Tregs in GBC patients and HI. Results are analyzed by Mann-Whitney test with $*p < 0.05$. (*d*) Representative figure depicting suppressive potential of Tregs ($CD4^{+}$ $CD25^{+}$) on CFSE labelled autologous responder cells ($CD4^{+}$ $CD25^{-}$) from GBC patients and HI ($n = 3$). First peak from right indicates mother population. Figures in the plot indicate division index at respective ratio. (*e*) Bar diagram summarizes the percent dividing responder T cells. (*f*) Scatter plot showing the ratios of Tγδ17/Treg (left), Th17/Treg (middle) and Tc17/Treg (right) in PBMCs ($n = 52$) and TIL ($n = 17$) of GBC patients and HI ($n = 30$). (*g*) Frequencies of Tγδ17, Th17, Tc17 and Treg were compared with clinical stages of GBC patients. Results are shown as mean ± SEM with $*p < 0.05$; $**p < 0.01$.

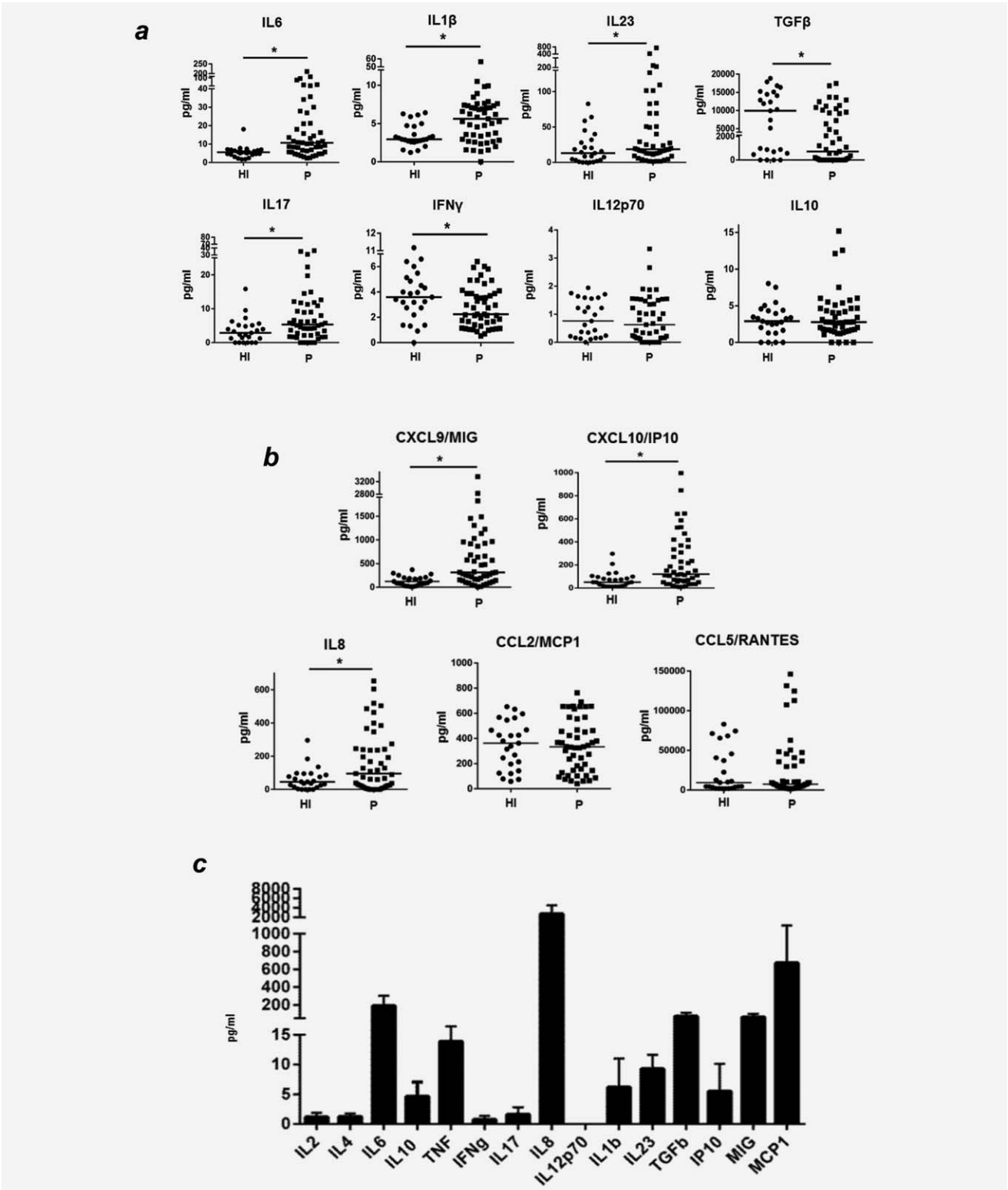

Figure 3. Cytokine profile in serum and tumor environment of GBC patients. (*a* and *b*) Scatter plots depict concentration of various cytokines (A) and chemokines (B) in sera of GBC patients ($n = 49$) and HI ($n = 25$) analyzed by cytometric bead array. Horizontal lines indicate median values. Data were analyzed by Mann–Whitney test with $*p < 0.05$. (*c*) Cytokines and chemokines were measured in cell-free tumor supernatants ($n = 15$) by cytometric bead array. Data presented as concentration in pg/ml.

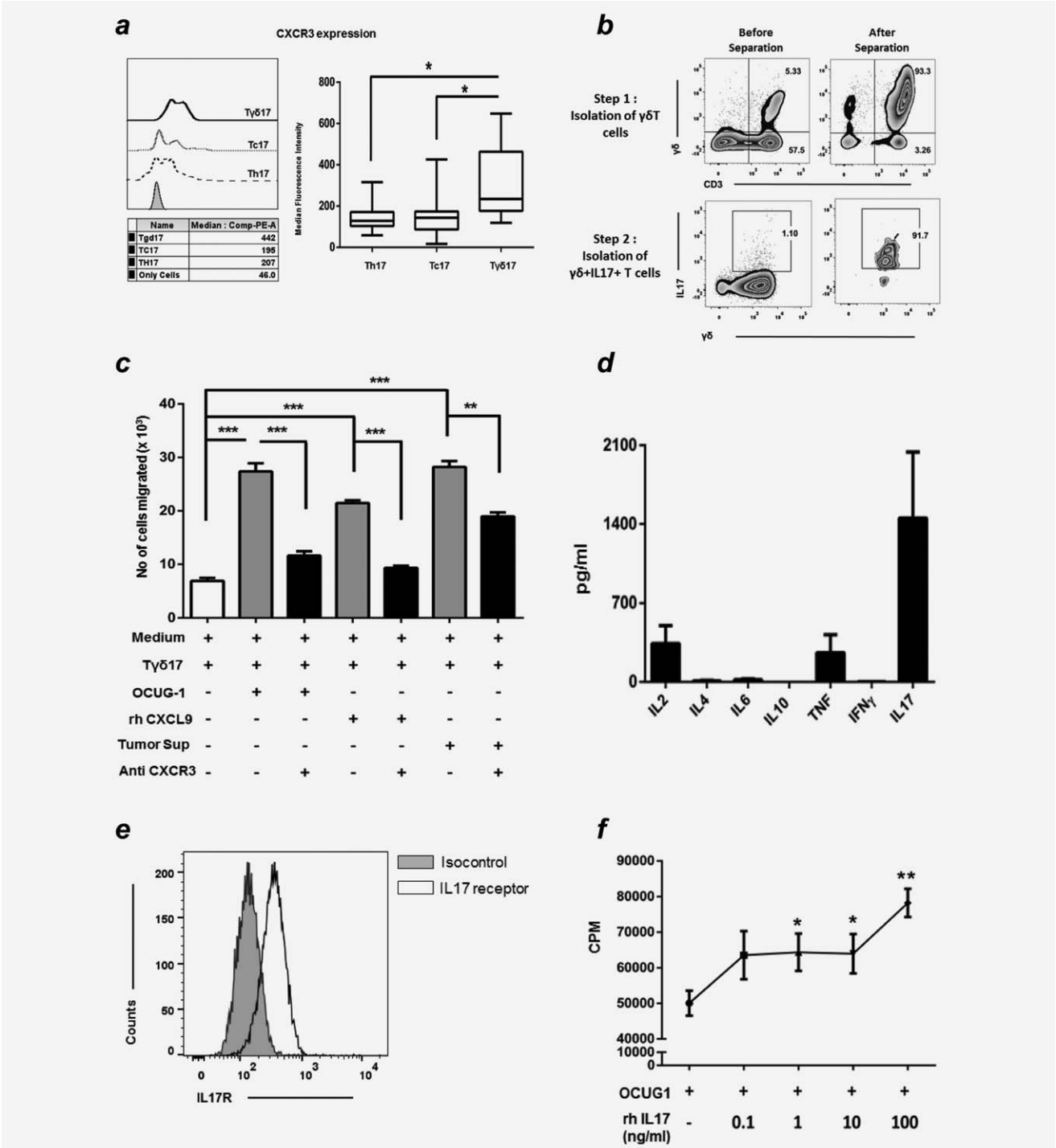

Figure 4. Tγδ17 cells migrate toward GBC tumor environment. (*a*) CXCR3 expression was analyzed on Th17, Tc17 and Tγδ17 cells by flow cytometry and depicted in a representative overlaid histogram. Data are presented as median fluorescence intensity ($n = 35$). (*b*) Representative figure of purity of sorted Tγδ17 cells. Upper panel shows purity of γδT cells. IL17 producing γδT cells were sorted to >90% purity as shown in lower panel. (*c*) Tγδ17 cells were cultured with OCUG-1 cells or rhCXCL9 or tumor supernatants in a trans-well assay in presence or absence of anti-CXCR3 mAb. Data presented as total number of migrated cells ($n = 3$). (*d*) Cytokines measured in culture supernatants of Tγδ17 cells stimulated with anti-CD3/anti-CD28 for 24 hr ($n = 5$). (*e*) A histogram indicates expression of IL17 receptor on OCUG-1 cells analyzed by flow cytometry. (*f*) rhIL17 induces proliferation of OCUG-1 cells measured by ^{3}H-Thymidine incorporation and represented as counts per minute ($n = 6$). Results are shown as mean ± SEM with $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

was observed that IL17 stimulated their proliferation in a dose-dependent manner indicating protumor role of IL17 (Fig. 4*f*).

Next to investigate whether Tγδ17 induces angiogenesis, OCUG-1 cells were cultured with cell-free supernatant of Tγδ17 and levels of VEGF were estimated in culture supernatants. As shown in Figure 5*a*, Tγδ17 cells secreted marginal amount of VEGF but upregulated secretion of VEGF by OCUG-1 cells compared to OCUG-1 cultured with medium alone. Addition of neutralizing anti-IL17 antibody significantly abrogated VEGF production by OCUG-1 cells. Further, the addition of rhIL17 to OCUG-1 cells induced VEGF production in a concentration-dependent manner (Fig. 5*b*).

Since, IL17 is known to regulate many downstream target genes associated with angiogenesis, proangiogenic action of Tγδ17 cells on GBC was studied by human protein profiler angiogenesis array. Cell-free supernatants from Tγδ17 cells significantly upregulated secretion of angiogenesis promoting factors from OCUG-1 cells such as VEGF, uPA, MMP9, MCP1, GM-CSF, CXCL16, coagulation factor III, angiogenin, *etc*. compared to OCUG-1 cells cultured with medium alone (Figs. 5*c* and 5*d*). This effect was abrogated by addition of anti-IL17 mAb. OCUG-1 cells secreted high IL8 and addition of Tγδ17 supernatant did not further increase its levels. Along with angiogenesis inducers, certain angiogenesis inhibitors like thrombospondin-1, TIMP-1, serpine-1, platelet factor 4, IGFBP-1, *etc*. were also secreted by OCUG-1 cells in presence of Tγδ17 supernatant.

Further to validate proangiogenic effects of Tγδ17, cell-free supernatants of Tγδ17 were *ex vivo* inoculated in CAM of 5-day-old chick embryos. It was observed that vascularization of CAM was enhanced in presence of Tγδ17 supernatant compared to medium alone. Similar pronounced effect of vasculogenesis was observed in presence of rhIL17. The blood vessels formed in presence of Tγδ17 supernatant showed tree-like branching with increased number of branching points from secondary blood vessels compared to parallel branching observed in presence of medium alone (Figs. 5*e* and 5*f*). Collectively, these results indicate that Tγδ17 cells are proangiogenic and may contribute to carcinogenesis of GBC.

Increased Tγδ17 cells associate with poor survival of GBC patients

To investigate the clinical significance of Tγδ17, Th17, Tc17 and Tregs, the survival time of patients was analyzed with frequency of these cells in peripheral blood of GBC patients ($n = 40$). Patients were divided into two groups based on mean percent values of respective cells (Tγδ17, Th17, Tc17 or Tregs). As shown in Figure 6*a*, cox proportional regression analysis revealed that patients with high Tγδ17 showed poor overall survival (median survival: 8.95 months) than patients with low levels of Tγδ17(median survival: 15.97 months). The individuals with high Tγδ17 levels were at higher risk compared to those with low Tγδ17 levels (Hazard ratio (HR): 2.4). In contrast, the patients with high levels of $\gamma\delta^+IFN\gamma^+$ had longer overall survival than patients with low levels of $\gamma\delta^+IFN\gamma^+$ cells (HR: 0.4; Fig. 6*e*).

Similarly, GBC patients with high levels of Th17 cells had shorter overall survival compared to patients with low levels (HR: 2.32; Fig. 6*b*). Tc17 were not associated with survival of patients (Fig. 6*c*). However, patients with increased Treg cells had poor overall survival (HR: 2.07; Fig. 6*d*). Altogether, the data suggest that Tγδ17, Th17 and Treg cells might serve as valuable factor for prediction of risk and prognosis of GBC.

Discussion

GBC is highly malignant cancer known for its aggressive biological nature and poor clinical presentation. As of today, prognostic biomarkers and effective adjuvant immunotherapy for GBC are unavailable. Therefore, efforts are needed to identify factors contributing to pathogenesis of GBC. Dissecting the complex network of immune cells in peripheral blood and inflammatory tumor environment is vital to design successful immunotherapeutic strategies. Studies in recent years have shown the importance of γδT cells in cell based therapies against cancer.[17,18] Studies from our lab and others have reported that γδT cells exhibit potent cytotoxicity against tumors.[19,20] Various clinical trials have been launched in breast, prostate, hepatocellular and renal cell carcinoma patients using *in vivo* activation of γδT cells or by adoptive transfer of activated γδT cells.[17] In contrast, γδT cells isolated from breast tumor biopsies were shown to mediate immunosuppression by inducing senescence in dendritic cells and $CD4^+$ T cells to suppress their antitumor response.[21] In a murine model, γδT cells are shown as major source of IL17. These cells inhibit $CD8^+$ T cell response and recruit myeloid-derived suppressor cells (MDSCs) thereby promoting development of hepatocellular carcinoma.[22] In a mouse model of breast cancer metastasis, Tγδ17 cells are shown to induce expansion and polarization of neutrophils which suppress cytotoxic antitumor response.[23] Thus, for successful application of γδT cell based immunotherapies in clinics, it is necessary to have a deeper insight into protumor role of γδT cells where data in humans are lacking.

In the present study, we report that Tγδ17 cells are increased in tumor environment and peripheral blood of GBC patients irrespective of clinical stage. Th17 and Tc17 cells were also elevated but $CD8^+$ $IFN\gamma^+$ and $\gamma\delta^+IFN\gamma^+$ cells were decreased in GB tumor environment. This supports the existence of chronic inflammation in GBC contributed by immune cells. A murine study in hepatocellular carcinoma has shown that the depletion of $V\gamma4^+$ γδ T cells (major source of IL17) resulted in significant reduction in tumor volumes in comparison with wild-type mice. Moreover, the infiltrations of effector $CD8^+$ $IFN\gamma^+$ cells in tumors were significantly increased in $V\gamma4^+$ γδT cell–depleted mice.[22] This suggests that Tγδ17 cells exhibit protumor functions by subsiding anti-tumor immune response.

As increased levels of Tγδ17 were observed in GBC patients, we reasoned that the cytokine milieu promoting Tγδ17 differentiation should be present in sera and tumor

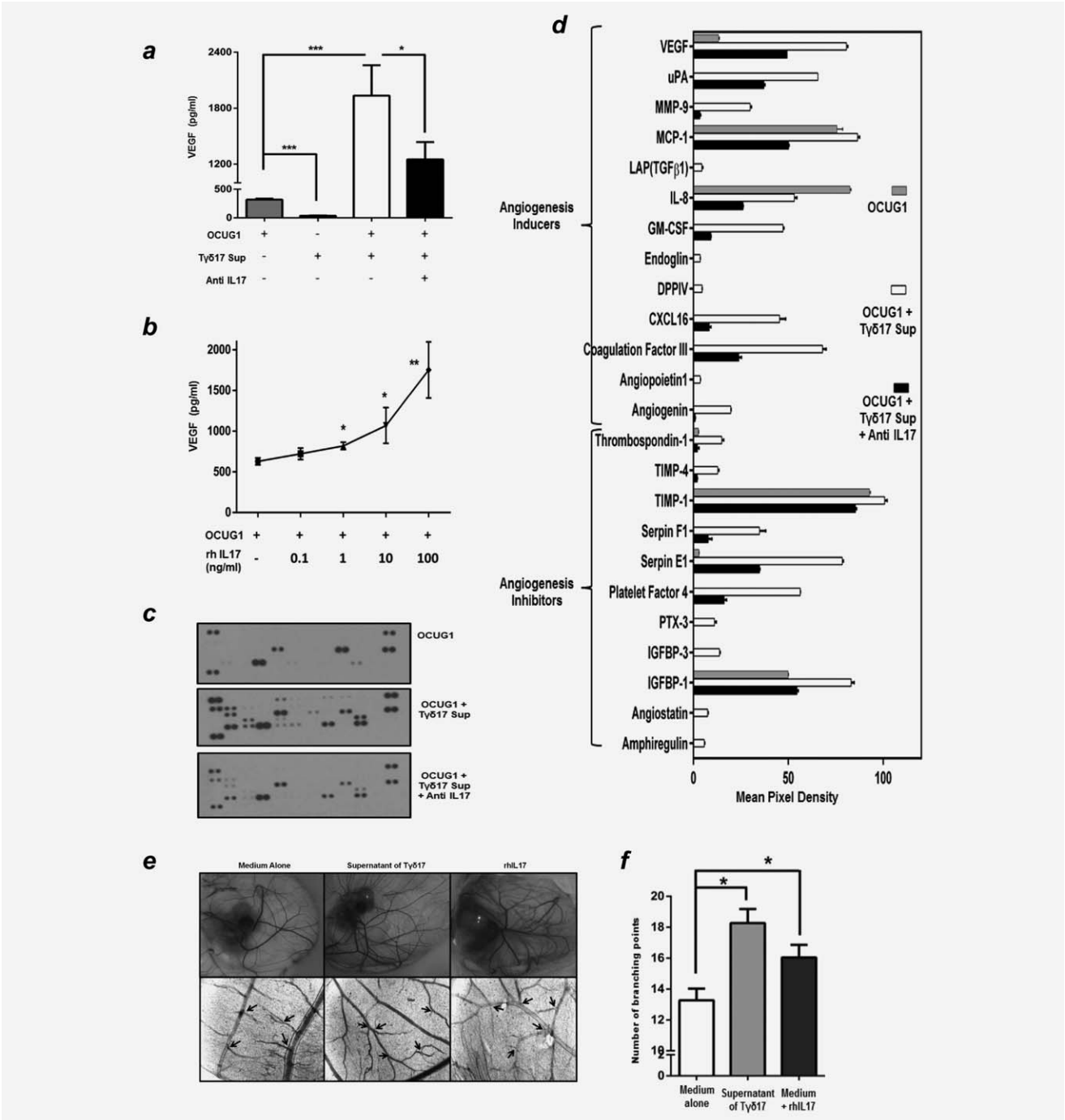

Figure 5. Tγδ17 cells induce angiogenesis in gallbladder cancer cells. (*a*) OCUG-1 cells were cultured with cell-free supernatants of Tγδ17 in presence or absence of neutralizing anti-IL17 antibody. VEGF levels in the supernatants were estimated using ELISA ($n = 6$). (*b*) OCUG-1 cells were cultured with rhIL17 in a serum-free medium for 48 hr. VEGF was estimated in supernatants by ELISA ($n = 6$). (*c* and *d*) Supernatant of Tγδ17 was incubated with OCUG-1 cells in presence or absence of anti-IL17. Angiogenesis related proteins in culture supernatants were analyzed by human angiogenesis proteome profiler array. (*c*) Representative membranes showing array blot developed using chemiluminescence. (*d*) The bar diagram showing densitometric analysis of angiogenesis array blots represented as mean pixel density ($n = 2$). (*e*) Representative images of CAM assay depicting enhanced vasculogenesis in presence of supernatant of Tγδ17 (middle) or rhIL17 (right) compared to medium alone (left). The lower panel shows images of CAM recorded using 4× objective. Arrows indicate branching points. (*f*) Bar diagram showing summarized data of number of branching points in CAM assay ($n = 3$). Results are shown as mean ± SEM with $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

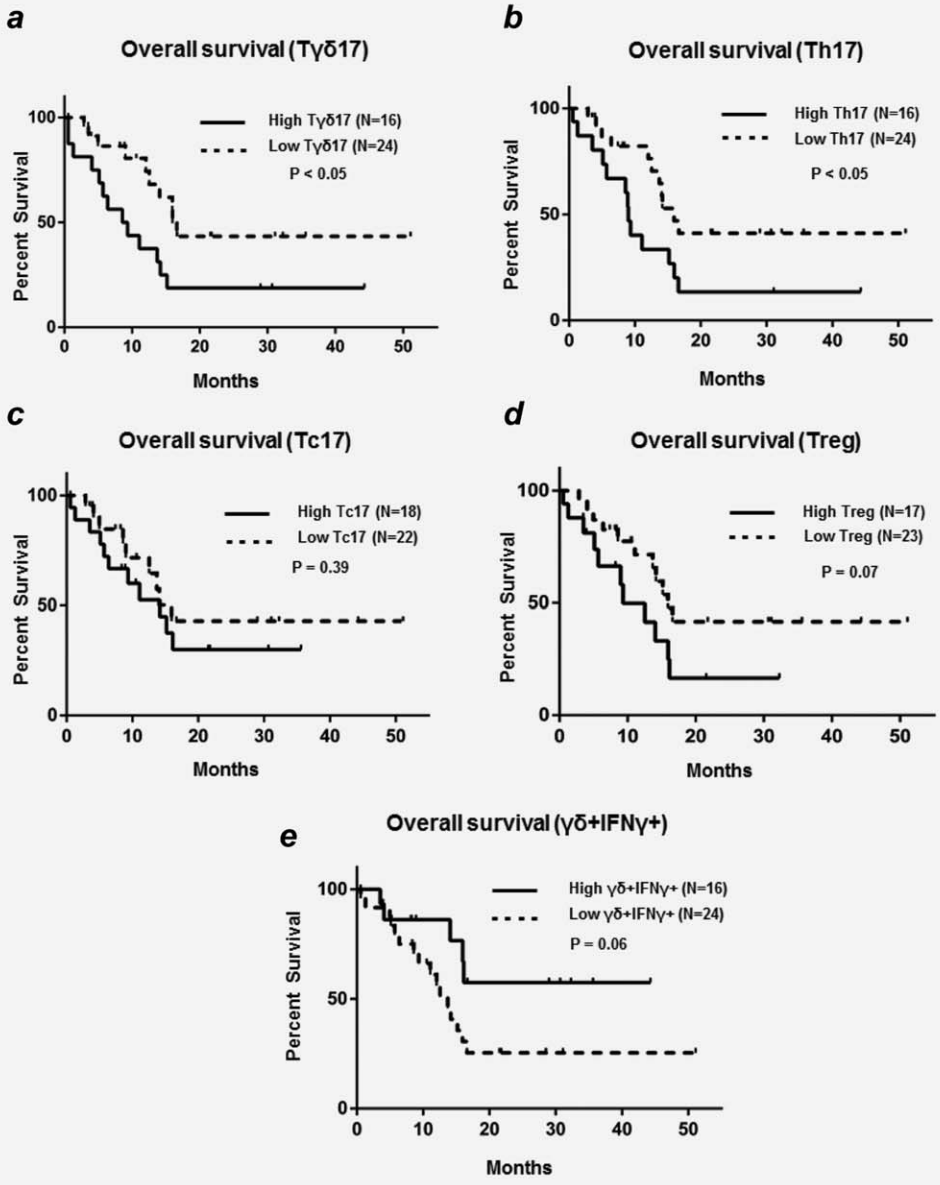

Figure 6. Increased Tγδ17 cells in GBC patients associate with poor survival. Overall survival of GBC patients ($n = 40$) was analyzed by Kaplan-Meier method for low or high levels of Tγδ17 cells (*a*), Th17 cells (*b*), Tc17 cells (*c*), Treg cells (*d*) and $\gamma\delta^{+}IFN\gamma^{+}$ cells (*e*). The curve statistics were compared by log-rank test with $p < 0.05$.

environment. It was documented that like Th17 cells, Tγδ17 also differentiate from naïve γδT cells in the presence of IL6, IL1β, IL23 and TGFβ upon antigenic stimulation.[16] Serum cytokine profile of GBC patients revealed that the levels of IL6, IL1β and IL23 were elevated and are also present in tumor compartment thus making the environment conducive for differentiation of Tγδ17 cells.

In the present study, we showed that IL17 enhanced proliferation of GBC cells highlighting its protumor role. IL17 is known to induce, chemoresistance, neoangiogenesis and activation of matrix metalloproteinases which in turn enhances tumor progression.[24,25] In GBC patients, we observed Tγδ17 and Th17 cells to be associated with poor survival. On the contrary, GBC patients with increased $\gamma\delta^{+}IFN\gamma^{+}$ experienced longer survival. These results strongly suggest that IL17 producing cells play a critical role in immune pathogenesis of human GBC. It appears that tumor environment selectively promotes IL17 producing cells as $CD8^{+}IFN\gamma^{+}$ and $\gamma\delta^{+}IFN\gamma^{+}$ cells were decreased in TILs. Collectively, these results highlight the pathogenic role of Tγδ17 in GBC.

Serum cytokine analysis revealed decreased levels of TGFβ in GBC patients. TGFβ is required for Treg differentiation and maturation.[26] We observed that compared to HI, Tregs were decreased in peripheral blood of GBC patients at all stages of disease. However, their suppressive potential was not compromised suggesting that Tregs in GBC patients are functionally normal. Low levels of TGFβ observed in serum may be

responsible for reduced levels of Tregs in peripheral blood of these patients. Similar observations were reported in multiple myeloma and pancreatic ductal adenocarcinoma patients which showed that Treg cells were significantly reduced in peripheral blood of patients compared to HI.[27,28] It is reported that the Th17 cells differentiate from FOXP3 expressing $CD4^+$ T cells[29] and there exists plasticity in the $CD4^+$ T cells in inflammatory environment.[30] Thus the decreased levels of Treg cells in peripheral blood of GBC patients would be because of skewing of $CD4^+$ T cell polarization toward Th17 phenotype in response to inflammatory cytokine milieu.

After categorizing the GBC patients having high or low levels of Tregs, we observed that patients with high peripheral blood Treg cells have decreased survival compared to those with low levels. We also noted that Tregs were increased in tumor compartment and express elevated levels of Foxp3 compared to peripheral blood of GBC patients. Given that the suppressive activity of Tregs is determined by Foxp3 expression,[31] the Tregs in TILs of GBC patients appear to be more immunosuppressive. A recent study in colorectal cancer patients demonstrated that Tγδ17 cells promote migration and survival of MDSCs which enhanced immunosuppression.[32] MDSCs are also known to induce Treg cells in cancer patients.[33] Thus, the increased levels of Tregs in tumor environment of GBC may be attributed to the Tγδ17 driven inflammation leading to accumulation of MDSCs and subsequent upregulation of Tregs which warrants further investigation. Moreover, it is reported that Th17 and Treg frequently colocalize at the same anatomic compartments and mutually promote each other's generation and function.[13] We observed the infiltration of Treg and IL17 producing cells in the tumor environment of GBC patients which corroborate the earlier observations in GBC patients reported by Zhang *et al.*[34] This suggests that although the ratios of Tγδ17/Treg, Th17/Treg and Tc17/Treg were increased in GBC patients, IL17 producing cells and Tregs may act cooperatively and eventually contribute to the poor survival observed in GBC patients.

Next we addressed the functional role of Tγδ17 cells on GBC tumor progression. We showed that Tγδ17 cells migrate toward tumor milieu through CXCL9-CXCR3 axis. The increased levels of CXCL9 and CXCL10 observed in sera of GBC patients, further supports the elevated levels of Tγδ17 cells observed in the tumor environment. Earlier it was reported that Th17 utilized this axis to migrate toward inflamed liver.[35] This is the first report demonstrating migration of Tγδ17 cells to the tumor environment using CXCL9-CXCR3 axis.

Angiogenesis is a critical step in the progression of solid tumors providing nutrients, growth factors and oxygen for growth of malignant cells. Th17 cells have been shown to be proangiogenic in human head and neck squamous cell carcinoma.[36] Proangiogenic functions of Tγδ17 in human cancer are not yet reported. Our data have shown that human Tγδ17 cells are proangiogenic and induce blood vessel formation as observed in *ex vivo* chick embryo CAM assay. Tγδ17 induced GBC cells to produce proangiogenic factors such as VEGF, uPA, MMP9, MCP1, GM-CSF, CXCL16, Coagulation factor III, angiogenin, etc. through secretion of IL17. A study in $IL17^{-/-}$ murine model has shown that γδT cells were the major source of IL17 and depletion of IL17 resulted in decreased vascular density and tumor growth.[37] Interestingly, we observed that Tγδ17 also induced anti-angiogenic factors (Thrombospondin-1, TIMP1, Serpine1, Platelet factor-4, IGFBP1, *etc.*). However, TIMP-1 and serpine-1 are also reported as markers of poor prognosis in cancer.[38,39] A recent study in glioblastoma showed that IGFBP1 secretion by microglial cells induced by MCSF is essential for angiogenesis.[40]

VEGF is a key mediator of tumor angiogenesis and metastasis. VEGF is reported as an independent prognostic marker and associates with poor survival of GBC patients.[41,42] Bevacizumab which targets VEGF, when combined with chemotherapy has shown direct antitumor effect and improved patient survival.[43] Chemotherapeutic drugs like oxaliplatin, doxorubicin, gemcitabine, 5-fluorouracil trigger cancer cell death which activate antitumor immune response. Gemcitabine (gem) and 5-fluorouracil (5-fu) were shown to induce apoptosis in MDSCs and release of IL1β which activates Th17 cells. Release of IL17 further compromised the antitumor effect of gem and 5-fu.[44] However, in a murine study, Tγδ17 cells improved the anti-tumor efficacy of anthracycline doxorubicin.[45] The studies suggest that the chemotherapeutic drugs should be combined with immunomodulatory agents for increasing efficacy of anticancer therapy. Recently, Secukinumab, monoclonal antibody that binds to human IL17A has been approved for treatment of psoriasis.[46] Other approaches to target IL17 inflammatory axis including monoclonal antibody targeting IL17 receptor (brodalumab), IL23 p40 subunit (ustekinumab), IL23 p19 subunit and small molecules with inverse agonist activity against RORγt are under Phase II/III clinical trials for inflammatory diseases.[47,48] Our data provide insights into proangiogenic role of Tγδ17 through IL17 production which could be a promising candidate for targeted therapy for treatment of GBC. Moreover, the increased levels of Tregs observed in GBC tumor could also be targeted using various immunotherapeutic strategies. Use of antibodies specific for CD25 (daclizumab), CTLA4 (ipilimumab), GITR, OX-40, PD-L1 or PD-1 (nivolumab) subvert the immunosuppression mediated by Treg cells and have demonstrated efficacy in clinical trials.[11,49] Disrupting tumor homing of Tregs by blocking CCR4 mediated migration is advantageous as it transiently inhibit Treg cells only during priming phase and avoid potential autoimmune complications caused by long-term depletion of Treg cells by mAbs.[49,50]

In conclusion, we report for the first time Tγδ17 and Th17 as predictive markers in GBC and provide evidence for the proangiogenic role of human Tγδ17. Our data strongly suggest that Tγδ17 mediated angiogenesis and Treg cells mediated immunosuppression may contribute to the negative clinical outcome of GBC patients. Thus, future immunotherapeutic treatment modality for GBC may use a combined approach to block the trafficking of Tγδ17 cells to the tumor, inhibit functions of IL17 and reverse the immunosuppression mediated by Treg cells.

Acknowledgements

The work is supported by Department of Biotechnology, Government of India, under Centre of Excellence grant (BT/01/CEIB/09/V/06). We acknowledge Department of Atomic Energy for providing research fellowship to R.S.P. We thank Prof. M. Indap (Ruparel College, Mumbai, India) for the help extended toward the chick chorioallantoic membrane assay.

References

1. Dutta U. Gallbladder cancer: can newer insights improve the outcome? *J Gastroenterol Hepatol* 2012; 27:642–53.
2. Randi G, Franceschi S, La Vecchia C. Gallbladder cancer worldwide: geographical distribution and risk factors. *Int J Cancer* 2006; 118:1591–602.
3. Shrikhande SV, Barreto SG, Singh S, et al. Cholelithiasis in gallbladder cancer: coincidence, cofactor, or cause!. *Eur J Surg Oncol* 2010; 36:514–9.
4. Wistuba II, Gazdar AF. Gallbladder cancer: lessons from a rare tumour. *Nat Rev Cancer* 2004; 4:695–706.
5. Zhu AX, Hong TS, Hezel AF, et al. Current management of gallbladder carcinoma. *The Oncologist* 2010; 15:168–81.
6. Sirohi B, Mitra A, Jagannath P, et al. Neoadjuvant chemotherapy in patients with locally advanced gallbladder cancer. *Future Oncol* 2015; 11:1501–9.
7. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010; 140:883–99.
8. Iwakura Y, Ishigame H, Saijo S, et al. Functional specialization of interleukin-17 family members. *Immunity* 2011; 34:149–62.
9. Wilke C, Zou W. TH17 cells in the tumor microenvironment. In: Jiang S, ed. TH17 cells in health and diseaseed. New York: Springer, 2011. 481–96.
10. Patil RS, Bhat SA, Dar AA, et al. The Jekyll and Hyde story of IL17-producing gammadeltaT Cells. *Front Immunol* 2015; 6:37
11. Nishikawa H, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Curr Opin Immunol* 2014; 27:1–7.
12. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 2009; 30:636–45.
13. Chen X, Oppenheim JJ. Th17 cells and Tregs: unlikely allies. *J Leukoc Biol* 2014;95:723–31.
14. Yamada N, Chung Y, Ohtani H, et al. Establishment and characterization of a new human gallbladder carcinoma cell line (OCUG-1) producing TA-4. *Int J Oncol* 1997; 10:1251–5.
15. Ganjalikhani Hakemi M, Ghaedi K, Andalib A, et al. Optimization of human Th17 cell differentiation in vitro: evaluating different polarizing factors. *In Vitro Cell Dev Biol Animal* 2011; 47: 581–92.
16. Caccamo N, La Mendola C, Orlando V, et al. Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9V-delta2 T cells. *Blood* 2011; 118:129–38.
17. Fisher JP, Heuijerjans J, Yan M, et al. gammadelta T cells for cancer immunotherapy: a systematic review of clinical trials. *Oncoimmunology* 2014; 3:e27572
18. Silva-Santos B, Serre K, Norell H. gammadelta T cells in cancer. *Nat Rev Immunol* 2015; 15: 683–91.
19. Dhar S, Chiplunkar SV. Lysis of aminobisphosphonate-sensitized MCF-7 breast tumor cells by Vgamma9Vdelta2 T cells. *Cancer Immun* 2010; 10:10
20. Hannani D, Ma Y, Yamazaki T, et al. Harnessing gammadelta T cells in anticancer immunotherapy. *Trends Immunol* 2012; 33:199–206.
21. Ye J, Ma C, Hsueh EC, et al. Tumor-derived gammadelta regulatory T cells suppress innate and adaptive immunity through the induction of immunosenescence. *J Immunol* 2013; 190: 2403–14.
22. Ma S, Cheng Q, Cai Y, et al. IL-17A produced by gammadelta T cells promotes tumor growth in hepatocellular carcinoma. *Cancer Res* 2014; 74: 1969–82.
23. Coffelt SB, Kersten K, Doornebal CW, et al. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* 2015; 522:345–8.
24. Zarogoulidis P, Katsikogianni F, Tsiouda T, et al. Interleukin-8 and interleukin-17 for cancer. *Cancer Invest* 2014; 32:197–205.
25. Cochaud S, Giustiniani J, Thomas C, et al. IL-17A is produced by breast cancer TILs and promotes chemoresistance and proliferation through ERK1/2. *Sci Rep* 2013; 3:3456
26. Yamagiwa S, Gray JD, Hashimoto S, et al. A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. *J Immunol* 2001; 166:7282–9.
27. Prabhala RH, Neri P, Bae JE, et al. Dysfunctional T regulatory cells in multiple myeloma. *Blood* 2006; 107:301–4.
28. Shibuya KC, Goel VK, Xiong W, et al. Pancreatic ductal adenocarcinoma contains an effector and regulatory immune cell infiltrate that is altered by multimodal neoadjuvant treatment. *PLoS One* 2014; 9:e96565
29. Valmori D, Raffin C, Raimbaud I, et al. Human RORgammat+ TH17 cells preferentially differentiate from naive FOXP3+Treg in the presence of lineage-specific polarizing factors. *Proc Natl Acad Sci USA* 2010; 107:19402–7.
30. Nakayamada S, Takahashi H, Kanno Y, et al. Helper T cell diversity and plasticity. *Curr Opin Immunol* 2012; 24:297–302.
31. Walker MR, Kasprowicz DJ, Gersuk VH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25− T cells. *J Clin Invest* 2003; 112:1437–43.
32. Wu P, Wu D, Ni C, et al. gammadeltaT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. *Immunity* 2014; 40:785–800.
33. Hoechst B, Ormandy LA, Ballmaier M, et al. A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+)Foxp3(+) T cells. *Gastroenterology* 2008; 135:234–43.
34. Zhang Y, Huang Y, Qin M. Tumour-infiltrating FoxP3+ and IL-17-producing T cells affect the progression and prognosis of gallbladder carcinoma after surgery. *Scand J Immunol* 2013; 78: 516–22.
35. Oo YH, Banz V, Kavanagh D, et al. CXCR3-dependent recruitment and CCR6-mediated positioning of Th-17 cells in the inflamed liver. *J Hepatol* 2012; 57:1044–51.
36. Kesselring R, Thiel A, Pries R, et al. Human Th17 cells can be induced through head and neck cancer and have a functional impact on HNSCC development. *Br J Cancer* 2010; 103: 1245–54.
37. Wakita D, Sumida K, Iwakura Y, et al. Tumor-infiltrating IL-17-producing gammadelta T cells support the progression of tumor by promoting angiogenesis. *Eur J Immunol* 2010; 40:1927–37.
38. Ma J, Wang J, Fan W, et al. Upregulated TIMP-1 correlates with poor prognosis of laryngeal squamous cell carcinoma. *Int J Clin Exp Pathol* 2014; 7:246–54.
39. Klein RM, Bernstein D, Higgins SP, et al. SERPINE1 expression discriminates site-specific metastasis in human melanoma. *Exp Dermatol* 2012; 21:551–4.
40. Nijaguna MB, Patil V, Urbach S, et al. Glioblastoma-derived macrophage colony-stimulating factor (MCSF) induces microglial release of insulin-like growth factor-binding protein 1 (IGFBP1) to promote angiogenesis. *J Biol Chem* 2015; 290: 23401–15.
41. Sun XN, Cao WG, Wang X, et al. Prognostic impact of vascular endothelial growth factor-A expression in resected gallbladder carcinoma. *Tumour Biol* 2011; 32:1183–90.
42. Letelier P, Garcia P, Leal P, et al. Immunohistochemical expression of vascular endothelial growth factor A in advanced gallbladder carcinoma. *Appl Immunohistochem Mol Morphol* 2014; 22:530–6.
43. Bizama C, Garcia P, Espinoza JA, et al. Targeting specific molecular pathways holds promise for advanced gallbladder cancer therapy. *Cancer Treat Rev* 2015; 41:222–34.
44. Ghiringhelli F, Apetoh L. Enhancing the anticancer effects of 5-fluorouracil: current challenges and future perspectives. *Biomed J* 2015; 38:111–6.
45. Ma Y, Aymeric L, Locher C, et al. Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy. *J Exp Med* 2011; 208:491–503.
46. Langley RG, Elewski BE, Lebwohl M, et al. Secukinumab in plaque psoriasis–results of two phase 3 trials. *N Engl J Med* 2014; 371:326–38.
47. Lebwohl M, Strober B, Menter A, et al. Phase 3 studies comparing brodalumab with ustekinumab in Psoriasis. *N Engl J Med* 2015; 373:1318–28.
48. Yang J, Sundrud MS, Skepner J, et al. Targeting Th17 cells in autoimmune diseases. *Trends Pharmacol Sci* 2014; 35:493–500.
49. Pere H, Tanchot C, Bayry J, et al. Comprehensive analysis of current approaches to inhibit regulatory T cells in cancer. *Oncoimmunology* 2012; 1: 326–33.
50. Bayry J, Tartour E, Tough DF. Targeting CCR4 as an emerging strategy for cancer therapy and vaccines. *Trends Pharmacol Sci* 2014; 35:163–5.

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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).

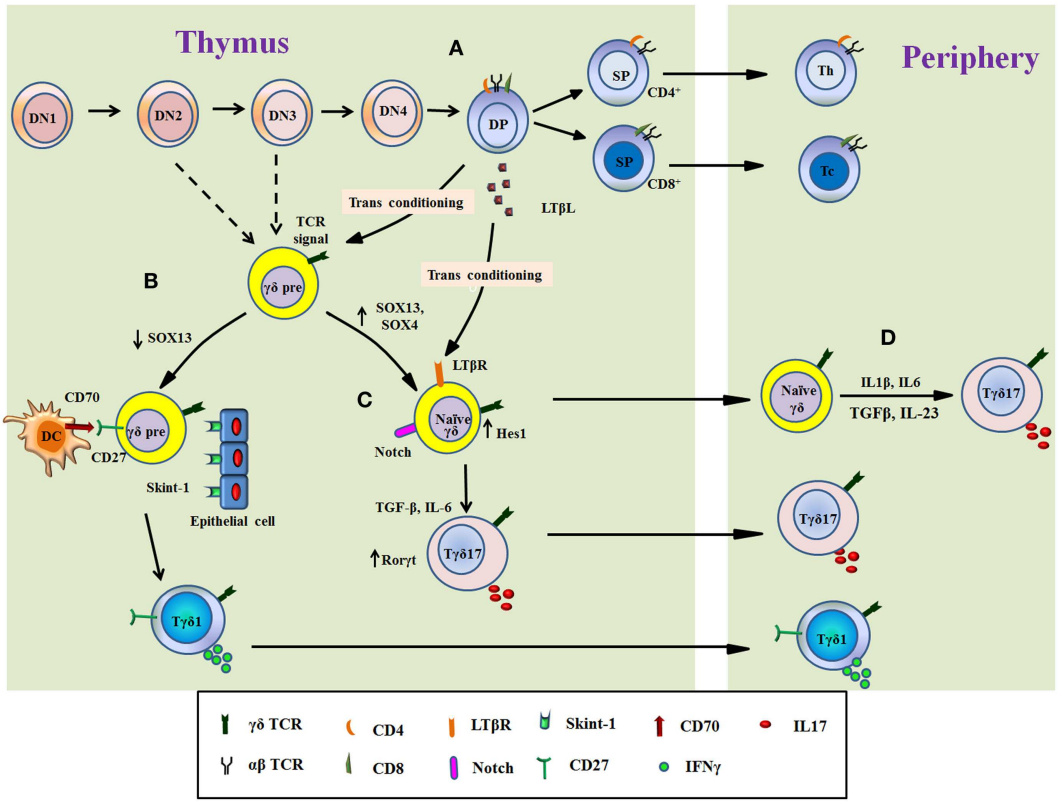

FIGURE 1 | Overview of Tγδ17 cells development. The figure illustrates the differentiation of Tγδ17 cells from T cell progenitors in the murine thymus **(A–C)** and from naïve γδT cells in periphery in human **(D)**. Progenitor T cells differentiate through double negative stage 1 (DN1) to DN stage 4 **(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

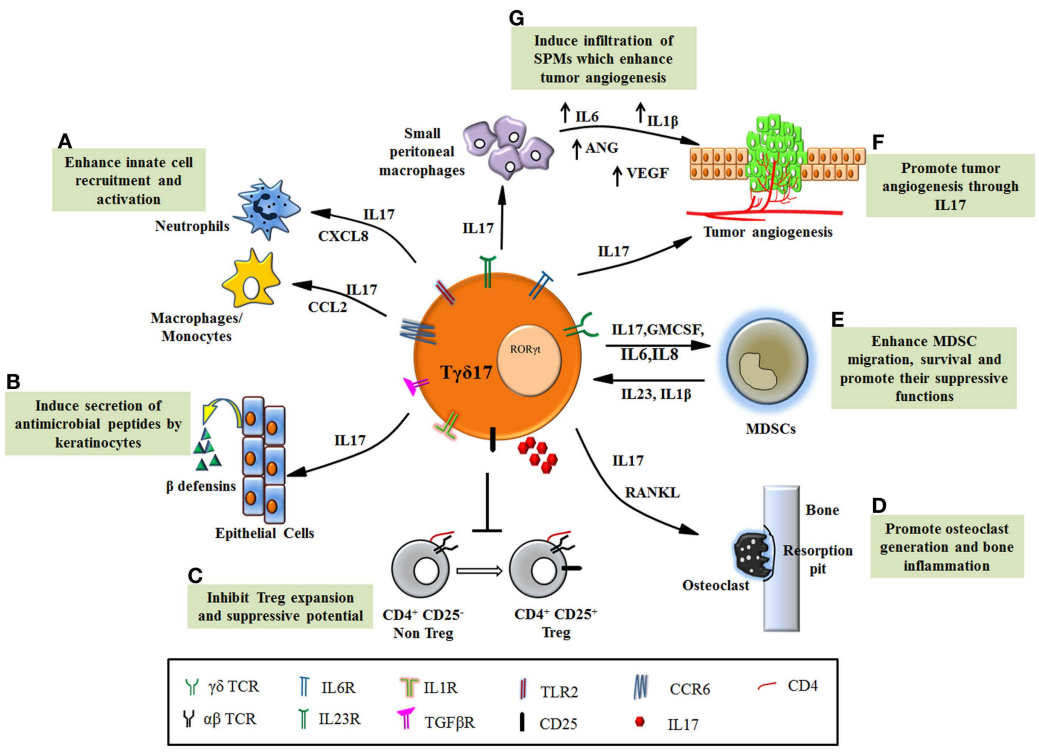

FIGURE 2 | Functions of Tγδ17 cells in pathological conditions. **(A)** Tγδ17 cells promote infiltration of neutrophils and monocytes/ macrophages to the site of inflammation through chemokines. **(B)** IL17 secreted by Tγδ17 cells induces keratinocytes to produce anti-microbial peptides such as β defensins 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

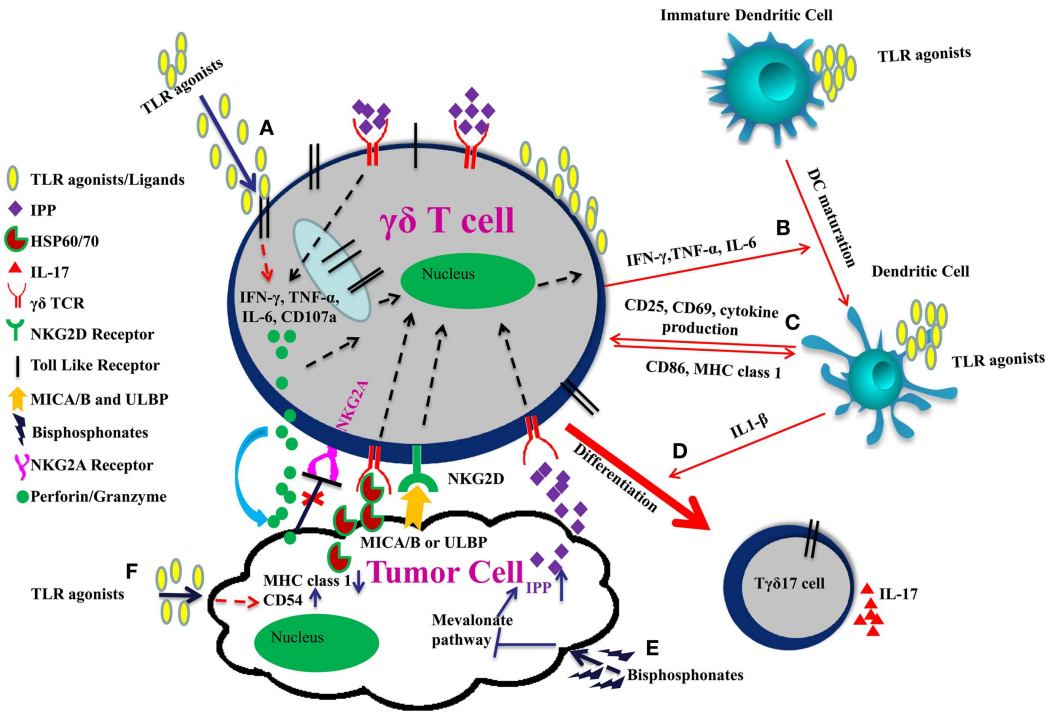

FIGURE 1 | Improving γδ T cell functions by TLRs in combinatorial therapy. **(A)** TLR agonists induce effector function of γδ T cells through IFN-γ, TNF-α, IL-6 secretion, and increased expression of CD107a. **(B)** IFN-γ, TNF-α, and IL-6 secreted by γδ T cells and TLR agonists promote the maturation 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.