Role of Notch in regulation of gamma delta T lymphocytes and regulatory T cell functions

By

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As members of the Viva Voce Board, we certify that we have read the dissertation prepared by Dimpu Gogoi entitled **"Role of Notch in regulation of gamma delta T lymphocytes and regulatory T cell functions"** and recommend that it may be accepted as fulfilling the thesis requirements for the award of Degree of Doctor of Philosophy.

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S.V.Chiplunkar

(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 / University.

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List of Publications arising from the thesis

Journal

1. "Involvement of Notch in activation and effector functions of gamma delta T cells", Dimpu Gogoi, Asif Amin Dar and Shubhada V. Chiplunkar, *The Journal of Immunology*, **2014**, 192(5):20542.

2. "Targeting gamma delta T cells for cancer immunotherapy: bench to bedside", Dimpu Gogoi and Shubhada V. Chiplunkar, *Indian Journal of Medical Research*, **2013**, 138(5):755-61

3. "Notch signaling regulates proliferation and IFN-γ production of $CD3^+$ T cells", Dimpu Gogoi and Shubhada V. Chiplunkar, *Scandavian journal of Immunology*, (Under review)

4. "Notch signal regulates the cross talk between oral tumors, γδ T cells and regulatory T cells" Dimpu Gogoi, Trupti N. Pradhan, Devendra A. Chaukar, Anil K. D'Cruz and Shubhada V. Chiplunkar. (Manuscript under preparation).

Conference proceedings

1. "Involvement of Notch in activation of γδ T cells", Dimpu Gogoi and Shubhada V. Chiplunkar, *The Journal of Immunology*, **2012**, 188:45.22 ("Immunology 2012, 99th Annual Meeting of The American Association of Immunologists", Boston).

Dimpu Gogoi

Presentations at conferences

1. "Role of Notch in regulation of γδ T cells", Oral presentation in '37th Annual Foundation Day celebration of RMRC Dibrugarh', **2014**, RMRC, ICMR, Dibrugarh.

2. "Association of Notch in human γδ T cells and its anti-tumor immunity", Dr. G.P. Talwar Young Scientist Award presentation in '39th Annual Conference of Indian Immunology Society (IMMUNOCON 2012)', **2012**, Banaras Hindu University, Varanasi.

3. "Involvement of Notch in activation of human gamma delta T cells", Poster presentation in Immunology 2012, 99th Annual Meeting of The American Association of Immunologists, **2012**, Boston, USA.

4. "Role of Notch in activation of human gamma delta T cells", Oral presentation in 'Graduate Student Meet' (currently known as 'National Research Scholar Meet in Life Sciences'), **2011**, ACTREC, Navi Mumbai.

5. "Involvement of Notch in effector functions of human gamma delta T cells", Oral presentation in '37th Conference of Indian Immunology Society', **2011**, Jammu University, Jammu.

6. "Role of Notch signaling in antigen specific response of γδ T-cells", Poster presentation in '4th Life sciences Symposium on Recent Advances in Immunomodulation in Stress and Cancer', **2008**, BARC, Mumbai.

7. "Notch signaling in activation of γδ T-cells", Poster presentation in 'Graduate Student Meet', **2008**, ACTREC, Navi Mumbai.

8. Participated in '5th Winter School in Immunology', **2011**, Taj Hari Mahal, Jodhpur.

9. Participated in 'Scientific Communication Workshop' organized by DBT-Welcome Trust, **2011**, IISER, Pune.

10. Served as a Faculty member in 'Basic Course in Flow Cytometers-Hands-on Workshop', organized by Mumbai Immunology Group, **2011**, Mumbai.

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Awards

1. 1st prize for oral presentation in '37th Foundation Day celebration of RMRC Dibrugarh', RMRC, ICMR, **2014**, Dibrugarh.

2. Dr. G.P. Talwar Young Scientist Award in '39th Annual Conference of Indian Immunology Society (IMMUNOCON 2012)', **2012**, Banaras Hindu University, Varanasi.

3. 1st prize for oral presentation in 'Graduate Student Meet', **2011**, ACTREC, Navi Mumbai.

4. Travel Bursary from Indian Immunology Society for oral presentation in '37th Conference of Indian Immunology Society', **2011**, Jammu University, Jammu.

5. Best Poster Presentation in '4th Life sciences Symposium on Recent Advances in immunomodulation in Stress and Cancer', **2008**, BARC, Mumbai.

6. Best Poster Presentation in 'Graduate Student Meet', **2008**, ACTREC, Navi Mumbai.

This thesis is dedicated to my mother.

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CONTENTS

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Ph. D. PROGRAMME

1. Name of the Student:	Dimpu Gogoi.
2. Name of the Constituent Institution:	Tata Memorial Centre, Advanced Centre for Treatment Research and Education in Cancer
3. Enrolment No. :	LIFE09200704002.
4. Title of the Thesis:	Role of Notch in regulation of gamma delta T lymphocytes and regulatory T cell functions.

SYNOPSIS

Introduction:

Notch signaling plays a critical role in several cell-fate decisions during the development and life span of multicellular organisms [1]. The Notch pathway is mediated by isoforms of the Notch receptor and ligands. A most important feature of Notch is that it acts as a transmembrane receptor and a transcription factor [2]. The interaction between Notch receptor and ligand pair cleaves the extracellular and transmembrane domain of Notch through activation of ADAM proteases and gamma secretase respectively resulting in the release of Notch intracellular domain (NICD) [3]. The NICD translocate into the nucleus where it binds to CSL (CBF1, Suppressor of Hairless, Lag-1) and this complex activates the transcription of target genes [4].

Integration of myriad signals (both extrinsic and intrinsic) is required for the activation of T cells. External stimuli emanate through various cell surface receptors that are then transduced and amplified through a coordinated circuitry of signaling cascades. Both extracellular and intracellular signaling components function to impart a fully activated state. Accumulating evidence suggest that Notch family of cell surface receptor plays an important role in T cell activation. The role of Notch in regulating effector functions of $CD8^+$ T cells and NK cells have been described [5, 6]. Signaling through the TCR in both $CD4^+$ and $CD8^+$ T cells induces the activation of Notch1 [6, 7]. Notch signaling was found to regulate both Foxp3 expression and maintenance regulatory T cells (Tregs) in vitro and in vivo [8]. However, the role of Notch in regulating the effector functions of human γδ T cells has not been reported earlier.

γδ T cells are involved in combating infectious diseases and have non-redundant capacities in the inhibition of tumor development and progression [9, 10]. Vγ9Vδ2 cells are predominantly present in the peripheral circulation and respond to non-peptide phosphoantigens [11, 12]. These T cells recognize self and microbial phosphorylated metabolites generated in the eukaryotic

mevalonate pathway and in the microbial 2-C-methyl-D-erythritol 4-phosphate (MEP) or non-mevalonate pathway [13]. One natural antigen from mycobacteria was isolated and identified as Isopentenyl pyrophosphate (IPP) [14]. Subsequent characterization of the microbial antigens recognized by human γδ T cells revealed that they are non-proteinaceous in nature and have critical phosphate residues [12, 15]. There are now number of synthetic phosphorylated compounds that are capable of stimulating γδ T cells like bromohydrin pyrophosphate (BrHPP), 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP or HDMAPP or picostim) and mono-ethyl pyrophosphate [16-18]. Vγ9Vδ2 T cells release copious amounts of INF-γ upon activation and exhibit potent cytotoxicity against virus infected and tumor cells. The unique features of human γδ T cells related to antigen recognition, tissue tropism, lack of antigen processing requirement and cytotoxic function make these ideal candidates for cancer immunotherapy.

Aim and Objectives:

In the present thesis we aimed at addressing the following:

1. Expression of Notch receptors and ligands in human peripheral blood γδ T cells.

2. Involvement of Notch signal in the activation of γδ T cells.

3. Role of Notch in mediating the crosstalk between γδ T cells and regulatory T cells (Tregs).

4. Role of Notch signaling in mediating the effector functions of peripheral γδ T cells from oral cancer patients.

Materials and Methods:

Study Group

Blood samples were collected from healthy individuals and patients with squamous cell carcinomas of the oral cavity TNM classification Stage I- IV. Heparinized blood was collected prior to surgery and tumor tissues were obtained from oral cancer patients after surgical excision. The study was approved by the institutional Ethics Committee

γδ T cell expansion

PBL were isolated from healthy individuals (HI) and oral cancer patients by Ficoll Hypaque density gradient centrifugation and were enriched by solid phase anti CD3 mAb and recombinant IL-2 (rIL-2) as described by Yamaguchi et al (1997).

Purification of γδ and regulatory T cells (Tregs)

γδ T cells were purified from the expanded PBMCs using MicroBeads. Tregs were purified from PBMCs using BD immunomagnetic separation kit. The separation procedure was conducted according to the manufacturer's instructions.

Isolation of tumor cells from surgically excised tumors

The tumor tissues were minced finely and were incubated in RPMI medium containing double strength antibiotics and enzyme mixture (0.05% collagenase, 0.02% DNase and 5U/ml hyaluronidase along with 1mg/ml trypsin) for 2 hours with intermittent shaking. The tumor tissue was then passed through a 200-gauge wire mesh to obtain single cell suspension.

Quantitative RT-PCR

RNA was extracted from immunomagnetically purified γδ T cells, oral cancer cells (AW13516, AW8507 and surgically excised oral tumors) and breast cancer cells (MCF-7, MDA-MB and surgically excised breast tumors) using Trizol reagent. Quantitative RT-PCR for different Notch receptor isoforms, ligands and its target genes was performed with PRISM 7700. Samples were analyzed using TaqMan primer sets purchased from Applied Biosystems. All values were normalized to the expression of the housekeeping gene β-actin.

Western blotting

$1X10^6$ γδ T cells were incubated with rIL-2 (100U/ml) and bromohydrin pyrophosphate (BrHPP/IPH1101) which was kindly provided by Innate Pharma at a concentration of 200nM for

24 h. These cells were pretreated for 30 min at 37°C with γ-secretase inhibitor-X, L-685,458 (GSI-X) at a concentration of 15 μM, or left untreated, before stimulation. The expression of N1ICD and c-Myc were analyzed by Western blot analysis. The expression of Notch ligands were analyzed on AW13516, AW8507, surgically excised oral and breast tumors using Jag1 and Dll1 antibodies. β-actin was used as a loading control. Immunostaining was performed using appropriate secondary antibody and developed with ECL plus Western blot detection system.

Flow cytometry

Purified γδ T cells were rested overnight at 37°C. Next day, these cells were rinsed in cold phosphate buffered saline (PBS) and cold-fixed in 1% paraformaldehyde in PBS for 10 min at 4^0C. The cells were washed and permeabilized for 15 mins with 0.1% saponin in PBS. Cells were stained with mouse anti-human γδ TCR antibody, sheep anti-human Notch1 intracellular domain (N1ICD) antibody or rabbit anti-human Notch2 intracellular domain (N2ICD) antibody for 45 min at 4°C. Thereafter, cells were washed and incubated with rhodamine-labeled goat anti-mouse IgG and FITC-labeled donkey anti-sheep IgG or FITC- labeled goat anti rabbit IgG respectively for another 45 min at 4°C. For cell surface markers non-permeabilized cells were stained with antibodies against CD14, CD15, CD19, CD33, CD56 CD69, CD25, Cd107a, Dll1 and Jag1 ligands. The Annexin PI staining was performed to determine the effect of GSI-X on the cell viability using propidium iodide (PI) and FITC-conjugated Annexin V. For this, γδ T cells were left untreated or were stimulated with rIL2 alone or rIL2 and BrHPP for 48 h at 37°C in round bottomed 96 well plates. GSI-X was added as described above. Data was analyzed using FlowJo software.

Confocal microscopy

As mentioned above, purified γδ T cells were stained for N1ICD and N2ICD. Similarly, the expression of HES1 was examined using mouse anti-human HES1 antibody and FITC-labeled donkey anti-sheep IgG. For nuclear staining, 4|, 6-diamino-2-phenylindole (DAPI) was used. The samples were visualized on Zeiss Laser-Scanning Microscope 510 (LSM510) META.

Proliferation assays and cytokine ELISA

Proliferation of γδ T cells was assayed by ^{3}H-Thymidine (^{3}H TdR) uptake assay. A total of $5X10^{4}$ γδ T cells were pretreated for 30 min at 37°C with γ-secretase inhibitor-X, L-685,458 (GSI-X) at different concentration ranging from 2.5-15μM, or left untreated, before cells were stimulated in round-bottom 96-well tissue culture plates with rIL-2 (0.1IU/ml) and plate bound anti CD3 (mAb) (1μg/well), for 72 h. Similarly, γδ T cells were also incubated in round-bottom 96-well tissue culture plates with rIL-2 (0.1IU/ml) plus BrHPP (200nM) or IPP (40μM) or 20nM of c-HDMAPP (IPH1201/picostim) or rDll1(0.25μg), rDll4(1.5μg) and rJag1 (0.5μg) (R&D Systems) for 72 h. For experiments using GSI-X, cells were pretreated with 15 μM of GSI-X. The cultures were pulsed with 1 μCi [^{3}H] thymidine (Board of Radiation and Isotope Technology, Mumbai) during the last 18 h of the assay. The radioactivity incorporated in the DNA was measured in a liquid scintillation counter.

For cytokine ELISA, γδ T cells were stimulated with anti CD3 (mAb) with or without GSI-X as described above. Likewise, γδ T cells were treated with different phosphoantigens (IPP, BrHPP and c-HDMAPP) or recombinant Notch ligands (rDll1, RDll4 and rJag1). After 24h, supernatants were collected, and IFN-γ concentration was assayed with an ELISA-based assay using anti-IFN-γ purchased from BD Biosciences.

Cytometric bead array (CBA)

Cytokines (IL2, IL4, IL6, IL10, IL17, IFN-γ and TNF-α) were quantitated in cell culture supernatant by Cytometric bead array kit. Cytokines were determined in the test samples according to the manufacturer instructions and acquired on BD FACS Aria cytometer. The CBA data were analyzed using FCAP Array software version 1.0.

Cytotoxicity assay

^{51}Chromium release assay was used to measure the cytotoxicity against oral cancer cell line (AW13516) as target cells. γδ T cells were left alone or treated with rIL2 (0.1U) overnight at 37^0C and AW13516 cells were treated for 18 h with zoledronate (100μM). For experiments using GSI-X, cells were pretreated as described above. Standard 4h ^{51}Chromium release assay was performed as previously described [19]. The radioactive chromium release was measured using 1470 Wallac automated gamma counter.

Small interfering RNA

γδ T cells isolated by MACS column (as above) were transfected with small interfering RNA (siRNA) specific for NOTCH1, NOTCH2 genes and fluorescent oligonucleotide SiGLO (transfection indicator). siRNA oligos were transfected at a concentration of 50 nM using X-treme GENE HP transfection reagent. The inhibition of Notch1 and Notch2 expression were assessed at 48 h.

Regulatory T cells (Tregs) suppression assay

Freshly isolated γδ T cells were labeled with 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester, (CFSE). CFSE labeled γδ T cells ($1X10^4$) were then co-cultured with purified regulatory T cells ($1X10^4$) and stimulated with rIL2 (0.5 IU/ml) and BrHPP (200 nm) for 5 days. CFSE labeled γδ without regulatory T cells in the presence of rIL2 and BrHPP were kept as control. Tregs cells were previously treated with either anti Jag1 antibody (0.5 μg) (to block Jag1 ligand) or Fc

control for 2 h at 37ºC. After 5 days cells were harvested and analyzed on FACS Aria flow cytometer. Data obtained was analyzed using FlowJo software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0. The student's t test was used as the test of significance.

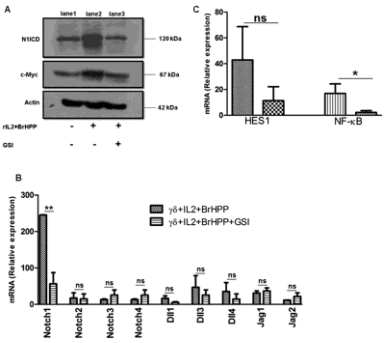
Results:

I. Expression of Notch receptors and ligands in human peripheral blood γδ T cells

It was observed that purified γδ T cells from healthy individuals express mRNA for Notch1 and Notch2 receptors along with lower level of Dll1 and Jag1 ligands. The interaction between Notch receptor and ligand pair cleaves the extracellular and transmembrane domain of Notch through activation of ADAM proteases and gamma secretase respectively [3]. The gamma secretase induced cleavage generates Notch intracellular domain (NICD) which translocates to the nucleus. The expression of NICD in γδ T cells was detected by confocal microscopy, flow cytometry and western blotting. Next, Notch target gene-HES1 was shown to express in both freshly isolated and ex-vivo expanded γδ T cells. Also, mRNA expression of other Notch target genes along with HES1 viz. NF-κB, Deltex and NRARP in γδ T cells was observed.

II. Involvement of Notch signal in activation of γδ T cells

Disruption of Notch signaling in activated γδ T cells reduces expression of Notch receptor and target genes

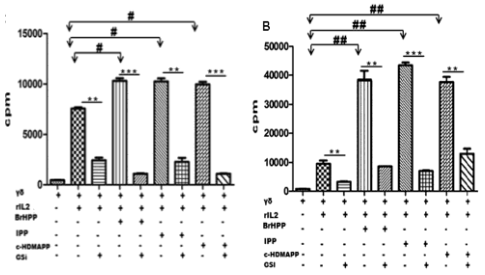


In the present study GSI-X was used to block γ-sectretase activity in γδ T cells [20]. Stimulation of γδ T cells with BrHPP and rIL2 triggered the activation of Notch signaling which was observed by abundant release of N1ICD in cell lysates as

analyzed by western blotting. Treatment of BrHPP and rIL2 activated γδ T cells with GSI-X inhibited the release of processed Notch1 (decreased NICD expression) compared to γδ T cells stimulated BrHPP and rIL2 alone (Figure). Stimulation of the Notch signaling pathway leads to the induction of c-Myc expression [21]. It was observed that Notch1 activation governs the downstream induction of c-Myc expression which was abrogated upon GSI-X treatment. Simultaneously, expression of mRNA for Notch receptors (1-4) and Notch ligands (Dll1, Dll3 and Dll4 and Jag1 and Jag2) in γδ T cells stimulated with BrHPP and rIL2 in the presence and absence of GSI-X were monitored. Following treatment with GSI-X a marked decreased in the expression of mRNA for Notch1 receptor was observed in antigen activated γδ T cells (Figure).

Inhibition of Notch signaling blocks γδ T cell activation and proliferation

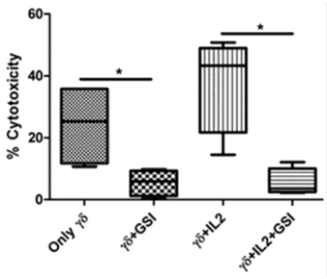
In order to determine the functional consequences of Notch signal in activated γδ T cells, γδ TCR mediated proliferation response was assessed by ^{3}H-TdR incorporation assay. A marked increase in the proliferation of γδ T cells was observed in the presence of anti CD3 and rIL2. However, blocking of Notch signal by GSI-X leads to decreased proliferative response in a concentration dependent manner. Ex-vivo expanded as well as freshly isolated γδ T cells were used to compare their ability to respond to phosphoantigens in the presence and absence of GSI-X (Figure). A significantly increased proliferative response of γδ T cells to phosphoantigens (BrHPP, IPP and picostim) in the presence of rIL2 was observed. Freshly isolated γδ T cells showed robust proliferative responses to antigens compared to ex-vivo expanded γδ T cells. However, in the presence of GSI-X the proliferative responses of γδ T cells to antigens were significantly reduced in both sets of isolated γδ T cells. Ex-vivo expanded γδ T cells alone (unstimulated) or after stimulation with



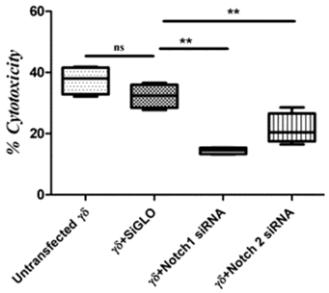
BrHPP and rIL2 showed higher expression of CD25 (late activation marker) compared to CD69 (early activation marker). Treatment with GSI-X showed reduction in CD25 and CD69 expression on γδ T cells.

Notch regulates cytolytic potential of γδ T lymphocytes

The effect of Notch signaling in cytolytic potential of γδ T lymphocytes was examined. Upon co-incubation with the oral cancer cells, few γδ T cells showed surface expression of CD107a a marker of degranulation (lysosome associated membrane protein 1, LAMP1). However, in the presence of rIL2, the proportion of CD107a+ γδ T cells increased. Addition of GSI-X leads to reduction in the percentage of CD107a+ in both unstimulated γδ T lymphocytes and also in rIL2 stimulated γδ T cells. Upon co-incubation with the oral cancer cells, few γδ T cells showed surface expression of CD107a (18.4%). The cytotoxic potential of γδ T cells against zoledronate treated tumor cells (AW13516) was determined by titration at different effector (E):



Target (T) ratio ranging from 5:1 to 40:1 maximum cytotoxicity of γδ T cells was observed at E: T ratio of 40:1. This ratio of E: T was used in further experiments where effect of GSI-X on cytolytic ability of γδ T cells was examined. At E: T ratio of 40:1, ex-vivo expanded γδ T cells in the presence of rIL2 efficiently lysed zoledronate treated oral tumor cells compared to untreated cells. Addition of GSI-X significantly reduced the cytotoxic ability of γδ T cells against zoledronate treated tumor targets (Figure).



The siRNA sequences targeting the Notch1 and Notch2 were transfected into the γδ T cells. Antitumor cytotoxic ability of γδ T cells transfected with siRNA specific for Notch1 and Notch2 against zoledronate treated tumor cells (AW13516) were

compared. Consistent with GSI-X treatment data silencing of both Notch1 and Notch2 in γδ T cells led to significant reduction in cytotoxic potential of γδ T cells against Zoledronate treated oral tumor cells (Figure). This finding supports the role of Notch signal in regulating cytotoxic potential of γδ T cells. Collectively the data proves that the cytotoxic action of γδ T cells is regulated by Notch signal.

Notch signaling regulates cytokines production in activated γδ T cells

To address whether the Notch signal has any role in the effector functions of activated γδ T cells, the effect of GSI-X on cytokines production was examined. The consequence of GSI-X treatment on the concentration of TH1 (IL2, IFN-γ and TNF-α), TH2 (IL4, IL6 and IL10) and TH17 (IL17) production by γδ T cells treated with rIL2 alone or along with BrHPP was assayed using cytometric bead array (CBA). It was observed that inhibition of Notch signaling leads to marked reduction in TNF-α, IFN-γ and IL17 production by activated γδ T cells. The inhibition was more pronounced in IFN-γ production. Therefore the effect of GSI-X on IFN-γ production by ELISA was confirmed. γδ T cells were stimulated with c-HDMAPP, IPP and BrHPP which leads to increased production of IFN-γ. The amount of IFN-γ decreased when cells were pretreated with GSI-X. Like phosphoantigens, anti-CD3 MAb stimulation of γδ T cells in the presence of GSI-X also showed a dose dependent decrease in IFN-γ production. Thus, a role of Notch in regulating IFN-γ production in γδ T cells is unconcealed which suggest that Notch plays a role in effector functions of γδ T cells.

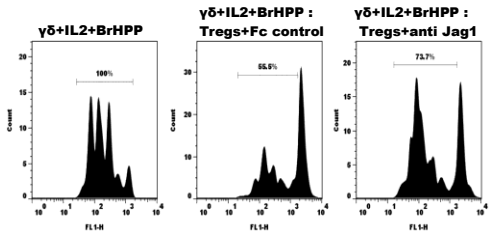
Effect of Notch ligands on effector functions of γδ T cells

The contribution of Notch ligands in effector functions of γδ T cells has not been reported earlier. It was observed that stimulation of γδ T cells with rDll1 and rDll41 augments whereas rJag1 significantly decreases phosphoantigen (BrHPP, IPP, Picostim) driven proliferation of γδ T cells.

Similarly, rJag1 significantly decreases IFN-γ secretion by phosphoantigen activated γδ T cells. It was also observed that rJag1 decreases anti CD3 stimulated proliferation and IFN-γ production by γδ T cells in a concentration dependent manner. The result indicates that stimulation with Notch ligands have different effects on γδ T cells with Dll1 and 4 having stimulatory and Jag1 having inhibitory effects on proliferation and IFN-γ production by γδ T cells.

III. Role of Notch in mediating crosstalk between γδ T cells and Tregs

$CD4^+CD25^+$ T cells (Tregs), but not $CD4^+CD25^-$ T cells, express cell surface Notch ligand Jag1[22]. However, as mentioned above, stimulation of γδ T cells with Jag1 inhibits the effector function of γδ T cells. Interestingly, anti Jag1 antibody pretreated Tregs have reduced suppressive potential as manifested by increased proliferation of antigen (BrHPP) stimulated γδ T cells (Figure). It was also observed that oral cancer cell lines (AW13516 and AW8507) and surgically excised oral tumors as well as breast cancer cell lines (MCF-7 and MDA-MB) and surgically excised breast tumor predominantly expressed Jag1 ligand as confirmed by western blotting experiments. This result thus indicates that notch signal plays a role in mediating the cross-talk between γδ cells and regulatory T cells.



IV. Role of Notch signaling in the effector functions of peripheral γδ T cells from oral cancer patients

Expression of Notch receptors in γδ T cells from oral cancer patients

Immune dysfunction is the hallmark of patients with oral cancer [23]. The expression of Notch1 and Notch2 genes were observed in γδ T cells from oral cancer patients. With lower expression of Dll1, Dll3 and Jag1 mRNA, it was observed that N1ICD was abundantly expressed on the cell membrane, cytoplasm and nucleus. Unlike γδ T cells from healthy individual, the N1ICD in oral cancer patients showed punctate staining. The expression of HES1 in the nucleus of γδ T cells

was also observed indicating the presence of active Notch signaling in γδ T cells from oral cancer patients.

Inhibition of Notch signaling blocks γδ T cell proliferation

The role of Notch in antigen driven proliferative response of γδ T cells from oral cancer patients was examined. It was also observed that the proliferation of γδ T cells was not significantly increased in the presence of the phosphoantigens which was in contrast to that observed in healthy individuals. Treatment with GSI-X leads to significant decrease in the proliferation of γδ T cells.

Notch regulates effector function of γδ T lymphocytes from oral cancer patients

Upon co-incubation with the zoledronate treated oral cancer cells (AW13516), γδ T cells of oral cancer patients showed surface expression of CD107a. Treatment with GSI-X leads to decreased expression of CD107a+ in γδ T cells cocultured with zoledronate treated AW13516. The cytotoxic potential of γδ T cells of oral cancer patients was assessed against ^{51}Cr- labelled zoledronate treated AW13516 cells in presence and absence of GSI-X. Addition of GSI-X significantly reduced the percent cytotoxicity against zoledronate treated oral tumor targets.

Blocking of the Notch signal in γδ T cells by GSI-X leads to marked decrease in production of TNF-α, IFN-γ and IL17 cytokines by γδ T cells. The data proves that the cytotoxic action of γδ T cells from oral cancer patients is regulated by Notch signal.

Discussion

These investigations, to our knowledge, have led to the first report that show expression and antigen-specific effector functions of Notch signal in peripheral human γδ T cells isolated from healthy individuals and oral cancer patients. The present study showed Notch1 and Notch2 expression in γδ T cells derived from healthy individuals and oral cancer patients at both mRNA and protein levels by real-time PCR, flow cytometry and confocal microscopy respectively.

Unlike γδ T cells from healthy individual, the N1ICD in oral cancer patients showed a characteristic punctate staining. Using BRHPP, a synthetic phosphoantigen, in the presence of pharmacological GSI-X, loss of Notch signaling in γδ T cells results in downregulation of mRNA for Notch1 and Notch2 receptors. Reduction in the expression of NICD by GSI-X in BrHPP-stimulated γδ T cells confirmed the involvement of Notch signaling in regulating antigen-specific responses of γδ T cells.

Further, inhibiting Notch signaling in anti-CD3mAb-stimulated γδ T cells resulted in marked decrease in proliferation of γδ T cells, confirming TCR engagement as a key initiating event affected by GSI-X treatment. Similarly, γδ T cells (freshly isolated and ex vivo expanded) activated with BrHPP, IPP, and c-HDMAPP as antigens resulted in a significantly decreased proliferation of γδ T cells in response to these antigens in both healthy individuals (HI) and oral cancer (OC) patients. The observation that Notch signaling is involved in regulating antigen-specific proliferative responses of γδ T cells prompted us to look at the expression of early and late activation markers CD69 and CD25 on T cells. Reduction in CD69 expression and a marked decrease in CD25 (IL-2R) expression on antigen-stimulated γδ T cells was observed when Notch signaling was inhibited.

It was observed that blocking of Notch signaling in γδ T cells by GSI-X inhibits the ability of γδ T cells to lyse tumor targets. Moreover, specific silencing of either Notch1 or Notch2 by siRNA led to the reduced cytotoxic potential of γδ T cells. This result suggests that both Notch1 and Notch2 are involved in the cytolytic activity of γδ T cells. IFN-γ plays crucial role in protective immune response against certain pathogens and tumors [9, 24, 25]. Blocking Notch signaling with GSI-X inhibited the IFN-γ secretion by γδ T cells stimulated with phosphoantigens BrHPP, IPP and c-HDMAPP and also anti CD3 activated γδ T cells. Thus notch signaling appears to play an

important role in modulating the antigen specific proliferation, IFN-γ secretion and ability to lyse the tumors properties of γδ T cells derived from healthy individuals as well as oral cancer patients.

Further experiment showed that rDll1 and rDll41 augments whereas rJag1 significantly decreases phosphoantigens driven proliferation of γδ T cells. Similarly, rJag1 significantly decreases IFN-γ secretion by phosphoantigen activated γδ T cells. It was also observed that rJag1 decreases anti CD3 stimulated proliferation and IFN-γ production by γδ T cells in a concentration dependent manner. The results indicate that delta like ligands (rDLL1 and rDLL4) and Jag1 ligands act in different ways, with Dll1 and 4 exhibiting stimulatory while Jag1 is showing inhibitory affect on γδ T cells. $CD4^+$ $CD25^+$ Tregs were found to predominantly express Jag1 ligand and, to a lesser extent, Dll4 on the cell surface [26]. Interestingly, blocking of Jag1 ligand on Tregs reduces their suppressive potential. It was observed that anti Jag1 antibody pretreated Tregs have reduced suppressive potential as manifested by increased dividing cells of γδ T cells. This result thus indicates cross-talk between γδ cells via notch signal. Oral cancer cell lines (AW13516 and AW8507) and surgically excised oral tumors as well as breast cancer cell lines (MCF-7 and MDA-MB) and surgically excised breast tumor predominantly express Jag1 ligand. Hence, it appears that upregulation of Jag1 on tumors and Tregs may inhibit γδ T cells and can be viewed as an immune evasion strategy that facilitates tumor progression.

Our findings describe that Notch is involved in regulating the effector functions of human γδ T cells. These studies identify Notch as an additional signal contributing to antigen specific effector functions of γδ T cells from HI and OC patients. Interestingly, suppression of antigen specific γδ T cell responses by Tregs is mediated by Jag1. These studies may have important implications in

clinical situations where new strategies for the manipulation of γδ T cells for cancer immunotherapy are being investigated.

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2) Dimpu Gogoi, Asif A. Dar and Shubhada V. Chiplunkar. Involvement of Notch in activa and effector functions of gamma delta T cells. Journal of Immunology 2014; 192(5):2054-62

3) Dimpu Gogoi & Shubhada V. Chiplunkar. Notch signaling regulates proliferation and IFN-production of $CD3^+$ T cells. Immunity, Inflammation and Disease (Under review).

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Date: 24/02/2014

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ABBREVIATIONS

γδ	Gamma delta
αβ	Alpha beta
Tregs	Regulatory T cells
NK	Natural killer
Th	T helper
CTL	Cytotoxic T lymphocyte
Ag	Antigeen
MEP	2-C-methyl-D-erythritol 4-phosphate
TCR	T-cell receptor
MHC	Major histocompatibility complex
HSPs	Heat shock proteins
MIC	MHC class I chain-related molecules
ADCC	Antibody dependent cellular cytotoxicity
TAA	Tumor associated antigen
IFN	Interferon
TNF	Tumor necrosis factor
IL	Interleukin
NICD	Notch intracellular domain
NECD	Notch extracellular doimain
CSL	CBF1, Suppressor of Hairless, Lag-1
Dll.	Delta like
Jag.	Jagged
HES	Hairy enhancer of split
HERP	Hes-related repressor protein
NRARP	Notch regulated ankyrin repeated protein
NF-κB	Nuclear factor kappa B
DTX	Deltex
PEST	A proline, glutamine, serine, threonine-rich domain

EGF	Epidermal growth factor
T-ALL	T-cell acute lymphoblastic leukemia/lymphoma
TLR	Toll like receptor
VEGF	Vascular endothelial growth factor
HIF	Hypoxia inducing factor
APC	Antigen presenting cell
LPS	Lipopolysaccharide
HMBPP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
IPP	Isopentenyl pyrophosphate
BrHPP	Bromohydrin pyrophosphate
HDMAPP	1-Hydroxy-2-methyl-2-buten-4-yl 4-diphosphate
NBP	Nitrogen-containing bisphosphonates
FPPS	Farnesyl pyrophosphate synthase
ULBP	UL-16 binding protein
mAbs	Monoclonal antibodies
rIL2	Recombinant interleukin 2
rDll	Recombinant delta like ligand
rJag	Recombinant jagged
CTLA4	Cytotoxic T lymphocyte associated protein 4
TIM3	T cell immunoglobulin mucin 3
IDO	Indoleamine 2,3-dioxygenase
SCC	Squamous cell carcinoma
WHO	World Health Organization
FCS	Fetal calf serum
FBS	Fetal bovine serum
DMSO	Dimethyl sulphoxide
HI	Healthy individual
PBL	Peripheral blood lymphocyte
GSI-X	γ-secretase inhibitor-X
RPMI	Roswell Park Memorial Institute
µl	Micro litre

µM	Micro molar
µg	Micro gram
FITC	Fluoresecein isothiocyanate
PE	Phycoerytin
DAPI	4,6-diamidino-2-phenylindole
SDS	Sodium dodecyl sulphate
RT	Room temperature
cDNA	Complementary deoxy ribo nucleic acid
siRNA	small interfering ribo nucleic acid
PBMC	Peripheralblood mononuclear cells
PCR	Polymerase chain reaction
CBA	Cytometric bead array
EDTA	Ethylenediaminetetraacetic acid
DTT	Dithiothreitol
DEPC	Diethylpyrocarbonate
KDa	Kilodalton
DAMP	Damage associated molecular patterns
bHLH	Basic helix loop helix

Chapter 1:

Introduction

The immune system has evolved to protect the host from infections and cancer. Typically, the immune system is divided into two categories- innate immunity and adaptive immunity. The innate immune system comes into play immediately after the appearance of antigen whereas the adaptive immune system provides antigen-specific response.

The innate immune system comprises a wide variety of mechanisms immediately available for combating infectious diseases. It represents the front line of host defence, involving physical barriers presented by epithelial layers, chemical defenses including antibacterial peptides, complement and lytic enzymes, as well as biological responses from innate immune cells patrolling the periphery [1]. The defensive arsenal employed by the innate immune system is effective in combating many pathogens, however this system is constrained by relying on a limited and invariable repertoire of pattern recognition receptors (PRRs) [2].

It is only when innate host defenses are impeded, avoided, or overwhelmed that the adaptive immune system is needed. In contrast to innate immunity, an adaptive immune response is specific for particular antigens (Ags) and is also capable of instilling immunological memory, so that prior infections trigger stronger and more immediate responses to subsequent infections [3, 4].

In addition to these defense mechanisms, there are unconventional T cells like the gamma delta (γδ) T lymphocytes and natural killer T (NKT) cells that functionally and phenotypically belong to both the innate and the adaptive immune system and are able to bridge the two [5-7]. In the peripheral circulation of humans, γδ T cells comprise about 1-10% of the circulating T cells, though this percentage can rise to as high as 50% at some mucosal sites [8]. These cells provide immediate action against pathogens and also show memory response. γδ T cells are involved in

combating infectious diseases and have non-redundant capacities in the inhibition of tumor development and progression [9, 10].

Vγ9Vδ2 cells are predominantly present in the peripheral circulation and respond to non-peptide phosphoantigens [11, 12]. These T cells recognize self and microbial phosphorylated metabolites generated in the eukaryotic mevalonate pathway and in the microbial 2-C-methyl-D-erythritol 4-phosphate (MEP) or non-mevalonate pathway [13]. Accumulation of mevalonate metabolites in tumor cells is a powerful danger signal that activates the γδ T cells [14, 15]. The unique features of human γδ T cells related to antigen recognition, tissue tropism, lack of antigen processing requirement and cytotoxic function make these ideal candidates for cancer immunotherapy. Vγ9Vδ2 T cells release copious amounts of INF-γ upon activation and exhibit potent cytotoxicity against virus infected and tumor cells. Unlike αβ T cells, γδ T cells do not require the help of conventional major histocompatibility complex (MHC) class I and class II molecules for recognizing the antigens [7]. Antigen recognition by γδ T cells is dependent upon the particular variable (V) region of the T cell receptor (TCR) as opposed to the entire rearranged TCR required by αβ T cells. In vitro and in vivo studies have demonstrated anti-tumor activity of γδ T cells, including against renal, prostate, colon and pancreatic cancers and melanoma, myeloma and lymphoma [16-21]. γδ TCR interacts with higher concentration of isopentenyl

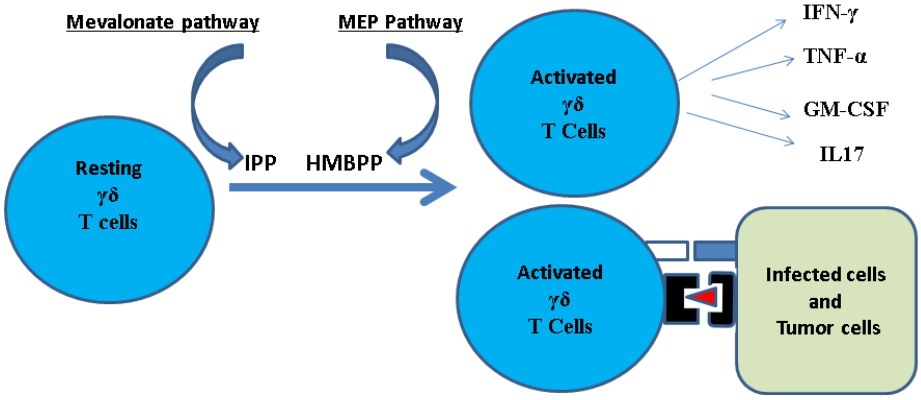

Figure 1: Activation of Vγ9Vδ2 T cells

pyrophosphate (IPP) generated through the dysregulated mevalonate pathway in tumors. Moreover, γδ T cells recognize heat shock proteins (HSPs) and MHC class I chain-related molecules (MICA/B) or UL-16 binding protein ULBP expressed on tumor cells via. their TCR and NKG2D receptors respectively. Perforin and granzyme released from activated γδ T cells lyse the tumor cell. γδ T cells can also kill tumor cells through antibody dependent cellular cytotoxicity (ADCC) . γδ T cells expressing CD16 (FCγRIII) interacts with tumor associated antigens (TAA) via. specific monoclonal antibodies and mediate ADCC. Upon actiation, γδ T cells secrete cytokines like IFN-γ and TNF-α that can recruit other immune cells (bystander effect).

However, tumors are known to directly interfere with the host immune system. Malignant progression is accompanied by profound immune suppression that interferes with an effective antitumor response and tumor elimination. Immune evasion by tumor cells can be attributed to the tumor microenvironment that helps tumor to dodge immune attack by preventing the expansion of tumor antigen-specific T cells and instead promote the production of proinflammatory cytokines and other factors inhibiting immunity. Further, changes in the tumor cells themselves (loss of tumor antigens, loss of human leukocyte antigen molecules, loss of sensitivity to complement, or T cell or natural killer (NK) cell lysis), helps them to escape immune recognition [22]. In cancer patients, γδ T cells have been shown to be numerically and functionally impaired [23, 24]. To exploit γδ T cells for cancer immunotherapy we need to understand the various signals/molecules that regulate their function.

Accumulating evidence suggest that Notch family of cell surface receptor plays an important role in T cell activation. The Notch pathway is mediated by isoforms of the Notch receptor and ligands. A most important feature of Notch is that it acts as a transmembrane receptor and a

transcription factor [25]. The interaction between Notch receptor and ligand pair cleaves the extracellular and transmembrane domain of Notch through activation of ADAM proteases and gamma secretase respectively resulting in the release of Notch intracellular domain (NICD) [26]. The NICD translocate into the nucleus where it binds to CSL (CBF1, Suppressor of Hairless, Lag-1) and this complex activates the transcription of target genes [27].

The role of Notch in regulating effector functions of $CD8^+$ T cells and NK cells have been described [28, 29]. Signaling through the TCR in both $CD4^+$ and $CD8^+$ T cells induces the activation of Notch1 [29, 30]. Notch signaling was found to regulate both Foxp3 expression and maintenance regulatory T cells (Tregs) in vitro and in vivo [31]. However, the role of Notch in regulating the effector functions of human γδ T cells has not been reported earlier.

In the present thesis we aimed at addressing the following:

1. Expression of Notch receptors and ligands in human peripheral blood γδ T cells.
2. Role of Notch in regulating effector functions of peripheral γδ T cells.
3. Activation of γδ T cells by Notch ligands.
4. Involvement of Notch signaling in effector functions of peripheral γδ T cells of oral cancer patients.

Chapter 2:

Review of literature

A century has passed since Dexter (1914) observed a mutant in Drosophila which generated serrations on the wing margin that lends the name Notch to the gene. In the early 1930s, Don Poulson while conducting work for his doctoral thesis under the supervision of A.H. Strutevant and Th. Dobzhansky discovered the allele of gene that was related to the phenotype of notch wing in the fruit fly. This work was done at a time when the relationship between genes and embryonic development was very much in doubt. Over the past 30 years Notch field has grown exponentially. We now know that Notch plays an essential role in both embryonic and adult life. Its function can be found in neuronal development, stem cell biology, wound healing, angiogenesis, immune system, etc. Perturbations in Notch signaling pathway activity have been linked to several human genetic disorders and cancers [32].

I. The structure of Notch

Notch is 300 kilodalton transmembrane receptor, which requires ligands to trigger the signaling events in the cell. The ligands for Notch are also transmembrane proteins [33]. In mammals, there are four Notch genes and five genes encoding ligands, three Delta-like (Dll1,3 and 4) and two Jagged (Jag1 and2) [25]. The Notch gene encodes members of family of receptors that spans the cell membrane with part of it inside and part outside (Figure 2). The Notch receptor is synthesized as a single large protein in endoplasmic reticulum. In golgi, Notch receptor undergoes first cleavage by furin like protease. This cleavage generates non-covalent linked heterodimeric Notch that then migrates to the cell membrane [34]. The heterodimeric Notch extracellular domain (NECD) is composed of conserved array of upto 36 epidermal growth factor (EGF) repeats, involved in ligand interaction. The membrane-tethered Notch intracellular domain (NICD) includes seven ankyrin repeats flanked by nuclear localizing signals, a proline, glutamine, serine, threonine-rich (PEST) domain and a transactivation (TAD) domain [35, 36].

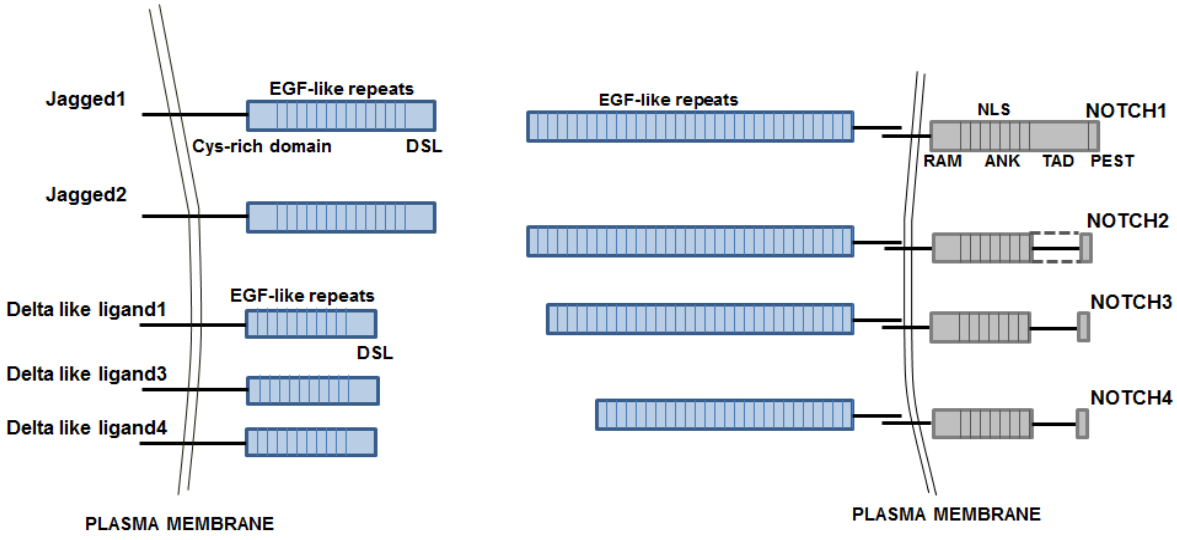

Figure 2: Structure of Notch receptor isoforms and its ligands

The differences in the notch receptors are present in their extracellular and intracellular regions. Notch1&2 have 36 extracellular epidermal growth factor repeats whereas Notch2 has 34 and Notch4 has 29 repeats. Notch1 and Notch2 are differentiated by the fact that Notch1 has a strong TAD region whereas Notch2 has a weak TAD region and Notch 3 and Notch 4 lack TAD region [37, 38]. Notch receptors are activated by five types of ligands in mammals (Jag 1 & 2 and Dll 1, 3& 4). These ligands are also transmembrane proteins with an extracellular region, transmembrane region and an intracellular region (Figure 2). The main structural difference between the Delta and Jagged/Serrate ligands is that the Jagged contain in the extracellular region a greater number of EGF repeats and also insertions within the EGF repeats [25]. Also, the Jagged contain a cysteine-rich region that is entirely absent from the Delta ligands [39].

II. Mechanism of action: The Notch signaling Pathway

A most important feature of Notch is that it acts as a transmembrane receptor and a transcription factor [25]. The initiation of Notch signal requires binding of a ligand to the 29-

36 tandems EGF repeats of NECD (Figure 3). The repeats 11-12 are required for productive interactions with ligands presented by neighboring cells (trans interactions). In contrast, cis-inhibition by ligands expressed in the same cell depend on EGF repeats 24–29 [40]. Many EGF repeats bind to calcium ions, which play an important role in determining the structure and affinity of Notch in ligand binding [41]. In addition, EGF-like repeat can be modified by O-linked glycans at specfic sites [42]. GDP-fucose protein *O*-fucosyltransferase 1 (POFUT1) adds O-fucose which is absolutely required for Notch function. Notch signaling can be also regulated by Fringe [N-acetylglucosaminyltransferase (GlcNAc-transferases) enzyme] [43]. Addition of GlcNAc to the O-fucose sugar leads to strong interaction of NECD to Delta ligand, but has markedly inhibited signaling when interacting with the Jagged ligand [44]. The other part of Notch, i.e., NICD is essentially a membrane-tethered transcription factor whose release is regulated by ligand binding [45]. The interaction between NECD and any of its ligands result in the shedding of the ectodomain and exposure of an extracellular metalloprotease site. The exposed site thus becomes susceptible to cleavage by transmembrane proteases of the ADAM/TACE (a disintigrin and metallopeptidase/tumor necrosis factor-α converting enzyme) family. This cleavage is followed by another cleavage at intramembrane position by γ-secretase enzyme [46-48]. The NICD of Notch is thus released and translocates into the nucleus where it binds to CSL (CBF1, Suppressor of Hairless, Lag-1) and this complex activates the transcription of target genes [47, 49]. To date, many Notch target genes have been identified. One of the best characterized is the hairy enhancer of split (HES) family of transcription factors [27]. The related Hes-related repressor protein (Herp) transcription factor family [50], the cell cycle regulator Cdkn1a [51], the gene for Notch-regulated ankyrin repeat protein (NRARP) [52], Deltex1 [53].

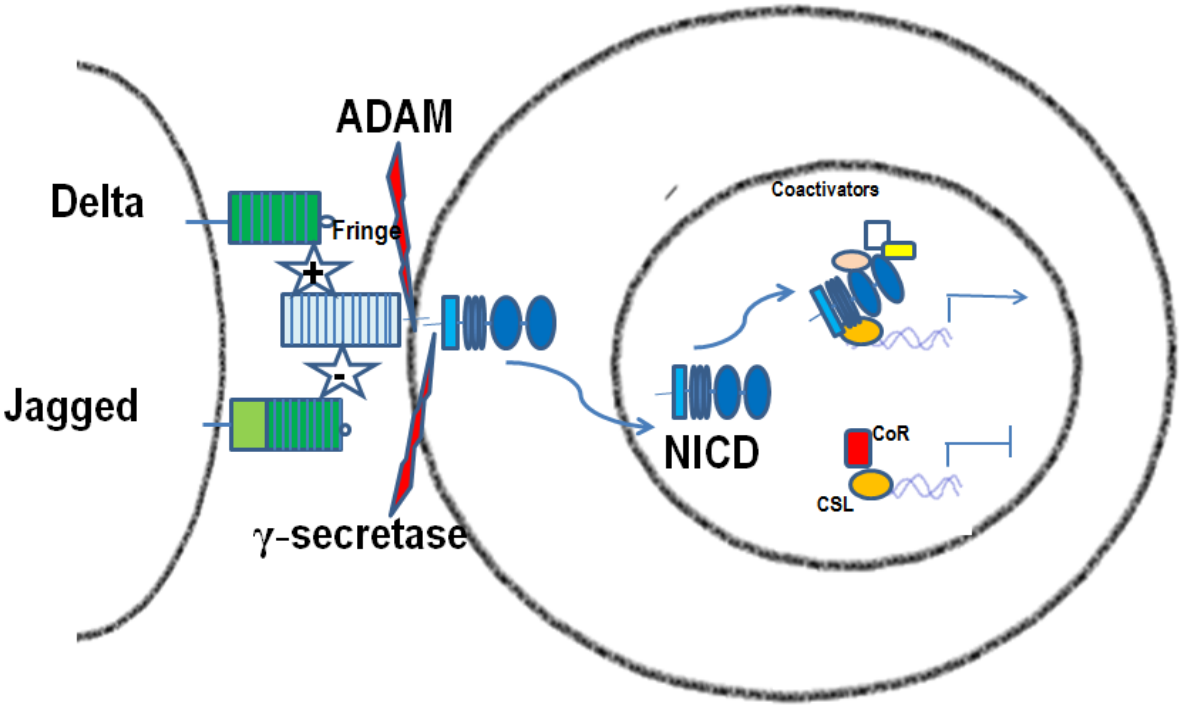

Figure 3: Canonical Notch signaling Pathway

This well-documented Notch signaling pathway is evolutionary conserved across species and occurs both in invertebrates and vertebrates to impart a wide variety of functions. Interestingly, non-canonical Notch signaling has also been reported that occur independent of CSL [54]. The non-canonical Notch signaling was also first reported in Drosophila. It was reported that a non-canonical Notch signaling is required for axon guidance in Drosophila [55]. Notch activity was implicated in dorsal closure during embryonic development which occurs independent of Su (Drosophila CSL) [56]. Similarly, it was found that that epithelial transformation by the transcription factor, KLF4 does not require CSL and therefore occurs through non-canonical Notch signaling [57]. In human peripheral T cells, it was observed that Notch regulates transcription independent of CSL by associating with NF-κB proteins, p50, or c-rel [29, 58]. There are reports of non-canonical non-nuclear Notch signaling where the effect of NICD in activation of mTOR and Akt was demonstrated to be localized in the cytosol [59, 60].

III. Notch in tumor cells

While Notch signaling is crucially involved in normal regulation of cell differentiation, proliferation, development, dysregulation of the signaling cascade often has profound effect on the cellular fate and may lead to tumor formation. In cancer, Notch signaling can function both in an ongogenic as well as in a tumor suppressive manner and the outcome seems dependent on its normal function in a given tissue. Generally, Notch may act as an oncogene in tissues where it is involved in stem cell self-renewal or in cell fate decisions and may have a tumor suppressive role in tissues where Notch promotes terminal differentiation [61-63]. Ellisen and his colleagues were the first to associate altered Notch function and cancer, as they defined a t(7:9)(q34;q34.3) chromosomal translocation in T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) which resulted in constitutive activation of Notch receptor [64]. Later on, activating mutations in the PEST domains were discovered in 55-60% of human T-ALLs [65]. In tumor, Notch signaling can function both in an ongogenic as well as in a tumor suppressive manner depending upon the cellular context [66]. An oncogenic role for Notch signaling has also been discovered in Hodgkin's lymphoma, anaplastic large-cell non-Hodgin's lymphoma, some acute myeloid leukemias, B-cell chronic lymphoid leukemias as well as several epithelial malignancies of the breast, cervix, lung, colon, prostate, head and neck, kidney, pancreas, as well as gliomas, medulloblastomas, and sarcomas [67-69]. However, several of the mechanisms underlying the deregulation in these malignancies are unclear but altered expression of the Notch receptors or other Notch signaling pathway components are often associated with poor prognosis or tumor metastasis [70]. Overactive Notch signaling in solid malignancies may lead to overexpression of genes important for proliferation e.g. CDK2 and cyclin D1 and repression of CDK inhibitors p27Kip1 and p57Kip2 [71, 72]. The proto-oncogenes Ras and c-myc are linked to Notch

signaling in tumorigenesis. Notch may function as a downstream target of Ras and in a positive feed-back loop act as an activator of Ras signalling [73]. C-Myc is directly regulated by Notch1 in T-ALLs [74]. In some tumor types, including human hepatocellular carcinoma, skin and small lung cancer, expression of Notch1, Notch2, Jagged1 and Hes1 are reduced [75, 76] and speculatively activated Notch signaling may function in a tumor suppressive manner [62, 69]. Also, exome sequencing of head and neck squamous cell carcinoma reveals that Notch1 acts as tumor suppressor in this type of tumor [77]. Another aspect of Notch signaling in cancer is its role in tumor angiogenesis. Notch1and Notch4 as well as the ligand Dll4 have been shown to interact with vascular endothelial growth factor (VEGF) and Hif-1α which are key controllers of angiogenesis [78].

IV. Notch in T cell lineage commitment

Notch signaling is required in lineage decisions in hematopoietic progenitor cells. Notch has been implicated in the T versus B lymphocyte differentiation from a common lymphocyte precursor [79]. It is also known that Notch signaling is intimately involved in γδ versus αβ lineage decision [80]. It is interesting to point out that the molecular events triggering T cell development are essentially different in human and mice (Figure 4). In mice several reports suggest that Notch favors αβ T cell lineage over γδ T cells [79-81]. The development of γδ T cells from γδ T cells receptor (TCR)-expressing T cell progenitors requires

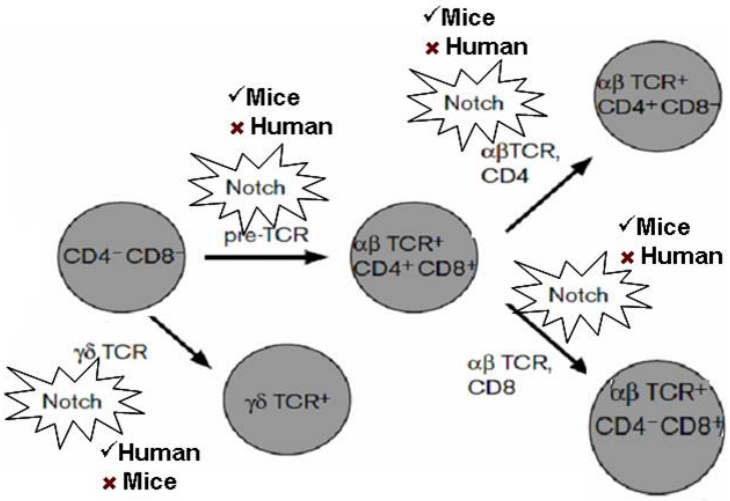

Figure 4: Notch signaling in αβ versus γδ T cells lineage

the absence of Notch ligand interaction [82]. In contrast, there is an opposing role for Notch signal in human αβ /γδ lineage decision. The induction of γδ-lineage precursors to split off from the αβ T cell program by Notch1 activity was observed in human [83]. It was also reported that high level of Notch activation generates T-lineage precursors and γδ T cells but inhibits differentiation towards αβ lineage [84]. Interestingly, in Hes1-deficient mice, γδ T-cell repertoires developed normally. However, IL-17–producing γδ T cells were strikingly decreased which indicates Notch signaling plays an important role in development of IL-17–producing γδ T cells in mice fetal thymi [85].

V. Notch signaling in effector functions of immune cells

Naive CD4+ and CD8+ T cells express Notch1 and Notch2 [86, 87]. Notch signaling is known to be involved in T cell activation. Upon TCR stimulation, Notch gene expression is rapidly induced and also, Notch signaling contributes to regulation of $CD4^+$ T cell immune responses by modulating CD25 expression [88]. Detection of NICD and canonical Notch target gene-HES1 indicate ongoing Notch signaling in stimulated T cells [30, 88]. In both mouse and human, Notch was shown to regulate Th17 differentiation. Blockade of Notch signaling significantly downregulates the production of Th17-associated cytokines. Promoter analysis and chromatin immunoprecipitation (ChIP) assays demonstrated regulation of both the IL-17 and RORγt promoters by Notch1 [89]. Signaling through the TCR in both $CD4^+$ and $CD8^+$ T cells induces the activation of Notch1 [29, 30]. Notch activation has been shown to be important for NF-κB activation and proliferation as well as IFN-γ production by αβ T cells [30]. Notch signaling is involved in cytolytic effector function in $CD8^+$ T cells by regulating the expression of key effector molecules, perforin and granzyme B [29]. Notch signaling was shown to be required for potent anti tumor immunity. Notch2 was found to be essential for mediating anti tumor immunity

by $CD8^+$ T cells [90]. In addition, Notch and cyclic AMP-responsive element (CRE) pathways were shown to intersect dynamically to regulate transcription of effector molecules in cytotoxic T lymphocytes [91]. Moreover, dendritic cells with lower expression of the Notch ligand Dll1 were less effective in inducing the differentiation of cytotoxic T lymphocytes [91]. Also, Notch signaling plays critical roles in the determination of M1 versus M2 polarization of macrophages. Activation of Notch signaling increased M1 macrophages which produce IL12 to promote tumoricidal responses. Whereas, when Notch signaling was blocked, the M1 inducers induced M2 response, produce IL10 and help tumor progression [92].

Accumulating data supports a role of Notch in regulating cross talk between antigen presenting cell (APC) and αβ T cells. It was observed that a stimulus that normally induces a Th1 response, such as lipopolysaccharide (LPS), results in upregulation of Dll4 on APCs. In contrast, type2 stimuli such as cholera toxin induced Jag2 expression [86]. Antigenic stimulation of naive $CD4^+$ T cells in the context of APCs engineered to express Dll-1 led to the secretion of Th1 cytokines, whereas Jagged1 promoted a Th2 cytokine profile. In human peripheral blood it was found that conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) expressed high levels of Notch-1 and Notch-2, and a variable proportion of the cells were positive for Jag1, Jag2, Dll1, and Dll4 [93].

Several reports suggest a role for Notch in modulating the function of regulatory T cells (Tregs). $CD4^+$ $CD25^+$ Tregs were found to predominantly express Jag1 ligand and, to a lesser extent, Dll4 on their cell surface [94]. It was also shown by the same group that phosphorylated Smad3 arising from TGF-β signaling binds to NICD arising from Notch signaling and that the complex formed facilitates the translocation of phosphorylated Smad3 to the nucleus. Notch signaling was found to be regulating Foxp3 expression and Tregs maintenance both in vitro and *in vivo* [31]. In

addition, Notch act as a molecular switch between proinflammatory and antiinflammatory Th1 cell function. Notch regulates IL-10 production in Th1 cells by a STAT4-dependent process that converts proinflammatory Th1 cells into T cells with regulatory activity [95].

Notch signaling can also be involved in NK cell development. Stimulation of human cord blood $CD34^+$ cells by Dll4 in a stroma-free culture induced the generation of cells with characteristics of functional NK cells, including CD56 and CD161 Ag expression, IFN-γ secretion, and cytotoxic activity against K562 and Jurkat cells [96]. Moreover, Pax5-deficient pro-B cells efficiently differentiate into NK cells upon transient Dll1 mediated signaling [97]. It was also demonstrated that Notch activation in peripheral blood NK cells leads to increased IFN-γ secretion [98].

However, the role of Notch in regulating the effector functions of human γδ T cells has not been reported earlier.

VI. γδ T lymphocytes

The majority of mature T cells express αβ TCR which recognizes peptides presented on the surface of APC to $CD4^+$ T cells by MHC class II molecules or to $CD8^+$ T cells by MHC class I. In addition, an alternative TCR heterodimer composed of γ and δ chains also exist [99]. In the peripheral circulation of humans, γδ T cells comprise about 1-10% of the circulating T cells, though this percentage can rise to as high as 50% at some mucosal sites [8]. γδ T cells are involved in combating infectious diseases and have non-redundant capacities in the inhibition of tumor development and progression [9, 10]. Six of the best γδ T cell functions were recently described by Adrian Hayday's group [100]. They are: (a) cytokine and chemokine production, (b) lysis of infected or stressed cells, (c) regulation of stromal cell function via growth factor production, (d) dendritic cell maturation, (e) priming of αβ T cells via antigen presentation and (f) B cell help and IgE production.

VI A. Antigen recognition and activation of γδ T lymphocytes

Unlike αβ T cells, γδ T cells do not require the help of conventional major histocompatibility complex (MHC) class I and class II molecules for recognizing the antigens [7]. Antigen recognition by γδ T cells is dependent upon the particular variable (V) region of the TCR as opposed to the entire rearranged TCR required by αβ T cells. γδ T cells expressing Vδ1 are abundantly found at mucosal sites and they respond to the expression of non-classical MHC molecules on the surface of virally-infected or tumor cells [101-103]. Vδ2+ (Vγ9Vδ2) cells are predominantly present in the peripheral circulation and respond to non-peptide phosphoantigens [11, 12]. Vγ9Vδ2 T cells recognize the pyrophosphorylated isoprenoid intermediates (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), an intermediate in the foreign 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, and isopentenyl pyrophosphate (IPP), an intermediate in the self-mevalonate pathway [13, 104, 105]. Various compounds like steroid hormones, cholesterol, many types of vitamins, rubber, etc. are derived from this pathway. It was observed that during bacterial and protozoan infections, Vγ9Vδ2 T cells expand to high levels which in some individuals represented the majority of circulating T cells [106]. The first chemically defined antigens for Vγ9Vδ2 were found to be alkyl phosphates [107]. One natural antigen from mycobacteria was isolated and identified as IPP [108]. Subsequent characterization of the microbial antigens recognized by human γδ T cells revealed that they are non-proteinaceous in nature and have critical phosphate residues [12, 109]. The Vγ9Vδ2 crystal structure confirmed the presence of a basic, positively charged region in the binding groove that could directly interact with the negatively charged pyrophosphate moiety of the antigen [11]. The MEP pathway's final intermediate, HMBPP is very similar in structure to IPP, but is much more potent [110]. The ED50 of IPP is ~20 μM, whereas that of HMBPP is ~70 pM, *i.e.* more than 105

times lower. Tumor cells accumulate IPP at concentrations at least 500 times lower than the ED50 measured using exogenous IPP, nevertheless they stimulate TCR Vγ9Vδ2 cells in a IPP-dependent manner, providing evidences that endogenous IPP is active at much lower concentrations than the ED50 measured with the exogenous one [14, 111, 112]. There are now number of synthetic phosphorylated compounds that are capable of stimulating γδ T cells like bromohydrin pyrophosphate (BrHPP), picostim and mono-ethyl pyrophosphate [107, 113, 114].

In addition to phosphoantigens, there are reports on additional ligands for human γδ T cells. Bisphosphonates, especially nitrogen-containing bisphosphonates (NBP) are widely used to treat postmenopausal osteoporosis and skeletal malignancies. NBP like pamidronate, alendronate, zoledronate,etc inhibit the key enzyme farnesyl pyrophosphate synthase (FPPS) of the mevalonate pathway, thereby upregulating the pool of endogenous IPP. The accumulated IPP activates Vγ9Vδ2 T cells to release inflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). Bisphosphonates have been used for the treatment of Paget's disease, tumor-associated bone diseases, and osteoporosis [115-119]. Evidence for the stimulation of γδ T cells by bisphosphonates was found when increased numbers of γδ T cells were observed in patients who had acute-phase reactions after their first bisphosphonates treatment [120]. The γδ T cells activated by bisphosphonates can secrete high levels of proinflammatory cytokines and kill tumor cells [121-123].

Another class of molecules that stimulate Vγ9Vδ2 T cells is alkylamines. Alkylamines are secreted by certain commensal bacteria. They are also present in edible plant products such as tea, wine, apples and mushrooms [124]. Alkylamines act in a manner similar to NBP, causing the subsequent intracellular accumulation of IPP [125]. This suggests that only pyrophosphomonoesters such as IPP are true Vγ9Vδ2-TCR agonists, whereas alkylamines and N-

BPs act in a similar, indirect manner to activate V 9V 2 T cells through inhibition of the mevalonate pathway. γδ T-cells discriminate transformed tumor cells from healthy cells by the upregulation of self-antigens like heat shock proteins (HSP). The expressions of these proteins are increased in tumor cells due to higher metabolism and serve as endogenous danger signals [10, 126]. Increased cytotoxicity of γδ T cells was observed against transformed cell lines expressing hsp60/70 [127]. Studies from our own laboratory have demonstrated that Vγ9Vδ2 T cells recognize hsp60 on oral tumor cells and have the ability to lyse autologous and allogenic oral and esophageal tumor targets via recognition of hsp60 and hsp70 [128, 129].

In addition to phosphoantigens, γδ T lymphocytes can also be activated by mitochondrial F1-ATPase-related structure expressed together with apolipoprotein A-I, which are expressed on the surface of some tumor cells [130]. ATP F1 synthase is an intracellular protein complex involved in ATP generation. It was also demonstrated that F1-ATPase displays characteristic of antigen presentation molecule by binding to the adenylated derivative of IPP and promoting TCR aggregation, cytokine secretion and cytotoxic activity [131].

Human γδ T cells also express certain Toll-like receptor (TLR) and directly respond to the corresponding ligands. TLR are pattern recognition receptors that specifically sense molecular patterns of microbes, leading to activation of immune cells [99].. Expression levels of TLR1-10 varied among donors, but expression of TLR1,2,3 was found to be more abundant than expression of other TLR in human γδ T cells [99]. TLR ligands including TLR3 ligand poly(I:C) and TLR9 ligand CpG enhance the in vitro activation of human γδ T cells via the stimulation of type I interferon production in dendritic cells [132, 133]. In human γδ T cells, TLR1/2/6, 3, and 5 ligands can act directly in combination with T cell receptor (TCR) stimulation to enhance cytokine/chemokine production [134]. Potent anticancer effects against established tumors in both

mice and humans after the administration of purified ligands for TLRs have been demonstrated as a result of local and systemic delivery [135, 136].

It was reported that peripheral blood γδ T cells express CD27 receptor which endows them with enhanced proliferative capacity upon ligation by its unique ligand CD70, a tumor necrosis factor superfamily member expressed on lymphoma B-cells as well as on TCR activated γδ T cells [137].

VI B. Antimicrobial activity of γδ T cells

γδ T cells sense 'danger' signals of invading pathogens (e.g. in tuberculosis, salmonellosis, listeriosis, tuberculosis, malaria, etc). This attributes γδ T cells an important role in immunity to pathogens [106]. Cellular proliferation is important for immune protection as the mounting of sufficient numbers of cells is important to be able to respond to microbial threats. A robust γδ T cells response has been demonstrated for a variety of bacterial and protozoan infections [13]. Studies in mice suggest that γδ T cells provide antibacterial effects in tuberculosis, listeriosis and protective immunity against malaria [138-140]. In human, increased activities of γδ T cells were shown to important during bacterial infections such as tuberculosis, listorisis, brucellosis, leprosis, Q-fever, meningitides [141-146]. Increased in human peripheral γδ T cells population were also shown in many protozoan as well as viral infections [147-150]. γδ T cells may also contribute to the integrity of the epithelium during infection or injury facilitating tissue healing [151, 152]. γδ T cells are further able to influence the adaptive immune response indirectly through cytokine production potentially contributing to the appropriateness of the response [153-155]. In psoriasis, dermal γδ plays a pivotal role in pathogenesis by secreting abundant amount of IL17 [156]. Recently, it was reported that mortality was associated with a significant decrease in γδ T cells in septic patients [157]. Most of the Eubacteria including mycobacteria, Gram-negative rods

including Pseudomonae, Salmonellae, Shigellae, Vibrios, Yersiniae, and Escherichiae, Neisseriae, Clostridia,and Brucellae, use the MEP pathway [106, 158]. The MEP pathway is also used by protozoa that cause malaria, toxoplasmosis, babesiosis, and cryptosporidiosis also use the MEP pathway [13, 159].

VI C. Migration of γδ T cells to the tumor site

The influx of TILs (tumor infiltrating lymphocytes) to the tumor site enhances the potential for anti-tumor immune responses. The numbers and types of lymphocytes present in the infiltrate are related to the chemokines produced by both the tumor cells and tissue stromal cells located at the tumor site. The infiltration of circulating lymphocytes to the tumor is facilitated by these chemokines. For example, breast, cervix and pancreatic tumors as well as ovarian tumors produce CC and CXC chemokines that are important mediators of macrophage and lymphocyte infiltration in these tumors [160-162]. Interestingly, both Vδ1 and Vγ9Vδ2 T cells display distinct chemokine receptor that bestow these cells the property to migrate to the tumor site. Vδ1 express CCR5 and Vγ9Vδ2 express both CCR5 and CXCR3[163]. In addition, Vγ9Vδ2 T cells express NK receptor P1A (NKR-P1A) platelet endothelial cell-adhesion molecules (PECAM) while Vδ1 use NK receptor P1A NKR-P1A for trans endothelial migration [164]. Vδ1 T cell subsets from the peripheral blood utilize a larger array of adhesion molecules, namely LFA-1, VLA-α4, VLA-α5, L-selectin and αEβ7, to bind to squamous cell carcinoma cells compared to the restricted usage of LFA-1, L-selectin and CD44v6 by the Vδ2 T cells[165]. The mutually exclusive pattern of chemokine receptor expression in both the subsets of γδ T cells indicates independent mechanism of homing to tumor site that might have an important implications in cancer immunotherapy.

VI D. Antitumor activity of γδ T lymphocytes

Ability of γδ T lymphocytes to produce abundant proinflammatory cytokines like IFN-γ, potent cytotoxic effector function and MHC-independent recognition of antigens makes them an important player of cancer immunotherapy [166]. γδ T cells kill many different types of tumor cell lines and tumors in vitro, including leukemia, neuroblastoma, esophageal tumors and various carcinomas [129, 167-170].

Accumulation of mevalonate metabolites in tumor cells is a powerful danger signal that activates the γδ T cells. In normal cells, IPP produced by mevalonate pathway are at a concentration that is insufficient to trigger γδ T cells response. However, dysregulation of mevalonate pathway in certain tumors leads to production of higher concentrations of IPP, which is sensed by γδ TCR as a tumor antigen [14, 15]. It was also shown that mRNA knockdown of IPP-consuming enzyme, FPPS, induced Vγ9Vδ2 T cell stimulation in otherwise non-stimulatory tumor cells [171]. γδ T cells are able to recognize and kill many differentiated tumors cells, either spontaneously or after treatment with bisphosphonates, including zoledronate. It was shown that human tumor cells can efficiently present aminobisphosphonate and pyrophosphomonoester compounds to γδ T cells, inducing its proliferation and IFN-γ production [172].

In addition, CD166 broadly expressed on the human tumor cell lines by engaging to CD6 on γδ T cells play a significant role in γδ T cell activation [173].

Combination treatment utilizing Vγ9Vδ2 T cells along with chemotherapeutic agents and zoledronate has been shown to induce increase cytotoxic function of γδ T cells against solid tumor [123, 174]. The ability of γδ T cells to efficiently kill bisphosphonates treated colon cancer stem cells and ovarian cancer stem-like cells have also been reported [170, 175].

VI E. NK receptors and anti-tumor activity of γδ T cells

Natural killer (NK) receptors expressed on γδ T cells play a crucial role in mediating the anti-tumor response of γδ T cells (Figure 5). Natural killer group2, member D protein (NKG2D) expressed on Vγ9Vδ2 T cells is critical for tumor recognition and provides activation signals upon binding to non-classical MHC molecules of the MHC class I chain-related molecules (MIC) and UL-16 binding protein (ULBP) families expressed on tumor cells [176-178]. This ligand binding to NKG2D can affect the release of TNF-α, IL-2 α receptor (CD25) upregulation and increase cytolytic potential of γδ T cells [177]. ULBP molecules are involved in Vγ9Vδ2 T cells recognition of leukemias and lymphomas [179] and also ovarian and colon carcinomas [180]. γδ T cells utilizing the Vδ1 chain isolated from tumor-infiltrating lymphocytes can also kill cancer cells. Vδ1 γδ T lymphocytes have been shown to mediate cytolytic activity by recognizing MICA, MICB or ULBP expressed on cancer cells [181, 182].

Moreover, γδ T cells resemble NK cells in as they express CD16 (FcγRIII) receptor. Upon recognition of phosphoantigens, a subset of Vγ9Vδ2 T cells upregulates CD16 [183]. It has been reported that CD16 represent activation/memory status of γδ T cells and these $CD16^{high}$ cells have specific phenotypic features that distinguish them from the $CD16^{low}$ subset. They constitutively express several natural killer receptors(NKG2A/CD94) and high amounts of perforin, but express low levels of chemokine receptors (CXCR3, CCR6) and IFN-γ [184]. CD16/FcγRIII receptor binds to Fc portion of immunoglobulin G (IgG) and engagement of CD16 by γδ T cells leads to antibody-dependent cellular cytotoxicity (ADCC) [185]. ADCC is a process in which CD16+ effector cells actively lyse tumor cells that have been bound by specific antibodies. Several reports have proven that in vitro γδ T cells respond to activation via. CD16 and mediate ADCC against tumor with therapeutic anti-tumor monoclonal antibodies (mAbs) like rituximab,

trastuzumab, ofatumumab and alemtuzumab [169, 186, 187]. It was also shown that stimulated γδ T cells increase the efficacy of trastuzumab *in vivo* in Her2+ breast cancer patients[188].

Additionally, the two NK receptors DNAX accessory molecule-1 (DNAM-1) were expressed by Vγ9Vδ2 T cells play a role in γδ T-cell cytotoxicity (Figure 5). It was shown that hepatocellular carcinoma expresses ligands Nectin-like-5 and Nectin-2 which binds to DNAM-1 and promotes hepatocellular carcinoma cell lysis by γδ T cells [189].

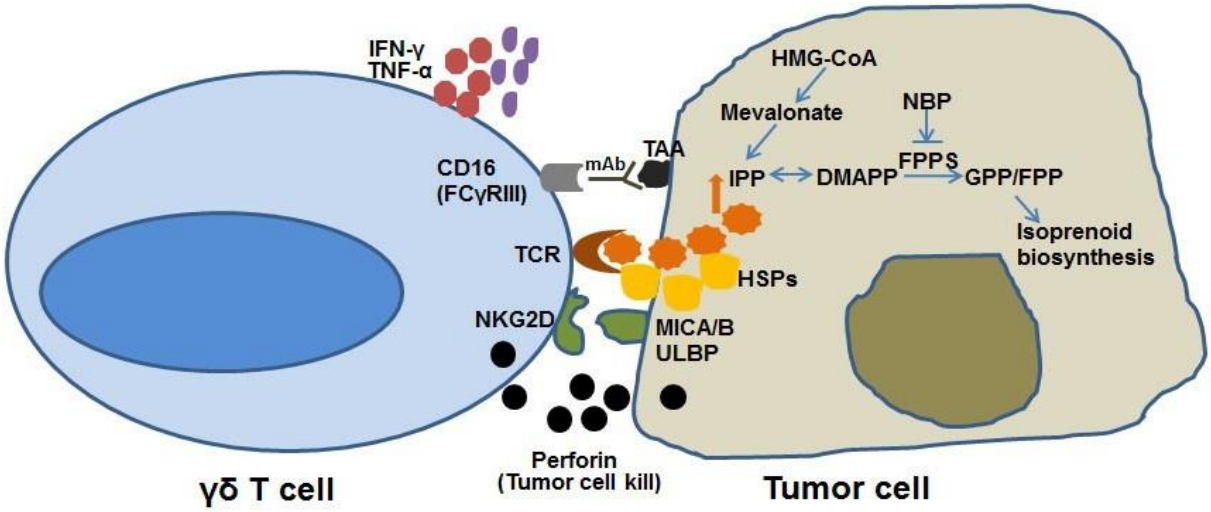

Figure 5: Mechanism underlying γδ T cell killing of tumors

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VI F. Application of γδ T cell immunotherapy in clinics

Given the potent antitumor effector function of γδ T cells and broad reactivity against many different types of tumors has raised a great interest to explore their therapeutic potential. An important feature of γδ T cells is that they favorably kill cancer cells and show low (if any) reactivity towards non-transformed cells which makes them very good candidates for cancer immunotherapy [180]. The safety and efficacy of γδ T cell- based immunotherapy have been evaluated in several clinical trials [111]. Presently, two strategies for γδ T cells in tumor

immunotherapy have been applied. They are the adoptive cell transfer of in vitro expanded γδ T cells and the in vivo therapeutic application of γδ-stimulating phosphoantigens or aminobisphosphonates together with low-dose recombinant interleukin2 (rIL2). Immunotherapy using γδ T cells alone shows promising clinical activity and combining this treatment modality with cancer-targeting antibodies can provide long-lasting protection [20].

Studies carried out in nude mice demonstrated that repeated infusion of γδ T cells leads to tumor growth arrest [190]. Another study carried out in SCID mice showed the anti-tumor effector functions of NK cells and γδ T lymphocytes against autologous melanoma cells [191]. In one of the pilot study, patients with B-cell malignancies that failed conventional therapy were treated with intravenous administration of pamidronate and rIL2 to stimulate Vγ9Vδ2 T cells *in vivo* [192]. It was observed that *in vivo* Vγ9Vδ2 T cells were expanded in five out of nine patients; three out of these five responding patients had partial remissions and one other had stable disease. Other trials with adoptive transfer of γδ T cells include patients with advanced cancer like metastatic renal cell carcinoma [17] and non-small cell lung carcinoma [193] where stable disease was found in 60% and 37% patients respectively. In these cases, the regimen consisted of ex vivo activation and expansion of autologous Vγ9Vδ2 T cells with either phosphoantigens, such as BrHPP or aminobsphosphnates, like zoledronate or pamidronate or their infusion into the patients. Aminobisphosphonates have also been used in clinical trials to treat metastatic prostate cancer [19] and advanced breast cancer [194] where partial remissions have been reported. Complete remission of lung metastasis in a patient with renal cell carcinoma has also been reported after adoptive transfer of γδ T cells [195]. It was shown that the patient was disease free for 2 years without any additional treatment following in vitro activation and expansion of autologous γδ T cells with HMBPP plus rIL2, combined with the infusion of zoledronate and rIL2 [195]. There is

also increasing evidence that stimulating γδ effector T cells can enhance monoclonal antibody-induced cytotoxicity and thereby improve the anticancer effects of mAbs. It was found that repeated infusions of phosphoantigens stimulated γδ T cells and trastuzumab increases the efficacy of trastuzumab against HER-2^+ breast carcinoma cell lines in vivo [188]. In addition, a survival advantage to patients with an increased γδ T cells following allogeneic stem cell transplantation (ASCT) has been reported. A long-term survival advantage in a group of high-risk acute leukemia patients who recovered with increased number of circulating γδ T cells following partially mismatched related hematopoietic stem cell transplantation was reported [196]. In another phase I clinical study of metastatic solid tumors, administration of in vitro activated Vγ9Vδ2 T cells with zoledronate and IL-2 was found to be safe and provided an additive effect [197].

Conversely, in vitro co-culture of human regulatory T cells (Tregs) and peripheral blood mononuclear cells from patients with cancer strongly inhibited phophoantigen induced proliferation of γδ T cells, whereas depletion of Tregs restored phosphoantigen induced γδ T cell proliferation [198]. These results might explain the frequently observed γδ T cell proliferative anergy in patients with cancer [199].

VII. Regulatory T cells (Tregs)

Tregs are professional suppressor cells that control immunological self-tolerance and immune responses to pathogens and tumor antigens. Tregs are characterized by the expression of $CD4^+$, $CD25^+$ and Foxp3 markers [200]. Sakaguchi and colleagues propose a two-mode of mechanism of suppression by Tregs under inflammatory or non-inflammatory conditions. Under normal condition, the activation of T cells can be suppressed by thymic derived Tregs via deprivation of activation signals including CD28 signal and IL-2 from antigen-reactive T cells. These

deprivation mechanisms are transiently abrogated in inflammatory conditions, allowing T cells to respond to antigen. In contrast, under inflammatory milieu, activated Tregs acquire the capacity to kill or inactivate effector T cells and antigen-presenting cells via granzyme/perforin pathway and IL-10 secretion, thereby actively damping excessive immune responses [201]. Tregs are required to suppress the activation of self-reactive lymphocytes and autoimmunity as well as also required to limit the immune response to chronic pathogens and commensal bacteria in the gut [202, 203]. Tregs has been harnessed as immunotherapy for various forms of human diseases. A clinical trial involving adoptive Treg transfer for prevention of acute graft versus host disease (GVHD) was reported [204]. Tregs can be used for successful allogeneic hematopoietic stem cell transplantation (HSCT) and solid organ transplantation [205]. However, these Tregs also limit beneficial responses by suppressing he activated immune cells and limiting antitumor immunity. A large number of Tregs are present in tumors and draining lymph nodes in tumor-bearing rodents and also patients with cancers [206-209]. As a mechanism of Treg recruitment to tumors, it has been proposed that tumor cells and tumor infiltrating macrophages produce the chemokine CCL22, which chemoattracts and recruits $CD25^{+}CD4^{+}$ Tregs expressing CCR4 [206, 210]. It is also likely that $Foxp3^{+}$Tregs are induced from non-Tregs because of high concentrations of TGF-β secreted by tumor cells and/or DCs present in tumors [211, 212]. Besides, Tregs cells can inhibit antigen-specific memory γδ T cells from producing IFN-γ [213]. It was also demonstrated that γδ T cell proliferation can be suppressed by Tregs [198, 214].

VIII. Mechanism of Tregs action

Over the past few years, significant progress has been made in delineating the molecules and mechanisms that Treg cells use to mediate suppression [215, 216] (Figure 6).

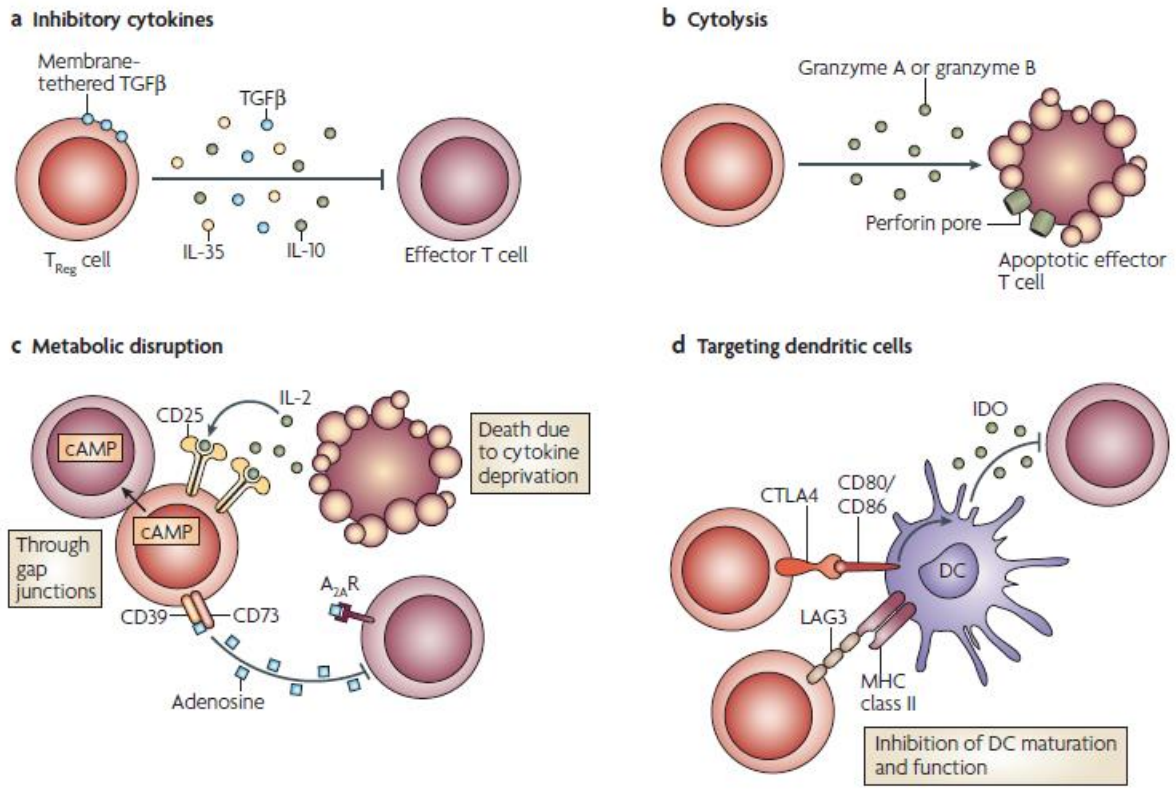

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Figure 6: Basic mechanisms used by Treg cells

VIII A. Inhibitory Cytokines

Inhibitory cytokines such as IL 10, TGF-β and IL35 secreted by Tregs act as a major mechanism for suppression. IL 10 is considered to be a key molecule for immunosuppression. It has been established that certain forms of immunity such as inflammation in colitis can be suppressed by $CD4^+$ $CD25^+$ T cells (Tregs) and require their secretion of IL 10 [217]. The suppression of $CD8^+$ that mediate tumor rejection or autoimmunity by $CD4^+$ $CD25^+$ T cells requires an intact TGF-β receptor II on $CD8^+$ [218, 219]. Naive CD4+ CD25 T cells can be converted into iTreg cells as a consequence of exposure to antigen in the presence of immunosuppressive conditions, including the presence of TGF-β or IL-10 [211, 220, 221]. IL-35 can also act as an autocrine Treg growth factor [222]. IL-35 is required for maximal Treg activity and ectopic expression of IL-35 confers

regulatory activity on naive T cells, whereas recombinant IL-35 suppresses T-cell proliferation [223].

VIII B. Cytolysis

It has been suggested that Tregs may use perforin/granzyme-mediated cytotoxicity as a mechanism of suppression. Human Tregs can be stimulated by combination of antibodies to CD3 and CD46 to express granzyme A and kill activated $CD4^+$ and $CD8^+$ T cells by a perforin dependent mechanism [224]. Tregs cells were reported to suppress the ability of NK cells and CTLs to clear tumors by killing these cells in a granzyme‐B-dependent and perforin-dependent manner [225]. In was also shown that Treg cells can kill B cells in a granzyme‐B dependent and partially perforin-dependent manner that results in the suppression of B cell function [226].

VIII C. Metabolic disruption

The high expression level of CD25 allows Tregs to consume local IL‐2 and therefore starve actively dividing effector immune cells by depleting the IL‐2 they need to survive [227, 228]. Treg cells induce cytokine (specifically IL 2)-deprivation mediated apoptosis of $CD4^+$ T cells [229]. The expression of CD39 and CD 73 on Tregs have been suppressed effector T‐cell function through activation of the adenosine receptor 2A ($A_{2A}R$) [230]. Tregs express ectoenzymes CD39 and CD73, which can cleave extracellular ATP to generate immunosuppressive adenosine that inhibits effector T cell function through activation of the adenosine receptor 2A [231, 232]. Tregs can also suppress effector T cells by transferring the second messenger cyclic AMP through gap junctions; which in turn potently inhibits IL-2 synthesis and T cell proliferation [233].

VIII D. Targeting of antigen presenting cells (APCs)

Tregs constitutively express CTLA4 (closely related to CD28) which is involved in binding to the B7 molecules (CD80 and CD86) present on APC. These interactions were proposed to function in attenuating effector T‑cell activation by APC [234, 235]. It was also shown that CTLA-4 expressed in Foxp3+ Tregs mediates down-regulation of CD80 and CD86 on antigen presenting cells [236]. In addition,CTLA-4-mediated interaction between Tregs and APCs may cause APCs to upregulate the production of indoleamine 2,3-dioxygenase (IDO), which is an enzyme that degrades the essential amino acid tryptophan [237]. Also, IDO-mediated degradation of tryptophan can generate toxic metabolites that cause apoptosis of T helper 1 (Th1) lymphocytes [238]. It was also found that Tregs selectively expresses LAG-3, a CD4-related molecule that binds MHC class II. LAG-3 regulates both the in vitro and in vivo suppressive function of Tregs, and ectopic expression of LAG-3 is sufficient to confer regulatory activity [239].

IX. Oral cancer

Oral cancer is often considered as malignant tumor (usually squamous cell carcinoma (SCC)) of the lip, mouth (oral cavity) and oral-pharynx. Oral cancer is a major health problem world-wide, accounting for 274 000 new cases and 145 000 deaths annually, of which two thirds occur in developing countries [240].

IXA. Clinical manifestation in oral cancer

Many oral SCCs develop from premalignant conditions of the oral cavity. Leukoplakia and erythroplakia are two clinical lesions widely considered to be premalignant [241, 242]. The term leukoplakia means a white plaque that does not rub off and cannot be clinically identified as another entity and an erythroplakia is a red lesion that cannot be classified as another entity. In contrast to leukoplakia which is often benign in nature, erythroplakia are generally either at a high risk for transformation to malignancy or already malignant. Currently, histological criteria

represent the gold standard for judging the risk of malignant transformation for oral premalignant lesions. The World Health Organization (WHO) has established criteria for dysplasia [243].

The WHO's criteria for architectural changes in the epithelium are

1. Irregular epithelial stratification
2. Loss of polarity of basal cells
3. Drop-shaped rete ridges
4. Increased number of mitotic figures
5. Abnormal mitoses not limited to basal or parabasal layers
6. Premature keratinization in single cells
7. Keratin pearls within rete ridges

The WHO's criteria for cytologic changes in the epithelium:

1. Abnormal variation in nuclear size
2. Abnormal variation in nuclear shape
3. Abnormal variation in cell size
4. Abnormal variation in cell shape
5. Increased nuclear–cytoplasmic ratio
6. Increased nuclear size
7. Atypical mitotic figures
8. Increased number and size of nucleoli
9. Hyperchromasia

Up to 71% of oral SCCs express antigens from at least 1 of 6 melanoma antigen genes (MAGEs), notably MAGE- 1 and MAGE-3 [244]. Antigen from NY-ESO-1, a gene expressed in normal ovary and testis, is highly expressed in a variety of tumor types including oral SCC [245].

IXB. Immune dysfunction

Tumor reactive CTLs may be ineffective because they remain in the periphery or in the draining lymph node without actually infiltrating the tumor [246], or they may disseminate to the tumor but are unable to mediate anti-tumour activity [247]. T-cell anergy can occur as a result of inadequate costimulation during priming, or can be acquired during later phases of clonal expansion after adequate initial activation [248, 249]. TCR signaling without costimulation leads to a state hyporesponsive known as anergy. Anergic T cells are characterized by the failure to produce IL-2, IFN-γ and consecutively diminished proliferation upon restimulation [250, 251]. T cell anergy can also be promoted by high IDO or ARG1 expression via deprivation of the essential amino acids tryptophan and arginine [252]. TCR associated ζ-chain is involved in the transduction of signals delivered via the receptor and therefore, its expression is important for the activation of T cells. Reduced TCR ζ chain expression in circulating lymphocytes of patients with cancer including melanoma, ovarian, breast, oral, as well as renal cell carcinomas has been reported [253]. Recent observations revealed that inhibitory receptors other than PD-1, such as lymphocyte activation gene 3 (LAG-3), T cell immunoglobulin mucin 3 (TIM-3), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), natural killer cell receptor 2B4 (2B4, CD244), leukocyte immunoglobulin-like receptor superfamily B member 3 (LILRB3, PIRB) and 4 (LILRB4, GP49), and CD160, are co-expressed on exhausted T cells during chronic viral infection and tumor progression [254-256]. A fraction of tumor infiltrating lymphocytes (TILs)

co-expressing PD-1 and TIM-3 exhibits a more severe exhausted phenotype in mice bearing solid tumors and in patients with advanced melanomas [257].

Evidence for the presence of functional defects and apoptosis of TILs as well as circulating T cells in patients with oral cancer emphasizes the fact that their antitumor responses are compromised [258]. The molecular mechanisms which account for the observed immune suppression in oral cancer patients include down-regulation of MHC class I, apoptosis induced by Fas ligand expressed on tumor cells, decreased production of cytokines, or the presence in the tumor of cytokines with known suppressive effects on T cell functions and presence of regulatory T cells [259]. It was also observed that T cells of oral cancer patients have decreased TCR ζ-chain expression and was inversely correlated with the stage of the tumor [260].

IXC. Treatment

Approaches using blockade of exhaustion as a potent rejuvenator of immune cells has been translated into clinical cancer trials [261-263]. An exciting approach being evaluated in clinical trials involves the use of monoclonal antibodies to blockade exhaustion markers such as CTLA-4 or PD-1 expressed by T cells. In the case of CTLA-4 blockade, a recent phase III clinical trial reported that therapy with CTLA-4–blocking antibodies imparted a significant survival benefit in approximately one third of patients with metastatic melanoma, making this drug a promising treatment for cancer [264]. A phase I/II clinical trial using anti-PD-1 human monoclonal antibody treatment induced clinical responses against renal cell carcinoma and melanoma with well-tolerable side effects [261].

Standard therapies for early stage oral cancer include surgery or primary radiotherapy, with adjuvant radiotherapy in selected cases with specific clinical or pathologic findings. Chemotherpay is used as combination therapy in locoregionally advanced oral cancer with

adverse features or in the setting of distant metastatic [265]. In spite of numerous advances in treatment, the 5-year survival has remained approximately 50% for the last 50 years [266]. Tumor development is a highly dynamic process which involves the complex interplay between the tumor and the immune system.

The recent promising results of clinical trials implementing programmed cell death 1 (PD1) and B7-H1-specific monoclonal antibodies and the recent approval of CTLA4-specific monoclonal antibodies (ipilimumab) for the treatment of patients with cancer by the US Food and Drug Administration provided clear evidence for the efficacy of costimulatory molecules as immunotherapeutic agents [267, 268].

Chapter 3:

Materials and methods

I. Culture medium

RPMI-1640 medium (Invitrogen Life-Technologies, Grand Island, N.Y.) or IMDM (Invitrogen Life -Technologies, USA.) media powder was dissolved in deionized water and supplemented with sodium bicarbonate (Sarabhai Chemicals, India) and if required with HEPES buffer (Sigma St. Louis, USA) as per manufacturer's instructions. The medium was sterilized by membrane filtration (0.45mM, Millipore Co, USA), checked for sterility and stored at -20ºC until use.

To prepare complete medium, RPMI/IMDM plain medium was supplemented with 10% inactivated human AB serum or fetal calf serum, (FCS; Invitrogen Life Technologies, USA), penicillin (100 IU/ml; AlembicChemicals India), streptomycin (100 mg/ml; Alembic chemicals India), mycostatin (5 mg/ml;Signa, USA), gentamycin (40mg/ml; Schering Corpn, India), LGlutamine (2mM, Hi Media, India) and β-mercaptoethanol (5x10-5M; Sigma USA).

II. Maintenance of cell lines

Media required for the maintenance of different cell cultures were as follows

Cell line	Type	Medium
AW13516	Adherent cell line	IMDM+10%FCS
AW8507	Adherent cell line	IMDM+10%FCS
MCF-7	Adherent cell line	RPMI+10%FCS
MDA-MB	Adherent cell line	RPMI+10%FCS

The adherent cell lines were maintained in 25cm^2 flasks and split when confluent. The cells were stripped 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 continuing the culture 0.5x10^{6}cells were seeded back into the flask with 5 ml of complete medium (containing 10% FCS).

For cryopreservation of the cultures, cells were pelleted by centrifugation at 1000 rpm. To the pellet, chilled freezing mixture (10% Dimethyl sulphoxide [DMSO] + 90% FCS) was added drop-wise with constant mixing. Two-three millioncells/ml of freezing mixture were transferred to cryotube (Nunc, Denmark) and frozen in liquid nitrogen.

For reviving cells, frozen vials were thawed quickly in a water bath at 37ºC and the cells were transferred to a centrifuge tube. Warm plain medium was added drop-wise with constant mixing to dilute the DMSO. Cells were washed thrice and checked for the viability using the vital dye trypan blue (0.4% trypan blue (Fluke AG, Buchs SG, Switzerland) in normal saline, sterilized by filtering and 0.01% thiomersal added (BDH Lab. Reagents, U.K)).

III. Antibodies used

Purified/conjugated antibodies	**Source**
Mouse anti-human CD3	In house preparation purified on sepharose protein A column.
Mouse anti-human TCR γδ	BD Pharmingen, USA

Sheep anti-human N1ICD	R&D Systems, USA
Sheep anti-human N2ICD	R&D Systems, USA
Rabbit anti-human Dll1	Calbiochem, USA
Rabbit anti-human Jag1	Calbiochem, USA
Rabbit anti-human HES1	Santa Cruz Biotechnology, USA
FITC conjugated mouse anti-human TCR γ9	BD Pharmingen, USA
PE conjugated mouse anti-human TCR δ2	BD Pharmingen, USA
FITC conjugated mouse anti-human TCR δ2	BD Pharmingen, USA
PE conjugated mouse anti-human CD3	BD Pharmingen, USA
PE conjugated mouse anti-human CD25	BD Pharmingen, USA
FITC conjugated mouse anti-human CD69	BD Pharmingen, USA
FITC conjugated mouse anti-human TCR γδ	BD Pharmingen, USA
PE conjugated mouse anti-human CD107a	BD Pharmingen, USA
FITC conjugated mouse anti-human Foxp3	BD Pharmingen, USA
Rabbit anti-human β-actin	Santa Cruz Biotechnology, Inc, USA
Annexin-V FITC	BD Pharmingen, USA

Secondary antibodies	Source
Donkey anti-sheep IgG FITC	Sigma Aldrich, USA
Donkey anti-sheep HRPO	Sigma Aldrich, USA
Goat anti-human HRPO	Sigma Aldrich, USA
Goat anti-mouse IgG Rhodamine Red-X	Molecular Probes, USA

IV. Recombinant proteins

Protein	Source
Recombinant interleukin 2 (rIL2)	Peprotech, USA
Recombinant delta like ligand1 (rDLL1)	R&D Systems, USA
Recombinant delta like ligand4 (rDLL4)	R&D Systems, USA
Recombinant jagged1 ligand (rJag1)	R&D Systems, USA

V. Study group

Blood samples were collected from healthy individuals (n=110). Patients with squamous cell carcinomas of the oral cavity (n=25) TNM classification Stage III and IV were included in the study. Heparinized blood was collected prior from oral cancer patients prior to surgery and tumor

tissues were obtained from oral cancer patients after surgical excision. The biological samples were collected after obtaining informed consent from the patients and healthy individuals.

VI. Separation of peripheral blood lymphocytes (PBLs)

Lymphocytes were separated from heparinized venous peripheral blood by Ficoll-Hypaque (FH, Sigma, U.S.A.) 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 (dd) water). 10 ml of diluted blood was loaded on 2.5ml of Ficoll-Hypaque [24 parts of 9% Ficoll 400 (Sigma, USA) + 10 parts 33.3% sodium diatrizoate (Sigma, USA), specific gravity adjusted to 1.077 ± 0.001] and centrifuged at 1,500rpm for 20min at room temperature (RT) using a swing-out rotor in Beckman centrifuge. PBL were collected from the interface between FH and plasma and washed thrice with sterile normal saline. Viability was checked using trypan blue dye.

VII. Enrichment and purification of γδ T cells from PBLs of healthy individuals and oral cancer patients

PBL were isolated from healthy individuals (HI) and oral cancer patients by Ficoll Hypaque density gradient centrifugation and were enriched by solid phase anti CD3 mAb and recombinant IL-2 (rIL-2) as described by Yamaguchi et al (1997).

A. Enrichment of γδ T cells from PBL

$10x10^6$ PBL were suspended in RPMI 1640 medium containing 10% heat inactivated human AB serum, glutamine (2mM), 2-mercaptoethanol ($5x10^{-5}$M) and antibiotics penicillin (100U/ml), streptomycin (100mg/ml), mycostatin (5mg/ml). rIL2 was supplemented at concentration of (10 IU/ml) in this growth medium. PBL were transferred to 25 cm^2 culture flasks (Nunc, Denmark)

coated with 1mg/ml anti CD3 MAb in PBS. Five ml of cell suspension ($5x10^6$/flask) was added to coated flask. Cells were fed daily for 4 days with 1 ml of growth medium containing 500U/ml rIL2 and then on the 5th day, cells were transferred to 75 cm^2 culture flask containing10 ml of growth medium containing 10 IU/ml of IL2. Cells were then sub-cultured after every 2 days with the addition of fresh growth medium till day 12.

B. Immunomagnetic purification of γδ T cells from expanded PBL

γδ T cells were purified from PBL using TCR-γδ Microbead Kit (Miltenyi Biotech, Germany).

Magnetic labeling of cells PBL were washed with buffer containing degassed 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 TCR-γδ hapten mAb was added per 10^7 total cells. Cells were mixed and incubated for 10 min at 4ºC. 30µl of buffer and 20µl of MACS anti FITC micro-beads were added per 10^7 cells, mixed well and incubated for 15 min at 4ºC. Cells were then washed by adding 10-20 times labeling volume of buffer and centrifuged at 1000 rpm for 10 min. Supernatant was removed and cells were suspended in 500 µl buffer per 10^8 cells.

C. Magnetic separation of positively selected cells

MS MACS column (for 10^7 cells) and LS MACS column (10^7 to $5x10^7$ cells) was placed in magnetic field of MACS separator and a column was washed thrice with 500µl of wash buffer. 500µl of cell suspension was applied onto the column. Negatively selected cells were washed out from the column and collected separately. Column was removed from the separator and positively selected cells were collected by flushing the cells from the column into 1 ml of buffer. Positively selected, FITC labeled cells were checked for the expression of γδ TCR by flow cytometry.

VIII. Confocal microscopy

Purified γδ T cells were rested overnight at 37ºC. Next day, these cells were rinsed in cold phosphate buffered saline (PBS) and fixed in 1% paraformaldehyde in PBS for 10 min at 4ºC. The cells were washed and permeabilized for 15 min with 0.1% saponin in PBS. Cells were stained with mouse anti-human γδ TCR antibody, sheep anti-human Notch1 intracellular domain (N1ICD) antibody or mouse anti-human HES1 antibody for 45 m at 4°C. Thereafter, cells were washed and incubated with rhodamine-labeled goat anti-mouse IgG, FITC-labeled goat anti-mouse IgG or FITC-labeled donkey anti-sheep IgG for another 45 min at 4°C. For nuclear staining, 4l, 6-diamino-2-phenylindole (DAPI) (Sigma Aldrich) was used. The samples were visualized on Zeiss Laser-Scanning Microscope 510 (LSM510) META (Carl Zeiss, Jena, Germany).

IX. Flow cytometry

A. Single color flow cytometry

A.1. Purity of immunomagnetically purified γδ T cells

Single color flow cytometery was performed on γδ T cells isolated by MACS by acquiring cells on a FACS Calibur flow cytometer (Becton Dickinson). γδ T cells stained with IgG FITC conjugated antibody was kept as isotype control. Cells were selectively gated for lymphocyte population on a Forward Scatter (FSC) and Side Scatter (SSC) plot. 10,000 events were acquired and data was analyzed using FlowJo software (Tree Star, USA).

A. 2. Analysis of expression of NICD in γδ and αβ T cells

The cells were cold-fixed in 1% paraformaldehyde in PBS for 10 min at 4ºC. Cells were washed and permeabilized for 15 m with 0.1% saponin in PBS. γδ T cells and αβ T cells were stained with sheep anti-human N1ICD or N2ICD antibody for 45 min at 4°C. Thereafter, cells were washed and incubated with FITC-labeled donkey anti-sheep IgG for another 45 min at 4°C. Cells were acquired on the flow cytometer and analyzed as described above.

A.3. Detection of activation markers on γδ T cells

For analyzing cell surface expression of activation markers, γδ T cells were left untreated or treated with rIL2 and BrHPP for 24 h with GSI-X (15µM) or left untreated as previously described. Cells were then incubated with FITC conjugated CD69 or PE conjugated CD25 (BD Biosciences) for 45 min in dark and subsequently washed with FACS buffer (0.01M PBS pH-7.4, 1%FCS, 0.01% Sodium azide), fixed with 1% paraformaldehyde and the intensity of fluorescence was measured using flow cytometer (FACS Calibur, BD Biosciences). Cells were acquired and analyzed as mentioned previously.

A.4. Measurement of Foxp3 and CD25 on Tregs

Purified Tregs cells were incubated with FITC conjugated Foxp3 and PE conjugated CD25 (BD Biosciences) for 45 min in dark and subsequently washed with FACS buffer (0.01M PBS pH-7.4, 1%FCS, 0.01% Sodium azide), fixed with 1% paraformaldehyde and the intensity of fluorescence was measured using flow cytometer (FACS Calibur, BD Biosciences). Cells were acquired and analyzed as mentioned previously.

B. Dual color flow cytometry

B.1. Determination of cell surface markers on phosphoantigens stimulated PBLs

PBLs ($1x10^6$) were stimulated with rIL2 alone or along with three different phosphoantigens (BrHPP, IPP and picostim) in 24 well plate (Nunc) for 12 days. Cells were harvested and incubated with FITC conjugated Vγ9 TCR and PE conjugated Vδ2 TCR or FITC conjugated Vδ1 and PE conjugated CD3 mAb for 45 min in dark. Subsequently washed with FACS buffer (0.01M PBS pH-7.4, 1%FCS, 0.01% sodium azide), fixed with 1% paraformaldehyde and the intensity of fluorescence was measured using flow cytometer (FACS Calibur, BD Biosciences). Cells were acquired and analyzed as mentioned previously.

B.2. Detection of apoptosis in γδ T cells

γδ T cells ($5x10^4$) were left untreated or were stimulated with rIL2 alone or rIL2 and BrHPP for 24 h at 37°C in round bottomed 96 well plate (Nunc). GSI-X was added as described above. Cells were then harvested, suspended in binding buffer (10mM HEPES pH 7.4, 150mM NaCl, 0.25mM CaCl2) and incubated with propidium iodide (PI) and FITC-conjugated Annexin V (BD Biosciences, San Diego, CA) in dark for 15 min at room temperature. After incubation, 400µl of binding buffer was added and cells were analyzed as mentioned previously.

B.3. Measurement of degranulation markers

For degranulation assay, purified γδ T cells were incubated alone or with rIL2 (0.1U) [PeproTech] overnight at 37ºC in round bottomed 96 well plates (Nunc) were taken as effector cells. The target cells were oral cancer cell line, AW13516, was treated for 18 h with zoledronate (100µM) (Panacea Biotech Ltd., India). Cells were co-cultured at an effector to target ratio of 4:1 in the presence of monensin (5µg/ml, Sigma Aldrich). Anti CD107a-PE antibody (BD Biosciences) was added at the start of co-culture assay. After 4h, cells were washed and γδ T cells were then stained

using anti-human TCR-γδ FITC antibody (BD Biosciences) and were acquired and analyzed on flow cytometer for the expression of CD107a on γδ T cells.

X. Separation of tumor cells from surgically excised oral and breast tumors

Tumors were collected in sterile plain RPMI (Invitrogen Life-Technology) medium supplemented with a double strength of antibiotics (double strengthened RPMI). The necrotic, hemorrhagic and fatty tissues were removed and tumor tissues were thoroughly washed with double strengthened RPMI. The tumor tissues were minced finely and incubated in double strength RPMI and enzyme mixture (0.05% collagenase, 0.02% DNase and 5U/ml hyaluronidase along with 1 mg/ml trypsin [Sigma Aldrich]), at 37ºC for 2 h with intermittent shaking. The tumor tissue was then passed through a 200-gauge wire mesh. The cells were washed with plain RPMI medium and were tested for cell viability. The cell suspension was layered on discontinuous gradient of 75% and 100% Ficoll-Hypaque and centrifuged at 1,500 rpm for 30 min. Tumor cells were collected from the 75% interface (100% interface contains lymphocyte-rich mononuclear cells and polymorphonuclear cells, erythrocytes and aggregated tumor cells were present in the bottom of the tube). The viability of cells was > 95%.

XI. Western blotting

$1X10^6$ γδ T cells were incubated with rIL-2 (100U/ml, PeproTech) and bromohydrin pyrophosphate (BrHPP/IPH1101) which was kindly provided by Innate Pharma (Marseille, France) at a concentration of 200nM for 24 h. These cells were pretreated for 30 min at 37°C with γ-secretase inhibitor-X, L-685,458 (GSI-X) (Calbiochem, La Jolla, CA) at a concentration of 15 μM, or left untreated, before stimulation. As a control $1X10^6$ γδ T cells were used. In

addition, $1X10^6$ αβ T cells, oral cancer cells (AW13516, AW8507 and CF4289 [surgically excised oral tumor]) and surgically excised breast cancer cells (CH6629) were also taken.

A. Cell lysate preparation

Cells were washed with ice cold 0.01M PBS and lysed in lysis buffer. The composition of lysis buffer was as follow:

1) 2X SDS buffer : 500 µl

2X SDS buffer composition

1M Tris HCL (pH 6.8) : 1.6 ml

10% SDS : 4 ml

Glycerol : 2 ml

β-mercaptoethanol (2ME) : 1 ml

Bromophenol blue : 4 mg

Double distilled water 1.4 ml

2) double distilled water : 390 µl

3) 1M dithiothritol (DTT) : 50 µl

4) Phenylmethylsulfonyl fluoride (PMSF) (50mM): 10 µl

5) Sodium flouride (0.5M) : 10 µl

6) Sodium Orthovanadate (0.5M) : 10 µl

7) Aprotinin (2mg/ml) **: 10 µl**

8) Leupeptin (2mg/ml) **: 10 µl**

9) Pepstatin (2mg/ml) **: 10 µl**

To the cell pellet 25 µl of SDS lysis buffer was added, cells were resuspended by vortexing and boiled for 10 m at 100^0C. The lysate was loaded immediately or stored at -80ºC. In case of frozen lysate, the samples were boiled once again at 100ºC for 5 m and then loaded.

B. **Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

The samples were loaded and run on 8% SDS PAGE at 150 V in the presence of the electrophoresis buffer (3.0275 g Tris, 14.413 g glycine and 1 g SDS in 1 liter dd water). The following solutions were mixed to cast 8% resolving polyacrylamide gel and 5% stacking gel.

Components	**8% resolving gel**	**5% stacking gel**
Double distilled water	4.6 ml	4.1 ml
30% acrylamide mixture (acrylamide:bisacrylamide=29:1)	2.7 ml	1 ml
1.5M Tris (pH 8.8) for resolving gel and 1M Tris (pH6.8) for stacking gel	2.5 ml	750 µl
10% SDS	100 µl	60 µl
10% ammonium per sulfate (freshly prepared)	100 µl	60 µl

TEMED	40 µl	25 µl

C. Western blotting

After electrophoresis under reducing conditions, the separated proteins were electrophoretically transferred onto mdi nitrocellulose membrane (Advanced microdevices Pvt. Ltd., India) in transfer buffer (3.0275 g Tris + 14.413 g Glycine + 200 ml Methanol + 800 ml dd water) at RT for 1h at a voltage of 100V using the vertical transfer apparatus (Biorad). The membrane containing the standard molecular weight marker along with protein lysates was stained with Ponceau S (10 ml dd water+0.3 ml glacial acetic acid+0.0033g Ponceau S; final volume adjusted to 30ml). The membranes containing the lysates were completely destained in wash buffer (0.01M PBS + 0.1% Tween 20) and blocked with 5% skimmed milk prepared in wash buffer for 2hr at RT. The membranes were then blotted with the appropriate concentration of the primary antibody (dilution buffer used was 5% skimmed milk) for 2 h at RT followed by an overnight incubation at 4ºC. After washings, the membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (HRPO) diluted in the dilution buffer for 2 h at RT. After washing off the excess of the secondary antibody, the protein bands were detected by enhanced chemiluminescence (ECL; Amersham, U.K.) detection kit.

XII. Real time Polymerase chain reaction (RT-PCR)

A. Extraction of RNA

γδ and αβ T cells from HI and γδ T cells from oral cancer patients were stored at –80 ºC in TRIzol (Invitrogen Life-Technologies, N.Y.) in a ratio of $1X10^6$ cells/100 µl TRIzol solution until further

use. At the time of RNA extraction, chloroform was added to make 1:5 ratio of chloroform: TRIzol (eg. 20µl choloroform in 100 µl TRIzol) mixed and centrifuged at 10,000 rpm for 15 min and the aqueous phase was collected and treated with chilled isopropyl alcohol (Qualigens, India) half the volume of TRIzol, mixed gently for 10 m and centrifuged at 10,000 rpm for 10 m. After centrifuging, the pellet obtained was washed with 75% ethanol (10,000 rpm for 5 m). The pellet was air dried and dissolved in appropriate volume of DEPC (Sigma)-treated water. Optical density (O.D.) readings were taken for quantitation of RNA by NanoDrop spectrophotometer (Thermo Scientific, DE). The RNA was run on a 1.5% agarose gel containing ethidium bromide to confirm its purity and integrity.

In case of surgically excised oral and breast tumors, tumors were collected and minced in double strengthen medium. Minced tumor tissues were weighed and collected in a tube containing TRIzol reagent (approx. 100 mg in 1 ml of the reagent). This was followed by homogenization and RNA was extracted as described above.

B. Complementary DNA (cDNA) synthesis by reverse transcription

Total RNA from the T cells was used for first strand cDNA synthesis using oligo dT primers (Invitrogen Life-Technologies, N.Y.). 5µg of RNA (10 µl volume with DEPC treated water) was reverse transcribed using 1µl of oligo dT and 1µl (10 mM) of dNTP; this mixture was heated at 65ºC for 10 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.

Component	Volume
5X 1st strand buffer	4 µl
0.1 M DTT	2 µl
RNase inhibitor	1 µl

This total mixture was heated at 37ºC for 2 min and then reverse transcribed using 1µl reverse transcriptase enzyme (200U/µl, Murine Moloney Leukemia Virus reverse transcriptase enzyme, Invitrogen Life-Technologies, N.Y.) at 37ºC for 50 min in PTC-100™ Programmable Thermal Controller (MJ Research Inc.). The mixture was heated at 70ºC for 15 min. The final cDNA volume was 20µl.

C. RT-PCR

Quantitative RT-PCR for different Notch receptor isoforms (Notch1-4), ligands (Dll1, Dll3, Dll4, Jag1 and Jag2) and its target genes (Hes1, DTX, NRARP and NF-κB) was performed with PRISM 7700 (PE Applied Biosystems, Foster City, CA). RT-PCR reaction mixture was prepared:

Component	Volume
100ng cDNA+DEPC water	2.25 µl
Primer probe mixture	0.25µl
Master-mix	2.5µl

Samples were analyzed using TaqMan primer sets purchased from Applied Biosystems (NOTCH1 Hs01062011_A1, NOTCH2 Hs01050719_A1, NOTCH3 Hs01128541_A1, NOTCH4 Hs00270200_A1, DLL1 Hs00194509_A1, DLL3 Hs00213561_A1, DLL4 Hs00184092_A1, JAG1 Hs01070036_A1, JAG2 Hs00171432_A1, HES1 Hs00172878, NF-κB Hs00765730_A1, DTX Hs00269995_A1, NRARP Hs01104102_S1, ACTB [β-actin] Hs99999903_A1). All values were normalized to the expression of the housekeeping gene β-actin.

XIII. Proliferation assay

Proliferation of cells was assayed by ^{3}H-thymidine (^{3}H TdR) uptake assay. A total of $5X10^{4}$ γδT cells were incubated in round-bottom 96-well tissue culture plates with 10µl of rIL-2 (0.1IU/ml, PeproTech) plus BrHPP (200nM, Innate Pharma) or IPP (40µM, Sigma Aldrich) or 20nM of c-HDMAPP (also known as IPH1201/picostim, Innate Pharma) for 72 h. For experiments using GSI-X (15µM), cells were pretreated as described above. When the effect of anti CD3 mAb was assessed in γδ T cells and PBLs, the wells were precoated overnight with purified anti-human CD3 mAb (1 mg/ml) (BD Biosciences). Similarly, to analyze the effect of Notch ligands, rDll1 (0.25 µg/ml), rDll4 (1.5 µg/ml) and rJag1 (0.5 µg/ml) were precoated on 96 well round bottom plates overnight for experiments showing effect of Notch ligands stimulation on proliferation of γδ T cells. The cultures were pulsed with 0.5 µCi [^{3}H] thymidine/well (Board of Radiation and Isotope Technology, Mumbai) during the last 18 h of the assay. The cells were harvested onto glass-fiber filter paper (Titertek, Norway) using a cell harvester (Titertek, Norway). The filter paper was dried at 50ºC and each disc corresponding to a single well was placed in 3 ml of scintillation fluid (0.5 g PPO, 7 g POPOP in 1 liter Toulene). The radioactivity incorporated in the

DNA was measured in a liquid scintillation counter (Packard, Meriden, CT) as counts per minute (cpm).

XIV. Estimation of cytokines

Immunomagnetically purified γδ T cells ($1x10^5$) from HI and OC patients were stimulated with BrHPP (200nM, Innate Pharma) or IPP (40μM, Sigma Aldrich) or 20nM of c-HDMAPP (IPH1201/picostim, Innate Pharma) or precoated anti CD3 mAb (1 mg/ml) (BD Biosciences) 0r Notch ligands (rDll1 (0.25 μg/ml), rDll4 (1.5 μg/ml) and rJag1 (0.5 μg/ml)) in the presence of IL-2 (0.1 IU/ml) for 24 h at 37ºC in round bottomed 96 well plates (Nunc, Denmark). As controls, only γδ T cells in complete medium (RPMI+10% FCS) were incubated at 37ºC for 24 h. After incubation supernatants were collected and stored at -20ºC. Cytokines levels were measured by the following two ways.

A. Cytometric bead array (CBA)

Cytokines (IL2, IL4, IL6, IL10, IL17, IFN-γ and TNF-α) were quantitated in cell culture supernatant by Cytometric bead array kit (BD Pharmingen). Cytokines were determined in the test samples according to the manufacturer instructions. Briefly, test samples (50 μl) and PE detection antibody were incubated with capture bead reagent for 3 h in the dark at room temperature. All unbound antibodies are washed (1.0 ml wash buffer), re-suspended in 300 μl before acquisition on BD FACS Aria cytometer (BD Bioscience, San Jose, CA, USA). Set-up bead was prepared by adding 50 μl of setup bead antibody and 450 μl of wash buffer. Samples were vortexed and acquired. The CBA data were analyzed using FCAP Array software version 1.0 (BD Biosciences).

B. Sandwich enzyme-linked immunosorbent assay (ELISA)

Levels of IFN-γ were quantitated in cell culture supernatant by sandwich ELISA using commercially available kit (Opt EIA human IFN-γ, BD Pharmingen, CA) according to manufacturer's instructions.

XV. Cytotoxicity assay

^{51}Chromium release assay was used to measure the cytotoxicity of γδ T cells (effector) against oral cancer cell line (AW13516) used as target cells. γδ T cells were left alone or treated with rIL2 (0.1U, PeproTech) overnight at 37ºC and AW13516 cells were treated for 18 h with zoledronate (100μM) (Panacea Biotech Ltd.). AW13516 cells were labeled with ^{51}Chromium for 90 min at 37ºC. Next, labeled target cells were incubated with effector cells at 40:1 effector to target (E:T) ratio at 37ºC in 5% CO_2 for 4h. After incubation, plates were centrifuged, supernatants were collected and radioactive chromium release was measured using 1470 Wallac automated gamma counter (Perkin-Elmer, Downers Grove). Initially, labeled target cells were cultured with γδ T cells at different E:T ratio (40:1, 30:1, 20:1, 10:1 and 5:1) and maximum cytotoxicity was observed with E:T ratio of 40:1. Spontaneous release was determined by incubating the target cells with medium alone and maximum release was determined by incubating target cells with 10% Triton X-100. The percent specific lysis was calculated as (experimental release-spontaneous release) / (maximum release-spontaneous release) x 100.

XVI. Small interfering RNA

γδ T cells isolated by MACS column (as above) were transfected with small interfering RNA (siRNA) specific for NOTCH1, NOTCH2 genes and fluorescent oligonucleotide SiGLO

(transfection indicator) (Thermo Fisher Scientific, Waltan, USA). siRNA oligos were transfected at a concentration of 50 nM using X-tremeGENE HP transfection reagent (Roche Diagnostics, Indianapolis, IN). The inhibition of Notch1 and Notch2 expression were assessed at 48 h. Briefly $2x10^5$ cells per well were seeded in 24 well plate in 400µL media supplemented with FBS. Then siRNA duplex was diluted in the 50µL of transfection media (Opti-MEM® I reduced serum medium) (Invitrogen Life Technologies, Inc., Gaithersburg, MD) such that each well gets the optimal level of siRNA (50nM). 2.5 µL of transfection reagent was gently mixed with 50µL of transfection media (Opti-MEM) and incubated for 5 min. After 5 minutes both the solutions were combined and incubated at room temperature for 30 minutes to allow the complex to form. Then mixed gently and overlaid the mixture onto the cells. Cells were incubated for 48h and monitored for siGLO by flow-cytometry for transfection efficiency (70±5%).

XVII. Purification of regulatory T cells

Regulatory T cells were isolated from PBLs of healthy individuals using BD Imag immunomagnetic separation kit. Cells were first labeled with cocktail of biotinylated 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-labeled mouse anti-human CD25 antibody. After peripheral blood lymphocytes were labeled 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. In the second immunomagnetic separation step, the Anti-APC Particles were used to select the $CD25^+$ cells, from the enriched $CD4^+$ cells, which were already labeled with the APC anti-CD25 mAb. The phenotype and purity of regulatory T cells was confirmed using flow cytometry.

XVIII. Regulatory T cells (Tregs) suppression assay

Purified freshly isolated γδ T cells were isolated from peripheral blood lymphocytes of healthy individuals using immunomagnetic separation kit. Freshly isolated γδ T cells were labeled with 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester, (CFSE [Invitrogen Life-Technologies, USA). Briefly, 1-5 X 10^6 cells in PBS + 5% fetal calf serum (FCS) were incubated with 5 µM CFSE for 5 m at room temperature in dark. After incubation cells were washed thrice with PBS + 5% FCS. CFSE labeled γδ T cells ($1X10^4$) were then co-cultured with purified regulatory T cells ($1X10^4$) and stimulated with rIL2 (0.5 IU/ml) and BrHPP (200 nm) for 5 days. CFSE labeled γδ without regulatory T cells in the presence of rIL2 and BrHPP were kept as control. Tregs cells were previously treated with either anti Jag1 antibody (0.5 µg) (to block Jag1 ligand) or Fc control for 2 h at 37ºC. After 5 days cells were harvested and analyzed on FACS Aria flow cytometer. Data obtained was analyzed using FlowJo software.

XIX. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0. The student's t test was used as the test of significance.

CHAPTER 4:

Expression of Notch receptors and ligands in human peripheral blood γδ T cells

Recently Notch family has been implicated in the regulation of effector functions of different kinds of immune cells. The role of Notch in regulating effector functions of $CD8^+$ T cells and NK cells have been described [28, 29] . Signaling through the TCR in both $CD4^+$ and $CD8^+$ T cells induces the activation of Notch1 [29, 30]. In addition Notch also facilitates TGF-β-mediated effector function of regulatory T cells [94]. Nevertheless, the involvement of Notch signal in activation of human γδ T cells has not been investigated. Experiments carried out in this chapter were aimed at analyzing expression of Notch receptor proteins (Notch1 and Notch2) and mRNA in γδ and $CD3^+$ T cells using flowcytometry, confocal microscopy, western blotting and real time PCR.

Isolation of γδ T cells

The percentage of γδ T cells present in peripheral blood is <10% and therefore, the cells were enriched before isolating γδ T cells directly from peripheral blood. Briefly, peripheral blood lymphocytes (PBLs, $10X10^6$) were expanded ex-vivo with solid phase anti CD3 mAb (1mg/ml) and rIL2 (10 IU/ml) for 12 days. This is followed by immunomagnetic purification to isolate γδ T cells. In order to check the purity of γδ T cells, cells collected from the positive fraction after immunomagnetic purification were analyzed for the γδ-TCR by flow cytometry. Table-1 summarizes the total expansion of lymphocytes from healthy individuals (n=84) using anti CD3 mAb and rIl2. $10X10^6$ PBL of each healthy individual were stimulated with anti CD3 mAb and rIL2 and the percentage of purified γδ T cells obtained from the same individuals after expansion is sho wn in Table-1,. Expansions γδ T cells range from $11X10^6$ to $238X10^6$ with mean of $96.5X10^6 \pm 5.8$ was observed from a starting population of $10X10^6$ PBLs. The immunomagneticaly

purified γδ T cells ranged from 2 to 52.5% (mean 13.32±3.7SE). Henceforth, in the thesis these γδ T cells will be referred to as "ex-vivo γδ T cells".

Table 1: Generation of γδ T cell lines from PBL

Healthy Donor	Cell count Day '0'	Cell count Day '12'	% recovery of γδ after immunomagnetic separation		Healthy Donor	Cell count Day '0'	Cell count Day '12'	% recovery of γδ after immunomagnetic separation
1	$10x10^6$	$132.5x10^6$	2.5%		30	$10x10^6$	$14.9x10^6$	8.4%
2	$10x10^6$	$68.7x10^6$	7.9%		31	$10x10^6$	$121x10^6$	8.8%
3	$10x10^6$	$131.6x10^6$	18.1%		32	$10x10^6$	$178x10^6$	16.7%
4	$10x10^6$	$25x10^6$	7.4%		33	$10x10^6$	$100x10^6$	9.8%
5	$10x10^6$	$92x10^6$	4.6%		34	$10x10^6$	$175x10^6$	33.7%
6	$10x10^6$	$145x10^6$	4.7%		35	$10x10^6$	$228x10^6$	18%
7	$10x10^6$	$136.5x10^6$	5.9%		36	$10x10^6$	$111x10^6$	15.7%
8	$10x10^6$	$74.7x10^6$	8.2%		37	$10x10^6$	$169x10^6$	18.6%
9	$10x10^6$	$90x10^6$	4.4%		38	$10x10^6$	$30x10^6$	20%
10	$10x10^6$	$35x10^6$	2%		39	$10x10^6$	$85x10^6$	8.2%
11	$10x10^6$	$120x10^6$	6.3%		40	$10x10^6$	$119x10^6$	33.3%
12	$10x10^6$	$97x10^6$	5.5%		41	$10x10^6$	$134x10^6$	14%
13	$10x10^6$	$177x10^6$	5.3%		42	$10x10^6$	$160x10^6$	15.9%
14	$10x10^6$	$124x10^6$	9.5%		43	$10x10^6$	$216x10^6$	34%
15	$10x10^6$	$147x10^6$	9.2%		44	$10x10^6$	$80x10^6$	34%
16	$10x10^6$	$135x10^6$	9.9%		45	$10x10^6$	$76x10^6$	24.9%
17	$10x10^6$	$30.6x10^6$	14%		46	$10x10^6$	7010^6	11.9%
18	$10x10^6$	$103x10^6$	21.3%		47	$10x10^6$	$45x10^6$	5.5%
19	$10x10^6$	$204x10^6$	2.8%		48	$10x10^6$	$18.5x10^6$	7.1%
20	$10x10^6$	$120x10^6$	3.8%		49	$10x10^6$	$132x10^6$	12.2%
21	$10x10^6$	$66x10^6$	8.3%		50	$10x10^6$	$96x10^6$	3.2%
22	$10x10^6$	$44.4x10^6$	5.8%		51	$10x10^6$	$75x10^6$	19%
23	$10x10^6$	$128x10^6$	11.7%		52	$10x10^6$	$66x10^6$	10%
24	$10x10^6$	$146x10^6$	3%		53	$10x10^6$	$98x10^6$	20%
25	$10x10^6$	$58x10^6$	15.4%		54	$10x10^6$	$138x0^6$	2.9%
26	$10x10^6$	$60x10^6$	32.2%		55	$10x10^6$	$155x10^6$	5.4%
27	$10x10^6$	$70x10^6$	1.3%		56	$10x10^6$	$61x10^6$	9.8%
28	$10x10^6$	$16x10^6$	20%		57	$10x10^6$	$126x10^6$	44.4%

Healthy Donor	Cell count Day '0'	Cell count Day '12'	% recovery of γδ after immunomagnetic separation		Healthy Donor	Cell count Day '0'	Cell count Day '12'	% recovery of γδ after immunomagnetic separation
29	$10x10^6$	$11x10^6$	5%		58	$10x10^6$	$20x10^6$	19%
59	$10x10^6$	$100x10^6$	5.3%		72	$10x10^6$	$12x10^6$	19.8%
60	$10x10^6$	$116x10^6$	52.5%		73	$10x10^6$	$165x10^6$	3%
61	$10x10^6$	$119x10^6$	4.7%		74	$10x10^6$	$42.5x10^6$	2.2%
62	$10x10^6$	$123x10^6$	10%		75	$10x10^6$	$71x10^6$	7.2%
63	$10x10^6$	$66x10^6$	19.8%		76	$10x10^6$	$135x10^6$	2.1%
64	$10x10^6$	$25x10^6$	23.5%		77	$10x10^6$	$119x10^6$	15.1 %
65	$10x10^6$	$124x10^6$	21.1%		78	$10x10^6$	$103x10^6$	11.5%
66	$10x10^6$	$116x10^6$	19.8%		79	$10x10^6$	$89x10^6$	14.3%
67	$10x10^6$	$158x10^6$	13.3%		80	$10x10^6$	$27x10^6$	15.7%
68	$10x10^6$	$238x10^6$	1.3%		81	$10x10^6$	$93x10^6$	19%
69	$10x10^6$	$22.5x10^6$	23.6%		82	$10x10^6$	$55.5x10^6$	1.4%
70	$10x10^6$	$57.6x10^6$	31.7%		83	$10x10^6$	$46.5x10^6$	10.8%
71	$10x10^6$	$15x10^6$	26.7%		84	$10x10^6$	$102x10^6$	2.7%

Mean cell count on Day 12= $96.5x10^6$ ±5.8
Mean % recovery of γδ T cells = 13.32 ±3.7 %

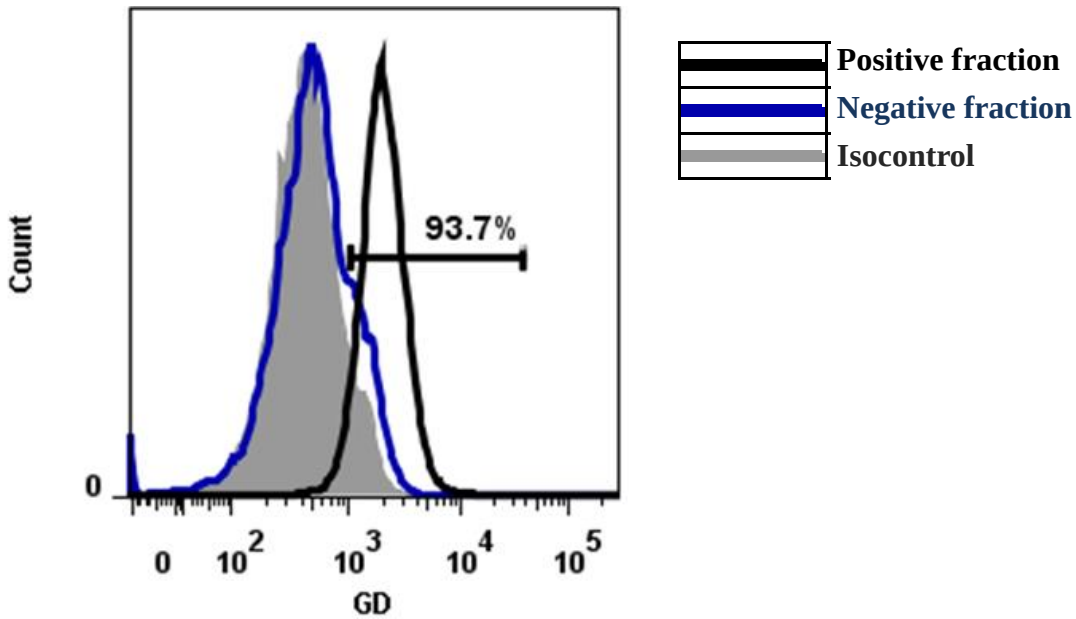

Figure 7: Purity of immunomagnetically separated γδ T cells

As shown in Figure 7, positive fraction obtained after immunomagnetic separation showed ≥90% of the cells positive for γδ-TCR. These cells were used in the subsequent experiments.

γδ T cells isolated directly from PBLs were also used for some of our experiments (Table-2). The lymphocyte population obtained from 30 to 50 ml of peripheral blood varied from 50 to 135 $x10^6$ cells and the percent recovery of purified γδ T cells ranged from 0.79-5.4% (mean3±0.3%SE). Henceforth, in the thesis these γδ T cells will be referred to as "freshly isolated γδ T cells".

Table 2: Isolation of fresh γδ T cells from PBL

Healthy Donor	**Blood Drawn volume**	**Total PBL**	**% recoveryof γδ after immunomagnetic separation**
85	**50 ml**	**$135x10^6$**	**1%**
86	**50 ml**	**$137x10^6$**	**1.3%**
87	**30ml**	**$70x10^6$**	**2.3%**
88	**50 ml**	**$100x10^6$**	**3%**
89	**50 ml**	**$120x10^6$**	**1.9%**
90	**50 ml**	**$110x10^6$**	**2.3%**
91	**30 ml**	**$60x10^6$**	**5%**
92	**30 ml**	**$110x10^6$**	**2.8%**
93	**30 ml**	**$86x10^6$**	**1%**
94	**30 ml**	**$66x10^6$**	**2.5%**
95	**30 ml**	**$64x10^6$**	**3%**
96	**20 ml**	**$50x10^6$**	**4.6%**
97	**30 ml**	**$81x10^6$**	**3.3%**
98	**30 ml**	**$56x10^6$**	**1.4%**

Mean % recovery of γδ T cells = 3 ±0.3%

Expression of mRNA for Notch receptors and ligands in peripheral human γδ T cells and CD3$^+$ T cells

To determine the expression of different Notch receptor isoform and their ligands in peripheral γδ T cells, the expression of mRNA for Notch genes (Notch1-4) and the ligands Dll1,3 and 4 and Jag1 and 2 were quantitated by real time PCR in ex vivo expanded and purified γδ T cells. β-actin was used as a house keeping gene control. As seen in Figure 8A, in γδ T cells, the expression of Notch2 gene was higher than Notch1. Lower expression of Dll1 and Jag1 mRNA was also observed. mRNA for Notch3, 4, Dll3, 4 and Jag.2 was not detected in γδ T cells. The results confirmed that Notch receptors (Notch1 and 2) and ligands (Dll1 and Jag1) are expressed on γδ T cells.

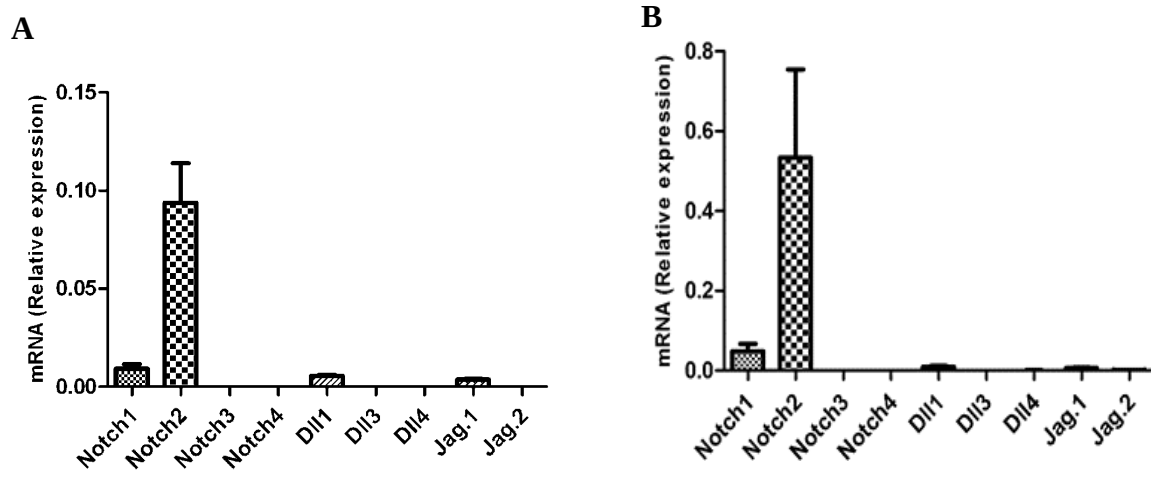

Figure 8: Real time quantitative PCR showed mRNA expression of Notch1, Notch2, Dll1 and Jag1 on γδ T cells and CD3$^+$ T cells. A. Total RNA was extracted from ex-vivo expanded γδ T cells. Real time quantitative PCR showed mRNA expression of Notch1, Notch2 along with lower level of Dll1 and Jag1 on γδ T cells. **B.** CD3$^+$ T cells were MACS purified from the peripheral blood. Real time quantitative PCR showed mRNA expression of Notch1 and Notch2 on CD3$^+$ T cells. Data were normalized to expression of GAPDH. The data represent mean of four independent experiments.

Besides γδ T cells, the involvement of Notch pathway components in peripheral blood $CD3^+$ T cells of healthy individuals was also studied. The expression of mRNA for Notch genes (Notch1-4) and its ligands Dll1, Dll3 and Dll4 and Jag1 and Jag2 in $CD3^+$ T cells were assesed. The expression of mRNA for Notch1 and Notch2 gene was observed (Figure 8B).

Expression of Notch intracellular domain (NICD) in γδ T cells and $CD3^+$ T cells

Following Notch activation, NICD enters the nucleus and regulates the expression of target genes [26]. The expression of N1ICD and N2ICD on the ex-vivo expanded γδ T cells was analyzed by flow cytometery (Figure 9A). Comparision of the mean fluorescence intensity (MFI) of N1ICD and N2ICD in γδ T cells was in the order of 561 and 353 respectively. A low level expression of ligands Dll1 and Jag1 are observed on these cells. Further the contamination of non T-cell was ruled out by staining isolated γδ T cells for expression of CD14, CD15, CD19, CD33 and CD56 markers (Figure 9B). Cells were positive for CD56 which is a marker known to be expressed by γδ T cells. The expression of NICD (N1ICD and N2ICD) was also determined in αβ T cells from peripheral blood using specific antibodies and flow cytometry (Figure 9C). The MFI of N1ICD and N2ICD in αβ T cells was in the order of 338 and 59.3 respectively.

The expression of NICD in γδ T cells was also visualized by confocal microscopy. Freshly isolated γδ T cells (without ex-vivo expansion) were stained for N1ICD and anti γδ TCR antibody and analyzed using confocal microscopy. It was observed that N1ICD was abundantly expressed on the cell membrane and cytoplasm of γδ T cells (Figure 10A). Similarly, the expression of N1ICD in the ex vivo expanded γδ T cells was analyzed. The expression of N1ICD was observed in the membrane, cytoplasm and the nucleus of γδ T cells (Figure 10B). Furthermore, as shown in

Figure 10C, the expression of N2ICD on the membrane was observed in ex vivo expanded γδ T cells.

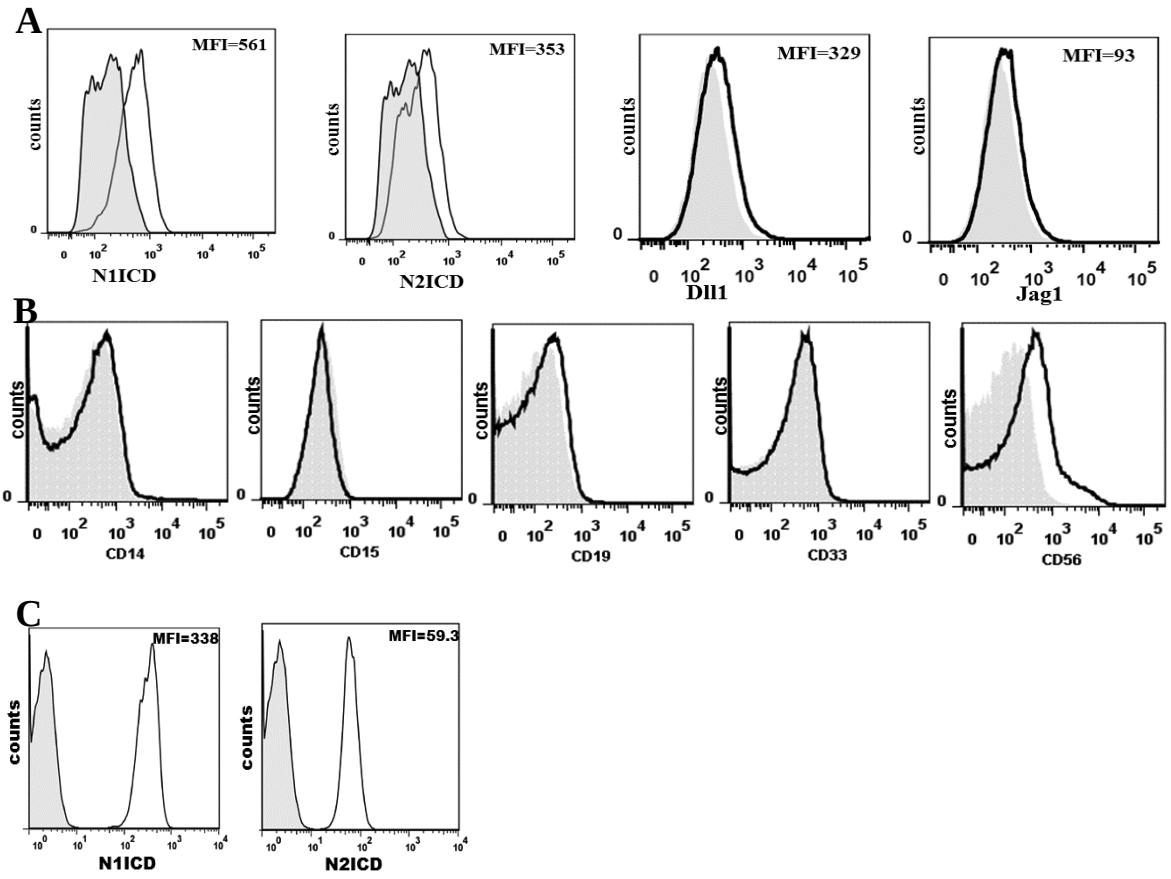

Figure 9: Expression of Notch intracellular domain (NICD) by flow cytometry. A. Expression of N1ICD, N2ICD, Dll1 and Jag1 on γδ T cells. **B.** Purified γδ T cells were negative for CD14, CD15, CD19, CD33 receptors and were positive for CD56 marker. **C.** Flow cytometric analysis of $CD3^+$ T cells showed the expression of N1ICD and N2ICD. The filled historam in each panel indicates the isotype control. The MFI in each case has been corrected with respect to for the MFI of the isotype control (Test MFI-Isotype MFI).

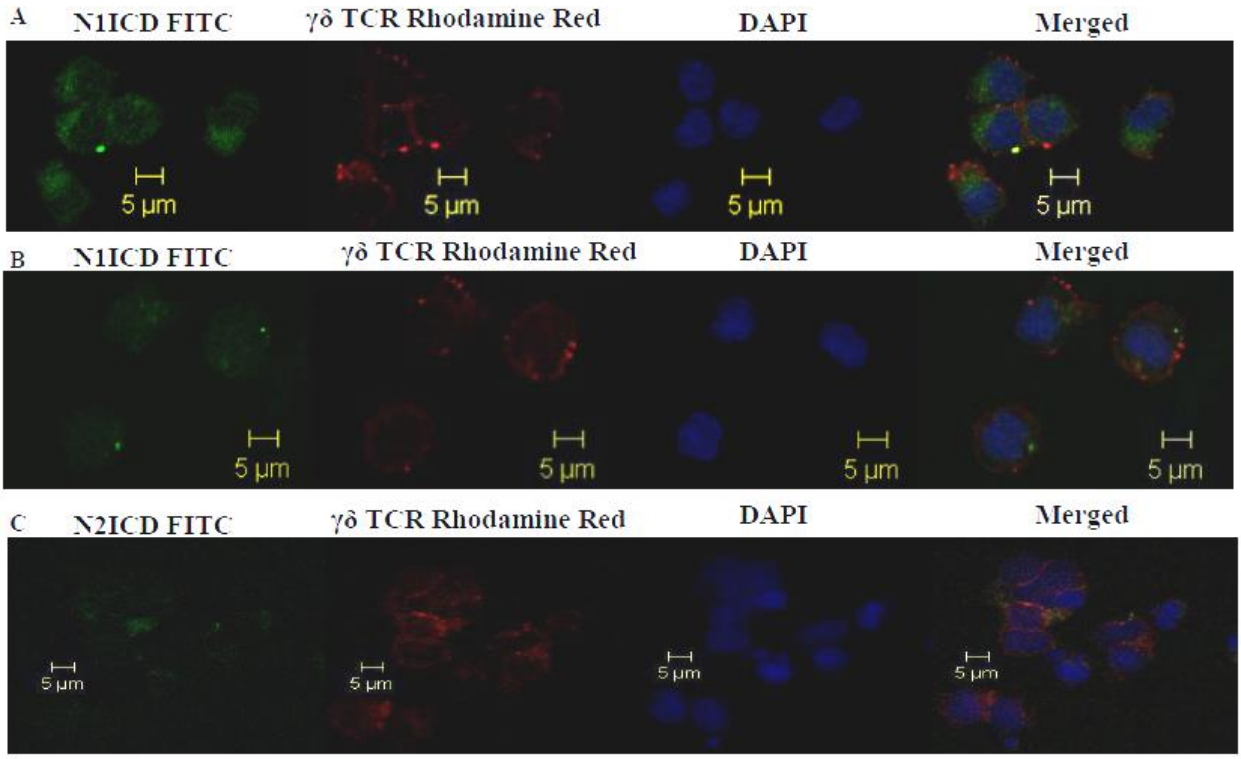

Figure 10: Expression of NICD by confocal microscopy. A. Immunofluorescence staining of freshly isolated γδ T cells showed the expression of N1ICD. **B.** Expression of N1ICD in ex-vivo expanded γδ T cells. **C.** Expression of N2ICD in ex-vivo expanded γδ T cells. Green fluorescence (FITC) shows pattern of N1ICD (**A** and **B**) and N2ICD (**C**), γδ TCR receptor is in red (rhodamine red) and nuclei are shown in blue (DAPI). Scale bar of 5 µm is shown at lower right of each image.

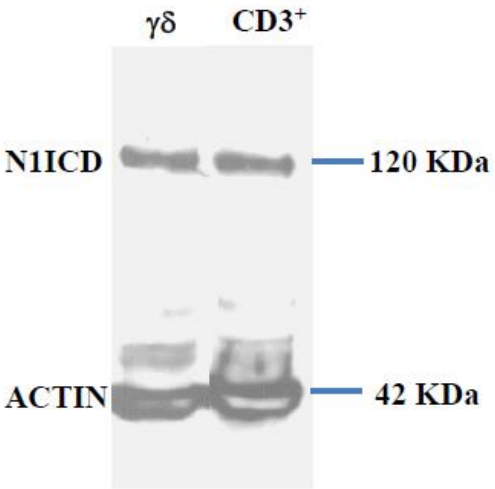

Figure 11: Expression of N1ICD in γδ T and CD3$^+$ T cells by western blotting. Detection of 120 KDa N1ICD and 42 KDa actin on ex-vivo expanded γδ T and CD3$^+$ T cells , using antibody that recognizes the cleaved active form of Notch1 (N1ICD) and β-actin respectively.

Further, the expression of NICD at protein level was confirmed using Western blotting (Figure 11). Whole cell lysates of ex-vivo expanded γδ T cells and αβ T cells were run on 8% SDS-PAGE and after transfer to nitrocellulose membrane probed with N1ICD. As shown in Figure 11, the expression of N1ICD in ex vivo expanded γδ T cells was found to be similar level to that observed in purified αβ T cells. The expression of NICD in γδ T cells confirmed that Notch signaling was active in γδ T cells.

Expression of Notch target genes in γδ T cells

In canonical Notch signaling pathway, the release of Notch intracellular domain (NICD) is followed by its translocation to the nucleus. In the nucleus, NICD associates with CSL to convert it from a repressor into an activation complex. The canonical Notch signaling pathway is conserved over evolutionary time and acts both in invertebrates as well as vertebrates to impart a wide variety of cell fate decisions [54]. The well-known canonical Notch target gene is Hairy and enhancer of split-1 (HES1). The presence of cleaved intracellular form of Notch (NICD) in the nucleus of γδ T cells leads to expression of HES1. Using confocal microscopy, the expression of HES1 was analysed in freshly isolated and ex-vivo expanded γδ T cells using anti HES1 FITC, anti γδ TCR Rhodamine Red labeled antibodies and DAPI. As seen in Figure 12A and B, the nuclear expression of HES1 was observed in freshly isolated and ex-vivo expanded γδ T cells respectively.

Other transcriptional targets of Notch signaling are Deltex-1 (DTX) and Notch-regulated ankyrin-repeat protein (NRARP) [269, 270]. In addition to the well-known canonical Notch signaling pathway evidence has emerged that non-canonical Notch signaling may also play an important role in T cells. In non-canonical Notch signaling, NICD associated with the Nuclear factor kappa

B (NF-κB) protein and Notch may regulate transcription independent of CSL [29, 58]. The mRNA expression profile of Notch target genes (HES1, NF-κB, DTX and NRARP) in ex-vivo γδ T cells were analyzed by real time PCR. As shown in Figure 12C, relatively higher level of mRNA for NF-κB was observed in γδ T cells. The expression of mRNA for HES1 and NRARP was higher than DTX.

Using real time PCR, the relative expression of mRNA for Hes1, NF-κB, Deltex and NRARP was also demonstrated in αβ T cells (Figure 12D).

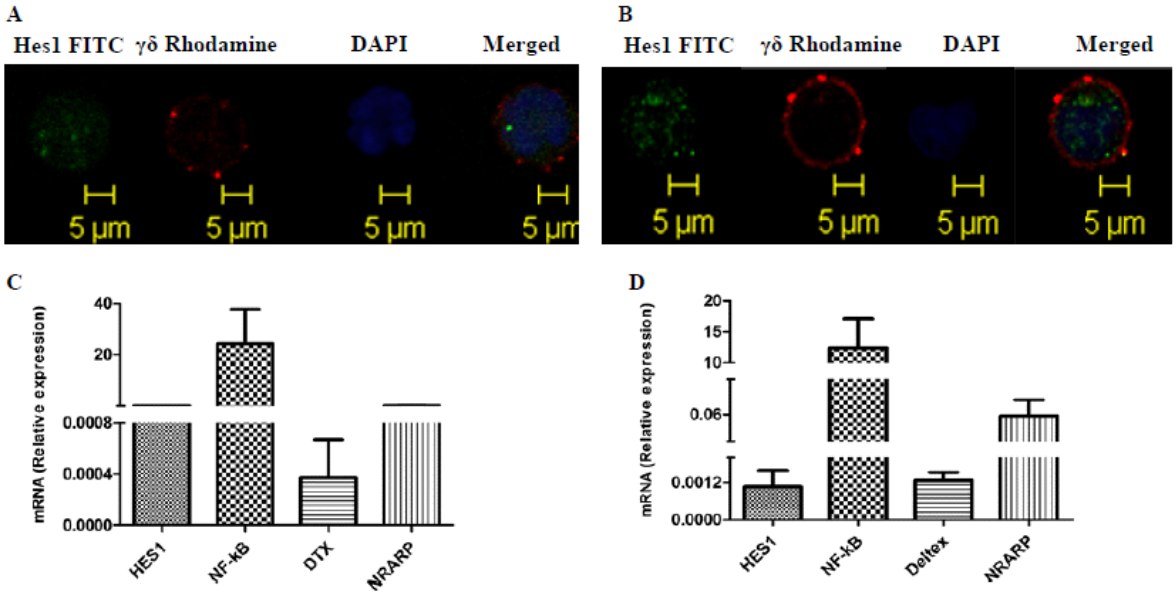

Figure 12: Expression of HES1 and other taget genes in γδ T cells. A. Immunofluorescence staining of freshly isolated γδ T cells showed the expression of HES1. **B.** Expression of HES1 in ex-vivo expanded γδ T cells. Green fluorescence (FITC) shows pattern of HES1, γδ TCR receptor is in red (rhodamine red) and nuclei are shown in blue (DAPI). Scale bar of 5 µm is shown at lower right of each image. **C.** Real time quantitative PCR showed mRNA expression of Notch target genes- HES1, NF-κB, DTX and NRARP on γδ T cells (n=3). Total RNA was extracted from ex-vivo expanded γδ T cells. **D.** Real time quantitative PCR showed mRNA expression of Notch target genes- HES1, NF-κB, DTX and NRARP on $CD3^+$ T cells (n=3). Data were normalized to expression of GAPDH.

CHAPTER 5:

Role of Notch in regulating effector functions of peripheral γδ T cells

Recently Notch has been implicated in the regulation of effector functions of immune cells. The role of Notch in regulating effector functions of $CD8^+$ T cells and NK cells have been described [28, 29] . Signaling through the TCR in both $CD4^+$ and $CD8^+$ T cells induces the activation of Notch1 [29, 30]. In addition, Notch also facilitates TGF-β-mediated effector function of regulatory T cells [94]. However, the role of Notch in regulating the effector functions of human γδ T cells has not been reported earlier. Experiments were aimed at analyzing expression of Notch receptor proteins (Notch1 and Notch2) and mRNA in γδ and αβ T cells using flowcytometry, confocal, western blotting and real time PCR. Further the ability of Notch in regulating effector functions of γδ T cells was also studied.

Selective expansion of Vγ9Vδ2 T cells from peripheral blood lymphocytes by phosphoantigens

Antigen recognition by γδ T cells is dependent upon the particular variable (V) region of the TCR as opposed to the entire rearranged TCR required by αβ T cells. γδ T cells expressing Vδ1 are abundantly found at mucosal sites and they respond to the expression of non-classical MHC molecules on the surface of virally-infected or tumor cells [101-103]. Vδ2+ (Vγ9Vδ2) cells are predominantly present in the peripheral circulation and respond to non-peptide phosphoantigens which are intermediates of the mevalonate pathway such as IPP and its synthetic analogues BrHPP and picostim. [11, 12].

PBLs obtained from healthy individuals ($1x10^6$/well) were cultured in 24 well plate for 12 days in the presence of rIL2 alone or along with one of three different types of phosphoantigens- Bromohydrin pyrophosphate (BrHPP), Isopentenyl pyrophosphate (IPP) or 1-Hydroxy-2-methyl-2-buten-4-yl 4-diphosphate (c-HDMAPP)/also known as picostim.

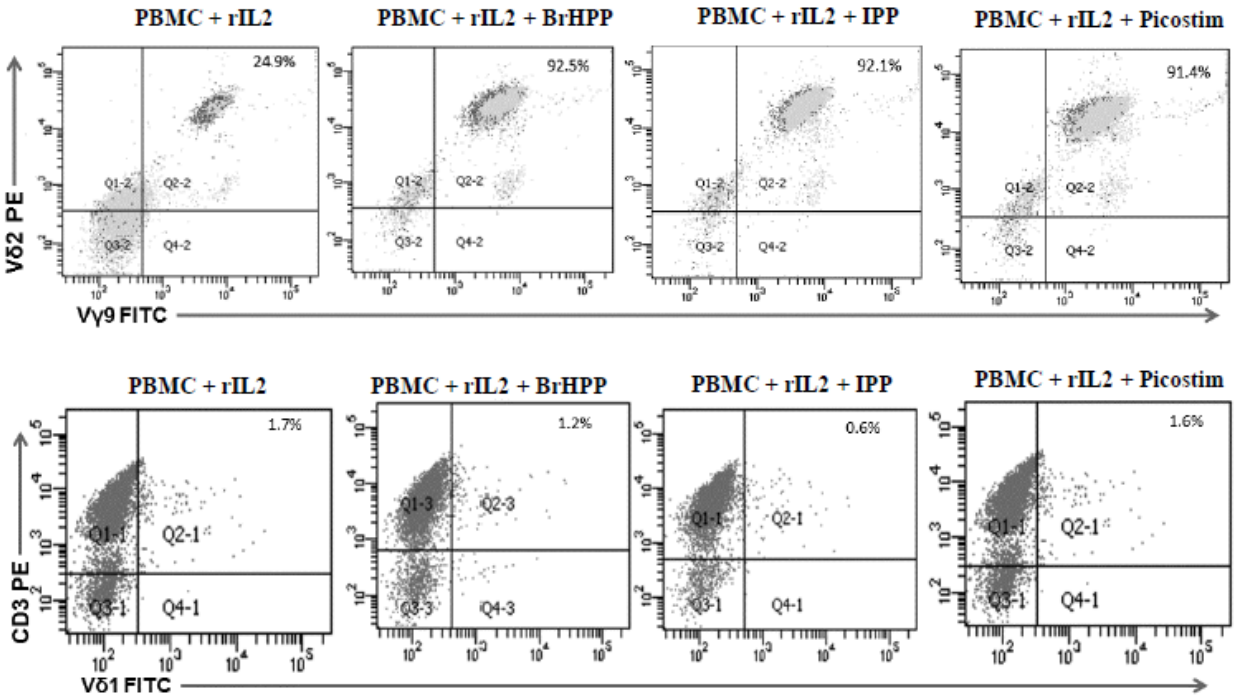

Figure 13: Selective expansion of Vγ9Vδ2 T cells from PBLs. A. PBLs were stained after culturing for 12 days and expression of Vγ9Vδ2 analyzed by flow cytometry. Treatment with phosphoantigens (BrHPP, IPP and picostim) results in selective outgrowth of Vγ9Vδ2 T cells. **B.** Flow ctometryic analysis showed that Vδ1 T cells were not responding to the phosphoantigens.

As shown in Figure 13, it was observed that culturing of PBLs with BrHPP, IPP and picostim leads to selective expansion of Vγ9Vδ2 T cells (>90%) compared to PBLs stimulated with rIL2 alone (24.9%). The percentage expansion of Vδ1 was negligible in PBLs stimulated with BrHPP, IPP and picostim (1.2%, 0.6% and 1.6% respectively) (Figure 13). This results show that phosphoantiges (BrHPP, IPP and picostim) selectively expand the pool of Vγ9Vδ2 T cells which constitute the dominant population of γδ T cells in the peripheral blood.

Disruption of Notch signaling in activated γδ T cells reduces expression of Notch receptor

The release of NICD mediated by γ-sectretase activity is required by all Notch receptors (Notch1-4) to initiate downstream signaling [271, 272]. In the present study γ-secretase inhibitor, GSI-X (L-685,458) was used to block γ-sectretase activity in γδ T cells [273]. γδ T cells were stimulated with rIL2 and BrHPP in the presence and absence of GSI-X. Stimulation of γδ T cells with BrHPP and rIL2 triggered the activation of Notch signaling which can be observed by abundant release of 120kd N1ICD (Figure 14A, lane 2) compared to expression of N1ICD in unstimulated γδ T cells (lane 1). A marked decrease in the release of N1ICD was observed when BrHPP and rIL2 activated γδ T cells were treated with GSI-X (compare lane 2 and 3). It has been reported that stimulation of the Notch signaling pathway leads to the induction of c-Myc expression [274]. It was, therefore, interesting to investigate whether activation of γδ T cells with BrHPP and rIL2 was necessary for the Notch-mediated induction of cell cycle regulator, c-Myc (67kd). The data show that Notch1 activation governs the downstream induction of c-Myc expression in γδ T cells which was abrogated upon GSI-X treatment (Figure 14A).

Simultaneously, expression of mRNA for Notch receptors (1-4) and Notch ligands (Dll1, Dll3 and Dll4 and Jag1 and Jag2) were monitored in γδ T cells stimulated with BrHPP and rIL2 in the presence and absence of GSI-X (Figure 14B). Following treatment with GSI-X a marked decrease in the expression of mRNA for Notch1 receptor was observed in antigen activated γδ T cells (Figure 14B). No significant changes in mRNA expression of other Notch receptors and Notch ligands were noted after treatment with GSI-X in antigen activated γδ T cells (Figure 14B).

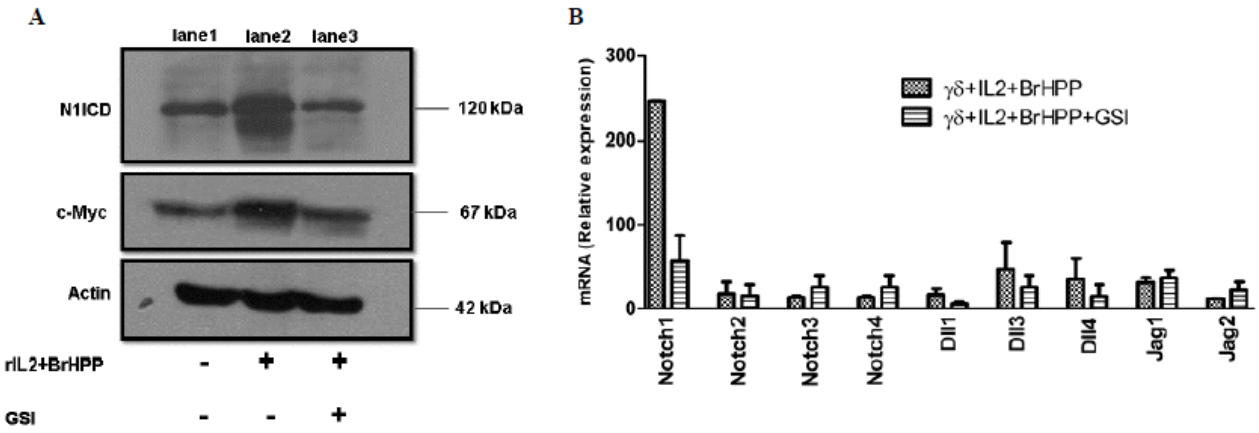

Figure 14: GSI-X treatment leads to decreased expression of Notch receptors and its target genes in antigen activated γδ T cells. A. Detection of 120 kDa NICD and 67 kDa c-Myc on unstimulated (lane1), BrHPP and rIL2 activated (24 h) γδ T cells in the presence (lane 3) or absence of GSI-X (lane2), using antibodies that recognizes the cleaved active form of Notch1 (N1ICD) and c-Myc. **B.** Real time PCR was performed for all the notch receptor isoforms (Notch 1-4) and its ligands (Dll1, Dll3 and Dll4; Jag1 and Jag2) on γδ T cells. Total RNA was extracted from BrHPP and IL2 stimulated γδ T cells (4 h) with or without GSI treatment. The expression of specific mRNA is relative to GAPDH and is normalized to that same ratio in unstimulated cells.

Effect of GSI-X on Notch target genes expression in activated γδ T cells

In order to investigate if Notch signaling is involved in antigen specific stimulation of γδ T cells, the expression of mRNA of Notch target genes were analyzed.in γδ T cells in the presence and absence of GSI-X. Using real time PCR, it was demonstrated that mRNA for Hes1, NF-κB, and DTX are expressed at higher level in BrHPP and rIL2 activated γδ T cells. Upon blocking of Notch signaling pathway by GSI-X, the expression of these target genes are down regulated (Figure 15). The expression of canonical Notch target gene-NF-κB was significantly decreased in BrHPP and rIL2 activated γδ T cells in the presence of GSI-X ($p<0.05$). However, the mRNA

expression for NRARP was very low in antigen activated γδ T cells in the presence and absence of GSI-X.

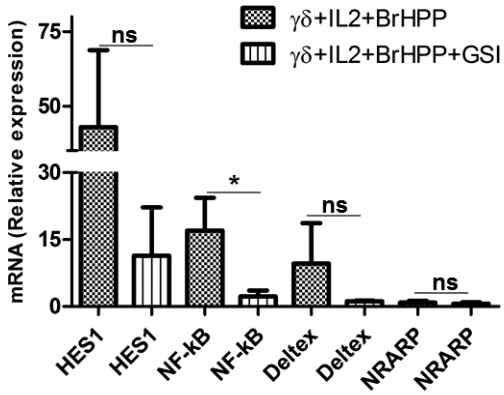

FIGURE 15: GSI treatment leads to decreased expression of HES1, NF-κB, and DTX and increased NRARP expression in antigen activated γδ T cells. Real time PCR was performed for the notch target genes in γδ T cells. Total RNA was extracted from BrHPP and IL2 stimulated γδ T cells (4 h) with or without GSI treatment. The expression of specific mRNA is relative to GAPDH and is normalized to that same ratio in unstimulated cells. These data are representative of three independent experiments (* $p<0.05$).

Inhibition of Notch signaling blocks γδ T cell activation

In order to determine whether Notch signal plays a role in the proliferative responses of γδ T cells, these cells were incubated with anti CD3 mAb alone or along with rIL2 in the presence and absence of GSI-X. γδ TCR mediated proliferation response was assessed by ^{3}H-TdR incorporation assay as described in materials and methods. A significant increase in the proliferation of γδ T cells was observed when cultured with anti CD3 mAb (mean cpm 1793±207) compared to γδ T cells cultured in the medium alone (mean cpm 690±9 SE, Figure 16A). A marked increased in the proliferation of γδ T cells was observed in the presence of anti CD3 and rIL2 (mean cpm 10718±550SE). However, as seen in Figure 16A, blocking of Notch signal by GSI-X leads to significantly decreased proliferative response to anti CD3 mAb in a concentration dependent (anti CD3 mAb concentration ranging from 15ug to 2.5 ug) manner as compared to the control cells (γδ T cells stimulated with anti CD3 mAb and rIL2).

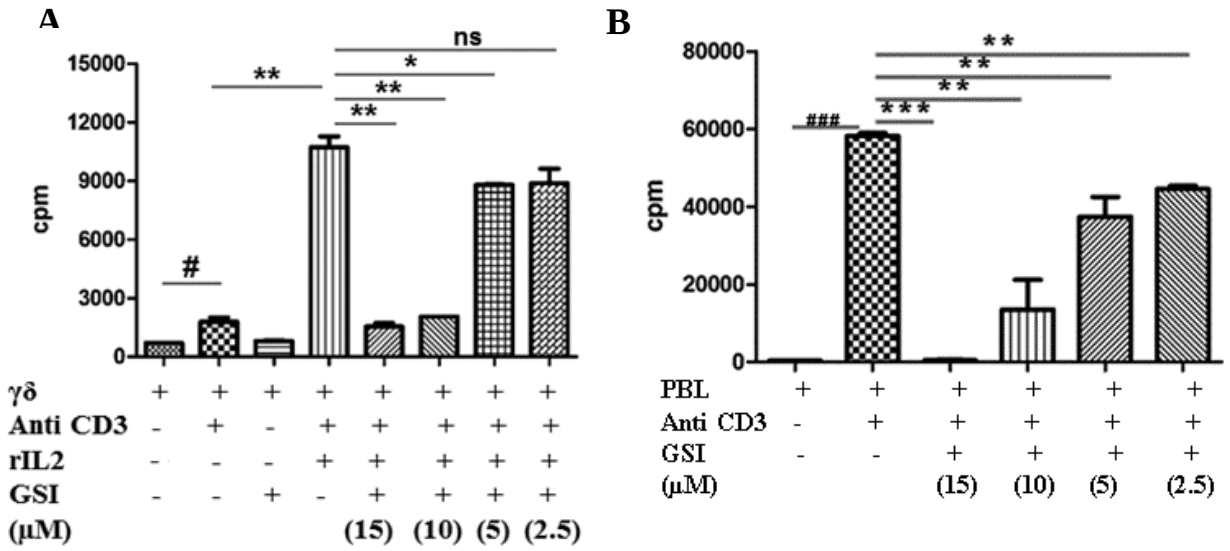

Figure 16: Treatment with GSI-X significantly reduces the anti-CD3 driven proliferative response of ex-vivo expanded γδ T cells and PBLs. Ex-vivo expanded γδ T cells ($5X10^4$ cells) were stimulated with anti CD3 (mAb) in the presence of rIL2, for 72h, with or without GSI-X treatment. Addition of GSI-X leads to decrease in the proliferative response of γδ T cells in a concentration dependent manner (15 μM, 10 μM, 5 μM and 2.5 μM). **B.** PBLs ($5X10^4$ cells) were stimulated with anti CD3 mAb in the presence and absence of different concentrations of GSI-X. Proliferation was measured by [^{3}H]TdR incorporation after 72 h. Values are measured as mean CPM (n=3). The horizontal bars indicate the levels of significance between different combinations used. (** $p<0.005$, *** $p<0.0005$, #$p<0.5$, ###$p<0.0005$).

To determine whether Notch signal plays a role in the proliferative response of $CD3^+$ T cells, PBLs were incubated with anti CD3 mAb in the absence or presence of different concentration of GSI-X. $CD3^+$ T cells present in PBLs showed robust proliferative responses to anti CD3 mAb (mean cpm=58293±771) compared to unstimulated PBLs (mean cpm=348±8). However, in the presence of various concentrations of GSI-X (15μM, 10μM, 5μM and 2.5 μM) the proliferative responses of $CD3^+$ T cells were significantly reduced in a concentration dependent manner (445.6± 147, 13525.7±7630, 37409.3±5116.3 and 44597±772 respectively (Figure 16B), with maximum decrease in proliferation of $CD3^+$ T cells observed at 15 μM concentration of GSI-X.

Purified γδ T cells were stimulated with BrHPP and rIL2 in the presence and absence of GSI-X. As shown in Figure 17A, an increased proliferation of γδ T cells was observed when γδ T cells were cultured with BRHPP+rIL2 compared to the γδ T cells stimulated with rIL-2 alone (mean cpm γδ+rIL2+BrHPP=4771±296.6 versus mean cpm γδ+rIL2=1561±99.9, $p<0.0005$). Treatment with GSI-X decreased the proliferation of antigen treated γδ T cells (Figure 17A). The maximum inhibition of BrHPP observed with 15μM GSI-X (76.9% inhibition). Lowering the concentration of GSI-X from 10μM to 0.5μM showed a reversal of inhibition (% inhibition of 25.4%, 11.6% and 9% for 10μm, 5μM and 0.5μM concentration of GSI-X respectively).

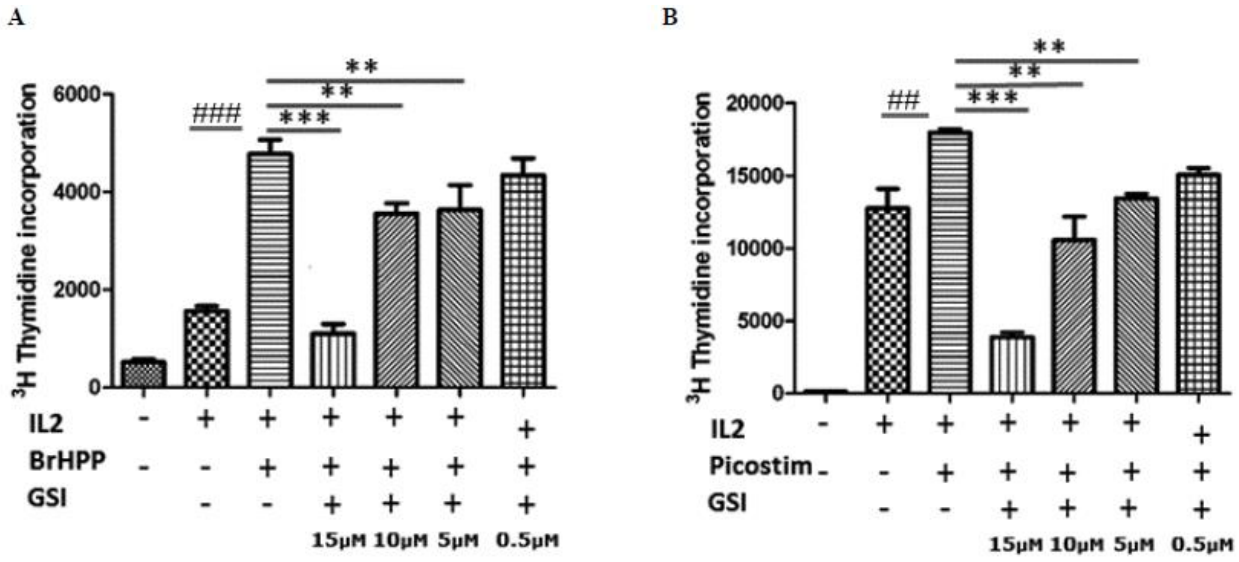

Figure 17A and B: Inhibition of antigen (BrHPP and picostim) driven proliferative response by GSI-X in a concentration dependent manner. γδ T cells ($5X10^4$ cells) were cultured in only medium or stimulated with rIL2 alone or along with the antigen in the presence or absence of GSI-X. Proliferation was measured by [^{3}H]TdR incorporation after 72 h. Values are measured as mean cpm (n=3). (** $p<0.005$, *** $p<0.0005$, ##$p<0.005$, ###$p<0.0005$).

Similarly, the involvement of Notch signal in picostim activated γδ T cell was studied (Figure 17B). An increased proliferation was observed when γδ T cells were cultured with picostim+rIL2 compared to the γδ T cells stimulated with rIL-2 alone (mean cpm γδ+rIL2+picostim=17938±269 versus mean cpm γδ+rIL2=12744±1355, $p<0.005$) (Figure 17B). As shown in Figure 17B, inhibition of notch signaling by GSI-X leads to reduction in antigen (picostim) driven proliferation of γδ T cells in a concentration dependent manner (% inhibition of 78.4%, 41.2%, 25.3% and 16.1% for 15μM, 10μm, 5μM and 0.5μM concentration of GSI-X respectively).

Next, the proliferative responses of ex-vivo expanded and freshly isolated γδ T cells to BrHPP, IPP and picostim in the presence of rIL2 and GSI were compared. As shown in Figure 18A, purified ex-vivo expanded γδ T cells were stimulated with rIL2 alone or along with three different phosphoantigens (BrHPP, IPP and picostim). Addition of the phosphoantigens (mean cpm γδ+rIL2+BrHPP=10302±266; γδ+rIL2+IPP=10246±307, γδ+rIL2+picostim=9953±258) induced increased propliferation of γδ T cells as compared to the γδ T cells stimulated with rIL-2 alone (mean cpm γδ+rIL2=7560±119, $p<0.05$). Treatment with GSI-X significantly reduced the proliferation of rIL2 stimulated γδ T cells (mean cpm γδ+rIL2+GSI-X=2426±67, $p<0.005$) as well as corresponding antigens treated γδ T cells (mean cpm γδ+rIL2+BRHPP+GSI-X=1090±48, $p<0.0005$; γδ+rIL2+IPP+GSI=2263±405, $p<0.0005$; γδ+rIL2+picosim+GSI=1091± 35.5, $p<0.0005$ (Figure 18A).The effect of GSI-X on phosphoantigen activated freshly isolated γδ T cells was ascertained. As shown in Figure 18B, freshly isolated γδ T cells showed robust proliferative responses (compared to ex vivo expanded γδ T cells) to BrHPP, IPP and picostim antigens over the base line i.e., γδ T cells stimulated with rIL-2 alone (mean cpm γδ+rIL2+BrHPP=38410±2766γδ+rIL2+IPP=43439±865, γδ+rIL2+picostim=37820±1297 versus γδ+rIL2=9564±992.3, $p<0.005$). In the presence of GSI-X the proliferative responses of γδ T cells

to rIL2 alone or along with phosphoantigens (BrHPP, IPP or picostim) were significantly reduced in freshly isolated γδ T cells (mean cpm γδ+rIL2+GSI-X=3279±130.6, $p<0.005$; γδ+rIL2+BrHPP+GSI-X=8637±7.8, $p<0.005$; γδ+rIL2+IPP+GSI-X=7065±165.5, $p<0.0005$; γδ+rIL2+picostim+GSI-X=12820±1450, $p<0.005$) (Figure 18B).

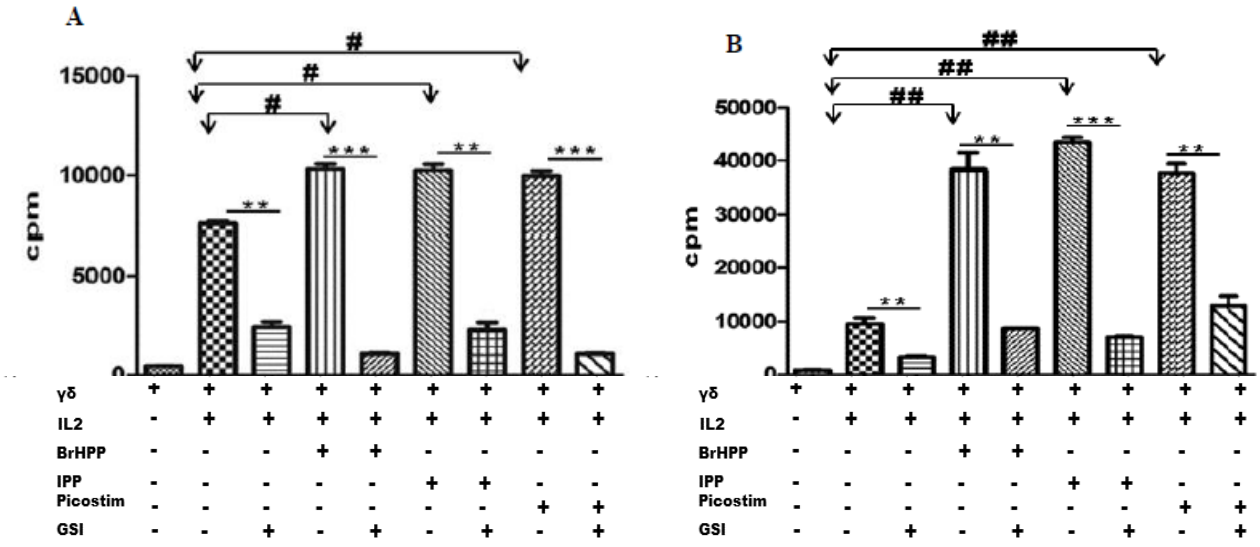

Figure 18A and B: Inhibition of antigens (BrHPP, IPP and picostim) driven prolifeartive response. Treatment with GSI-X significantly reduces the antigens (BrHPP, IPP and picostim) driven proliferative response of ex-vivo expanded γδ T cells (Figure18A) and freshly isolated γδ T cells (Figure 18B). γδ T cells ($5X10^4$ cells) were cultured in only medium or stimulated with rIL2 alone or along with the antigen in the presence or absence of GSI-X. Proliferation was measured by [^{3}H]TdR incorporation after 72 h. Values are measured as mean cpm (n=3). (** $p<0.005$, *** $p<0.0005$, # $p<0.05$, ## $p<0.005$).

Inhibition of Notch signaling pathway by GSI does not induce apoptotic or necrotic cell death

In order to confirm that inhibition of antigen specific proliferative response of γδ T cells in the presence of GSI-X is not due to loss of cells because of apoptosis or necrosis, apoptotic and necrotic cells were monitored in unstimulated, antigen stimulated γδ T cells and γδ T cells

stimulated with rIL-2 alone in the presence and absence of GSI-X. The effects of GSI-X on the frequency of apoptotic cells in unstimulated γδ T cells and after stimulation with IL2 alone or with both IL2 and BrHPP for 24 hrs was compared. The percentages of γδ T cells in early apoptotic (annexin V-positive, propidium iodide-negative), late apoptotic (annexin V-positive propidium iodide-positive) and necrotic (propidium iodide-positive, annexin V-negative) stage remained comparable in the absence (A, B and C) and presence (D, E and F) of GSI-X (Figure 19).

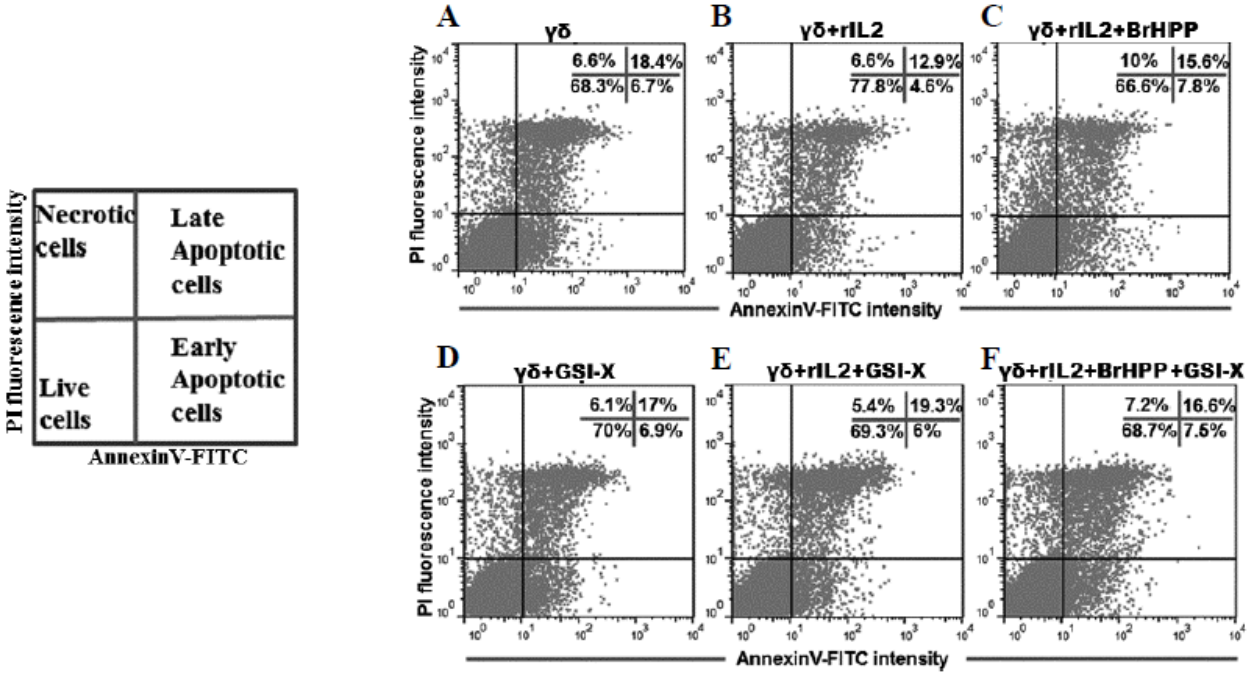

FIGURE 19: Inhibition of Notch signaling pathway by GSI does not induce apoptotic or necrotic cell death. Annexin V and PI staining of unstimulated γδ T cells (γδ), rIL2 activated γδ T cells (γδ+IL2) and γδ T cells stimulated with BrHPP in the presence of rIL2 (γδ+IL2+BrHPP) without GSI (*upper panel*) or with GSI (*lower panel*). These cells were stained after culturing for 24 h and analyzed by flow cytometry. The unaffected, early apoptotic, late apoptotic, and necrotic cells are present in the lower left, lower right, upper right, and upper left quadrant, respectively. Dot plot show the mean percentage of positive cells. Results shown are representative of three independent experiments.

Expression of activation markers is regulated by Notch signaling

Multiple proteins are known to be upregulated on the surface of lymphocytes during immune activation process. The most common of these include CD25 and CD69, the early and late activation markers respectively. The expression of these markers were analysed on unstimulated γδ T cells stimulated with BrHPP and IL-2 with or without GSI-X treatment. Ex-vivo expanded γδ T cells stimulated with BrHPP and rIL2 showed higher expression of CD25 compared to CD69. Treatment with GSI-X showed a moderate reduction in CD69 expression. However, a marked reduction in the expression of late activation marker-CD25 was observed on antigen stimulated γδ T cells (Figure 20). γδ T cells are known to express high affinity IL-2R and it was observed IL2Rα (CD25) expression is regulated by Notch signaling in γδ T cells.

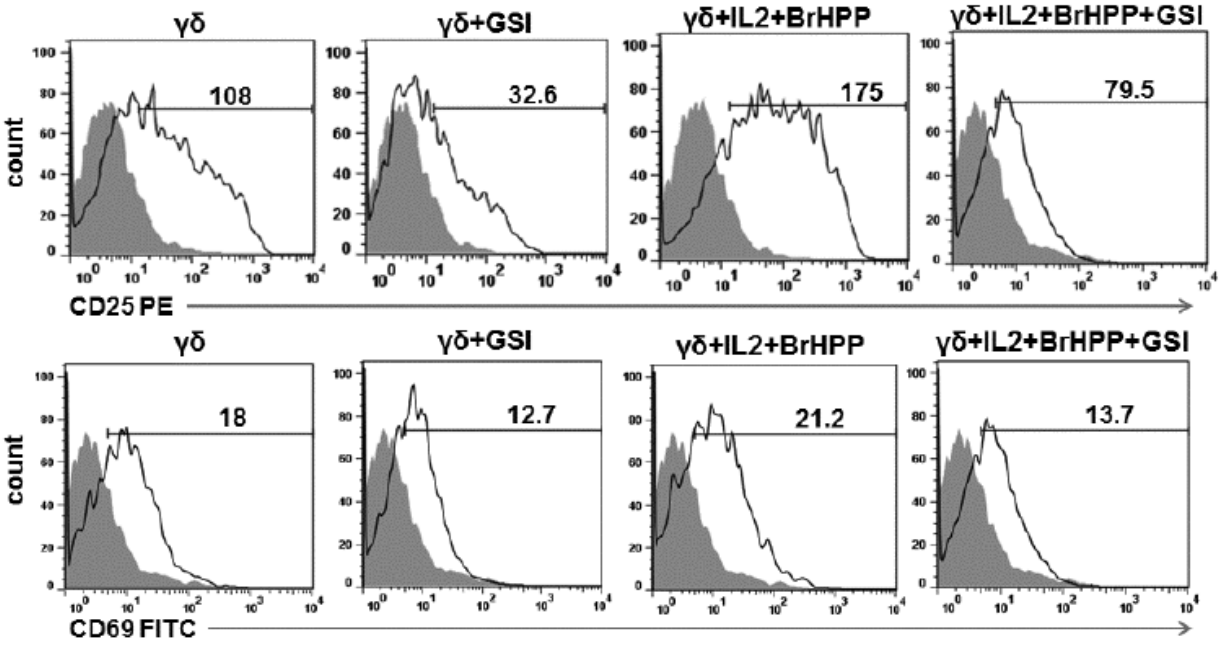

FIGURE 20: The effect of GSI on cell surface expression of activation markers. Blocking of Notch signaling by GSI-X inhibits the surface expression of activation markers. The effect of GSI on cell surface expression of late (CD25) and early (CD69) activation markers on unstimulated

and BrHPP stimulated (24 h) γδ T cells was analyzed by flow cytometry. Blocking of Notch signaling by GSI-X inhibits the surface expression of activation markers. Data indicates the MFI of the activation markers. Dark shaded histogram indicates isotype control.

Notch regulates cytolytic effector function of γδ T lymphocytes

During degranulation of cytotoxic T cells, CD107a (lysosome associated membrane protein-1) is expressed on the extracellular cell membrane of the lymphocytes. CD107a expression was examined on γδ T cells cocultured with oral squamous carcinoma cell line, AW13516 cells.. Before co-culture the AW13516 were pretreated with zoledronate (100μM) for 18h in order to increase their susceptibility to γδ T cells mediated killing (Dhar & Chiplunkar, 2010). Purified γδ T cells were co-incubated with zoledronnate treated AW13516 (oral tumor cells) at a ratio of 4:1.

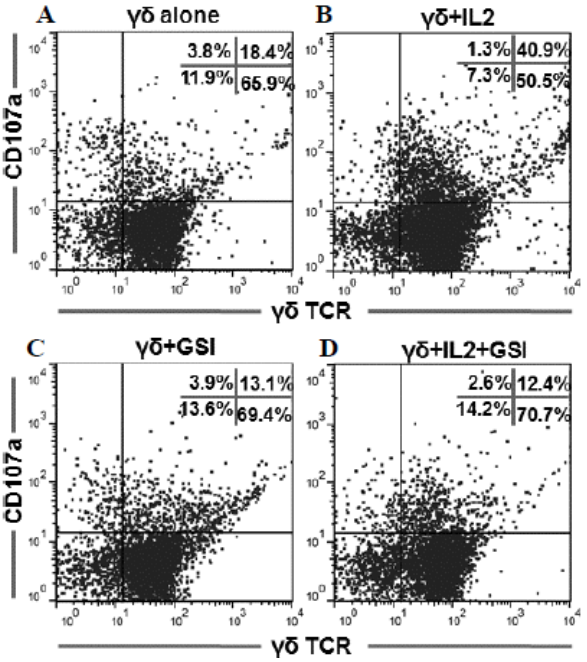

FIGURE 21: Expression of CD107a or LAMP1 (marker for degranulation) on γδ T cells. Effector γδ T cells alone or in the presence of rIL2 were co-cultured with target oral cancer cell line (AW13516) at the ratio of 4:1 and incubated for 4 h. AW13516 were previously treated with zoledronate (100μM) for 16 h. Decreased expression of CD107a was observed when the cells were treated with GSI (*lower panel*). Figure is representative of three experiments.

After 4h the cells were stained with FITC labeled γδ TCR and CD107a PE labeled antibodies. Upon co-incubation with the zoledronate treated oral cancer cells (AW13516), few γδ T cells showed surface expression of CD107a (18.4%, Figure 21A). The proportion of CD107a+ γδ T

cells increased in the presence of rIL2 (40.9%, Figure 21B). However, in the presence of GSI, the expression of CD107a+ was reduced in γδ T cells co-cultured with zoledronate treated AW13516 (13.1%, Figure 21C) and also in γδ T cells cultured with zoledronate treated oral tumor cells in the presence of rIL2 (12.4%, Figure 21D).

In order to investigate if Notch signaling is involved in the anti tumor cytotoxic effector function of γδ T cells, ^{51}Cr labelled zoledronate treated oral tumor cells, AW13516 (target cells; T) were incubated with γδ T cells (effector cells; E) at E:T ratio of 40:1, 30:1, 20:1, 10:1 and 5:1.^{51}Cr-release was determined after 4h of coculture and percent cytotoxicity was determined as described in materials and methods. A dose response in percent cytotoxicity was observed at E:T ratio ranging from 40:1 to 5:1 (Figure 22A). The effect of GSI on cytotoxic potential of γδ T cells against zoledronate treated tumor targets was investigated at E:T ratio of 40:1.

As shown in Figure 22B, the mean percent cytotoxicity (mean cytotoxicity=24.3±6%) γδ T cells against zoledronate treated tumor targets.was significantly reduced in the presence of GSI-X (mean cytotoxicity=5.5±2%, $p<0.05$) The cytotoxic effect of γδ T cells against zoledronate treated tumor targets was more pronounced in the presence of rIL2 (mean cytotoxicity=38±8%) at E:T of 40:1. Addition of GSI-X significantly reduced percent cytotoxicity of γδ T cells against zoledronate treated tumor targets (mean cytotoxicity=5.4±2%), $p<0.05$) (Figure 22B). Collectively the data show that the cytotoxic potential of γδ T cells against tumors is regulated by Notch signal.

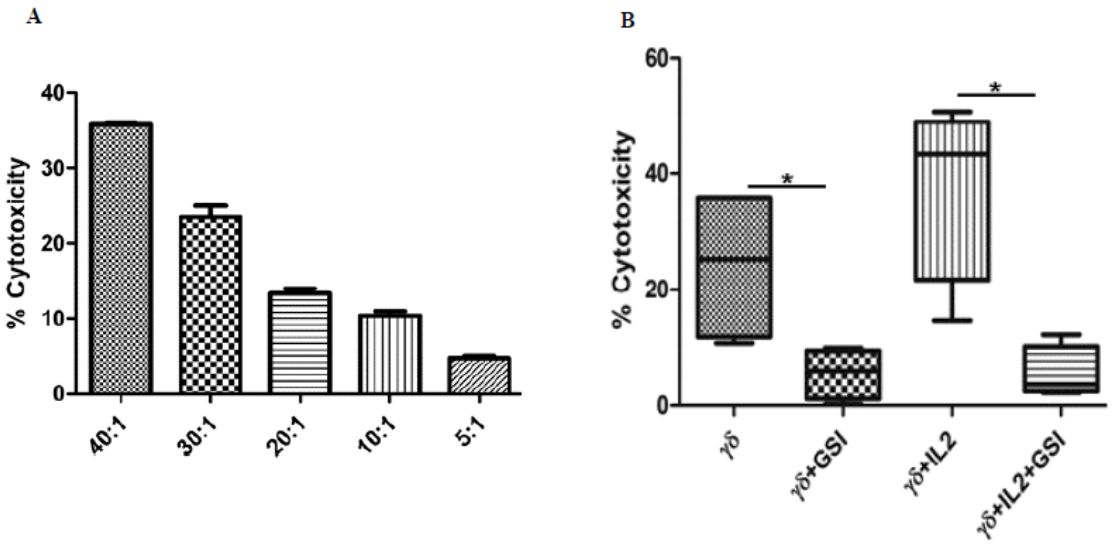

FIGURE 22: Involvement of Notch signaling in regulating cytolytic effector functions of γδ T cells. A. Zoledronate treated AW13516 cells labelled with ^{51}Cr were used as target cells and were co-cultured with γδ T cells at different E:T ratios in a standard 4 h ^{51}Cr release assay. Data represents % cytotoxicity as described in materials and methods. Representative data of an independent experiment. **B.** Cytolytic ability of γδ T cells alone or in the presence of rIL2 against zoledronate treated AW13516 was determined by ^{51}Cr release assay at the ratio of 40:1. Treatment of γδ T cells with GSI blocked the target cell lysis. *, $p<0.05$ compared with % cytotoxicity of both γδ T cells and γδ T cells with rIL2 without GSI-X. The results are mean % cytotoxicity of γδ T cells from five healthy individuals.

Further, the contribution of the Notch pathway in regulating the cytotoxic potential γδ T cells against tumor targets was assessed using small interfering RNA-mediated knockdown of individual Notch receptors.. The synthetic small interfering RNA (siRNA) sequences targeting the Notch1 and Notch2 receptors were transfected into γδ T cells .In parallel, γδ T cells were also transfected with 50nM control (SiGLO) or different Notch specific siRNA duplexes. After 48 hours cells were harvested and lysates were prepared. Western blot analysis for the presence of Notch1 and Notch2 showed that γδ T cells transfected with SiGLO (control) showed the presence of N1ICD and N2ICD (120 kDa) protein in the cell lysates as analysed by western blotting.

siRNA transfection led to the reduced expression of Notch1 (N1ICD) and Notch2 (N2ICD) receptors (120kDa) compared to fluorescent oligonucleotide SiGLO (Figure 23A). Next, antitumor cytotoxic ability of γδ T cells transfected with siRNA specific for Notch1 and Notch2 against zoledronate treated tumor cells (AW13516) was compared. Maximum cytotoxicity of γδ T cells against zoledronate treated AW13516 oral tumor cells was observed at E:T ratio of 40:1(mean 38%) (Figure 23B), silencing of both Notch1 and Notch2 receptors using SiRNA led to significant reduction in cytotoxicity of γδ T cells against AW13516 oral tumor cells (Figure 23B).Transfection of γδ T cells with SiGLO showed cytotoxicity that was comparable to untransfected γδ T cells. This finding confirms that Notch signal regulates cytotoxic potential of γδ T cells.

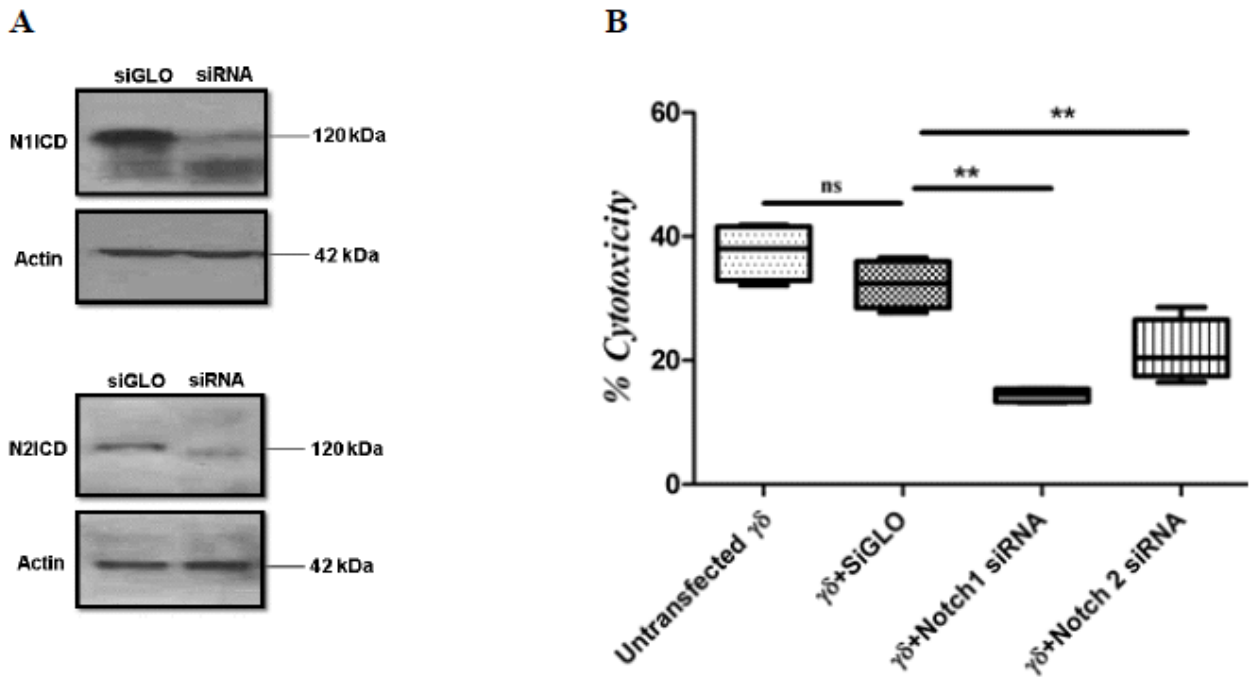

FIGURE 23. Notch signaling regulates cytolytic effector functions of γδ T cells. γδ cells were transfected with 50nM control (SiGLO) or different Notch specific SiRNA duplexes. 48 hours posttransfection, cells were harvested, and samples were subjected to western blot analysis for Notch1 and Notch2 (top panel and bottom panel respectively). β-Actin was included as

loading/normalization control. **B.** γδ T cells were transfected with 50 nM control (SiGLO) or specific Notch1 and Notch2 siRNA duplexes, Forty-eight hours posttransfection, the cytolytic effector function of rIL2 activated γδ T cells against zoledronate treated AW13516 was determined by ^{51}Cr release assay at the ratio of 40:1. Silencing of either Notch1 or Notch2 inhibits target cell lysis by γδ T cells. **, $p<0.005$ compared with % cytotoxicity of Notch-specific siRNA with SiGLO treated γδ T cells. The results are mean % cytotoxicity of γδ T cells from five healthy individuals.

Notch signaling regulates cytokine production by activated γδ T cells

To address whether Notch signal plays a role in the effector functions of activated γδ T cells, the effect of GSI-X on cytokine production of activated γδ T cells was examined using cytometric bead array. It is well established that activated γδ T cells release copious amounts of IFN-γ and TNF-α in response to stimulation with non-peptidic antigens [121, 122]. γδ T cells are a potent source of IL17 [275, 276]. The consequence of GSI-X treatment of BrHPP stimulated γδ T cells t on the secretion of TH1 (IL2, IFN-γ and TNF-α), TH2 (IL4, IL6 and IL10) and TH17 (IL17) production in the presence and absence of rIL2 was monitored. As shown in Figure 24A and C γδ T cells stimulated in the presence of BrHPP showed higher level of IFN-γ, TNF-α and IL17 compared to γδ T cells stimulated with rIL2 alone (control). BrHPP stimulated γδ T cells did not produce IL4, IL6 and IL10 (Figure 24B). A low level of IL2 was secreted by BrHPP activated γδ T cells. Addition of GSI-X markedly reduced IFN-γ, TNF-α and IL17 production by BRHPP+rIL2 stimulated γδ T cells and in γδ T cells stimulated wirh rIL2 alone (Figure 24). Among all these cytokines analyzed addition of GSI dominantly inhibited IFN-γ production of γδ T cells.

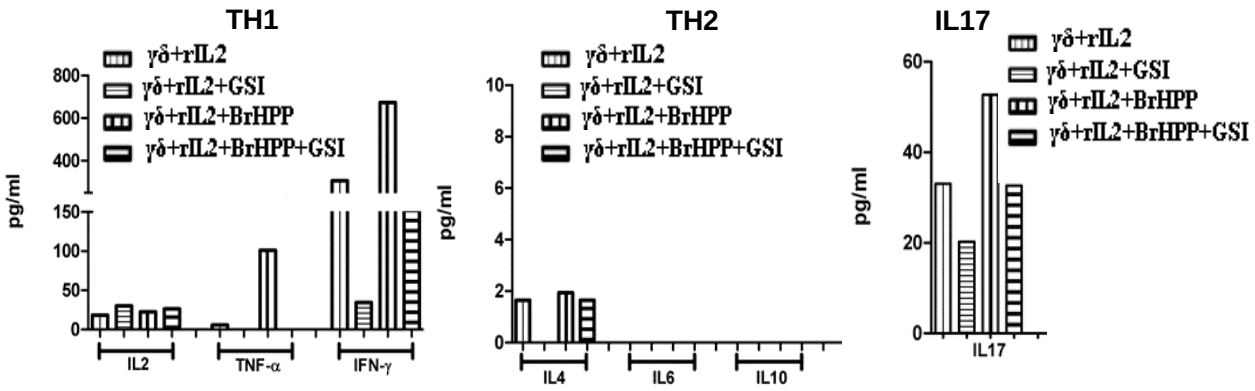

FIGURE 24: Effect of GSI-X on the secretion of different cytokines. Concentrations of different cytokine proteins (TH1, TH2 and TH17) were determined in the culture supernatant of γδ T cells stimulated with rIL2 alone or with BrHPP in the presence of rIL2 by Cytometric Bead Array. γδ T cells ($5X10^4$ cells) were cultured with rIL2 alone or along with BrHPP in the presence or absence of GSI-X for 24 h. Culture supernatants were collected after 24 h and level of secreted cytokines was measured by CBA. Addition of GSI leads to marked reduction in TNF-α, IFN-γ and IL17 production by activated γδ T cells. Results shown are representative of three independent studies.

Next, the effect of GSI-X on IFN-γ production of γδ T cells stimulated with all the three phosphoantiges (BrHPP, IPP and picostim) was also examined by ELISA. γδ T cells were stimulated with BrHPP, IPP and picostim in the presence and absence of GSI-X and after 24h of incubation, cell-free supernatants were collected and analyzed by ELISA. γδ T cells stimulated with BrHPP ($p<0.05$), IPP ($p<0.05$) and picostim ($p<0.005$) released significantly increased amounts of IFN-γ compared to γδ T cells stimulated with rIL2 alone. Addition of GSI-X significantly inhibited IFN-γ production of γδ T cells stimulated with rIL2 alone ($p<0.005$) and γδ T cells stimulated with BrHPP, IPP and picostim and rIL-2 ($p<0.05$) (Figure 25A). Further the effect of different concentration of GSI-X (15 µM, 10 µM, 5 µM, 2.5 µM and 1.3 µM) on IFN-γ production by anti CD3 activated γδ T cells was compared. Ex-vivo expanded γδ T cells were stimulated with anti CD3 alone or along with rIL2 in the presence and absence of above

mentioned concentrations of GSI-X. After 24h of incubation, cell-free supernatants were collected and analyzed by ELISA. γδ T cells activated with anti CD3 alone or along with rIL2 showed significantly increased IFN-γ production ($p<0.005$). However, treatment with GSI-X leads to significant decrease in the level of IFN-γ production by anti CD3 activated γδ T cells (Figure 25B). Thus, a role of Notch in regulating IFN-γ production in γδ T cells is unconcealed which unequivocally establishes the role of Notch in regulating the effector functions of γδ T cells.

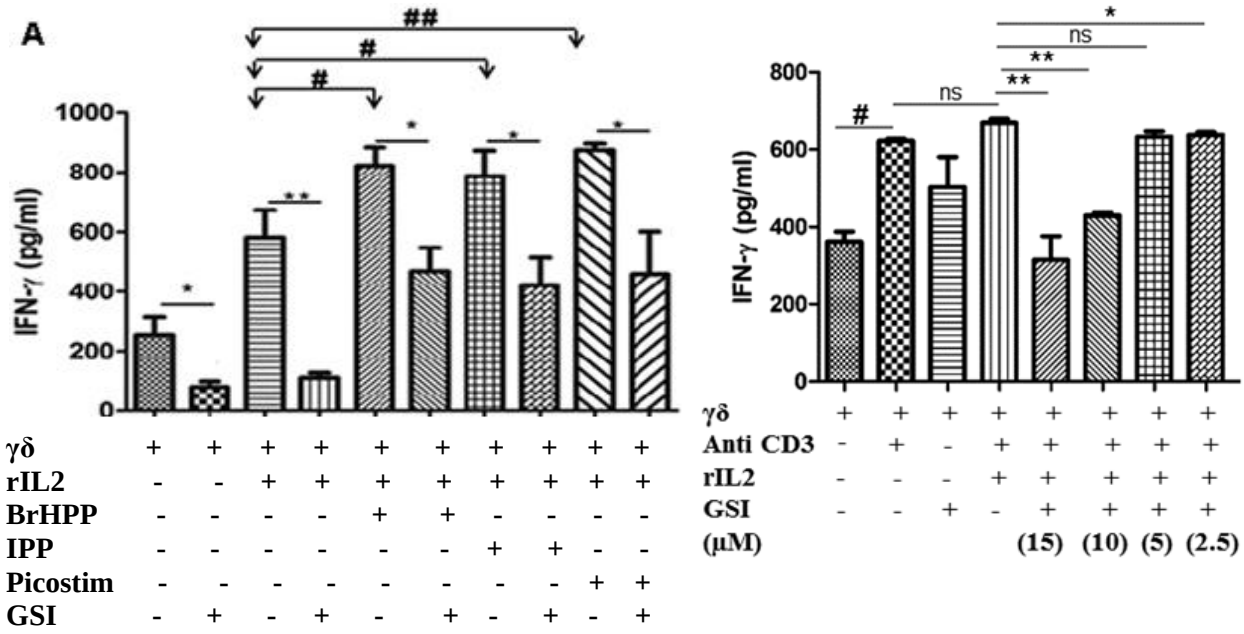

FIGURE 25. Treatment with GSI blocks IFN-γ production by γδ T cells. A. γδ T cells alone, γδ T cells with rIL2 or along with three different (BrHPP, IPP and picostim) were cultured in the presence or absence of GSI-X (15 μM) for 24h in 96 well plate. **B.** γδ T cells were either cultured alone, or in the presence of anti CD3 (mAb) or with rIl2 and in the presence or absence of different concentration of GSI (15 μM, 10 μM, 5 μM and 2.5 μM) for 24h in 96 well plate. Supernatants were collected and level of IFN-γ was measured by sandwich ELISA. GSI treatment decreases IFN-γ production by both unstimulated and stimulated γδ T cells. Results shown are mean of three experiments. *$p<0.05$, **$p<0.005$ compared with IFN-γ production of the cells without GSI-X treatment and #$p<0.05$, ##$p<0.005$ compared IFN-γ of cells treated with rIL2 alone.

CHAPTER 6:

Activation of γδ T cells by Notch ligands

A cell in which Notch signaling has been activated by one or more of its several ligands will have a different fate compared to a cell in which Notch activation is absent. Notch ligands Delta and Jagged are known to transmit distinct signals to T cell precursor. In the absence of Notch signals, a subset of progenitors found within the DN1 population differentiate into rapidly proliferating B-lineage cells, whereas potent Dll1 signals promote T-cell maturation. However, Jag1 signals favor NK cell development by not allowing the development of B-lineage precursors and preventing T-cell maturation [277]. Activation of Notch by DLL1 and DLL4 results in increased human γδ T cell development at the cost of TCR-αβ lineage cells [84]. It has been reported that overexpression of Jag1 induces allogen specific regulatory T cells [278].

A role of Notch in regulating cross talk between APCs and αβ T cells has also been studied. It was observed that a stimulus that normally induces a Th1 response, such as lipopolysaccharide (LPS), results in upregulation of Dll4 on APCs. In contrast, type2 stimuli such as cholera toxin induced Jag2 expression [86]. Antigenic stimulation of naive $CD4^+$ T cells in the context of APCs engineered to express Dll-1 led to the secretion of Th1 cytokines, whereas Jagged1 promoted a Th2 cytokine profile. Studies on Tregs reported that these cells predominantly express Jag1 ligand and, to a lesser extent, Dll4 on their cell surface [94].

In this chapter, the influence of Notch ligands in regulating γδ T cells function has been addressed. Furthermore, the cross-talk between γδ T cells and Tregs via Notch signal was studied; and finally the expression of Notch receptor isoforms and its ligands in oral and breast cancer cell lines were examined.

Effect of Notch ligands in regulating γδ T cells proliferation

To verify whether ligation of Notch receptor has any effect on the response of γδ T cells to phosphoantigens, the effect of rDll1, rDll4 and rJag1 were assessed by ^{3}H-TdR incorporation assay as described in materials and methods.

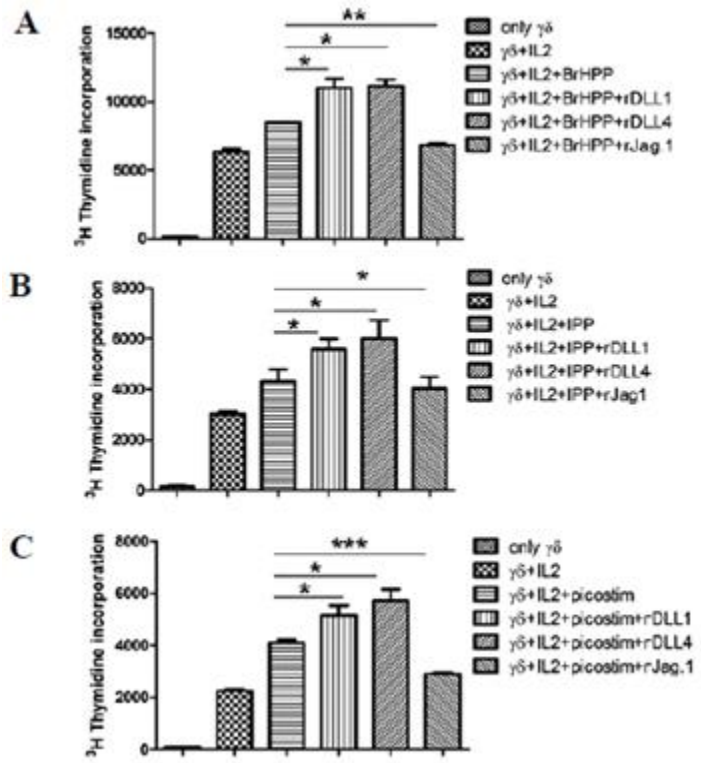

FIGURE 26. Effect of Notch ligands in antigen driven proliferation of γδ T cells. rDll1 and rDll4 ligands increases antigen specific (BrHPP, IPP & picostim) proliferation of γδ T cells, whereas rJag1 ligand inhibits the proliferative response. γδ T cells ($5X10^{4}$ cells) were cultured and proliferation of the antigen stimulated γδ T cells was compared to the rligand+antigen treated cells. Proliferation was measured by [^{3}H]TdR incorporation after 72 h. Values are measured as mean cpm. (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$).

As shown in Figure 26A, γδ T cells stimulated with BrHPP+rIL2 showed increased proliferation (7391±533) which was significantly amplified upon ligation of the cells with rDll1 (10144±679, $p<0.05$) and rDll4 (11569±827, $p<0.05$). However, a decrease in the BrHPP driven proliferation of γδ T cells upon ligation with rJag1 (6830±448, $p<0.005$) was observed. .The decrease was significant compared to that observed with γδ T cells stimulated with BrHPP + rIL2 which served as the control (7391±533). Similarly, stimulation of γδ T cells with IPP increases the proliferation

of γδ T cells (4301±482). Treatment with rDll1 and rDll4 augmented the proliferative response to 5589±397 and 5999±730 ($p<0.05$) respectively but ligation of rJag1 decreased the proliferative response to 4032±432 ($p<0.005$) compared to control (Figure 26B**)**. Likewise, as shown in Figure 26C, stimulation with rDll1 and rDll4 increased the proliferative response of picostim treated γδ T cells to 5157±372 ($p<0.05$) and 5732±419 ($p<0.05$) respectively as compared to picostim+rIL2 treated γδ T cells (4088±125). Also, rJag1 treatment leads to decreased in proliferation of picostim treated γδ T cells (2898±55, $p<0.0005$) compared to control (Figure 26C).

Similarly, the involvements of Jag ligand in anti CD3 activated γδ T cells were also studied. Treatment with rJag1 leads to significant inhibition of anti CD3 driven proliferation of γδ T cells in a concentration dependent manner (14843±2624, $p<0.05$; 13003±62.5, $p<0.005$; and 12132±1246, $p<0.005$ for 0.25 µg, 0.5 µg and 1 µg of rJag1 respectively) as compared to anti CD3+rIL2 treated cells (220182±1256) which are the controls (Figure 27).

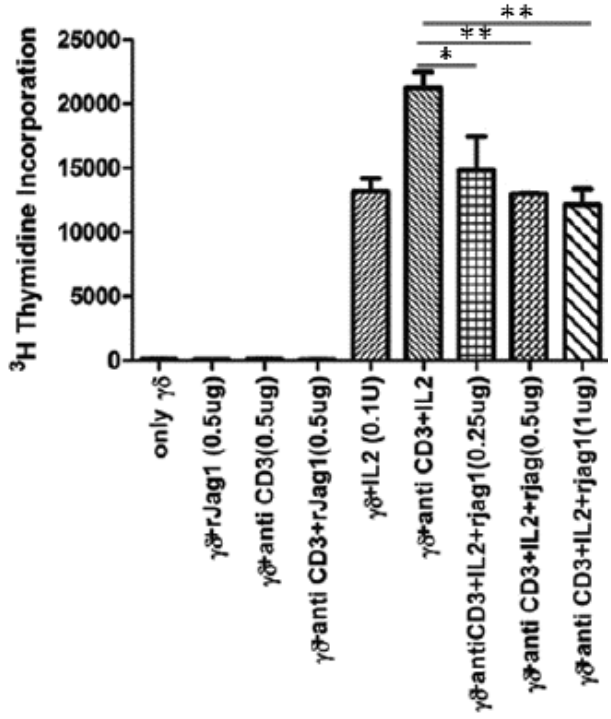

FIGURE 27. Ligation of rJag1 significantly reduces the anti-CD3 driven proliferative response of ex-vivo expanded γδ T cells. E*x-vivo* expanded γδ T cells ($5X10^4$ cells) were stimulated with anti CD3 (mAb) in the presence of rIL2, for 72h, with or without GSI-X treatment. Addition of rJag1 leads to decrease in the proliferative response of γδ T cells in a concentration dependent manner (0.25 µg, 0.5 µg and 1 µg). Proliferation was measured by [^{3}H]TdR incorporation after 72 h. Values are measured as mean cpm (n=3). *, $p<0.05$ and **, $p<0.005$) .

Stimulation with Notch ligands induces IFN-γ production by γδ T cells

IFN-γ produced by γδ T cells plays a crucial role in protective immune response against certain pathogens and tumors [9, 279, 280]. The effect of notch ligands on IFN-γ production by γδ T cells was studied. It was observed that rDLL1 and rDLL4 increases the production of IFN-γ whereas treatment with rJag1 leads to significant decrease in the secretion of IFN-γ by γδ T cells ($p<0.0005$) (Figure 28).

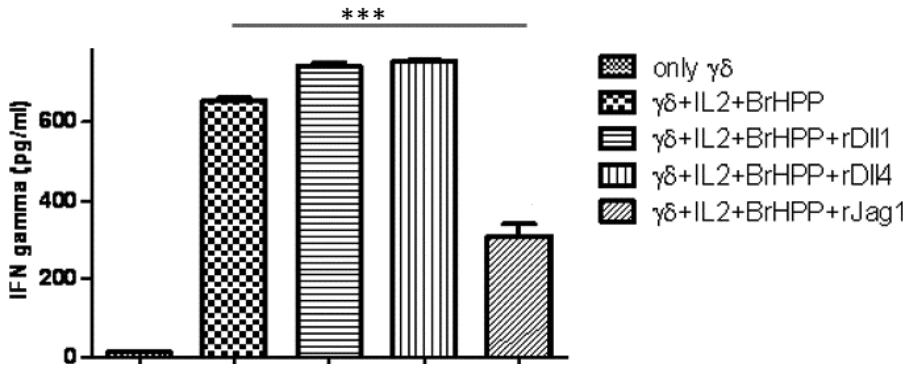

FIGURE 28. Effect of Notch ligands on the secretion of IFN-γ by γδ T cells. rDLL1 and rDLL4 ligands increases BrHPP stimulaed IFN-γ secretion by γδ T cells, whereas rJag1 ligand inhibits the secretion. γδ T cells ($5X10^4$ cells) were cultured and proliferation of the antigen stimulated γδ T cells was compared to the rligand+BrHPP treated cells. IFN-γ production by γδ T cells was determined by ELISA *** $p<0.0005$ of rJag1 treated cells compared with BrHPP and rIL2 stimulated cells without any ligands.

Similarly, the effect of Jag1 ligand on anti CD3 stimulated proliferation of γδ T cells was studied. As shown in Figure 29, in a concentration dependent manner (0.25ug, 0.5ug and 1ug) , ligation of Jag1 on γδ T cells leads to significantly decreased proliferation and IFN-γ production compared to the respective control cells (γδ+anti CD3+ rIL2).

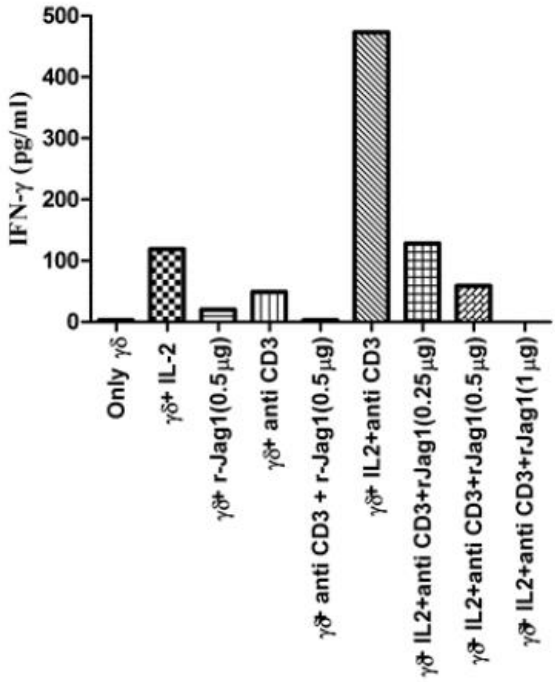

FIGURE 29. Treatment with rJag1 prevents IFN-γ production by anti CD3 stimulated γδ T cells in a concentration dependent manner. γδ T cells ($5X10^4$ cells) were cultured and IFN-γ production of the anti CD3 stimulated γδ T cells was compared to the rJag1+anti CD3 treated cells. Addition of rJag1 leads to decrease in IFN-γ release by γδ T cells in a concentration dependent manner (0.25 µg, 0.5 µg and 1 µg). IFN-γ released was measured by sandwich ELISA after collecting supernatant at 24 h.

Isolation of Regulatory T cells (Tregs)

Regulatory T cells were isolated from PBLs of healthy individuals using BD Imag immunomagnetic separation kit. PBLs were first labeled with cocktail of biotinylated antibodies that recognizes antigens on erythrocytes, platelets and peripheral leukocytes that are not CD4 T lymphocytes (CD8, CD11b, CD16, CD19, CD36, CD41a, CD56, CD123, γδ TCR and Glycophorin A) and APC-labeled mouse anti-human CD25 antibody. Then, the $CD4^+$ T lymphocytes were enriched by negative selection (depletion of the non- $CD4^+$ cells) using the Streptavidin Particles. This is followed by a second immunomagnetic separation step and this time it was a positive selection. The anti-APC particles were used to select the $CD4^+$ cells, from among the enriched $CD4^+$ cells, which already had been labeled with the APC anti-CD25 mAb. Table-3 shows the % recovery of Tregs obtained after immunomagnetic separation from PBLs of eight healthy individuals. Treg cells are characterized by the expression of high $CD25^+$ and Foxp3 markers [200]. As shown in Figure 30, purified cells were positive for both $CD25^+$ and Foxp3

markers (Figure 30A represent dual $CD25^+$ PE and Foxp3 FITC dual positive, 99.9%); histograms showing Tregs positive for both $CD25^+$ and Foxp3 markers are represented (Figure 30B and C).

Table 3: Isolation of Tregs from PBL

Blood Drawn	Total PBL	% recovery of Tregs
30-50 ml	$87x10^6$	0.98%
	$66x10^6$	0.86%
	$81x10^6$	0.2%
	$64x10^6$	0.81%
	$50x10^6$	0.32%
	$81x10^6$	0.92%
	$56x10^6$	0.99%
	$42x10^6$	0.73%

Mean % recovery of Tregs =0.73±0.1

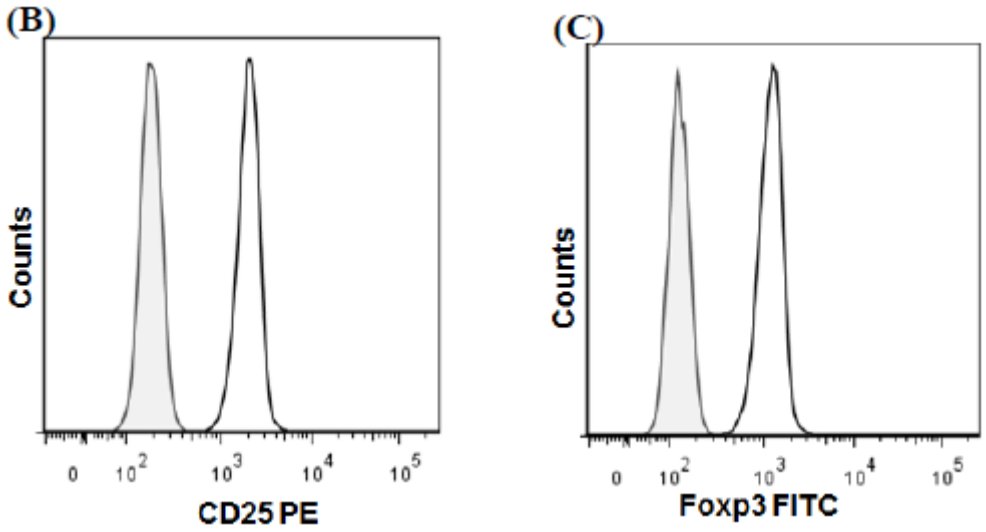

FIGURE 30. Immunomagnetically purified Tregs: Flow cytometric analysis of Tregs cells showed the expression of CD25 and Foxp3.

Cross-talk between γδ T cells and Tregs via Notch signal

$CD4^+CD25^+$ Tregs have been reported to express higher level of Jag1 ligand [94] and it was also demonstrated that overexpression of Jag1 in the alloantigen-presenting LCL induces Tregs [278]. However, as described above proliferation and IFNg production of γδ T cells is inhibited after stimulation with rJag1 ligand. In order to check whether Tregs with higher level of Jag1 ligand on the cell surface are able to inhibit γδ T cells, co-culture assay was set up. Freshly isolated γδ T cells were labeled with CFSE dye and then co-cultured with Tregs at 1:1 ratio for 5 days. Tregs cells were previously treated with either anti Jag1 antibody (to block Jag1 ligand) or Fc control antibody. Proliferation of γδ T cells was induced by adding rIL2 and BrHPP to the co-culture. As control, γδ T cells were cultured in the presence of rIL2 and BrHPP. As shown in Figure 31A, B and C. γδ T cells stimulated with rIL2 and BrHPP showed increased frequency of dividing cells (100%, 53.6% and 71.2% for three different individuals). The addition of Tregs in the presence of Fc control to the culture inhibited the antigen specific proliferation of γδ T cells as evident by decrease in frequency of dividing cells (55.5%, 44.2% and 63.2% respectively for the three different individuals analysed). However, blocking of Jag1 ligand on Tregs reduced the suppressive potential of Tregs on antigen specific proliferation γδ T cells. It was observed that anti Jag1 antibody rescued the suppressive potential of Tregs as manifested by increased numbers of dividing γδ T cells s (73.7%, 54% and 70.5% respectivelyafter BrHPP+rL2 stimulation compared cultures where untreared Tregs were added.

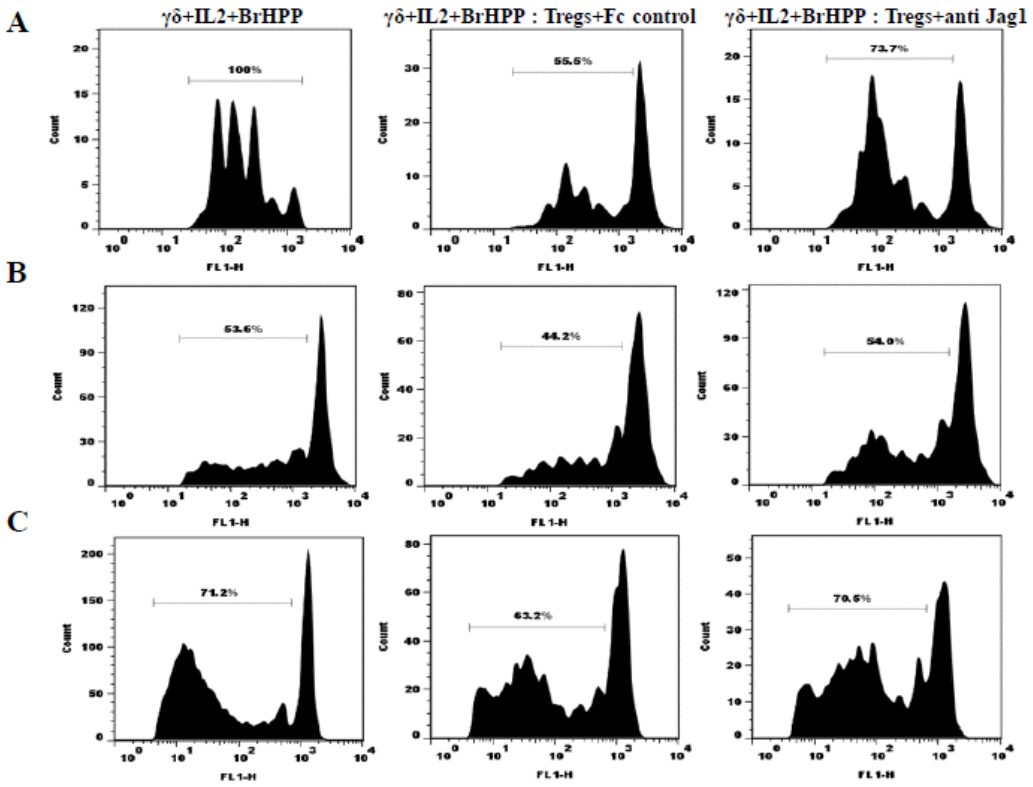

FIGURE 31. Blocking of jagged1 ligand on Tregs inhibits their suppressive effect on antigen driven proliferation of γδ T cells. γδ T cells were labeled with CFSE and were treated with rIL2 and BrHPP. The cells were cultured alone or co-cultured with Tregs (1:1 ratio). Tregs were pre-treated with anti Jag1 Ab or Fc control Ab for 2 h. Proliferation of CFSE labeled γδ T cells were measured on day 5.

Expression of Notch receptor isoforms and its ligands in oral cancer cells

The expression of Notch receptors and their ligands was examined in oral cancer cells (AW13516, AW8507) and surgically excised oral tumors from oral SCC patients. As seen in Figure 32, the expression of mRNA for Notch genes (Notch1-4) and its ligands Dll1, 3 and 4 and Jag1 and 2 were quantitated using real time PCR method in γδ T cells GAPDH was used as house keeping gene control. In AW13516 cell line, the expression of Notch2 and Jag1 genes was at a higher level. Lower expression of Notch1, 3, 4, Dll1 and Dll4 mRNA was also observed. The mRNA profile thus shows that Jag1 ligand is predominantly expressed by oral cancer cells.

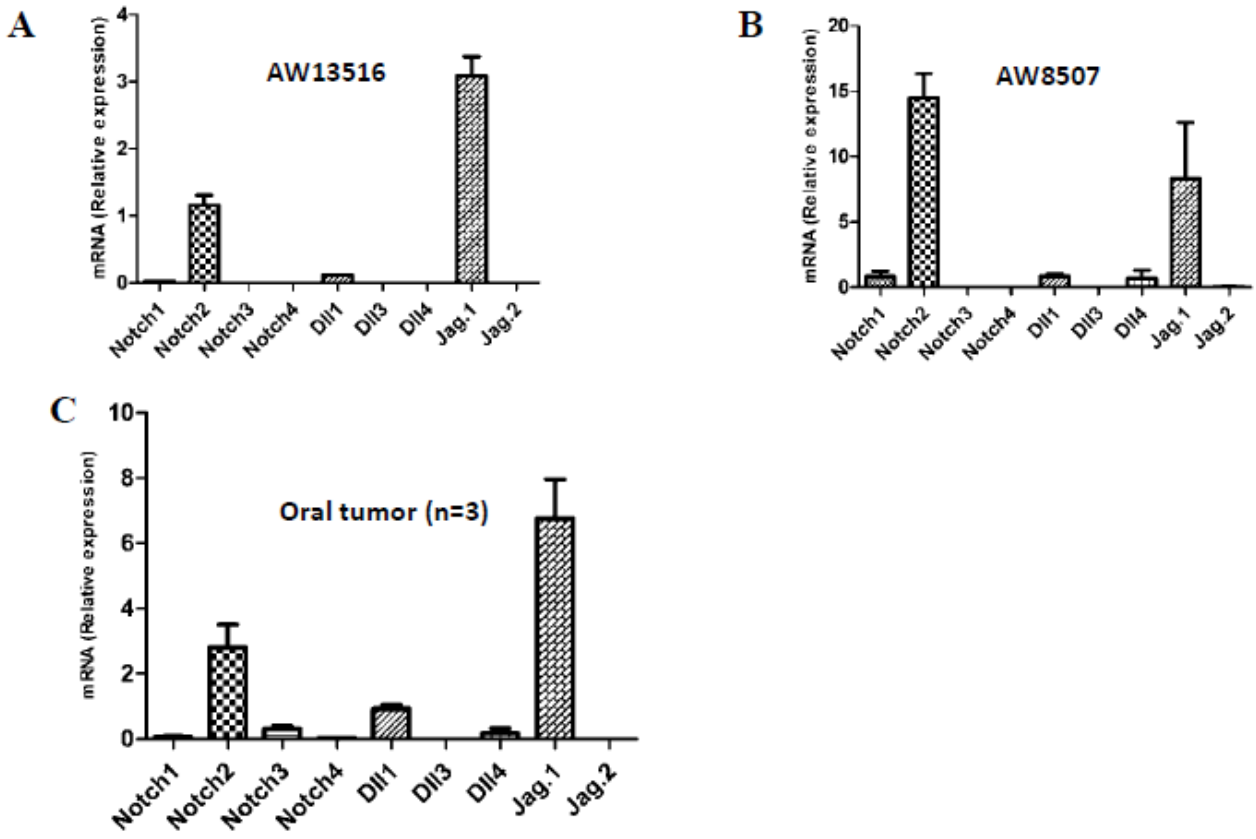

FIGURE 32. mRNA expression analysis of Notch and its ligands in AW13516, AW8507 cell lines and oral tumor by Real Time PCR. Total RNA was extracted from AW13516, AW8507 and surgically excised oral tumors (A, B and C respectively) .**A.** Real time quantitative PCR showed mRNA expression of Notch2 and Jag1 with lower level of Notch1 and Dll1on AW13516 cell line. **B.** AW8507 cell line showed mRNA expression of Notch2 and Jag1 with lower level of Notch1, Dll1 and Dll4. **C.** Surgically excised tumors also express higher level of Notch2 and Jag1 along with lower level of Notch1, Notch3, Notch4, Dll1 and Dll4.

The expression of Jag1 on oral cancer cells (AW13516, AW8507 and excised oral tumor) was then confirmed by immunoblotting experiment as shown in Figure 33A, B and C respectively. The expression of Jag1 at 180 KDa was observed without any detection of Dll1 (78 KDa).

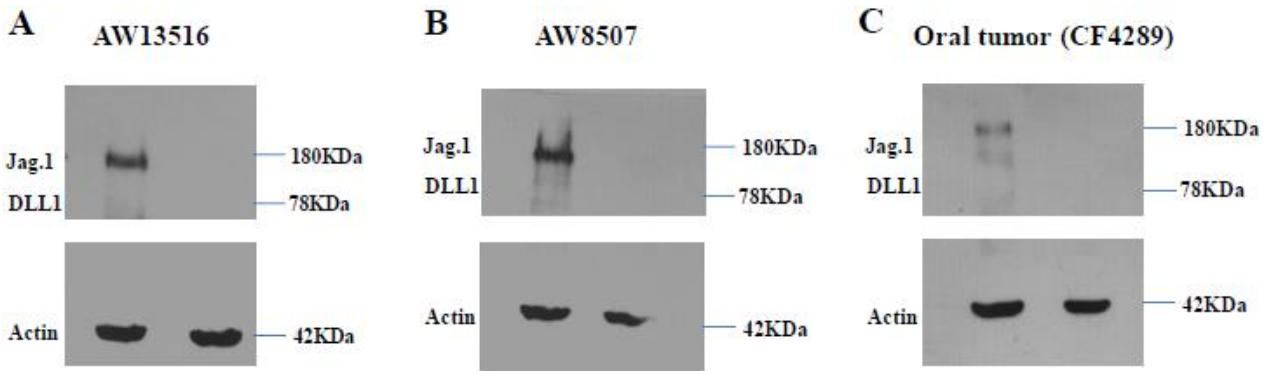

FIGURE 33. Immunoblotting of Jag1 and Dll1 in AW13516, AW8507 cell lines and oral tumor. Detection of 180 KDa Jag1 on AW13516, AW8507 and oral tumor (A, B and C respectively) using anti-Jag1 antibody. Dll1 was not deteceted in the oral cancer cell lines (AW13516 and Aw8507) and surgically excised oral tumor.

Expression of Notch receptor isoforms and its ligands in breast cancer cells

Similarly, the expression pattern of different Notch receptors and ligands in breast cancer cells was also examined. As shown in Figure 34A and B, Jag1 gene was predominantly expressed in both MCF-7 and MDA-MB cell lines. Lesser expression of mRNA for Notch2, Notch3 and Dll1 was observed in both the breast cancer cell lines. In surgically excised breast tumor, the dominant expression of, Notch2,Notch 3 anf Jag1 followed by relatively lower expression of Notch1, Dll4,Notch4, Dll1 and Jag2 genes was observed (Figure 34C).

Figure 34D confirms Jag1 expression on surgically excised breast tumor (CH6629) by immunoblotting. Hence similar to oral cancer cells, breast cancer cells also express Jag1 ligand.

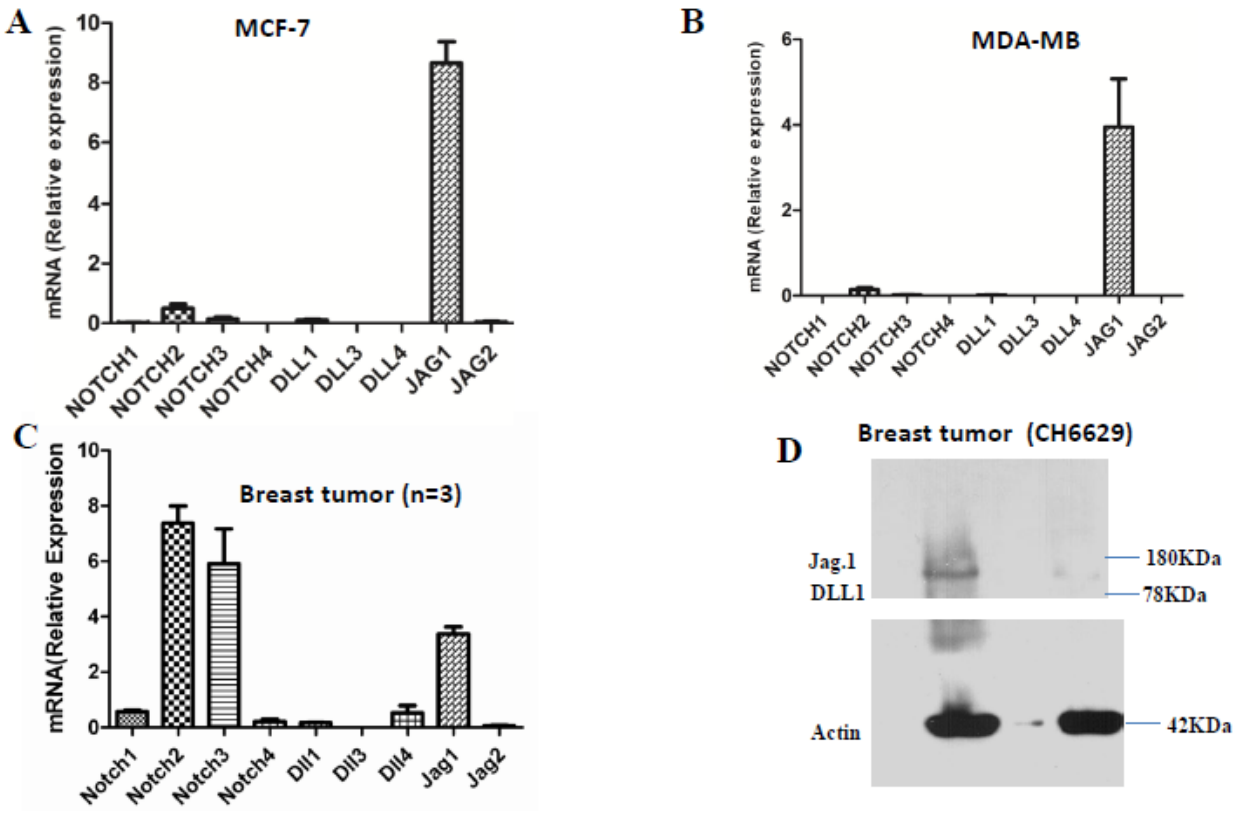

FIGURE 34. Expression of Notch and its ligands in breast cancer cell lines and breast tumor **A.** Real time quantitative PCR showed mRNA expression of Jag1 with lower level of Notch2, Notch3 and Dll1on MCF-7 cell line. **B.** MDA-MB also cell line showed mRNA expression of Jag1 with lower level of Notch2, Notch3 and Dll1. **C.** Surgically excised breast tumors express higher level of Notch2, Notch3 and Jag1 along with lower level of Notch1, Notch4, Dll1, Dll4 and Jag1.**D.** Detection of 180 KDa Jag1 on surgically excised breast tumor using antibody that recognizes the Jag1. Dll1 was not deteceted in the surgically excised oral tumor.

CHAPTER 7:

Involvement of Notch signaling in effector functions of peripheral γδ T cells of oral cancer patients

Oral cancer patient's immune system is an important element in the development of the disease and, in many cases, in the response to treatment [281]. The production by oral SCC and some other cancers of chemo-attractive factors such as VEGF can attract immunosuppressive $CD34^+$ progenitor cells that inhibit the capacity of intratumoral lymphoid cells to become activated [282]. Moreover, TGF-β induces expansion of Tregs and inhibits T-cell effector function [211, 283]. The shedding of MICA and MICB by ADAMs 10 and 17 from cancer cells prevents binding of the corresponding NKG2D receptor of natural killer cells and γδ T cells [284].The data indicates that γδ T cells of oral cancer patient may differ from those of healthy individuals with respect to antigen spectic effector functions.

In the previous chapters it was demonstrated that Notch signaling plays an important role in regulation of effector function of peripheral γδ T cells of healthy individuals. Experimental data in this Chapter aims to investigate if Notch signal is also involved in regulating the effector function of γδ T cells isolated from oral cancer patients.

Isolation of γδ T cells from oral cancer patients

$10X10^6$ PBLs were ex-vivo expanded with solid phase anti CD3 mAb (1mg/ml) and rIL2 (10 IU/ml) for 12 days. This was followed by immunomagnetic purification to isolate γδ T cells. The purity of γδ T cells as checked by flow cytometer was >90%. (Figure 35). Table-3 summarizes the total expansion of lymphocytes from oral cancer patients (n=15) using anti CD3 mAb and rIl2 and also the percentage of purified γδ T cells obtained from the same individuals after expansion. The expanded lymphocytes after anti CD3 stimulation ranged from $12X10^6$ to $153X10^6$ with mean of $77.5X10^6$±12.3 from a starting population of $10X10^6$ PBL. The immunomagneticaly purified γδ T cells ranged from 2.2 to 46.4% (mean16.2±3.2%).

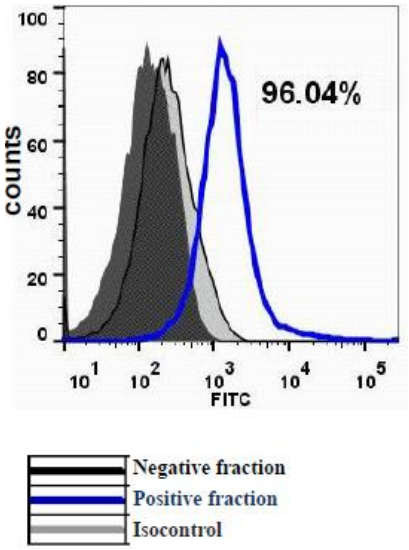

Figure 35. Purity of immunomagnetically separated γδ T cells from oral cancer patients.

Table 4: Generation of γδ T cell lines from PBL of oral cancer patients

Oral cancer patients	Cell count Day '0'	Cell count Day '12'	% recovery of γδ after immunomagnetic separation
1	$10x10^6$	$136x10^6$	10%
2	$10x10^6$	$24x10^6$	2.7%
3	$10x10^6$	$53x10^6$	19.8%
4	$10x10^6$	$120x10^6$	21.7%
5	$10x10^6$	$21x10^6$	6.2%
6	$10x10^6$	$100x10^6$	15%
7	$10x10^6$	$83x10^6$	21.1%
8	$10x10^6$	$153x10^6$	46.4%
9	$10x10^6$	$16x10^6$	23.8%
10	$10x10^6$	$122x10^6$	9%
11	$10x10^6$	$120x10^6$	21.7%
12	$10x10^6$	$102x10^6$	32.2%
13	$10x10^6$	$12x10^6$	5%
14	$10x10^6$	$57.5x10^6$	2.2%
15	$10x10^6$	$46x10^6$	7.2%

Mean cell expansion on Day 12= $77.6x10^6$ ±12.3
Mean % recovery of γδ T cells = 16.2±3.2%

Expression of Notch receptors in γδ T cells from oral cancer patients

To determine the expression of different Notch receptor isoforms and its ligands in peripheral γδ T cells from oral cancer patients, the expression of mRNA for Notch genes (Notch1-4) and its ligands Dll1, 3 and 4 and Jag1 and 2 were quantitated by real time PCR. β-actin was used as

house keeping gene control. As seen in Figure 36A, the expression of mRNA for Notch1 and Notch2 genes were dominantly observed in γδ T cells with lower expression of Dll1, Dll3 and Jag1 mRNA. mRNA for Notch3, 4, Dll3 and Jag.2 was not detected in γδ T cells.

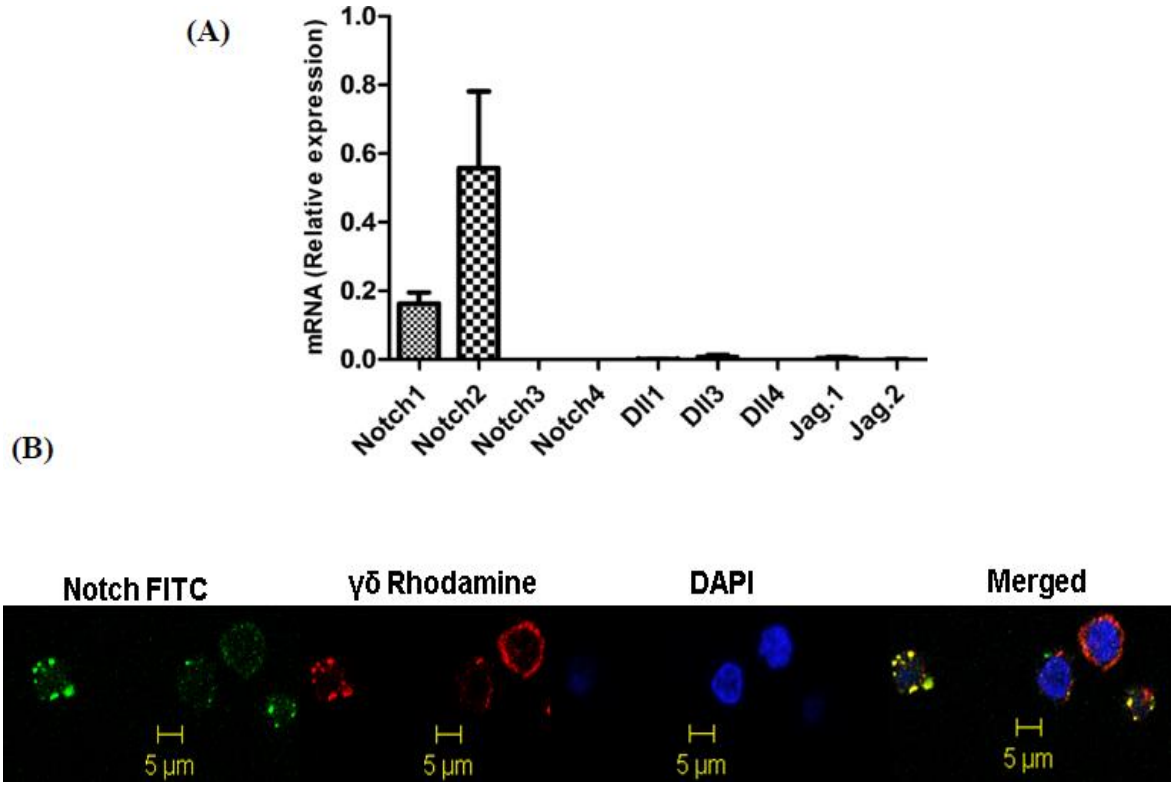

Figure 36A and B. Expression of Notch receptors on γδ T cells from oral cancer patients. Real time quantitative PCR showed mRNA expression of Notch1 and Notch2 on γδ T cells (A). Total RNA was extracted from ex-vivo expanded γδ T cells obtained from oral cancer patients. Data were normalized to expression of GAPDH. The data represent mean of four independent experiments. Panel B shows N1ICD expression by confocal microscopy.

The expression of NICD in the γδ T cells was confirmed by confocal microscopy. Ex-vivo expanded and purified γδ T cells were stained for N1ICD and anti γδ TCR antibody and analyzed using confocal microscopy. As shown in Figure 36B, it was observed that N1ICD was abundantly expressed on the cell membrane, in the cytoplasm and nucleus of γδ T cells isolated from oral cancer patients. Unlike γδ T cells of healthy individuals, the N1ICD in oral cancer patients showed more of punctate staining pattern.

Inhibition of Notch signaling blocks γδ T cell proliferation

The role of Notch in antigen driven proliferative response of γδ T cells from oral cancer patients was examined. γδ T cells were cultured with rIL2 alone or along with three different phosphoantigens (BrHPP, IPP and picostim) in the presence and absence of GSI-X. Cells were stimulated for 72 h and proliferation was measured by the uptake of [^{3}H]TdR during the last 18 h of culture period. As shown in Figure 37, a significant increased in the proliferation of γδ T cells was observed in the presence of rIL2 (mean cpm 1617±91.6). It was also observed that the proliferation of γδ T cells was not significantly increased in the presence of the phosphoantigens (mean cpm: BrHPP+rIL2=1673±111.8, IPP+rIL2±1777±1.5, picostim+rIL2=1766±226.5) indicating a poor response to antigens, which was in contrast to that of healthy individuals (Figure 18). Analogous to γδ T cells from healthy individuals, treatment with GSI-X leads to significant decrease in the proliferation of γδ T cells (mean cpm: γδ+rIL2+GSI-X=186±22.9, $p<0.005$; γδ+BrHPP+IL2+GSI=198±46.7, $p<0.005$; γδ+IPP+rIL2+GSI-X=26±66, $p<0.005$ and γδ+picostim+rIL2+GSI=208±44, $p<0.005$) as compared to the control cells (response BrHPP, IPP, picostim in the presence of rIL2).

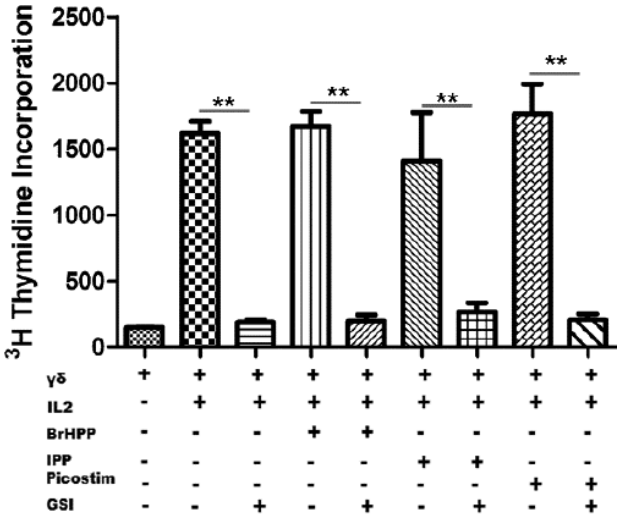

Figure 37. Treatment with GSI-X significantly reduces the antigens (BrHPP, IPP and picostim) driven proliferative response of ex-vivo expanded γδ T cells from oral cancer patients. γδ T cells ($5X10^4$ cells) were cultured in only medium or stimulated with rIL2 alone or along with the antigen in the presence or absence of GSI-X. Proliferation was measured by [^{3}H]TdR incorporation after 72 h. Values are measured as mean CPM (n=3); (** $p<0.005$).

Notch regulates cytolytic effector function of γδ T lymphocytes from oral cancer patients

The effect of Notch signaling on the cytolytic potential of γδ T lymphocytes from oral cancer paients was then determined. Purified γδ T cells were co-incubated with zoledronate treated AW13516 at a ratio of E;T 4:1.Before co-culture the AW13516 were pretreated with zoledronate (100μM) for 18h in order to increase the susceptibility towards γδ T cells mediated killing [123]. After 4h the cells were stained with FITC labeled γδ TCR and CD107a PE antibodies. Upon co-incubation with the zoledronate treated oral cancer cells (AW13516), γδ T cells showed surface expression of CD107a (12.41%, Figure 38A). The proportion of CD107a+ γδ T cells was slightly increased in the presence of IL2 (15.86%, Figure 38B). However, in the presence of GSI, the expression of CD107a+ was reduced in γδ T cells cocultured with zoledronate treated AW13516 (4.75%, Figure 38C) and also in γδ T cells cultured with zoledronate treated oral tumor cells in the presence of rIL2 (6.3%, Figure 38D).

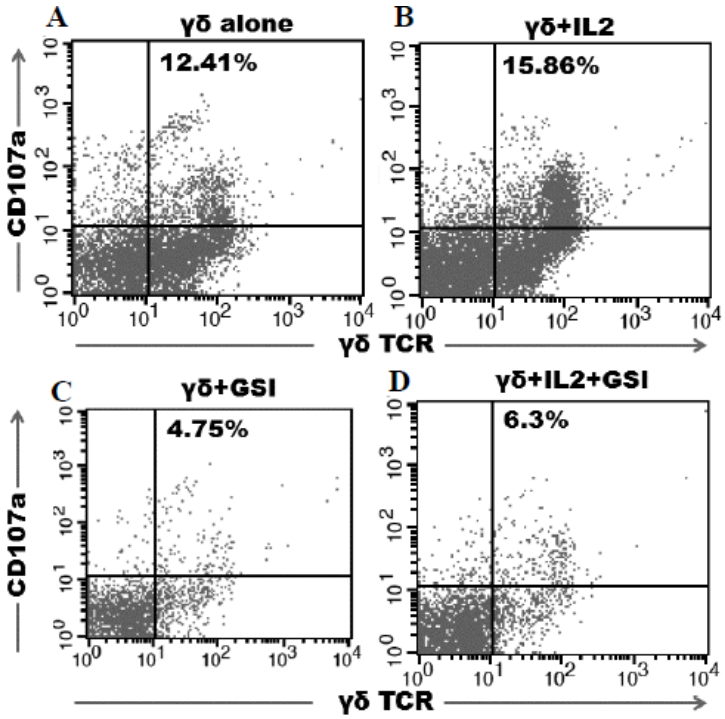

FIGURE 38. Expression of CD107a or LAMP1 (marker for degranulation) on γδ T cells from oral cancer patients. Effector γδ T cells alone or in the presence of rIL2 were co-cultured with target oral cancer cell line (AW13516) at the ratio of 4:1 and incubated for 4 h. AW13516 were previously treated with zoledronate (100μM) for 16 h. Decreased expression of CD107a was observed when the cells were treated with GSI (*lower panel*).

The cytotoxic potential of γδ T cells of oral cancer patients against zoledronate treated AW13516 in their presence and absence of GSI were confirmed using ^{51}Cr- release assay. ^{51}Cr release was determined after 4 h of co-culture at E:T ratio of 40:1. The mean percent cytotoxicity (mean cytotoxicity=±32.5%) was significantly reduced in the presence of GSI (mean cytotoxicity=±3.1%, $p<0.05$) against zoledronate treated tumor targets. The cytotoxic effect of γδ T cells against zoledronate treated tumor targets increased in the presence of rIL2 (mean cytotoxicity=±44.1%). Addition of GSI significantly reduced percent cytotoxicity against zoledronate treated tumor targets (mean cytotoxicity=±4.2%), $p<0.005$) (Figure 39). Collectively these data proves that the cytotoxic action of γδ T cells is regulated by Notch signal.

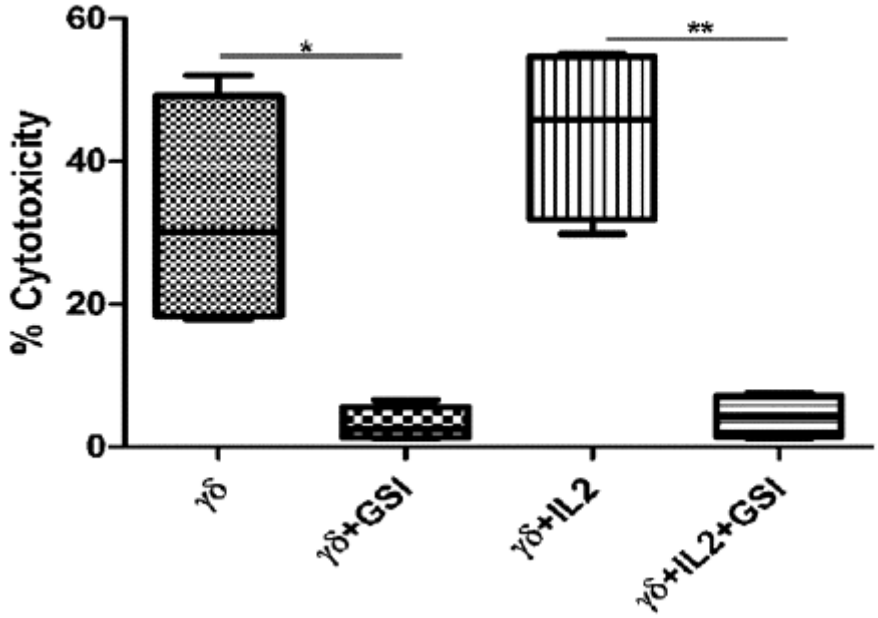

FIGURE 39. Involvement of Notch signaling in regulating cytolytic effector functions of γδ T cells from oral cancer patients. Cytolytic ability of γδ T cells alone or in the presence of rIL2 against zoledronate treated AW13516 was determined by ^{51}Cr release assay at the ratio of 40:1 in a standard 4 h ^{51}Cr release assay. Treatment of γδ T cells with GSI blocked the target cell lysis. Data represents % cytotoxicity as described in materials and methods. *, $p<0.05$ and **, $p<0.005$ compared with % cytotoxicity of both γδ T cells and γδ T cells with rIL2 without GSI-X respectively. The results are mean % cytotoxicity of γδ T cells from five healthy individuals.

Notch signaling is involved in cytokines production by activated γδ T cells from oral cancer patients

The effect of blocking Notch signaling on cytokine production of γδ T cells from oral cancer patients was analyzed. γδ T cells were cultured with rIL2 alone or with BrHPP in the presence or absence of GSI. Secreted cytokines were collected at 24h and measured by cytometric bead array. The cytometric bead array used measures Th1 (IL2, TNF-α and IFN-γ), Th2 (IL4, IL6 and IL10) and IL17 cytokines. As shown in Figure 40, addition of GSI leads to marked decreased in production of TNF-α, IFN-γ and IL17 cytokines by γδ T cells.

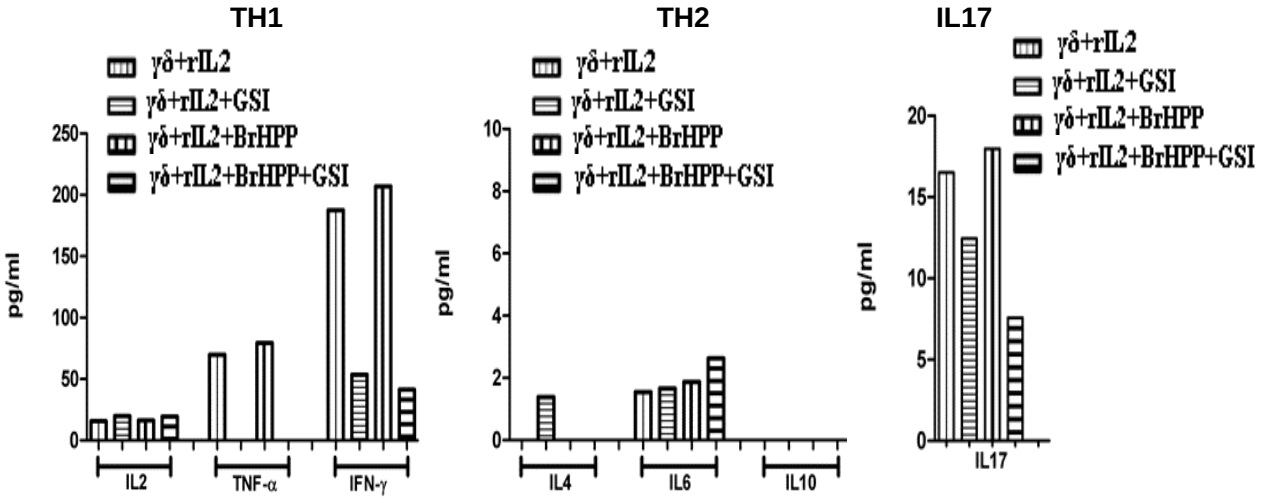

FIGURE 40. Effect of GSI-X on the secretion of different cytokines. Concentrations of different cytokine proteins (TH1, TH2 and TH17) were determined in the culture supernatant of γδ T cells stimulated with rIL2 alone or with BrHPP in the presence of rIL2 by CBA. γδ T cells ($5X10^4$ cells) from oral cancer patients were cultured with rIL2 alone or along with BrHPP in the presence or absence of GSI-X for 24 h. Culture supernatants were collected after 24 h and level of secreted cytokines was measured by Cytometric bead array. Addition of GSI leads to marked reduction in TNF-α, IFN-γ and IL17 production by activated γδ T cells. Results shown are representative of three independent experiments.

The IFN-γ release by γδ T cells and its inhibition by GSI were further confirmed by ELISA. Purified γδ T cells from oral cancer patients were stimulated with three different phosphoantigens (BrHPP, IPP and picostim) in the presence of rIL2 with or without GSI. As shown in Figure 41, γδ T cells stimulated with phosphoantigens showed increased production of IFN-γ over γδ T cells stimulated with rIL2 alone. Treatment with GSI inhibits the level of IFN-γ production by these cells (Figure 41).

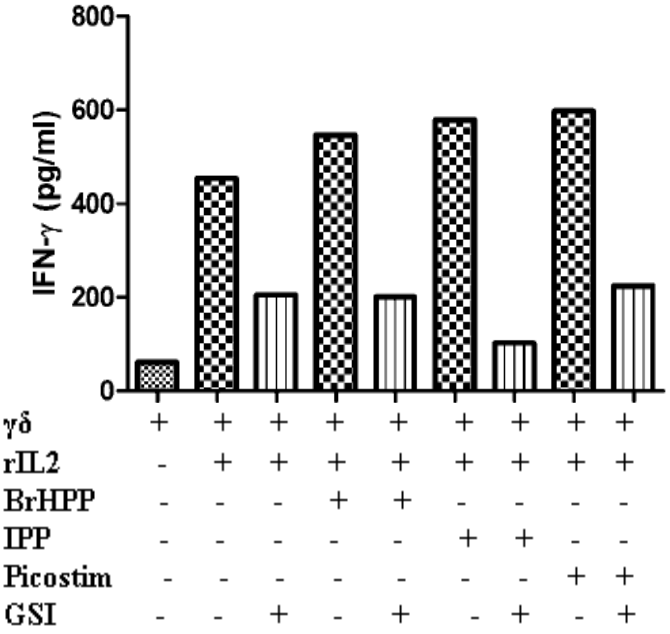

FIGURE 41. Treatment with GSI blocks IFN-γ production by γδ T cells from oral cancer patients. γδ T cells alone, γδ T cells with rIL2 or along with three different (BrHPP, IPP and picostim) were cultured in the presence or absence of GSI for 24h in 96 well plate. Supernatants were collected and level of IFN-γ was measured by sandwich ELISA. GSI treatment decreases IFN-γ production by both unstimulated and stimulated γδ T cells.

Expression of Notch target gene (HES1) in γδ T cells

The release of NICD into the nucleus leads to expression of Notch target genes. Next, the presence of well-known Notch target gene, HES1, in γδ T cells from oral cancer patients was examined by confocal microscopy. As shown in Figure 42, the expression of HES1 was observed in the nucleus of γδ T cells. Thus, it indicates the presence of active Notch signaling in γδ T cells from oral cancer patients.

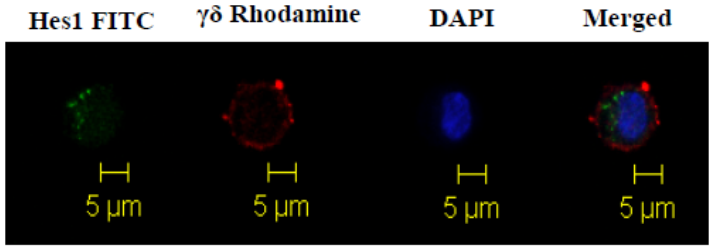

Figure 42: Expression of HES1 in the nucleus of γδ T cell from oral cancer patient. Expression of HES1 in ex-vivo expanded γδ T cells from oral cancer patient. Green fluorescence (FITC) shows pattern of HES1, γδ TCR receptor is in red (rhodamine red) and nuclei are shown in blue (DAPI).

Chapter 8:

Discussion

The immune system plays at least three distinct roles in preventing cancer: (i) it eliminates tumor cells in certain tissues. The nascent transformed cells often co-express ligands for activating receptors on innate immune cells and also, expresses tumor antigens that are recognized by immune receptors on lymphocytes of the adaptive immune system.; (ii) it prevents the establishment of an inflammatory environment that facilitates tumorigenesis by eliminating pathogens and by prompt resolution of inflammation; and (iii) it protects the host against viral infection and hence suppresses virus-induced tumors [262].

Tumors typically express two types of antigen: neoantigens and self-antigens. Neo-antigens (tumour-specific antigens) occur as a result of genomic instability are derived from mutated self-proteins or oncogenic viruses, and are not expressed in normal tissue [285, 286]. Tumors can also express normal self-proteins, but in abnormal quantities or locations (tumour-associated antigens) [287-289]. Up to 71% of oral SCCs express antigens from at least 1 of 6 melanoma antigen genes (MAGEs), notably MAGE- 1 and MAGE-3 [244]. Antigen from NY-ESO-1, a gene expressed in normal ovary and testis, is highly expressed in a variety of tumor types including oral SCC [245]. For effective elimination of cancerous cells, tumor antigens must also be encountered in the context of 'danger signals', such as those from microorganisms (pathogen-associated molecular patterns) or from dying or damaged cells (damage associated molecular patterns; DAMP) [290, 291]. The classical 'danger signals' such as IFN-γ activate dendritic cells and promote induction of anti-tumor immune responses. DAMPs from dying tumor cells (such as high mobility group box 1) or from damaged tissues (such as hyaluronan fragments), as solid tumors begin to grow invasively also activate immune system [292]. Ligands that are frequently expressed on tumor cells bind to activating receptors on innate immune cells, leading to release of pro-inflammatory and immunomodulatory cytokines, which in turn establish a microenvironment that facilitates the

development of a tumor-specific adaptive immune response [293]. A number of chemotherapy drugs have now been shown to induce tumor cell death in a way which looks dangerous to the immune system [294-296]. This act of killing of tumor cell is known as “elimination phase”.

Some tumor cell variants may survive the elimination phase and enter the “equilibrium phase”. In this phase, the immune system maintains residual tumor cells in a functional state of dormancy; the outgrowth of occult tumors is controlled by immunity that may reside in patients for decades before eventually resuming growth as either recurrent primary tumors or distant metastases [297]. In a primary tumorigenesis experiments, it was shown that immunocompetent mice treated with low-dose carcinogen [3′-methylcholanthrene (MCA)] harbored occult cancer cells for an extended time period even when the mice did not develop any apparent tumors. When monoclonal antibodies (mAbs) that deplete T cells and IFN-γ was administered, tumors rapidly appeared at the original MCA injection site in half of the mice [298].

Tumor cells may evade immune recognition or killing and emerge as progressively growing tumors. This phase is known as “escape”. Malignant progression is often accompanied by profound immune suppression that interferes with an effective antitumor response and tumor elimination.

It has been reported that specific genetic, immunochemical, or functional ablations of NKT, γδ T cells, NK cells or αβ T cells lead to increased susceptibility of host to tumors [299]. Studies from cancer-immune interaction have revealed that every known innate and adaptive immune effector mechanism participates in anti-tumor activity [300]. Among these is class of effector immune cells known as γδ T cells. These cells represent a unique subset of unconventional lymphocytes, because they display features of both conventional αβ T cells and NK cells [7]. γδ T cells play an

important role in mediating antitumor activity by direct killing the cancerous cells and also by producing cytokine and chemokine [100].

The presence of efficient costimulation is essential for effective antitumor immunity. The lack of proper costimulation in the tumor microenvironment could be responsible for absence of an appropriate antitumor immune response [301]. In this thesis, the role of Notch signal as an additional costimulatory signal in the regulation of γδ T cell effector functions has been investigated.

In mammals, there are four Notch receptors (Notch1-4) and five Notch ligands, three Delta-like (Dll1, Dll3 and Dll4) and two Jagged (Jag1 and Jag2) [302]. In the immune system, Notch signaling is involved in T and B cell development [303, 304] and in myeloid lineage commitment [305]. Notch signaling is known to be involved in immune Naive $CD4^+$ and $CD8^+$ T cells express Notch1 and Notch2 [87, 306]. Dendritic cells (DCs) have been shown to express Notch1, Notch2, Jagged1, Jagged2, Dll-1, and Dll-4 [306, 307]. Furthermore, Tregs expresses Notch1, Jagged1 and, to a lesser extent, Delta-like4 [94]. In the present study it was reported for the first time that purified γδ T cells from healthy individuals express mRNA for Notch1 and Notch2 receptors along with lower level of Dll1 and Jag1 ligands. mRNA of Notch3, Notch4, Dll3, Dll4 and Jag2 was not detected in γδ T cells. Similarly, real time PCR in αβ T cells shows expression of mRNA for Notch1 and Notch2 with lower level of Dll1, Dll4, Jag1 and Jag2.

The interaction between Notch receptor and ligand pair cleaves the extracellular and transmembrane domain of Notch through activation of ADAM proteases and gamma secretase respectively [26]. The gamma secretase induced cleavage generates Notch intracellular domain (NICD) which translocates to the nucleus. Next, the expression of NICD was visualized in γδ T

cells by confocal microscopy, flow cytometry and western blotting. Confocal microscopic analysis of γδ T cells showed that N1ICD is predominantly expressed in both freshly isolated and ex-vivo expanded γδ T cells. Also, the expression of N1ICD and N2ICD on the ex-vivo expanded γδ T cells was examined by flow cytometery. The expression of NICD at protein level was finally confirmed using Western blotting. Thus the expression of NICD in γδ T cells indicated that Notch signaling was active in γδ T cells. The expression of NICD in αβ T cells was also determined in order to compare with that of γδ T cells.

Once liberated from the membrane and translocated into the nucleus, NICD regulates the expression of target genes. A frequent target in Drosophila and mammals is the family of bHLH-type transcriptional repressors, known as Hairy/Enhancer of Split (HES) [27]. It was found that HES1 is expressed in both freshly isolated and ex-vivo expanded γδ T cells. Next, mRNA expression of other Notch target genes along with HES1 viz. NF-κB, Deltex and NRARP in were observed γδ T cells as well as in αβ T cells.

Unlike αβ T cells, γδ T cells do not require the help of conventional major histocompatibility complex. (MHC) class I and class II molecules for recognizing the antigens [166]. γδ T cells expressing Vδ1 are abundantly found at mucosal sites and these respond to the expression of non-classical MHC molecules on the surface of virally-infected or tumour cells [101, 103]. $V\delta2^{+}$ (Vγ9Vδ2) cells are predominantly present in the peripheral circulation and respond to non-peptide phosphoantigens [11, 12]. The Vγ9Vδ2 crystal structure confirmed the presence of a basic, positively charged region in the binding groove that could directly interact with the negatively charged pyrophosphate moiety of the antigen [11]. These phosphoantigens are generated during the non-mevalonate and mevalonate pathways utilized by prokaryotic and eukaryotic cells,

respectively [13, 104, 105]. Our study demonstrates selectively expansion of Vγ9Vδ2 T cells from peripheral bood lymphocytes in response to three different phosphoantigens (BrHPP, IPP and picostim).

It is apparent that T cells exist in an environment rich in cell types, such as antigen presenting cells, known to express Notch ligands, it also is well documented that signals through the T cell receptor (TCR) are sufficient to induce γ-secretase-mediated cleavage of Notch [30, 88]. In the present study, it was observed that stimulation of γδ T cells with BrHPP (a synthetic analog of IPP) and rIL2 triggered the activation of Notch signaling which can be observed by abundant release of N1ICD. As described previously, the final step in Notch activation is accomplished through the action of the enzyme, γ-secretase. Because of the complete dependence of Notch activation upon γ-secretase, inhibitors of this enzyme have proved a convenient means by which Notch signaling may be inhibited. Our results demonstrate reduction in the expression of N1ICD by GSI (γ-secretase inhibitor) in BrHPP stimulated γδ T cells showing the involvement of Notch signaling in regulating antigen specific responses of γδ T cells. Simultaneously, treatment with GSI-X results in marked decreased in the expression of mRNA for Notch1 receptor and its target gene-NF-κB expression in antigen activated γδ T cells.

Activation of the Notch signaling pathway leads to the induction of c-Myc expression in immune cells [274] which was also observed in γδ T cells. It was also found that activation of γδ T cells with BrHPP and rIL2 was necessary for the Notch-mediated induction of cell cycle regulator, c-Myc which was abrogated upon GSI-X treatment.

Further experiments to determine the role of Notch signaling in co-stimulating the effector functions of γδ T cells were carried out. The role of Notch signaling in terms of proliferation,

cytokine production and cytotoxicity of γδ T cells were determined. It was observed that inhibiting the Notch signaling in anti CD3 (mAb) stimulated γδ T cells resulted in marked decrease in proliferation of γδ T cells confirming TCR engagement as a key initiating event affected by GSI-X treatment. Similarly, γδ T cells (freshly isolated and *ex-vivo* expanded) activated with BrHPP, IPP and c-HDMAPP as antigens resulted in a significantly decreased proliferation of γδ T cells in response to these antigens. The observation that Notch signaling is involved in regulating antigen specific proliferative responses of γδ T cells prompted us to look at the expression of early and late activation markers CD69 and CD25 on γδ T cells stimulated with the antigen (BrHPP) in the presence and absence of GSI. Although substantial changes in CD69 expression on γδ T cells was not detected, a marked decrease in CD25 expression on antigen stimulated γδ T cells was observed when Notch signaling was inhibited. Notch signaling is known to contribute to regulation of $CD4^+$ T cell immune responses by modulating CD25 expression [88]. γδ T cells are dependent on IL-2 for their growth and survival and express high affinity IL-2R [308]. It is therefore not surprising that CD25 expression in γδ T cells is also regulated by Notch which may provide a positive feedback loop through IL2 signaling.

The importance of Notch signaling in mediating cytotoxic responses in immune cells has been well documented. Earlier studies carried out in murine $CD8^+$ T lymphocytes and NK cells have demonstrated the importance of Notch signaling in regulating their effector functions [29, 309]. Notch signaling was shown to directly regulate granzyme B expression in CD8+ cytotoxic T lymphocytes [91]. The involvement of Notch in anti-tumor immunity was further supported by studies which showed that deficiency of Notch2 decreased the anti-tumor responses of $CD8^+$ T cells in mice models [90]. Notch signaling also contributes to dendritic cell mediated NK cell activation, which enhanced the killing activity of NK cells. Murine NK cells exhibit enhanced

cytokine expression and cytotoxic function in response to signaling from tumor cells or dendritic cells transduced with Jag2 [309].

Therefore, it was expected that Notch signaling may similarly regulate cytotoxic effector functions of γδ T cells. Blocking of Notch signaling in γδ T cells by GSI inhibits the ability of γδ T cells to lyse tumor targets. Oral tumor cell line AW13516 treated with zoledronate was used as target cell line. Earlier data from our lab has demonstrated that tumor cells treated with zoledronate are aggressively killed by γδ T cells [123, 128]. Our data demonstrates that treatment with GSI blocks the ability of γδ T cells to lyse both untreated and zoledronate treated tumor cells. Moreover, it was found that specific silencing of either Notch1 or Notch2 by siRNA led to the reduced cytotoxic potential of γδ T cells. This result suggests that both Notch1 and Notch2 are involved in the cytolytic activity of γδ T cells. Cho and colleagues [29] demonstrated that Notch1 regulates expression of eomesodermin (EOMES), perforin and granzyme B through direct binding to the promoters of these effector molecules. In CD8+ T cells, Notch 2 signaling was shown to directly control CTL effector molecules including granzyme B, by integrating RBP-J and CREB1 (41). It was observed that GSI treatment of γδ T cells alone or γδ T cells activated with IL2 results in a reduction of CD107a expression in these cells. CD107a or LAMP1 is a marker of degranulation in cytotoxic T lymphocytes [310-312].

γδ T cells are known to secrete copious amounts of IFN-γ. IFN-γ plays crucial role in protective immune response against certain pathogens and tumors [9, 279, 280]. It was showed that blocking Notch signaling with GSI inhibited the IFN-γ secretion by γδ T cells stimulated with phosphoantigens BrHPP, IPP and c-HDMAPP. Likewise, GSI inhibited IFN-γ production by anti CD3 (mAb) activated γδ T cells. γδ T cells provide an early source of IFN-γ in tumor immunosurveillance and against viral challenge [9, 313]. Epigenetic program that regulates IFN-γ

gene transcription in γδ T cells is different from $CD4^+$ and $CD8^+$ T cells [314]. EOMES contributes to T bet independent IFN-γ production in γδ T cells [314]. It was observed that BrHPP stimulated γδ T cells produced IL17 and its secretion was inhibited in the presence of GSI-X. γδ T cells have been reported to be a potent source of IL17 [275, 276]. It has been reported that DLL4 upregulates RORC expression in T cells and both RORC and IL17 gene promoters are direct transcriptional notch targets and enhance Th17 cell population [315].

Within the immune system, Notch ligands are mainly expressed on antigen presenting cells (APCs), whereas the Notch receptors are expressed on T cells [86, 307]. Depending on the predominant Notch ligand expressed on APCs, different target genes are activated by Notch signaling. As an example, Delta1-expressing dendritic cells (DCs) have increased ability to activate naive $CD4^+$ T cells and promote Th1 cell development in vitro via upregulation of T-bet and IFN-γ, and inhibition of IL-4R signaling [86, 316]. In contrast, Jagged-expressing APCs lead to Th2 cell differentiation via enhancement of GATA3 transcription [86]. Finally, transfected Delta1 on DCs are also able to direct differentiation of naive $CD8^+$ T cells into CTLs by promoting granzyme B and eomesodermin (Eomes) transcription [29, 91]. Nevertheless, the contribution of Notch ligands in effector functions of γδ T cells has not been reported earlier. Therefore, it was decided to study the effect of different Notch ligands on activation of γδ T cells. This study has demonstrated that rDll1 and rDll4 augments whereas rJag1 significantly decreases phosphoantigen driven proliferation of γδ T cells. rJag1 significantly decreased IFN-γ secretion by phosphoantigen activated γδ T cells. It was also observed that rJag1 decreased anti CD3 stimulated proliferation and IFN-γ production by γδ T cells in a concentration dependent manner. Therefore, the result indicates that delta like ligands (rDLL1 and rDLL4) and Jag1 ligands acts in a different way, with Dll1 and 4 having stimulatory and Jag1 is having inhibitory effects on γδ T

cells. Our results showing inhibitory effect of Jag1 supports previous observation by Anderson group where it was shown that Jag1 mediated signal inhibits activation of both $CD4^+$ and $CD8^+$ T cells [317].

$CD4^+CD25^+$ T cells (Tregs), but not $CD4^+CD25^-$ T cells, express cell surface Notch ligand Jag1 [94]. It has also been reported that stimulation of $CD45RA^+$ naive T cells by Jag1 reduces production of IFN-γ, IL2, and IL5, but upregulates TGF-β synthesis, consistent with induction of a regulatory T-cell phenotype [278]. However, as mentioned above, Jag1 inhibits the effector function of γδ T cells. Interestingly, blocking of Jag1 ligand on Tregs reduces its suppressive potential against γδ T cells. It was observed that anti Jag1 antibody pretreated Tregs have reduced suppressive potential as manifested by increased dividing γδ T cells. This data thus indicates cross-talk between γδ cells and Tregs via. Notch signal.

Emerging evidence has suggested that Notch signaling pathway may be involved in the development, progression and metastasis of certain cances[318]. Upregulation of Jag.1 notch signaling pathway plays a role in prostatic cancer progression and metastasis and is a useful marker in distinguishing indolent and aggressive prostate cancer [319]. Jagged 1 expression was observed as the primary Notch 3 ligand in ovarian carcinoma and Jagged 1/ Notch 3 interaction constituted a juxtacrine loop promoting proliferation and dissemination of ovarian cancer cells within the intraperitoneal cavity [320]. Interestingly, Wnt/β-Catenin pathway activation also upregulated Jag.1 expression in ovarian cancer [321]. In the present study expression of Notch receptor isoforms and its ligands was examined in oral and breast cancer cells. Several reports have confirmed Jag. 1 mRNA in embryonic stem cells, neural tissues, lung carcinoid, gastric cancer, pancreatic cancer colon cancer oral cancer also in SCC of the skin, oral cavity esophageas, head and neck [322].

In the present study mRNA and protein expression of Jagged 1 was observed in oral cancer tumors and oral cancer cell lines and also in breast tumors and breast cancer cell lines. Lin et al. showed that high level co expression of jagged 1 and Notch 1 is associated with poor oral all survival in patient with head and neck cancer [323].

Our own observations revealed that Jag.1 expression is high in oral tumors and also Jag.1 delivers an inhibitory signal to γδ T cells. We hypothesized that upregulation of Jag.1 on tumors may be an immune evasion strategy adapted by the tumors to evade immune regulatory γδ T cells and other data from our own laboratory and others have shown that human γδT cells mediate potent cytotoxicity against SCC of oral cavity [128, 324]. γδ T cell infiltration in oral SCC have been reported earlier [325]. A significantly higher proportion of γδ T cells were found in patient with recurrent or metachoroneus second primary SCC.

The existence of T regulatory cells (Tregs) in humans and mice is well accepted as these control several immunological disorders [326]. The mechanism by which Tregs cause immunesupression is area of intense investigation and several ideas have been put forward [327]. Among the implicated molecules,, TGF-β plays an important role in the maintenance of FoxP3 expression and suppressor function of $CD4^{+}CD25^{+}$ Tregs [211, 220, 221]. A second molecule which plays an important role in Tregs cells are Notch ligands but the differentiation is not Notch dependent because Notch loss of function mutant mice do not lack Tregs cells [278, 328]. Notch signaling is involved in the development and function of Tregs through regulating Foxp3 expression [329, 330].

Intergration of the differentiation of TGF-β and Notch pathways in antigen induced peripheral tolerance has been reported [31]. Notch 1 ligand Jag.1 is known to be expressed on Tregs and

blockade of Notch signaling with anti Jag.1 or blocking anti Notch 1 antibody inhibits Tregs suppressor function in vitro [94]. In the present study it was demonstrated that blocking of Jagged 1 expression of Tregs inhibits their ability to suppress antigen specific proliferative responses of γδ T cells. The result indicates that Jag.1 expressed on Tregs plays an important role in regularating antigen specific responces of γδ T cells.

Thus tumor cells evade immune recognition by γδ T cells in two possible ways

1) Upregulating the expression of Jag.1 on tumors to inhibit the antigen specific proliferation and IFN-γ production of γδ T cells.

2) Facilitating the cross talk between antigen specific γδ T cells and Tregs through Jag.1.

It was reported that Notch activation on effector T cells increases their sensitivity to Treg cell-mediated suppression through upregulation of TGF-βRII expression and phosphorylated form of smad3 [331]. N1ICD (Notch1 intracellular domain) was shown to be involved in Tregs suppressor function by binding to phosphorylated Smad3 arising from TGF-signaling and facilitating the translocation of phosphorylated Smad3 to the nucleus [94]. Therefore, it is likely that the crosstalk between Notch and Jag.1-TGF- β may lead to the abserval potentiation of the suppressive effect of Treg cells on γδ T cells.

Several lines of evidence support a broad role for γδ T cells in tumor immunosurveillance [164, 332]. In clinical studies, γδ T cells have been shown to infiltrate a variety of tumors including renal cell carcinoma [333], seminoma [334], ovarian cancer [335], and colon cancer [336]. In many instances, γδ T cells that are cytotoxic to a specific tumor type will cross-react with other tumors but not with the tumor's non transformed counterpart [24, 335, 336]. Notch signaling

plays an important role in regulation of effector function of peripheral γδ T cells from healthy individuals was previously demonstrated. Next, the involvement of Notch signal in the effector function of γδ T cells from oral cancer patients was assesed.

The expression of Notch1 and Notch2 genes was observed in γδ T cells from oral cancer patients. Lower expression of Dll1, Dll3 and Jag1 mRNA was also observed. It was observed that N1ICD was abundantly expressed in the cell membrane, cytoplasm and nucleus. Unlike γδ T cells from healthy individual, the N1ICD in oral cancer patients showed lots of punctate staining. The expression of HES1 in the nucleus of γδ T cells was also observed indicating the presence of active Notch signaling in γδ T cells from oral cancer patients.

The role of Notch in antigen driven proliferative response of γδ T cells from oral cancer patients was examined. It was also observed that the proliferation of γδ T cells was not significantly increased in the presence of the phosphoantigens which was in contrast to that of healthy individuals. Analogous to γδ T cells from healthy individuals, treatment with GSI-X leads to significant decrease in the proliferation of γδ T cells.

Upon co-incubation with the zoledronate treated oral cancer cells (AW13516), γδ T cells showed surface expression of CD107a. Treatment of GSI-X leads to decreased expression of CD107a+ in γδ T cells cocultured with zoledronate treated AW13516. The cytotoxic potential of γδ T cells of oral cancer patients against zoledronate treated AW13516 in their presence and absence of GSI-X were confirmed using ^{51}Cr- release assay. Addition of GS-XI significantly reduced percent cytotoxicity against zoledronate treated tumor targets.

The effect of blocking Notch signaling on cytokine production by γδ T cells from oral cancer patients was analyzed. Addition of GSI-X leads to marked decreased in production of TNF-α,

IFN-γ and IL17 cytokines by γδ T cells. The effect of GSI-X on IFN-γ release by ELISA was further confirmed. Collectively these results prove that Notch signal is involved in regulation of effector function of γδ T cells from oral cancer patients.

Our findings, therefore, describe for the first time that Notch is involved in regulating the effector functions of human γδ T cells. Notch signaling appears to play an important role in modulating the antigen specific proliferation of γδ T cells, their ability to lyse tumor targets and secrete IFN-γ upon stimulation. Taken together, these studies identify Notch as an additional signal contributing to antigen specific effector functions of γδ T cells. Interestingly, blocking of Jag1 on Tregs reduces its suppressive potential against γδ T cells. These studies may have important implications in clinical situations where new strategies for the clinical manipulation of γδ T cells for cancer immunotherapy are being investigated.

Chapter 9:

Summary & conclusion

Combinatorial use of number of signaling pathway contributes diversity among different cell types and enable cell to execute functions. Among these, the Notch signaling pathway facilitates short range communication between cells. Indeed, transmission of Notch signals requires physical contact between cells under most circumstances. In mammals four Notch single-pass transmembrane receptors (Notch-1–4) and five transmembrane ligands divided into two main families, Jagged (Jag1, Jag2) and Delta-like ligands (Dll1, Dll3, Dll4), have been described. In a context dependent manner, Notch signals can promote or suppress acquisition of specific cell fates, cell differentiation, cell death, proliferation, or effector functions. Aberrant gain or loss of Notch signaling components has been directly linked to multiple disorders. Early in the history of Notch, Poulson demonstrated that absence of Notch activity in flies causes embryonic neuronal hyperplasia and deficiency of non-neuronal cells in the brain. Notch related human disorders include developmental syndromes (for example, Alagille syndrome, Tetralogy of Fallot, syndactyly, spondylocostal dysostosis, familial aortic valve disease), adult onset diseases (such as CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy and cancer. In addition to its importance in disease, Notch signal play an important role in effector functions of immune cells. It is becoming increasingly clear that Notch signaling regulates peripheral $CD4^+$, $CD8^+$, Tregs and NK cells responses.

In the present study, we evaluated the role of Notch in regulation of peripheral γδ T cells and regulatory T cell functions. It was described for the first time, to our knowledge, the role of Notch in regulating the effector functions of human γδ T cells. γδ T cells are a minor fraction in the peripheral blood but are known to play a major role in defense against pathogens and tumors. The results revealed that human peripheral blood γδ T cells highly expressed Notch receptors (Notch 1

and Notch2). The expression of Notch target gene established the existence of Notch signaling pathway in γδ T cells.

Notch receptors become activated by two successive proteolytic cleavages upon cell-to-cell contact as a result of ligand binding. The first cleavage is catalyzed by the ADAM family of metalloproteases, and the second cleavage is mediated by the enzyme γ-secretase. This second cleavage releases the NICD, which may function as transcriptional cofactor. Activation of γδ T cells with phosphoantigen triggered the stimulation of Notch signaling which was observed by abundant release of NICD. Nevertheless, a marked decrease in CD25 expression (activation marker) and c-myc (cell cycle regulator) on antigen stimulated γδ T cells was observed when Notch signaling was inhibited. Thus, Notch signaling appeared to be involved in the activation of γδ T cells.

Inhibition of Notch signaling pathway in phosphoantigen/anti CD3 mAb activated γδ T cells by γ-secretase inhibitor resulted in decrease in proliferation of γδ T cells confirming TCR engagement as a key initiating event affected by GSI-X treatment. It was also observed that blocking of Notch signaling in γδ T cells by GSI-X as well specific silencing of either Notch1 or Notch2 by siRNA inhibits the ability of γδ T cells to lyse tumor targets. Similarly, hindering Notch signaling inhibited the IFN-γ secretion by γδ T cells stimulated with phosphoantigens/anti CD3 mAb. These results showed that Notch signaling pathway plays an important role in regulating effector functions of γδ T cells (Figure 43). Taken together, the present study has identified Notch as an additional signal contributing to antigen-specific effector functions of γδ T cells.

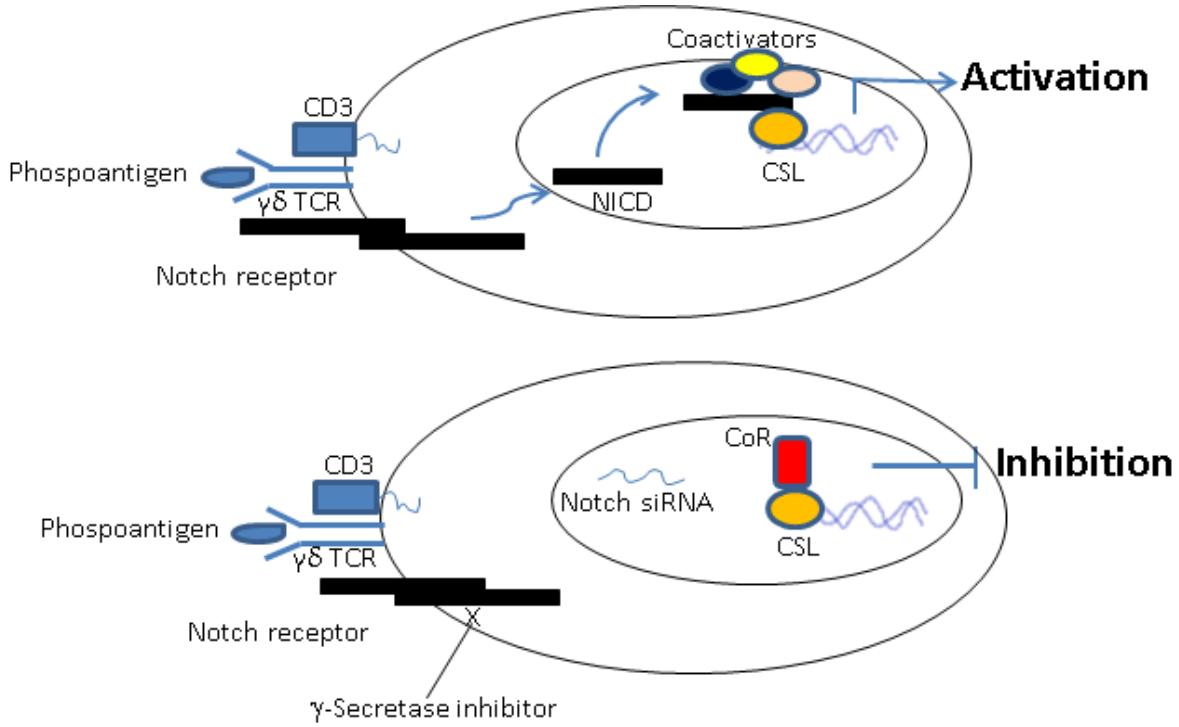

Figure 43: Notch signal is involved in activation of γδ T cells.

Similarly, the expression of Notch in γδ T cells from oral cancer patients was detected. Unlike γδ T cells from healthy individual, the N1ICD in oral cancer patients showed punctate staining. The expression of Notch target gene-HES1 in the nucleus of γδ T cells was also observed indicating the presence of active Notch signaling in γδ T cells from oral cancer patients. Notch signaling pathway was similarly found to be involved in regulating effector functions of γδ T cells from oral cancer patients.

Depending on the predominant Notch ligand expressed on the 'signal-sending' cell, different target genes are activated by Notch signaling. In the present study, it was observed that rDll1 and rDll4 augments whereas rJag1 significantly decreases phosphoantigens driven proliferation of γδ T cells (Figure 44). Similarly, rJag1 significantly decreased IFN-γ secretion by phosphoantigen/anti CD3 mAb activated γδ T cells. Remarkably, the cross-talk between

regulatory T cell and γδ cells via notch signal was detected. Tregs are known to express Jag1 ligand on their cell surface. It was observed that anti Jag1 antibody pretreated Tregs have reduced suppressive potential as manifested by increased dividing cells of γδ T cells. Oral cancer cell lines (AW13516 and AW8507) and surgically excised oral tumors as well as breast cancer cell lines (MCF-7 and MDA-MB) and surgically excised breast tumor predominantly express Jag1. Hence, it appears that upregulation of Jag1 on Tregs and tumors may inhibit γδ T cells and can be viewed as an immune evasion strategy that facilitates tumor progression.

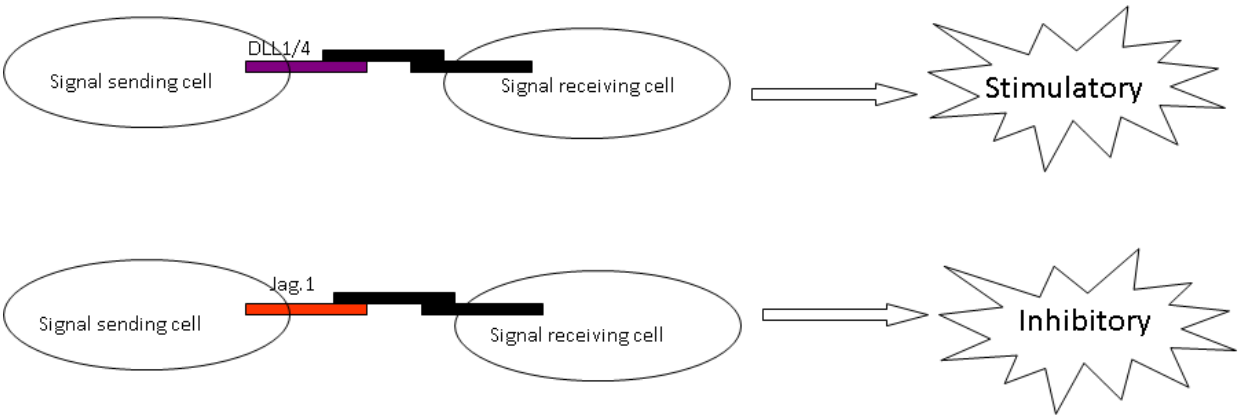

Figure 44: Effect of Notch ligands on activation of γδ T cells.

Thus, the studydemonstrated that Notch is involved in regulating the effector functions of human γδ T cells and identified Notch as an additional signal contributing to antigen specific effector functions of γδ T cells from HI and OC patients. We also identified that notch ligands (Dll and Jag) induce opposing signals in γδ T cell. Dll1 and Dll4 augment while Jag1 inhibits γδ T cell responses. Interestingly, suppression of antigen specific γδ T cell responses by Tregs is mediated by Jag1. These studies may have important implications in clinical situations where new strategies for the manipulation of γδ T cells for cancer immunotherapy are being investigated.

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Publications

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Involvement of Notch in Activation and Effector Functions of γδ T cells

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Involvement of Notch in Activation and Effector Functions of γδ T cells

Dimpu Gogoi, Asif A. Dar, and Shubhada V. Chiplunkar

Notch signaling plays a pivotal role in cell fate decision and lineage commitment of lymphocytes. Although the role of Notch in $CD4^+$ and $CD8^+$ αβ T cells is well documented, there are no reports on how Notch signaling regulates effector functions of γδ T cells. γδ T cells are a minor fraction in the peripheral blood but are known to play a major role in defense against pathogens and tumors. In this study, we show that Notch receptors (mRNA and protein) are expressed in peripheral γδ T cells. Inhibition of Notch signaling by γ-secretase inhibitor inhibited the proliferation and IFN-γ secretion of γδ T cells in response to stimulation with phosphoantigens and anti-CD3 mAb. In the presence of γ-secretase inhibitor, the antitumor cytolytic ability of γδ T cells was inhibited with a decreased CD107a expression. Knockdown of Notch1 and Notch2 genes in γδ T cells using small interfering RNA inhibited their antitumor cytotoxic potential. Our study describes for the first time, to our knowledge, the role of Notch as an additional signal contributing to Ag-specific effector functions of γδ T cells. ***The Journal of Immunology*****, 2014, 192: 2054–2062.**

Compared with αβ T cells (>90%), γδ T cells are a minor fraction of T lymphocytes in the peripheral blood (<10%). γδ T cells differ from classical αβ T cells with respect to Ag recognition, tissue localization, and the use of TCR gene repertoire (1). Vγ9Vδ2 represents the dominant subset of the peripheral blood in humans (2). γδ T lymphocytes play a major role in defense against pathogens and tumors (3–5). γδ T lymphocytes are activated by phosphoantigens-isopentyl pyrophosphate (IPP) or 4-hydroxy-3-methyl-but-2-eneyl pyrophosphate, which is produced through the mevalonate pathway in mammalian cells or nonmevalonate/rohmer pathway in non-mammalian cells, respectively (6). Aminobisphosphonates are synthetic analogs of inorganic pyrophosphates and are widely used in the treatment of skeletal disorders (7). Nitrogen-containing bisphosphonates such as risedronate and zoledronate inhibit farnesyl pyrophosphate synthase, a key enzyme of the mevalonate pathway leading to accumulation of IPP pool in the cells (8). Upon activation, γδ T cells release copious amounts of IFN-γ and TNF-α (2, 5, 9). Earlier data from our own laboratory and others have shown that γδ T cells isolated from cancer patients can mediate potent antitumor immunity (10–12). Tumor cells treated with bisphosphonate zoledronate are actively lysed by activated γδ T cells (13, 14).

The Notch signaling pathway, originally described in drosophila, controls the development and activation of a variety of immune cells (15). Notch signaling is suggested to play a role in cell fate decisions and has been implicated in γδ versus αβ lineage decisions (16, 17). The molecular events triggering T cell development (γδ versus αβ lineage) are essentially different in human and mice. In mice, it was reported that the development of γδ T cells from γδ TCR-expressing T cell progenitors requires the absence of Notch ligand interaction (16–18). In contrast, there is an opposing role for Notch signal in human αβ/γδ lineage decision. The induction of γδ-lineage precursors to split off from the αβ T cell program by Notch1 activity was observed in humans (19). It was also reported that high level of Notch activation generates T lineage precursors and γδ T cells but inhibits differentiation toward αβ lineage (20).

The role of Notch in regulating effector functions of $CD8^+$ T cells and NK cells have been described (21, 22). Signaling through the TCR in both $CD4^+$ and $CD8^+$ T cells induces the activation of Notch1 (22, 23). However, the role of Notch in regulating the effector functions of human γδ T cells has not been reported earlier.

Notch proteins are single-pass transmembrane receptors that require multiple enzymatic cleavages to produce the full-length heterodimer expressed on the cell surface. In mammals, there are four Notch receptors (Notch1-4) and five Notch ligands, three delta-like (Dll1, Dll3, and Dll4) and two Jagged (Jag1 and Jag2) (24). The interaction between Notch receptor and ligand pair cleaves the extracellular and transmembrane domain of Notch through activation of ADAM proteases and γ-secretase, respectively (25). The γ-secretase–induced cleavage generates Notch intracellular domain (NICD), which translocates to the nucleus. In the nucleus, NICD binds to cofactors like CBF-1/suppressor of hairless/Lag1, mastermind like1, and p300/CBP to create a complex that acts as a transcriptional coactivator. Notch signaling then induces the expression of target genes, for example, HES1 (hairy and enhancer of split-1), HES-related repressor protein, and so on (26).

In this report, we describe the expression of Notch receptors on human γδ T cells in the peripheral blood of healthy individuals and further demonstrate the importance of Notch pathway in Ag-specific responses of γδ T cells. Our data demonstrate that Notch pathway also regulates the cytotoxic effector functions of γδ T cells against tumor cells. These results suggest that Notch signaling can be viewed as an additional mechanism regulating antitumor effector functions of γδ T cells.

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Abbreviations used in this article: BrHPP, bromohydrin pyrophosphate; Dll, delta-like; GSI-X, γ-secretase inhibitor-X; HES1, hairy and enhancer of split-1; IPP, isopentyl pyrophosphate; Jag, Jagged; LAMP1, lysosome-associated membrane protein-1; NICD, Notch intracellular domain; N1ICD, Notch1 intracellular domain; PI, propidium iodide; siRNA, small interfering RNA.

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Materials and Methods

γδ T cell expansion and purification

Blood samples were collected from healthy individuals. The study was approved by the institutional Ethics Committee, and written informed consent was obtained from the donor before collection of blood samples. PBMCs were isolated by differential density gradient centrifugation (Ficoll Hypaque; Sigma-Aldrich, St. Louis, MO), and γδ T cells were enriched from peripheral blood using plate-bound anti-CD3 (OKT3) mAb and rIL-2 (Peprotech, Rocky Hill, NJ). In brief, lymphocytes were cultured with RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum plus rIL-2 (100 U/ml), 2 mM glutamine, and antibiotics. Five milliliters of cell suspension (1×10^6/ml) was added to 25-cm^2 culture flasks (Nunc, Roskilde, Denmark) precoated with 1 mg/ml anti-CD3 mAb, as described in earlier study (27). Cells were incubated at 37˚C and fed daily with 1 ml growth medium containing 100 U/ml rIL-2. On the fifth day, cells were transferred to 75-cm^2 culture flask containing10 ml growth medium containing 500 U/ml IL-2. Cells were then subcultured after every 2 d with the addition of fresh growth medium until day 12. γδ T cells were purified from the expanded PBMCs using MicroBeads (Miltenyi Biotec, Bergish Gladbach, Germany). The separation procedure was conducted according to the manufacturer's instructions. The purity of separated cells was >95% as determined by flow cytometry (BD Biosciences, San Jose, CA).

Quantitative RT-PCR

RNA was extracted from immunomagnetically purified γδ T cells using TRIzol reagent (Invitrogen Life Technologies, Grand Island NY) in accordance with the company's instructions. Quantitative RT-PCR for different Notch receptor isoforms, ligands, and its target genes was performed with PRISM 7700 (PE Applied Biosystems, Foster City, CA). Samples were analyzed using TaqMan primer sets purchased from Applied Biosystems (NOTCH1 Hs01062011_m1, NOTCH2 Hs01050719_m1, NOTCH3 Hs01128541_m1, NOTCH4 Hs00270200_m1, DLL1 Hs00194509_m1, DLL3 Hs00213561_m1, DLL4 Hs00184092_m1, JAG1 Hs01070036_m1, JAG2 Hs00171432_m1, HES1 Hs00172878_m1, NF-κB Hs00765730_m1, ACTB [β-actin] Hs99999903_m1). All values were normalized to the expression of the housekeeping gene β-actin.

Western blotting

A total of 1×10^6 γδ T cells was incubated with rIL-2 (100 U/ml; Peprotech) and bromohydrin pyrophosphate (BrHPP/IPH1101), which was kindly provided by Innate Pharma (Marseille, France) at a concentration of 200 nM for 24 h. These cells were pretreated for 30 min at 37˚C with γ-secretase inhibitor-X, L-685,458 (GSI-X) (Calbiochem, La Jolla, CA) at a concentration of 15 μM, or left untreated, before stimulation. The expression of Notch1 intracellular domain (N1ICD) and c-Myc were analyzed by Western blot analysis. Whole-cell lysates (1×10^6 cells) were prepared in SDS sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue), vortexed to reduce sample viscosity, denatured by boiling, and then cooled on ice. Samples were resolved on 8% SDS-PAGE gels, transferred onto Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The primary Abs to N1ICD (R&D Systems, Minneapolis, MN), c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma-Aldrich) as loading control were added at 1:5000, 1:1000 and 1:1000 dilution, respectively. Immunostaining was performed using appropriate secondary Ab at a dilution of 1:1000 and developed with ECL plus Western blot detection system (Amersham Pharmacia).

Flow cytometry

Purified γδ T cells were rested overnight at 37˚C. Next day, these cells were rinsed in cold PBS and cold-fixed in 1% paraformaldehyde in PBS for 10 min at 4˚C. The cells were washed and permeabilized for 15 min with 0.1% saponin in PBS. Cells were stained with allophycocyanin-labeled mouse anti-human γδ TCR Ab (BD Bioscience, San Diego, CA), sheep anti-human N1ICD Ab, or goat anti-human Notch2 intracellular domain Ab (R&D Systems) for 45 min at 4˚C. Thereafter, cells were washed and incubated with FITC-labeled donkey anti-sheep IgG or FITC-labeled rabbit anti-goat IgG, respectively, for another 45 min at 4˚C. For cell-surface markers, nonpermeabilized cells were stained with labeled Abs for CD14-PerCP, CD15-Pacific Blue, CD19-PE, CD33-PECF594, and CD56-FITC (BD Biosciences, San Diego, CA). γδ T cells were also stained with rabbit anti-human Dll1 and Jag1 ligands (Calbiochem) for 45 min at 4˚C. Thereafter, cells were washed and incubated with FITC-labeled goat anti-rabbit IgG (Sigma Aldrich) for another 45 min at 4˚C. Appropriate isotype controls were used. The Annexin propidium iodide (PI) staining was performed to determine the effect of GSI-X on the cell viability. In brief, γδ T cells were left untreated or were stimulated with rIL-2 alone or rIL-2 and BrHPP for 48 h at 37˚C in round-bottomed, 96-well plates (Nunc). GSI-X was added as described earlier. Cells were then harvested, suspended in binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.25 mM $CaCl_2$), and incubated with PI and FITC-conjugated Annexin V (BD Biosciences) in dark for 15 min at room temperature. After incubation, 400 μl binding buffer was added and cells were analyzed. For analyzing cell-surface expression of activation markers, γδ T cells were left untreated or treated with rIL-2 and BrHPP for 24 h with GSI-X or left untreated as previously described. Cells were then incubated with FITC-conjugated CD69 or PE-conjugated CD25 (BD Biosciences) for 45 min in dark and subsequently washed with FACS buffer (0.01 M PBS pH 7.4, 1% FCS, 0.01% sodium azide), fixed with 1% paraformaldehyde, and the intensity of fluorescence was measured using flow cytometer (FACSAria; BD Biosciences).

For degranulation assay, purified γδ T cells were incubated alone or with rIL-2 (0.1 U; Peprotech) overnight at 37˚C in round-bottom, 96-well plates (Nunc) and were taken as effector cells. The target cells were oral cancer cell line, AW13516 (28), and were treated for 18 h with zoledronate (100 μM; Panacea Biotech, New Delhi, India). Cells were cocultured at an E/T ratio of 4:1 in the presence of monensin (5 μg/ml; Sigma-Aldrich). Anti–CD107a-PE Ab (BD Biosciences) was added at the start of coculture assay. After 4 h, cells were washed and γδ T cells were then stained using anti-human TCR-γδ FITC Ab (BD Biosciences), and were acquired and analyzed on flow cytometer for the expression of CD107a on γδ T cells.

For the above experiments, γδ T cells were gated on the basis of their forward and side scatter characteristics and the fluorescence intensity was measured. Cells were analyzed using FlowJo software (Tree Star, Ashland, OR).

Proliferation assays and cytokine ELISA

Proliferation of γδ T cells was assayed by [^{3}H]thymidine uptake assay. A total of 5×10^4 γδ T cells was pretreated for 30 min at 37˚C with GSI-X (Calbiochem, La Jolla, CA) at different concentration ranging from 2.5 to 15 μM, or left untreated, before cells were stimulated in round-bottom, 96-well tissue culture plates with rIL-2 (0.1 IU/ml; Peprotech) and plate-bound anti-CD3 mAb (1 μg/well; BD Biosciences) for 72 h.

Similarly, γδ T cells were also incubated in round-bottom, 96-well tissue culture plates with rIL-2 (0.1 IU/ml; Peprotech) plus BrHPP (200 nM; Innate Pharma) or IPP (40 μM; Sigma-Aldrich) or 20 nM c-HDMAPP (IPH1201/picostim; Innate Pharma) for 72 h. For experiments using GSI-X, cells were pretreated with 15 μM GSI-X. The cultures were pulsed with 1 μCi [^{3}H]thymidine (Board of Radiation and Isotope Technology, Mumbai, India) during the last 18 h of the assay. The radioactivity incorporated in the DNA was measured in a liquid beta scintillation counter (Packard, Meriden, CT).

For cytokine ELISA, γδ T cells were stimulated with anti-CD3 mAb with or without GSI-X as described earlier. Likewise, γδ T cells were treated with different phosphoantigens (IPP, BrHPP, and c-HDMAPP). After 24 h, supernatants were collected, and IFN-γ concentration was assayed with an ELISA-based assay using anti–IFN-γ purchased from BD Biosciences.

Cytotoxicity assay

[^{51}Cr] release assay was used to measure the cytotoxicity of γδ T cells against oral cancer cell line (AW13516) as target cells. γδ T cells were left alone or treated with rIL-2 (0.1 U; Peprotech) overnight at 37˚C, and AW13516 cells were treated for 18 h with zoledronate (100 μM; Panacea Biotech). For experiments using GSI-X, cells were pretreated as described earlier. Standard 4 h [^{51}Cr] release assay was performed as previously described (13). AW13516 cells were labeled with [^{51}Cr] for 90 min at 37˚C. Labeled target cells (AW13516) were incubated with effector cells (γδ T cells) at 40:1 E/T ratio at 37˚C in 5% CO_2 for 4 h. After incubation, plates were centrifuged, supernatants were collected, and radioactive chromium release was measured using 1470 Wallac automated gamma counter (Perkin-Elmer, Downers Grove, IL). Spontaneous release was determined by incubating the target cells with medium alone, and maximum release was determined by incubating target cells with 10% Triton X-100. The percent specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Small interfering RNA

γδ T cells isolated by MACS column (as described earlier) were transfected with small interfering RNA (siRNA) specific for NOTCH1, NOTCH2

genes, and fluorescent oligonucleotide SiGLO (transfection indicator; Thermo Fisher Scientific, Waltham, MA). siRNA oligos were transfected at a concentration of 50 nM using X-tremeGENE HP transfection reagent (Roche Diagnostics, Indianapolis, IN). The inhibition of Notch1 and Notch2 expression were assessed at 48 h.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0. The Student *t* test was used as the test of significance.

Results

Expression of Notch receptors and ligands in human peripheral blood γδ T cells

The mRNA expression of Notch genes (Notch1-4) and its ligands Dll1, Dll3, and Dll4 and Jag1 and Jag2 were quantitated in ex vivo expanded and purified γδ T cells. The mRNA expression of Notch2 gene was higher than Notch1 (Fig. 1A). Relatively very low expression of Dll1 and Jag1 mRNA was observed. mRNA of Notch3, Notch4, Dll3, Dll4, and Jag2 was not detected in γδ T cells. After Notch activation, NICD enters the nucleus and then regulates the expression of target genes (25). Flow cytometry showed that Notch receptors (Notch1 and Notch2) are dominantly expressed on γδ T cells (Fig. 1B), although a low-level expression of ligands Dll1 and Jag1 are observed. The purity of isolated γδ T cells was analyzed by flow cytometry (95 ± 5%) and further, the contamination of non–T cell was ruled out by staining isolated γδ T cells for CD14, CD15, CD19, CD33, and CD56 markers (Fig. 1C). Cells were positive for CD56, which is a marker expressed by γδ T cells. The expression of NICD in γδ T cells further confirmed that Notch signaling may be active in γδ T cells.

Disruption of Notch signaling in activated γδ T cells reduces expression of Notch receptor and target genes

The release of NICD mediated by γ-secretase activity is required by all Notch receptors (Notch1-4) to initiate downstream signaling (29). In this study, we used GSI-X to block γ-secretase activity in γδ T cells (30). γδ T cells were stimulated with rIL-2 and BrHPP in the presence and absence of GSI-X. Stimulation of γδ T cells with BrHPP and rIL-2 triggered the activation of Notch signaling, which can be observed by abundant release of N1ICD by Western blot analysis (Fig. 2A). Treatment of BrHPP and rIL-2 activated γδ T cells with GSI-X decreased release of processed Notch1 (decreased NICD expression) compared with γδ T cells stimulated with BrHPP and rIL-2 alone (Fig. 2A). Stimulation of the Notch signaling pathway leads to the induction of c-Myc expression (31). Finally, we assessed whether activation of γδ T cells with BrHPP and rIL-2 was necessary for the Notch-mediated induction of cell-cycle regulator, c-Myc. We observed that Notch1 activation governs the downstream induction of c-Myc expression, which was abrogated upon GSI-X treatment (Fig. 2A). Simultaneously, we also monitored expression of mRNA for Notch receptors (1–4) and Notch ligands (Dll1, Dll3, Dll4, Jag1, and Jag2) in γδ T cells stimulated with BrHPP and rIL-2 in the presence and absence of GSI-X (Fig. 2B). After treatment with

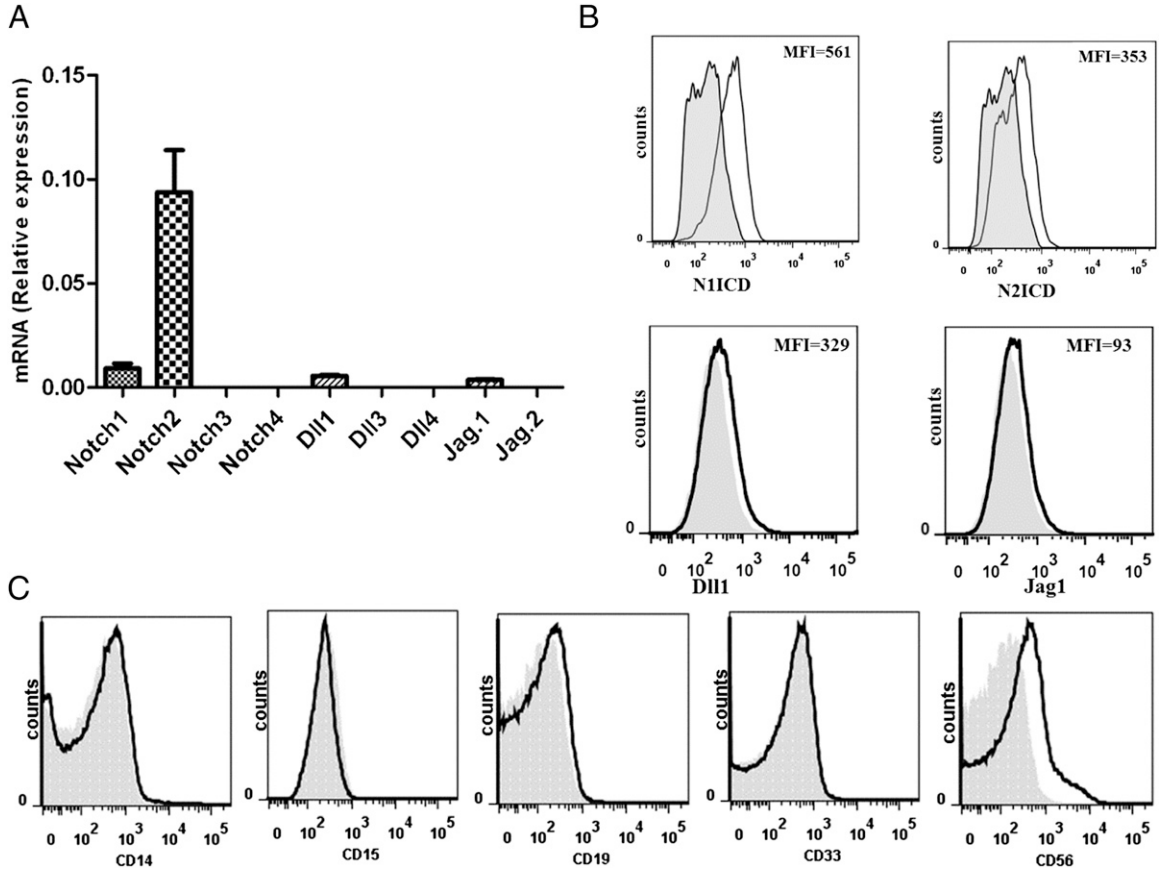

FIGURE 1. Notch receptors are expressed on peripheral γδ T cells. γδ T cells were MACS purified from the peripheral blood. (**A**) Real-time quantitative PCR showed mRNA expression of Notch1, Notch2, Dll1, and Jag1 on γδ T cells. Data were normalized to expression of GAPDH. Data represent mean of four independent experiments. (**B**) Expression of N1ICD, Notch2 intracellular domain, Dll1, and Jag1 on γδ T cells. The MFI in each case has been corrected for the MFI of the isotype control. (**C**) Purified γδ T cells were negative for CD14, CD15, CD19, and CD33 receptors, and were positive for CD56 marker. Filled histogram indicates the isotype control.

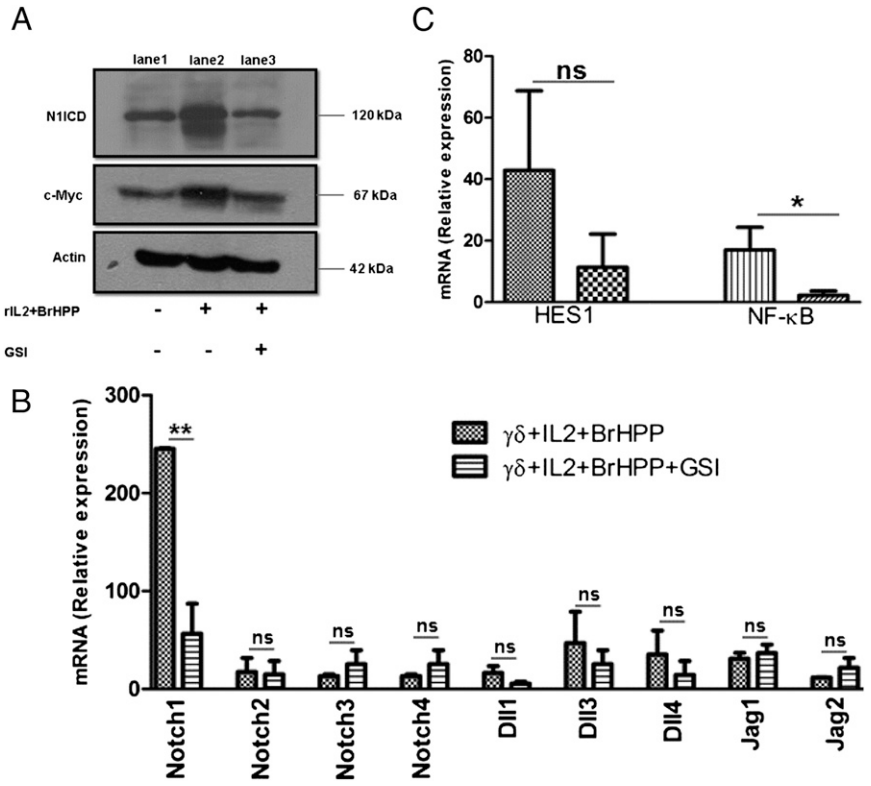

FIGURE 2. GSI-X treatment leads to decreased expression of Notch receptors and its target genes in Ag-activated γδ T cells. (**A**) Western blotting for detection of 120-kDa NICD and 67-kDa c-Myc in unstimulated (*lane 1*), BrHPP, and rIL-2–activated (24 h) γδ T cells in the presence (*lane 3*) or absence of GSI-X (*lane 2*), using Abs that recognize the cleaved active form of Notch1 (N1ICD) and c-Myc. (**B**) Real-time PCR was performed for all the notch receptor isoforms (Notch1-4) and its ligands (Dll1, Dll3, and Dll4; Jag1 and Jag2) on γδ T cells. (**C**) γδ T cells were analyzed for both canonical (HES1) and noncanonical (NF-κB) target gene expression. In both (B) and (C), total RNA was extracted from BrHPP and IL-2–stimulated γδ T cells (4 h) with or without GSI-X treatment. The expression of specific mRNA is relative to GAPDH and is normalized to that same ratio in unstimulated cells. The Student *t* test was used as the test of significance (*$p < 0.05$, **$p < 0.005$).

GSI-X, a marked decrease in the expression of mRNA for Notch1 receptor was observed in Ag-activated γδ T cells (Fig. 2B).

In addition to the well-known canonical Notch signaling pathway, evidence has emerged that noncanonical Notch signaling may also play an important role in T cells (32). We therefore investigated the expression of two target genes Hes1 and NF-κB in γδ T cells, which are representatives of canonical and noncanonical Notch signaling pathway, respectively. Using real-time PCR, we demonstrated that relative expression of mRNA for Hes1 and NF-κB were reduced in BrHPP and rIL-2 activated γδ T cells upon treatment with GSI-X (Fig. 2C).

Inhibition of Notch signaling blocks γδ T cell activation and proliferation

To determine the functional consequences of Notch signal in activated γδ T cells, we assessed γδ TCR-mediated proliferation response by [^{3}H]thymidine incorporation assay. Purified γδ T cells were pretreated with different concentrations of GSI-X (15, 10, 5, and 2.5 μM) or left untreated, before cells were activated with plate-bound anti-CD3 mAb and rIL-2. A marked increase in the proliferation of γδ T cells was observed in the presence of anti-CD3 mAb and rIL-2. However, blocking of Notch signal by GSI-X leads to decreased proliferative response

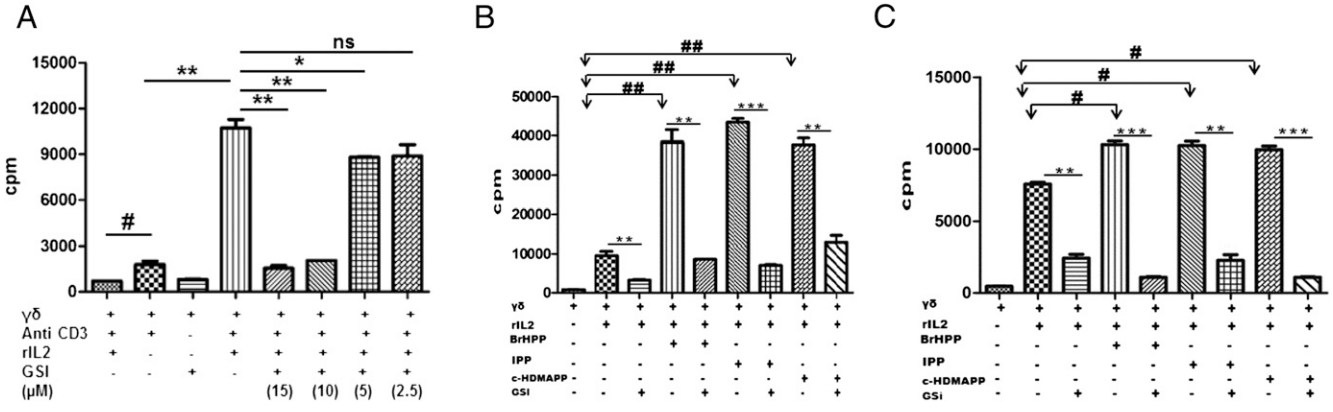

FIGURE 3. Inhibition of Ag-driven proliferation of γδ T cells by GSI-X. (**A**) Ex vivo–expanded γδ T cells were stimulated with anti-CD3 mAb in the presence of rIL-2, for 72 h, with or without GSI-X treatment. Addition of GSI-X leads to decrease in the proliferative response of γδ T cells in a concentration-dependent manner (15, 10, 5, and 2.5 μM). (**B**) Freshly isolated γδ T cells were stimulated with phosphoantigens (BrHPP, IPP, and c-HDMAPP), for 72 h, in the presence of rIL-2 with or without GSI-X treatment (**C**) Ex vivo–expanded γδ T cells were stimulated with BrHPP, IPP, and c-HDMAPP, for 72 h, in the presence of rIL-2 with or without GSI-X treatment. Addition of GSI-X leads to decrease in the proliferative response of γδ T cells. Proliferation was determined by [^{3}H]thymidine incorporation assay. Results represent the mean ± SE of cpm of three replicates studied in each experiment. Data shown are representative figures from three independent experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, compared with cpm of the cells without GSI-X treatment. $^{\#}p < 0.05$, $^{\#\#}p < 0.005$, compared cpm of cells treated with rIL-2 alone.

in a concentration-dependent manner (Fig. 3A). We also used freshly isolated, as well as ex vivo–stimulated and expanded, γδ T cells to compare their ability to respond to phosphoantigens in the presence and absence of GSI-X (15 μM). We observed a significantly increased proliferative response of γδ T cells to phosphoantigens (BrHPP, IPP, and c-HDMAPP) in the presence of rIL-2 (Fig. 3B, 3C). Freshly isolated γδ T cells showed robust proliferative responses to Ags compared with ex vivo–expanded γδ T cells. However, in the presence of GSI-X, the proliferative responses of γδ T cells to Ags were significantly reduced in both sets of isolated γδ T cells (Fig. 3B, 3C).

The expression of early and late activation markers, CD69 and CD25, respectively, were analyzed on unstimulated and Ag-stimulated

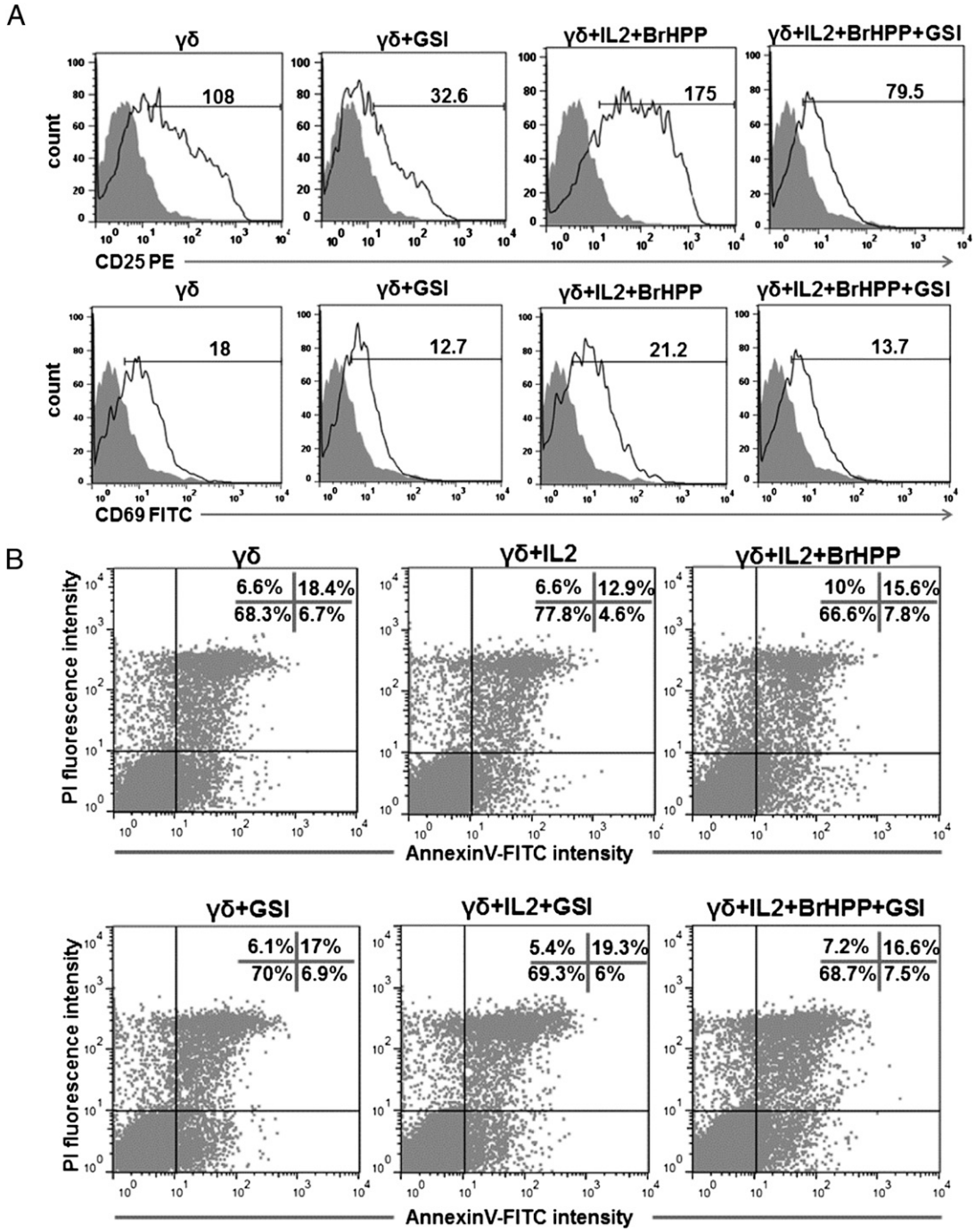

FIGURE 4. GSI-X inhibits the expression of activation markers on γδ T cells but does not induce apoptotic or necrotic cell death in γδ T cells. (**A**) The effect of GSI-X on cell-surface expression of late (CD25) and early (CD69) activation markers on unstimulated and BrHPP-stimulated (24 h) γδ T cells was analyzed by flow cytometry. Blocking of Notch signaling by GSI-X inhibits the surface expression of activation markers. Data indicate the MFI of the activation markers. Dark shaded histogram indicates isotype control. (**B**) Annexin V and PI staining of unstimulated γδ T cells (γδ), rIL-2–activated γδ T cells (γδ+IL-2), and γδ T cells stimulated with BrHPP in the presence of rIL-2 (γδ+IL-2+BrHPP) without GSI-X (*upper panel*) or with GSI-X (*lower panel*). These cells were stained after culturing for 24 h and analyzed by flow cytometry. The unaffected, early apoptotic, late apoptotic, and necrotic cells are present in the lower left, lower right, upper right, and upper left quadrant, respectively. Dot plots show the mean percentage of positive cells. Results shown are representative of three independent experiments.

γδ T cells with or without GSI-X treatment. Ex vivo–expanded γδ T cells alone (unstimulated) or after stimulation with BrHPP and rIL-2 showed higher expression of CD25 compared with CD69. Treatment with GSI-X showed a moderate reduction in CD69 expression. A marked reduction in the expression of late activation marker CD25 was observed on both unstimulated and BrHPP-stimulated γδ T cells (Fig. 4A).

We then investigated whether treatment with GSI-X results in apoptotic/necrotic cell death of Ag-activated γδ T cells. To verify, we compared the effects of GSI-X on the frequency of apoptotic cells in unstimulated γδ T cells and after stimulation with rIL-2 alone or with both rIL-2 and BrHPP. We did not observe differences in the frequency of early apoptotic (Annexin V^+), late apoptotic (apoptotic V^+ PI^+), and necrotic (PI^+) γδ T cells in untreated compared with GSI-X–treated cells (Fig. 4B).

Notch regulates cytolytic potential of γδ T lymphocytes

Notch signaling has been reported to regulate cytotoxic responses in both CTL and NK cells (33, 34). The effect of Notch signaling in cytolytic potential of γδ T lymphocytes was examined. Purified γδ T cells were coincubated for 4 h with zoledronate-treated oral cancer cells. We evaluated CD107a (lysosome-associated membrane protein-1 [LAMP1]) expression, a marker of degranulation in unstimulated and rIL-2–stimulated γδ T cells in the presence and absence of GSI-X. Upon coincubation with the oral cancer cells, few γδ T cells showed surface expression of CD107a (18.4%). However, in the presence of rIL-2, the proportion of $CD107a^+$ γδ T cells increased (40.9%; Fig. 5A). Addition of GSI-X leads to reduction in the percentage of $CD107a^+$ in both unstimulated γδ T lymphocytes (13.1%) and rIL-2–stimulated γδ T cells (12.4%). The cytotoxic potential of γδ T cells against zoledronate-treated tumor cells (AW13516) was determined by

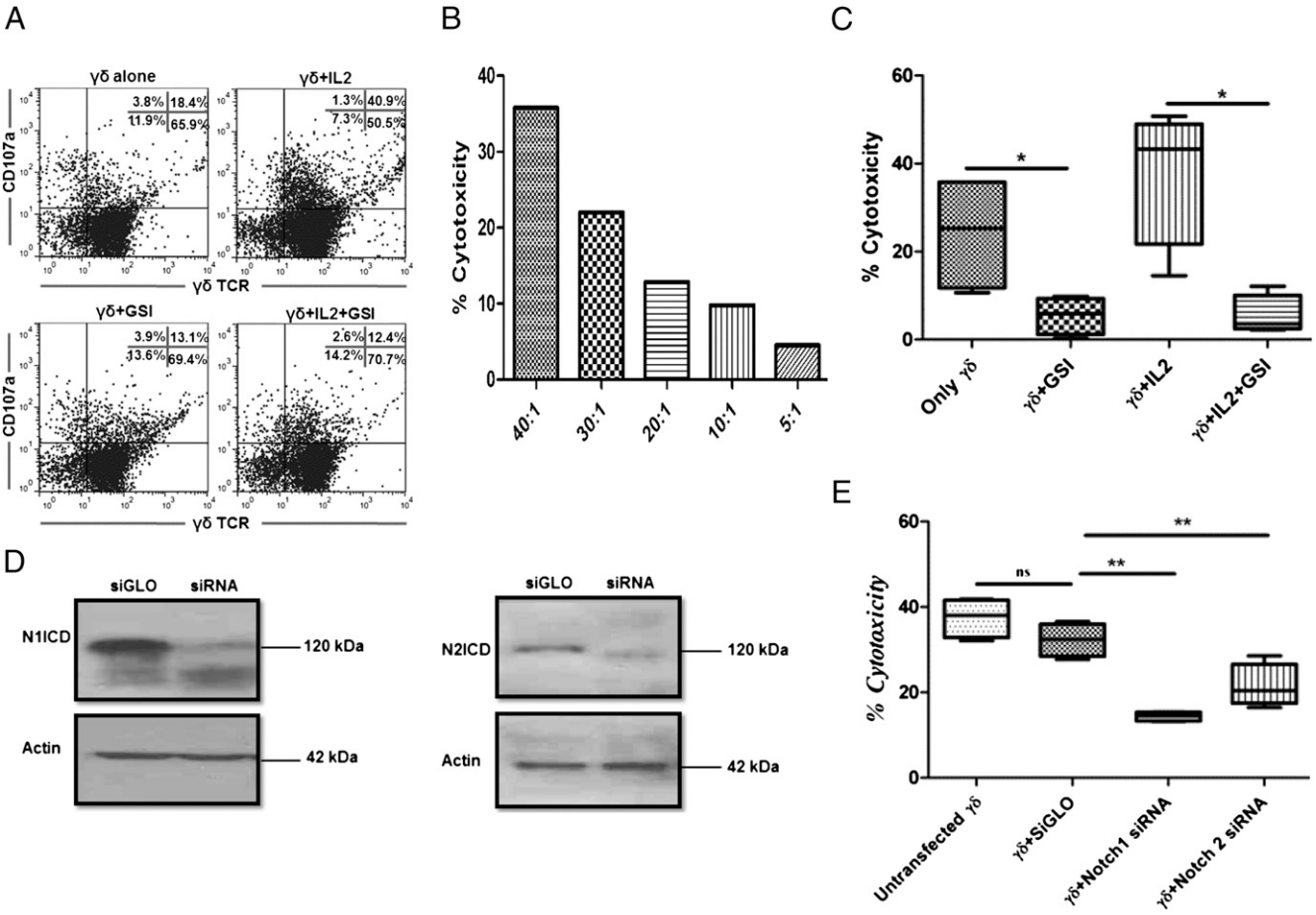

FIGURE 5. Notch signaling regulates cytolytic effector functions of γδ T cells. (**A**) Expression of CD107a or LAMP1 (marker for degranulation) on γδ T cells. Effector γδ T cells alone or in the presence of rIL-2 were cocultured with target oral cancer cell line (AW13516) at the ratio of 4:1 and incubated for 4 h. AW13516 were previously treated with zoledronate (100 μM) for 16 h. Decreased expression of CD107a was observed when the cells were treated with GSI-X (*lower panel*). Panel is representative of three experiments. (**B**) Zoledronate-treated AW13516 cells labeled with [^{51}Cr] used as target cells were cocultured with γδ T cells at different E/T ratios in a standard 4-h [^{51}Cr] release assay. Data represent percentage cytotoxicity as described in *Materials and Methods*. Representative data of an independent experiment. (**C**) Cytolytic ability of γδ T cells alone or in the presence of rIL-2 against zoledronate-treated AW13516 was determined by [^{51}Cr] release assay at the ratio of 40:1. Treatment of γδ T cells with GSI-X blocked the target cell lysis. *$p < 0.05$ compared with percentage cytotoxicity of both γδ T cells and γδ T cells with rIL-2 without GSI-X. Results are mean percentage cytotoxicity of γδ T cells from five healthy individuals. (**D**) γδ cells were transfected with 50 nM control (SiGLO) or different Notch-specific SiRNA duplexes. Forty-eight hours post-transfection, cells were harvested and samples were subjected to Western blot analysis for Notch1 and Notch2 (*top panel*) or β-Actin (*bottom panel*). The latter was included as loading/normalization control. (**E**) γδ T cells were transfected with 50 nM control (SiGLO) or specific Notch1 and Notch2 siRNA duplexes, Forty-eight hours posttransfection, the cytolytic effector function of rIL-2–activated γδ T cells against zoledronate-treated AW13516 was determined by [^{51}Cr] release assay at the ratio of 40:1. Silencing of either Notch1 or Notch2 inhibits target cell lysis by γδ T cells. **$p < 0.005$ compared with percentage cytotoxicity of Notch-specific siRNA with SiGLO-treated γδ T cells. The results are mean percentage cytotoxicity of γδ T cells from five healthy individuals.

titration at different E/T ratio ranging from 5:1 to 40:1. As seen in Fig. 5B, maximum cytotoxicity of γδ T cells was observed at E/T ratio of 40:1. This ratio of E/T was used in further experiments where effect of GSI-X on cytolytic ability of γδ T cells was examined. At E/T ratio of 40:1, ex vivo–expanded γδ T cells in the presence of rIL-2 efficiently lysed zoledronate-treated oral tumor cells compared with untreated cells. Addition of GSI-X significantly reduced the cytotoxic ability of γδ T cells against zoledronate-treated tumor targets (Fig. 5C).

Because pharmacological inhibition of the γ-secretase complex may have nonspecific effects, we assessed the contribution of the Notch pathway in regulating the cytotoxic potential using siRNA-mediated knockdown of individual Notch receptors. The synthetic siRNA sequences targeting the Notch1 and Notch2 were transfected into the γδ T cells. γδ T cells were transfected with 50 nM control (SiGLO) or different Notch-specific siRNA duplexes. After 48 h, cells were harvested and lysates were prepared. Western blot analysis for Notch1 and Notch2 showed that siRNA transfection led to the reduced expression of Notch1 and Notch2 receptors compared with fluorescent oligonucleotide SiGLO (Fig. 5D). Next, we compared antitumor cytotoxic ability of γδ T cells transfected with siRNA specific for Notch1 and Notch2 against zoledronate-treated tumor cells (AW13516). Cytotoxicity of γδ T cells was observed at E/T ratio of 40:1 and consistent with GSI-X treatment data (Fig. 5C); silencing of both Notch1 and Notch2 led to significant reduction in cytotoxic potential of γδ T cells (Fig. 5E). This observation confirms that cytotoxic potential of γδ T cells is regulated by Notch signaling.

Notch signaling regulates cytokines production in activated γδ T cells

To address whether Notch signal has any role in the effector functions of activated γδ T cells, we examined the effect of GSI-X on cytokine production. The consequence of GSI-X treatment on the concentration of TH1 (IL-2, IFN-γ, and TNF-α), TH2 (IL-4, IL-6, and IL-10), and TH17 (IL-17) production by γδ T cells treated with rIL-2 alone or along with BrHPP was assayed. It was observed that inhibition of Notch signaling leads to marked reduction in TNF-α, IFN-γ, and IL-17 production by activated γδ T cells (data not shown). The inhibition was more pronounced in IFN-γ production. We therefore confirmed the effect of GSI-X on IFN-γ production by ELISA. Activated human γδ T cells secrete IFN-γ, and Notch signaling regulates IFN-γ secretion in activated $CD4^+$ T cells (5, 23). γδ T cells were stimulated with c-HDMAPP, IPP, and BrHPP, which leads to increased production of IFN-γ. The amount of IFN-γ decreased when the cells were pretreated with GSI-X (Fig. 6A). Like phosphoantigens, anti-CD3 mAb stimulation of γδ T cells was also inhibited by GSI-X, which is depicted by dose-dependent decrease in IFN-γ production (Fig. 6B). Thus, a role of Notch in regulating IFN-γ production in γδ T cells is unraveled, which suggests that Notch plays a role in effector functions of γδ T cells.

Discussion

The role of Notch signaling in T cell differentiation, activation, and effector functions have been well documented in CD4 and CD8 T cells (22, 23). However, there is a lacuna of studies addressing the role of notch signaling in effector functions of human γδ T cells. A recent report showed that Hes1 is critically involved in the development of IL-17–producing γδ T cells (35). In these investigations, we report for the first time, to our knowledge, that peripheral human γδ T cells express notch receptors and notch signaling is required in Ag-specific responses of γδ T cells. We report Notch1 and Notch2 expression in γδ T cells at mRNA and protein level as analyzed by real-time PCR and flow cytometry. γδ T cells are known to be activated by phosphoantigens, for example, IPP and 4-hydroxy-3-methyl-but-2-eneyl pyrophosphate, which are intermediates of the mevalonate pathway of cholesterol metabolism in eukaryotic cells and rohmer pathway in prokaryotic cells (6). Using BRHPP, a synthetic analog of IPP in the presence of pharmacological GSI-X, we show that loss of Notch signaling in γδ T cells results in downregulation of mRNA for Notch1 and Notch2 receptors. Reduction in the expression of NICD by GSI-X in BrHPP-stimulated γδ T cells further confirmed the involvement of Notch signaling in regulating Ag-specific responses of γδ T cells. Activation of the Notch signaling pathway leads to the induction of c-Myc expression in immune cells (36), which was also observed in γδ T cells. Recent reports suggest that Notch signaling involves both canonical and noncanonical pathways in T cells, and these interactions will influence cell fate decisions and functions (32, 37). We therefore analyzed the expression of

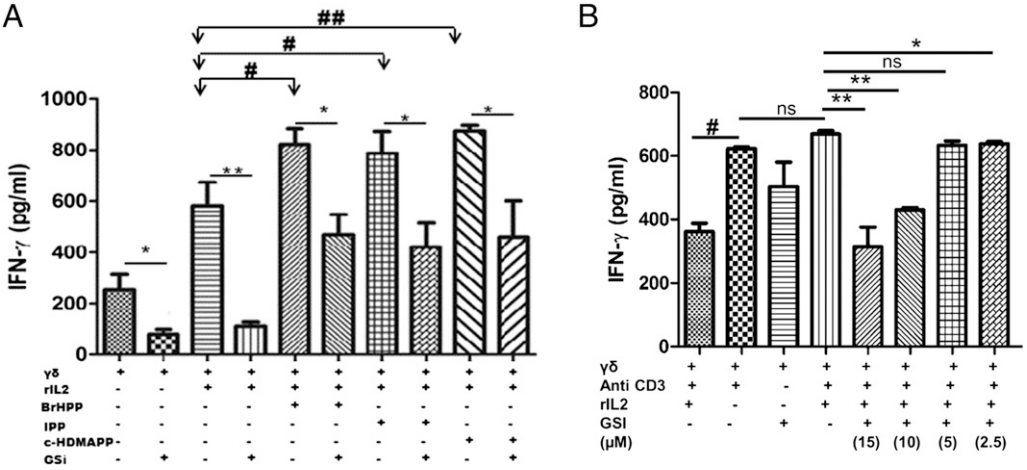

FIGURE 6. Treatment with GSI-X blocks IFN-γ production by γδ T cells. (**A**) γδ T cells alone, γδ T cells with rIL-2, or along with three different phosphoantigens (BrHPP, IPP, and c-HDMAPP) were cultured in the presence or absence of GSI-X for 24 h in 96-well plates. (**B**) γδ T cells were either cultured alone, or in the presence of anti-CD3 mAb or with rIl2 and in the presence or absence of different concentration of GSI-X (15, 10, 5, and 2.5 μM) for 24 h in 96-well plates. Supernatants were collected, and level of IFN-γ was measured by sandwich ELISA. GSI-X treatment decreases IFN-γ production by both unstimulated and stimulated γδ T cells. Results shown are mean of three experiments. $^*p < 0.05$, $^{**}p < 0.005$, compared with IFN-γ production of the cells without GSI-X treatment. $^{\#}p < 0.5$, $^{\#\#}p < 0.05$, compared with cpm of cells treated with rIL-2 alone.

mRNA for Hes1 and NF-κB in BrHPP-stimulated γδ T cells after inhibiting the Notch signaling by GSI-X. A decrease in the Notch target gene NF-κB indicates that noncanonical Notch signaling pathway is active in activated γδ T cells.

Further, inhibiting the Notch signaling in anti-CD3 mAb-stimulated γδ T cells resulted in marked decrease in proliferation of γδ T cells, confirming TCR engagement as a key initiating event affected by GSI-X treatment. Similarly, γδ T cells (freshly isolated and ex vivo expanded) activated with BrHPP, IPP, and c-HDMAPP as Ags resulted in a significantly decreased proliferation of γδ T cells in response to these Ags. The observation that Notch signaling is involved in regulating Ag-specific proliferative responses of γδ T cells prompted us to look at the expression of early and late activation markers CD69 and CD25 on γδ T cells stimulated with the Ag (BrHPP) in the presence and absence of GSI-X. Reduction in CD69 expression and a marked decrease in CD25 (IL-2R) expression on Ag-stimulated γδ T cells was observed when Notch signaling was inhibited. Various reports have shown that Notch signaling induces the expression of CD25 in immune cells (38–40). NF-κB, which is downstream of Notch signaling, also regulates the expression of CD25 (41). γδ T cells are dependent on IL-2 for their growth and survival, and express high-affinity CD25 (42). It is therefore not surprising that CD25 expression in γδ T cells is also regulated by Notch signaling, and IL-2 stimulation will thereby enhance the proliferative response and cytokine production of γδ T cells via IL-2R (CD25).

The importance of Notch signaling in mediating cytotoxic responses in immune cells has been well documented. Earlier studies carried out in murine $CD8^+$ T lymphocytes and NK cells have demonstrated the importance of Notch signaling in regulating their effector functions (22, 33). Notch signaling was shown to directly regulate granzyme B expression in $CD8^+$ cytotoxic T lymphocytes (34). The involvement of Notch in antitumor immunity was further supported by studies that showed that deficiency of Notch2 decreased the antitumor responses of $CD8^+$ T cells in mice models (43). Notch signaling also contributes to dendritic cell–mediated NK cell activation, which enhanced the killing activity of NK cells. Murine NK cells exhibit enhanced cytokine expression and cytotoxic function in response to signaling from tumor cells or dendritic cells transduced with Jag2 (33).

We therefore expected that Notch signaling may similarly regulate cytotoxic effector functions of γδ T cells. We showed that blocking of Notch signaling in γδ T cells by GSI-X inhibits the ability of γδ T cells to lyse tumor targets. We used oral tumor cell line AW13516 treated with zoledronate as target cell line. Earlier data from our laboratory have demonstrated that tumor cells treated with zoledronate are aggressively killed by γδ T cells (10, 13). Our data demonstrate that treatment with GSI-X blocks the ability of γδ T cells to lyse both untreated and zoledronate-treated tumor cells. Moreover, we found that specific silencing of either Notch1 or Notch2 by siRNA led to the reduced cytotoxic potential of γδ T cells. This result suggests that both Notch1 and Notch2 are involved in the cytolytic activity of γδ T cells. Cho and colleagues (22) demonstrated that Notch1 regulates expression of eomesodermin, perforin, and granzyme B through direct binding to the promoters of these effector molecules. In $CD8^+$ T cells, Notch2 signaling was shown to directly control CTL effector molecules including granzyme B, by integrating RBP-J and CREB1 (40). We observed that GSI-X treatment of γδ T cells alone or γδ T cells activated with IL-2 results in a reduction of CD107a expression in these cells. CD107a or LAMP1 is a marker of degranulation in cytotoxic T lymphocytes (44–46).

γδ T cells are known to secrete copious amounts of IFN-γ. IFN-γ plays a crucial role in protective immune response against certain pathogens and tumors (47–49). We showed that blocking Notch signaling with GSI-X inhibited the IFN-γ secretion by γδ T cells stimulated with phosphoantigens BrHPP, IPP, and c-HDMAPP. Likewise, we observed that GSI-X inhibits IFN-γ production by anti-CD3 mAb–activated γδ T cells. γδ T cells provide an early source of IFN-γ in tumor immunosurveillance and against viral challenge (47, 50). Epigenetic program that regulates IFN-γ gene transcription in γδ T cells is different from $CD4^+$ and $CD8^+$ T cells (51). Eomesodermin contributes to T bet–independent IFN-γ production in γδ T cells (51). We observed that BrHPP-stimulated γδ T cells produced IL-17, and its secretion was inhibited in the presence of GSI-X (data not shown). γδ T cells have been reported to be a potent source of IL-17 (52, 53). It has been reported that DLL4 upregulates RORC expression in T cells, and both RORC and IL-17 gene promoters are direct transcriptional notch targets and enhance Th17 cell population (54).

In this study, we describe for the first time, to our knowledge, the role of Notch in regulating the effector functions of human γδ T cells. Notch signaling appears to play an important role in modulating the Ag-specific proliferation of γδ T cells, their ability to lyse tumor targets and secrete IFN-γ upon stimulation. Taken together, these studies identify Notch as an additional signal contributing to Ag-specific effector functions of γδ T cells. These studies may have important implications in clinical situations where new strategies for the clinical manipulation of γδ T cells for cancer immunotherapy are being investigated.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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Review Article

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Targeting gamma delta T cells for cancer immunotherapy: bench to bedside

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γδ T lymphocytes represent a minor subset of peripheral blood in humans (<10%). γδ T cells expressing Vγ9Vδ2 T cell receptor recognise the endogenous pool of isopentenyl pyrophosphate (IPP) that is overproduced in cancer cells as a result of dysregulated mevalonate pathway. Aminobisphosphonates increase the endogenous pool of IPP in cells by blocking the enzyme farnesyl pyrophosphate synthase (FPPS) of the mevalonate pathway. Activated γδ T cells release copious amounts of interferon (IFN)-γ and tumour necrosis factor (TNF)-α and exhibit potent anti-tumour activity. Combination of γδ T cells with therapeutic monoclonal antibodies can efficiently mediate antibody dependent cellular cytotoxicity against tumours. These features makes γδ T cells attractive mediator of cancer immunotherapy. We review here, the basic properties and importance of γδ T cells in tumour immunity, and highlight the key advances in anti-tumour effector functions of γδ T cells achieved over the last few years and also summarize the results of the clinical trials that have been done till date. Future immunotherapeutic approach utilizing γδ T cells holds considerable promise for treatment of different types of cancer.

Key words Aminobisphosphonates - anti-tumor cytotoxicity - clinical trials - immunotherapy - γδ T cells - phosphoantigens

Introduction

The immune system has evolved to protect the host from infections and cancer. Typically, the immune system is divided into two categories- innate immunity and adaptive immunity. The innate immune system comes into play immediately after the appearance of antigen whereas the adaptive immune system provides antigen-specific response. In addition to these defense mechanisms, there are unconventional T cells like the gamma delta (γδ) T lymphocytes and natural killer T (NKT) cells that functionally and phenotypically belong to both the innate and the adaptive immune system and are able to bridge the two[1-3]. In the peripheral circulation of humans, γδ T cells comprise about 1-10 per cent of the circulating T cells, though this percentage can rise to as high as 50 per cent at some mucosal sites[4]. γδ T cells are involved in combating infectious diseases and have non-redundant capacities in the inhibition of tumour development and progression[5,6].

Antigen recognition and activation of γδ T lymphocytes

Unlike αβ T cells, γδ T cells do not require the help of conventional major histocompatibility complex

(MHC) class I and class II molecules for recognizing the antigens[1]. Antigen recognition by γδ T cells is dependent upon the particular variable (V) region of the T cell receptor (TCR) as opposed to the entire rearranged TCR required by αβ T cells. γδ T cells expressing Vδ1 are abundantly found at mucosal sites and these respond to the expression of non-classical MHC molecules on the surface of virally-infected or tumour cells[7-9]. Vδ2+ (Vγ9Vδ2) cells are predominantly present in the peripheral circulation and respond to non-peptide phosphoantigens[10,11]. Vγ9Vδ2 T cells recognizes self and microbial phosphorylated metabolites generated in the eukaryotic mevalonate pathway and in the microbial 2-C-methyl-D-erythritol 4-phosphate (MEP) or non-mevalonate pathway[12]. It was observed that during bacterial and protozoan infections, Vγ9Vδ2 T cells expand to high levels which in some individuals represented the majority of circulating T cells[13]. The first chemically defined antigens for Vγ9Vδ2 were found to be alkyl phosphates[14]. One natural antigen from mycobacteria was isolated and identified as isopentenyl pyrophosphate (IPP)[15]. Subsequent characterization of the microbial antigens recognized by human γδ T cells revealed that these are non-proteinaceous in nature and have critical phosphate residues[11,16]. The Vγ9Vδ2 crystal structure confirmed the presence of a basic, positively charged region in the binding groove that could directly interact with the negatively charged pyrophosphate moiety of the antigen[10]. These phosphoantigens are generated during the non-mevalonate and mevalonate pathways utilized by prokaryotic and eukaryotic cells, respectively[12,17,18]. Various compounds like steroid hormones, cholesterol, many types of vitamins, rubber, *etc.* are derived from this pathway. There are now many synthetic phosphorylated compounds that are capable of stimulating γδ T cells like bromohydrin pyrophosphate (BrHPP), 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) and mono-ethyl pyrophosphate[14,19,20]. In addition to phosphoantigens, there are reports on additional ligands for human γδ T cells. Bisphosphonates, especially nitrogen-containing bisphosphonates (NBP) are widely used to treat postmenopausal osteoporosis and skeletal malignancies. NBP like pamidronate, alendronate, zoledronate, *etc.* inhibit the key enzyme farnesyl pyrophosphate synthase (FPPS) of the mevalonate pathway, thereby upregulating the pool of endogenous IPP. The accumulated IPP activates Vγ9Vδ2 T cells to release inflammatory cytokines interferon (IFN)-γ and tumour necrosis factor (TNF)-α. Another class of molecules that stimulate Vγ9Vδ2 T cells is alkylamines. Alkylamines are secreted by certain commensal bacteria. These are also present in edible plant products such as tea, wine, apples and mushrooms[21]. Alkylamines act in a manner similar to NBP[22]. γδ T cells discriminate transformed tumour cells from healthy cells by the upregulation of self-antigens like heat shock proteins (HSP). The expression of these proteins are increased in tumour cells due to higher metabolism and serves as endogenous danger signals[6,23]. Increased cytotoxicity of γδ T cells was observed against transformed cell lines expressing hsp60/70[24]. Studies from our laboratory have demonstrated that Vγ9Vδ2 T cells recognize hsp60 on oral tumour cells and have the ability to lyse autologous and allogenic oesophageal tumour targets via recognition of hsp60 and hsp70[25,26].

Migration of γδ T cells to the tumour site

The influx of TILs (tumour infiltrating lymphocytes) to the tumour site enhances the potential for anti-tumour immune responses. The numbers and types of lymphocytes present in the infiltrate are related to the chemokines produced by both the tumour cells and tissue stromal cells located at the tumour site. The infiltration of circulating lymphocytes to the tumour is facilitated by these chemokines. For example, breast, cervix and pancreatic tumours as well as ovarian tumour produce CC and CXC chemokines that are important mediators of macrophage and lymphocyte infiltration in those tumours[27-29]. Interestingly, both Vδ1 and Vγ9δ2 T cells display distinct chemokine receptors that bestow these cells the property to migrate to the tumour site. Vδ1 express CCR5 and Vγ9δ2 express both CCR5 and CXCR3[30]. In addition, Vγ9δ2 T cells express NK receptor P1A (NKR-P1A) platelet endothelial cell-adhesion molecules (PECAM) while Vδ1 use NK receptor P1A NKR-P1A for transendothelial migration[31]. Vδ1 T cell subsets from the peripheral blood utilize a larger array of adhesion molecules, namely LFA-1, VLA-α4, VLA-α5, L-selectin and αEβ7, to bind to squamous cell carcinoma cells compared to the restricted usage of LFA-1, L-selectin and CD44v6 by the Vδ2 T cells[32]. The mutually exclusive pattern of chemokine receptor expression in both the subsets of γδ T cells indicates independent mechanism of homing to tumour site that might have an important aspect in cancer immunotherapy.

Anti-tumour activity of γδ T lymphocytes

Ability of γδ T lymphocytes to produce abundant proinflammatory cytokines like IFN-γ, potent cytotoxic effector function and MHC-independent recognition of antigens makes it an important player of cancer immunotherapy. γδ T cells kill many different types

of tumour cell lines and tumours *in vitro*, including leukemia, neuroblastoma and various carcinomas[33-36].

Accumulation of mevalonate metabolites in tumour cells is a powerful danger signal that activates the γδ T cells. In normal cells, IPP produced by mevalonate pathway are at a concentration that is insufficient to trigger γδ T cells response. However, dysregulation of mevalonate pathway in certain tumours leads to production of higher concentrations of IPP, which is sensed by γδ TCR as a tumour antigen[37,38]. It was also shown that mRNA knockdown of IPP-consuming enzyme, FPPS, induced Vγ9Vδ2 T cell stimulation in otherwise non-stimulatory tumour cells[39]. γδ T cells are able to recognize and kill many different differentiated tumours cells, either spontaneously or after treatment with different bisphosphonates, including zoledronate. It has been shown that human tumour cells can efficiently present aminobisphosphonate and pyrophosphomonoester compounds to γδ T cells, inducing its proliferation and IFN-γ production[40].

Combination treatment utilizing Vγ9Vδ2 T cells along with chemotherapeutic agents and zoledronate has been shown to induce an increase in the cytotoxic function of γδ T cells against solid tumour[41,42]. The ability of γδ T cells to efficiently kill bisphosphonates treated colon cancer stem cells and ovarian cancer stem-like cells has also been reported[36,43].

In addition to phosphoantigens, γδ T lymphocytes can also be activated by mitochondrial F1-ATPase-related structure expressed together with apolipoprotein A-I, which are expressed on the surface of some tumour cells[44]. ATP F1 synthase is an intracellular protein complex involved in ATP generation. F1-ATPase displays characteristic of antigen presentation molecule by binding to the adenylated derivative of IPP and promoting TCR aggregation, cytokine secretion and cytotoxic activity[45].

NK receptors and anti-tumour activity of γδ T cells

Natural killer (NK) receptors expressed on γδ T cells play a crucial role in mediating the anti-tumour response of γδ T cells. Natural killer group 2, member D protein (NKG2D) expressed on Vγ9Vδ2 T cells is critical for tumour recognition and provides activation signals upon binding to non-classical MHC molecules of the MHC class I chain-related molecules (MIC) and UL-16 binding protein (ULBP) families expressed on tumour cells[46-48]. This ligand binding to NKG2D can affect the release of TNF-α, interleukin (IL)-2 α receptor (CD25) upregulation and increase cytolytic potential of γδ T cells[47]. ULBP molecules are involved in Vγ9Vδ2 T cells recognition of leukemias and lymphomas[49] and also ovarian and colon carcinomas[50]. γδ T cells utilizing the Vδ1 chain isolated from tumour-infiltrating lymphocytes can also kill cancer cells. Vδ1 γδ T lymphocytes have been shown to mediate cytolytic activity by recognizing MICA, MICB or ULBP expressed on cancer cells[51,52].

γδ T cells resemble NK cells as these also express CD16 (FcγRIII) receptor. Upon recognition of phosphoantigens, a subset of Vγ9Vδ2 T cells upregulates CD16[53]. It has been reported that CD16 represent activation/memory status of γδ T cells and these $CD16^{high}$ cells have specific phenotypic features that distinguish these from the $CD16^{low}$ subset. These constitutively express several natural killer receptors (NKG2A/CD94) and high amounts of perforin, but express low levels of chemokine receptors (CXCR3, CCR6) and IFN-γ[54]. CD16/FcγRIII receptor binds to Fc portion of immunoglobulin G (IgG) and engagement of CD16 by γδ T cells leads to antibody-dependent cellular cytotoxicity (ADCC)[55]. ADCC is a process in which CD16+ effector cells actively lyse tumour cells that have been bound by specific antibodies. Several reports have proven that *in vitro* γδ T cells respond to activation via CD16 and mediate ADCC against tumour with therapeutic anti-tumour monoclonal antibodies (mAbs) like rituximab, trastuzumab, of atumumab and alemtuzumab[35,56,57]. It has also been shown that stimulated γδ T cells increase the efficacy of trastuzumab *in vivo* in Her2+ breast cancer patients[58].

Application of γδ T cell immunotherapy in clinics

Given the potent antitumour effector function of γδ T cells and broad reactivity to many different types of tumours has raised a great interest to explore their therapeutic potential. An important feature of γδ T cells is that these favourably kill cancer cells and show low (if any) reactivity towards non-transformed cells which makes these very good candidates for cancer immunotherapy[50]. The safety and efficacy of γδ T cell-based immunotherapy have been evaluated in several clinical trials[59]. Presently, two strategies for γδ T cells in tumour immunotherapy have been applied. These are the adoptive cell transfer of *in vitro* expanded γδ T cells and the *in vivo* therapeutic application of γδ-stimulating phosphoantigens or aminobisphosphonates together with low-dose recombinant IL2 (rIL2).

Studies carried out in nude mice demonstrated that repeated infusion of γδ T cells leads to tumour growth arrest[60]. Another study carried out in SCID mice showed

the anti-tumour effector functions of NK cells and γδ T lymphocytes against autologous melanoma cells[61]. In one pilot study, patients with B-cell malignancies that failed conventional therapy were treated with intravenous administration of pamidronate and rIL2 to stimulate Vγ9Vδ2 T cells *in vivo*[62]. It was observed that *in vivo* Vγ9Vδ2 T cells were expanded in five out of nine patients; three out of these five responding patients had partial remissions and one had stable disease. Other trials with adoptive transfer of γδ T cells include patients with advanced cancer like metastatic renal cell carcinoma[63] and non-small cell lung carcinoma[64] where stable disease was found in 60 and 37 per cent patients, respectively. In these cases, the regimen consisted of *ex vivo* activation and expansion of autologous Vγ9Vδ2 T cells with either phosphoantigens, such as BrHPP or aminobsphosphnates, like zoledronate or pamidronate or their infusion into the patients. Aminobisphosphonates have also been used in clinical trials to treat metastatic prostate cancer[65] and advanced breast cancer[66] where partial remissions have been reported. Complete remission of lung metastasis in a patient with renal cell carcinoma has also been reported after adoptive transfer of γδ T cells[67]. It was shown that the patient was disease free for two years without any additional treatment following *in vitro* activation and expansion of autologous γδ T cells with HMBPP plus rIL2, combined with the infusion of zoledronate and rIL2[67]. There is also increasing evidence that stimulating γδ effector T cells can enhance monoclonal antibody-induced cytotoxicity and thereby improve the anticancer effects of mAbs. It was found that repeated infusions of phosphoantigens stimulated γδ T cells and trastuzumab increased the efficacy of γδ T cells against HER-2^{+} breast carcinoma cell lines *in vivo*[58]. In addition, a survival advantage to patients with an increased γδ T cells following allogeneic stem cell transplantation (ASCT) has been reported. A long-term survival advantage in a group of high-risk acute leukemia patients who recovered with increased number of circulating γδ T cells following partially mismatched related haematopoietic stem cell transplantation was reported[68].

Conclusions

The unique features of human γδ T cells related to antigen recognition, tissue tropism, lack of antigen processing requirement and cytotoxic function make these ideal candidates for cancer immunotherapy. γδ T cells recognize increased pool of endogenous IPP (a consequence of dysregulated mevalonate pathway) in cancer cells, release IFN-γ/TNF-α and mediate cytolyic effector functions. Expression of NKG2D receptors provides a selective advantage to γδ T cells to recognize tumours that express stress induced molecules like MICA/B. This property of γδ T cells can be exploited for immunotherapy as tumours downregulate MHC molecules to evade immune recognition (Fig.). Human γδ T cells show potent cytotoxic effector functions

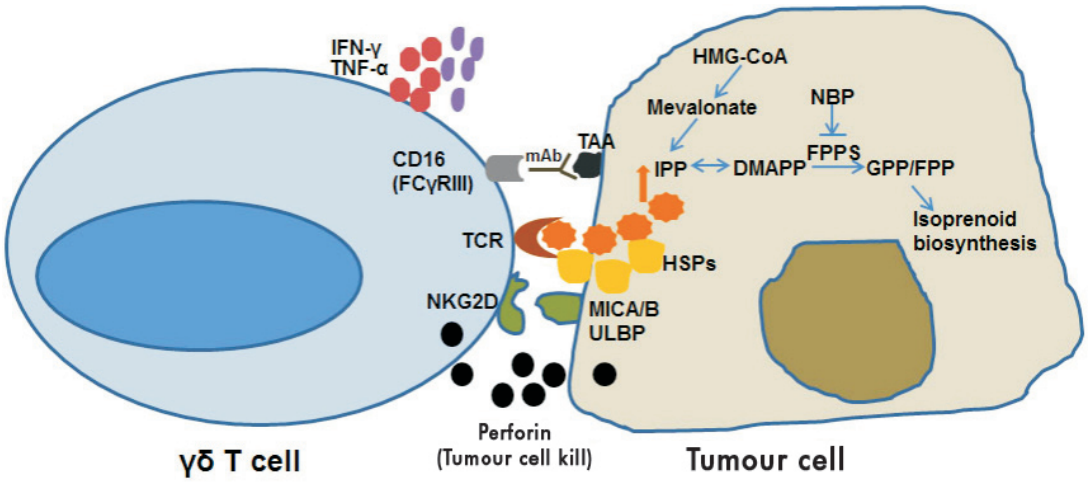

Fig. Mechanism underlying γδ T cell killing of tumours: γδ T cell receptor (TCR) interacts with isopentenyl pyrophosphate (IPP) generated through the mevalonate pathway in tumours. Bisphosphonates inhibits farnesyl pyrophosphate synthase (FPPS) leading to increased endogenous pool of IPP and dimethylalleyl pyrophosphate (DMAPP) in tumour cells. γδ T cells recognize heat shock proteins (HSPs) and MHC class I chain-related molecules (MICA/B) or UL-16 binding protein ULBP expressed on tumour cells via their TCR and natural killer group 2, member D protein (NKG2D) receptors, respectively. Perforin released from activated γδ T cells lyse the tumour cell. γδ T cells can also kill tumour cells through antibody dependent cellular cytotoxicity (ADCC). γδ T cells expressing CD16 (FCγRIII) interacts with tumour associated antigens (TAA) via specific monoclonal antibodies and mediate ADCC. Cytokines like interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) released by γδ T cells can recruit other immune cells (bystander effect).

against various types of tumours. One way to exploit γδ T cells for cancer immunotherapy is the use of synthetic phosphoantigens like BrHPP or HMBPP which can act as γδ TCR agonists. Future trials should harness bisphosphonate activated γδ T cells in combination with chemotherapy or monoclonal antibodies for treatment of solid tumours and haematologic malignancies.

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