“Epigenetic regulation and anti-tumor effector functions of Gamma Delta (γδ) T cells”

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As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Mr. Sajad Ahmad Bhat entitled "Epigenetic regulation and anti-tumor effector functions of Gamma Delta (γδ) T cells" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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LIST OF PUBLICATIONS ARISING FROM THE THESIS

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2. Asif A Dar*, **Sajad A Bhat***, Dimpu Gogoi, Abhiram Gokhale, Shubhada V Chiplunkar "Inhibition of Notch signalling has ability to alter the proximal and distal TCR signalling events in human CD3+ αβ T-cells" *Molecular Immunology 2017 Dec;* **doi 10.1016/j.molimm.2017.10.013** *(* **Equal contribution**)*
3. Rushikesh S Patil, **Sajad A Bhat**, Asif A Dar, Shubhada V Chiplunkar "The Jekyll and Hyde story of IL17 producing γδT (Tγδ17) cells" *Frontiers in Immunology* 2015 Jan*;* 6:37. **doi: 10.3389/fimmu.2015.00037**

Manuscripts in preparation

1. **Sajad A. Bhat**, Disha Mohan Vedpathak, Sanjeev Galande and Shubhada V. Chiplunkar " Involvement of histone methyltransferase Ezh2 in TCR mediated anti-tumour effector functions of γδ T cells"(Manuscript under Preparation)
2. **Sajad A. Bhat**, Sanjeev Galande and Shubhada V. Chiplunkar "Notch signaling modulate the effector functions of human γδ T cells through TCR and IL-2 driven signalling pathways" (Manuscript under Preparation)

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2. **Sajad Bhat and Shubhada Vivek Chiplunkar S45 "Notch signaling augments the TCR driven effector functions of human γδ T cells through IL2 signaling pathway**" *Journal of Carcinogenesis*. 2015; 14 (Suppl 1): S21–S38. Published online 2015 Feb 10

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1. Presented a Poster titled "Epigenetic regulation and anti-tumor effector functions of Gamma Delta (γδ) T-cells" at **"33rd Annual Convention of Indian Association for Cancer Research"** Jointly Organized by Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram & Srinivasa Ramanujan Institute for Basic Sciences, Kottayam (February 2014).
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mechanisms **"New Ideas in cancer challenging the dogmas"** organized by Tata Memorial Centre, India (February 2016)

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SYNOPSIS OF Ph. D. THESIS

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SYNOPSIS

Introduction

Gamma delta (γδ) T cells, the enigmatic brethren of alpha beta (αβ) T cells were discovered coincidently during cloning the αβ T-cell receptor (TCR) locus [1]. This small subset of T cells, constitute about 5–10% of the circulating T cell population, which express the variant form of TCR heterodimer [2]. γδ T cells form the connecting bridge between innate and adaptive immunity and show the features of both innate and adaptive immunity [3]. The Vγ9Vδ2 T cell subset of γδ T cells predominate in the peripheral blood, and these cells play an important role in the defense against microbial pathogens, stressed cells, and tumor cells of various origin [4, 5]. γδ T cells differ from αβ T cells by their TCR gene usage, tissue tropism, and MHC-independent antigen recognition [6, 7]. They display broad functional plasticity,

like regulatory potential, antigen-presenting capacity, B-cell helper activity, and have the potential for diverse cytokine production [8]. γδ T cells recognize non-peptide phosphoantigens such as isopentenyl pyrophosphate (IPP) or 4-hydroxy-3-methyl but-2-eneyl pyrophosphate (HMBPP), which are produced through the mevalonate pathway in eukaryotes and by non-mevalonate pathway or Rohmer pathway in bacteria [9]. There are now a number of synthetic phosphorylated compounds commercially available that are capable of stimulating γδ T cells like bromohydrin pyrophosphate (BrHPP), 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP or HDMAPP) [10, 11]. γδ T cells are also activated indirectly by aminobisphosphonates such as Zoledronate which inhibits the key enzyme of the mevalonate pathway, farnesyl pyrophosphate synthase and leads to accumulation of isopentenyl pyrophosphate (IPP). Tumors treated with aminobisphosphonates are easily targeted by γδ T cells as accumulated IPP is recognized as an antigen by γδ T cells [12, 13]. TCR mediated activation of γδ T cells by phosphoantigens in the presence of co-stimulatory signal provided by rIL2 leads to increase in the expression pro-inflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [14]. In addition to these cytokines γδ T cells also express the effector genes Perforin and Granzyme-B which mediate the cytolytic functions of γδ T cells, upon TCR mediated activation [15]. Besides the elevated level of cytokines and effector molecules, γδ T cells also show increased expression of different activation markers and other co-stimulatory molecules like NKG2D and Notch receptors [16].

Notch signaling plays a critical role in several cell-fate decisions during development. In mammals, there are four Notch receptors (Notch1–4) which are activated by five different ligands that belong to either the Delta-like (DLL1,3 and 4) or Jagged family (jagged 1 and 2). Activation of T cells via TCR accompanied by co-stimulation

generates intra-cellular Notch domain in $CD4^+$ T cells and $CD8^+$ T cells while inhibition of Notch signaling with γ-secretase inhibitors (GSI-X) decreases T cell activation. TCR mediated signaling in both $CD4^+$ and $CD8^+$ T cells induce the activation of Notch1 [17, 18]. Notch activation has been shown to be important for Nf-κB activation and proliferation as well as IFN-γ production by αβ T cells [18]. Notch signaling is involved in regulating the cytolytic effector function in $CD8^+$ T cells through the expression of key effector molecules, Perforin and Granzyme B. It is well known that TCR mediated activation in $CD8^+$ cytotoxic T cells leads to expression of different transcription factors like Eomes and T-bet which regulate their effector functions [17]. Previously our lab has shown that the γδ T cells show increased expression of Notch receptors and ligands upon TCR mediated activation [19]. However, the exact mechanism of how Notch signaling regulates the effector functions of human γδ T cells are not well understood.

Downstream events of different signaling pathways are regulated by epigenetic modifications of the histones. The tails of histone proteins undergo different complex and coordinated posttranslational modifications like histone acetylation, methylation, phosphorylation, and ubiquitination. Histone modifications are reversible in nature and influence many fundamental biological processes. Histone acetylation is directed by histone modifying enzymes, histone acetyl transferases (HAT), and histone deacetylases (HDAC) and histone methylation by methyltransferases which participate in potential cross-talk between different modifications (15). Impact of HDAC inhibitors on tumor cells is well studied. Currently, different HDAC inhibitors are in clinical trials and some have been approved for the treatment of different tumors. HDAC inhibitors also modulate the immune cells, but they have a dual effect either leading to activation or suppression that is distinct for each immune cell subset

[20]. However, the impact of HDAC inhibitors on the functional responses of human γδ T cells is not well understood.

Post-transcriptional modification of histones by methylation plays important roles in regulating antigen-driven T-cell responses. TCR mediated activation in $CD8^+$ T leads to the expression of Ezh2 along with the Notch receptor expression. T-cell responses regulated by the histone methyltransferase Ezh2, which catalyzes histone H3 lysine 27 trimethylation, are well studied. Published studies suggest that Ezh2 is involved in differentiation of type 1 and type 2 helper T cells (TH1 and TH2 cells) in mice. In human CD8 T cells, Ezh2 controls the effector functions by regulating the Notch signaling. However, how Ezh2 controls the effector functions of γδ T cells is not well understood.

Aims and Objectives

1) To study the role of HDAC inhibitors on immune phenotype, proliferation and cytotoxic functions of γδ T cells.
2) To study the mechanism of how Notch regulates cytotoxic effector molecules in γδ T cells
3) To investigate how epigenetic changes modulate effector functions of γδ T cells.

Methodology

γδ T Cell Separation: Heparinized peripheral blood was collected from healthy individuals. Peripheral blood mononuclear cells (PBMCs) were isolated by differential density gradient centrifugation using Ficoll Hypaque (Sigma-Aldrich, St. Louis, MO, USA). The study was approved by the Institutional Ethics Committee (TMC-IECIII Project no. 166) and written informed consent was obtained from the donors prior to collection of blood samples. γδ T cells were purified from PBMCs

using immunomagnetic MicroBeads (Miltenyi Biotech, Bergish Gladbach, Germany) by positive selection, as per manufacturer's instructions. The purity of separated γδ T cells was >95% as confirmed by flow cytometry (FACS Aria, BD Biosciences, USA).

Proliferation Assay: Proliferation of γδ T cells was analyzed using 3H-Thymidine (3HTdR) incorporation assay. A total of 5×10^4 γδ T cells were treated with γ-secretase inhibitor (GSI-X), HDAC inhibitors (TSA, VPA, and SAHA) in presence and absence HDMAPP or plate-bound anti-CD3 and rIL2 72 h in 96-well tissue culture plates. The cultures were pulsed with 1 μCi [3H] thymidine 18 h prior to termination of the assay. The radioactivity incorporated into the DNA was measured in a liquid beta scintillation counter (Packard, Meriden, CT, USA). Data were expressed as counts per minute (cpm).

Flow cytometry: The γδ T cells were stained with live–dead (LD) fixable dead cell stain kit (Thermo Fischer) as per the manufacturer's protocol. After staining with LD dye, the cells were fixed with paraformaldehyde and permeabilized with 1% saponin. Cells were washed and stained with different fluorophore-tagged antibodies for 30 min at 4°C. Further, the cells were washed and acquired on FACS Aria (BD Biosciences, San Jose, CA, USA) and analysis was done by using FlowJo software (Tree Star, Ashland, OR, USA).

Small interfering RNA: γδ T cells were transfected with small interfering RNA (siRNA) specific for different target genes (Notch1, Notch2 and Ezh2) and fluorescent oligonucleotide control siRNA (Cell Signaling Technology, USA) at a concentration of 100 nM using X-treme GENE HP transfection reagent (Roche Diagnostics, Indianapolis, IN).

Western blotting: Cells were washed and lysed using 1% Nonidet P40 (NP 40) lysis buffer containing 10mM Tris, 50mM NaCl, 5mM EDTA, 1mM PMSF, 10μg

aprotinin, 10μg leupeptin and 1% NP 40. Samples were resolved on SDS-PAGE and then transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was probed with the primary antibodies. Immunostaining was performed using appropriate secondary antibody and developed with ECL plus Western blot detection system (Amersham Pharmacia).

Quantitative Real-Time PCR (qPCR): RNA was extracted from different cells using Trizol reagent (Invitrogen Life Technologies, N.Y) in accordance with the company's instructions. RNA obtained from cells was reverse transcribed into cDNA. The cDNA was used to probe different targets genes by using specific primers

Cytotoxicity Assay: ^{51}Chromium release assay or LDH colorimetric based cytotoxicity assay was used to measure the cytotoxicity against a panel of tumor cell lines (AW13516 Oral cancer cell line, COLO-205 Colon cancer cell line and Raji B lymphoblastic cell line) as target cells. Standard 4 hour 51Chromium release assay or lactate dehydrogenase (LDH) cytotoxicity assay was performed as previously described [19]. The radioactive chromium release was measured using an automated gamma counter. The percent specific lysis was calculated as [(experimental release – spontantious)/ (maximum release-spotanious release)] X100

Chromatin Immunoprecipitation: Chromatin Immunoprecipitation assays were performed using MAGnify TM Chromatin Immunoprecipitation System (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Specific antibodies against different histone modifications (Abcam) were used to determine histone modifications of different genes. Normal rabbit IgG was used as a negative control. DNA was extracted and analyzed by quantitative real-time PCR (qPCR) with specific primers for target sequences.

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

Results

Objective1: To study the role of HDAC inhibitors on immune phenotype, proliferation and cytotoxic functions of γδ T cells.

There has been growing interest in combining T cell-based immunotherapy with alternative anti neoplastic treatment in order to increase their clinical efficacy. Histone deacetylases (HDAC) are one of the key epigenetic modifiers that control chromatin accessibility and gene expression and HDAC inhibitors are well established as anti-cancer drugs in clinical settings. Although the HDAC inhibitors have shown promising results in clinical trials and some of them have been approved for the treatment of different cancers, the impact of HDAC inhibitors on the immune cells has recently surfaced. Interestingly, studies have demonstrated that HDAC inhibitors affect each immune cell distinctly. However, their role in modulation of effector responses of γδ T cells is poorly understood. In the present thesis, we have investigated the effect of three different HDAC inhibitors Trichostatin A (TSA), Sodium Valproate (VPA) and Suberoylanilidehydroxamic acid (SAHA) on the effector functions of human γδ T cells. γδ T cells show robust proliferation when activated with phosphoantigen HDMAPP and rIL2. However, in the presence of HDAC inhibitors VPA, TSA and SAHA γδ T cells show less antigen-specific proliferative capacity. This decrease in the proliferative responses of γδ T cells was also reflected in the cell cycle arrest in antigen-activated γδ T cells in the presence of HDAC inhibitors. Antigen-activated γδ T cells cell showed a decrease in the

expression of activation markers (CD25, CD69, and CD71) and cytokines (IFN-γ and TNF-α).

Eomes and T-bet are two main transcription factors, which regulate the effector functions of CD8 T cells and γδ T cells through the expression of effector genes Perforin and Granzyme B. γδ T cells activated with phosphoantigen HDMAPP and rIL2 in presence of HDAC inhibitors TSA,SAHA and VPA show decrease in the expression of transcription factors Eomes, T-bet and also the effector molecules Perforin and Granzyme B. Due to decrease in the expression of effector molecules HDAC inhibitor-treated γδ T cells show decreased anti-tumor cytotoxic potential against panel of Zoledronate treated tumor cell lines (AW13516 Oral cancer cell line, COLO-205 Colon cancer cell line and Raji B lymphoblastic cell line). These results confirm that HDAC inhibitors modulate the effector functions of γδ T cells.

We further observed that HDAC inhibitor-treated γδ T cells showed increased expression of immune checkpoints programmed death-1(PD-1) and its ligand (PD-L1). However, blockade of PD-1 in HDAC inhibitor-treated γδ T cells rescued the anti-tumor cytotoxic potential against a panel of Zoledronate treated tumor cell lines (AW13516, COLO-205, and Raji). These results provide a rationale for designing efficacious combination of HDAC inhibitors and γδ T cell-based immunotherapy using immune checkpoint blockade as a treatment modality for cancer

Objective2: To study the mechanism of how Notch regulates cytotoxic effector molecules in γδ T cells

The Notch signaling pathway is identified as an important regulator of T cell function. Human γδ T cells from healthy individuals express Notch1, Notch2 receptors along with Dll1 and Jag1 both at mRNA and protein level. TCR mediated activation of γδ T cells by phosphoantigen HDMAPP or with anti-CD3 leads to

further increase the expression of Notch1, Notch2, Dll1, and Jag1. TCR mediated activation also leads to an increase in the expression of the Notch intracellular domain. Our data shows that there is a crosstalk between Notch and TCR signaling in γδ T cells. Pharmacological inhibition of Notch signaling by a γ-secretase inhibitor (GSI-X) leads to a decrease in the expression of Notch receptors and ligands in γδ T cells. GSI-X treatment in antigen-activated γδ T cells led to a decrease in the expression of downstream Notch signaling targets Hes1, Hey2 and Nf-κB. To further elucidate the mechanism of how Notch signaling mediated the effector functions of γδ T cells, we checked the effect of GSI-X on the expression of transcription factors Eomes, T-bet and Nf-κB. γδ T cells activated with HDMAPP or plate-bound anti-CD3 upon treatment with GSI-X showed a decrease in the expression of these principal transcription factors. Inhibition of Notch signaling by GSI-X in γδ T cells also decreases the level of effector molecules Perforin and Granzyme B and which leads to abrogation of anti-tumor cytotoxic potential of γδ T cells. Thus Notch signaling regulates the TCR mediated effector functions of γδ T cells through the regulation of transcription factors and effector molecules. GSI-X treatment in γδ T cells decreases the antigen-specific proliferative capacity of γδ T cells which was due to an increase in the expression of cell cycle regulators p53 and p21. Inhibition of Notch signalling also abrogates the proximal and distal signalling events of co-stimulatory IL-2 signaling in γδ T cells.The study shows how Notch modulates the effector functions of γδ T cells through TCR and IL-2 signaling and thus controls the anti-tumor effector functions of human γδ T cells.

<u>Objective3:</u> To investigate how epigenetic changes modulate effector functions of γδ T cells

γδ T cells when activated with phospho antigen (HDMAPP) or with plate-bound anti-CD3 MAb express increased level of effector molecules Perforin and Granzyme B. γδ T cells activated with phosphoantigen HDMAPP and rIL2 show increased level of histone modification H3K9 acetylation which is active histone modification on the promoter regions of Perforin and Granzyme B as compared to unstimulated γδ T cells. Inhibition of Notch signaling by GSI-X in γδ T cells decreases the level of H3K9 acetylation on the promoter regions of Perforin and Granzyme B. However inhibition of Notch signaling in antigen-activated γδ T cells also shows increase in the level of H3K9 trimethylation which is a repressive histone modification on the promoter regions of Perforin and Granzyme B. Thus our results explain that Notch signaling controls the TCR mediated effector functions of γδ T cell through epigenetic mechanisms. These histone modifications are due to different mediators like methyltransferases or acetyltransferases. One such mediator is Ezh2 methyltransferase which is the positive regulator of Notch signaling. The methyltransferase Ezh2 is the catalytic subunit of the polycomb-group family that trimethylates histone H3 on Lys27 (H3K27me3). Activation of γδ T cells with phospho antigen HDMAPP or with anti-CD3 showed an increase in the expression of Ezh2 both at mRNA and protein level. These γδ T cells also show a decrease in the level of NUMB and FBXW7, two important Notch repressors. This confirms that Notch regulates the expression of Notch repressors NUMB and FBXW7 epigenetically. To further confirm our findings we analysed the expression of NUMB and FBXW7 in γδ T cells activated with HDMAPP or anti-CD3 in the presence and absence of Ezh2 inhibitor 3-Deazaneplanocin A (DZnep). We found that blockade with DZNep led to elevated

expression of NUMB and FBXW7 as compared to untreated γδ T cells. ChIP assays revealed high occupancies of H3K27me3 in the proximal promoter areas of NUMB and FBXW7, whereas inhibition of Ezh2 by DZnep abrogated these occupancies. These data suggest that Ezh2 targets Notch repressors and promotes activation of Notch signaling in antigen activated γδ-T cells. Further pharmological inhibition of Ezh2 by DZnep in antigen activated γδ-T cells led to decrease in the expression of cytokines IFN-γ and TNF-α and effector molecules Perforin and Granzyme B. Treatment with DZnep decreased the anti-tumor cytotoxic potential of γδ T cells against a panel of Zoledronate treated tumor cell lines. These results confirm that TCR mediated Notch signaling regulates the anti-tumor effector functions of γδ T cells through an epigenetic mechanism.

Summary and Conclusion

T cell-based immunotherapies are widely investigated for cancer immunotherapies due to their distinctive immune features and potent antitumor effector functions. Gamma delta (γδ) T cells are crucial mediators of anti-tumor immunity and an emerging class of cancer immunotherapy. The present investigation has unveiled the impact of the different signaling pathways and epigenetics modulators on the anti-tumor effector functions of γδ T cells. For the first time, our study has shown the impact of HDAC inhibitors on the effector functions of human γδ T cells. In antigen-activated γδ T cells, HDAC inhibitors inhibit the expression of transcription factors (Eomes and T-bet) and effector molecules (Perforin and Granzyme B). Treatment with HDAC inhibitors attenuated the antitumor cytotoxic potential of γδ T cells, which correlated with the enhanced expression of immune checkpoints programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) in the γδ T cell. The interesting observation was that blockade of immune checkpoints in the presence of

HDAC inhibitors improves the anti-tumor cytotoxic potential of γδ T cells. Our results implicate that checkpoint blockade along with HDAC inhibitors can be used as a combination treatment modality with γδ T cell therapies for cancer patients.

Our study has also investigated the role of Notch signaling in the anti-tumor functions of γδ T cells. Antigenic activation of γδ T cells leads to an increase in the expression of Notch receptors and ligands. The Notch signaling was found to be one of the positive regulators of TCR mediated antitumor effector functions in γδ T cells. Abrogation of Notch signaling decreases the ability of γδ T cells to lyse tumor targets. Inhibition of Notch signaling leads to decreased expression of transcription factors Eomes, T-bet, Nf-κB; and effector molecules like Perforin and Granzyme in γδ T cells. Inhibition of Notch signaling in antigen-activated γδ T cells also abrogates both proximal and distal IL-2 signaling in γδ T cells. Our study also showed that Notch signaling in γδ T cells regulates the effector functions through an epigenetic mechanism by histone acetylation and methylation. Further, it was observed that TCR mediated activation in γδ T cells led to an increase in the expression of methyltransferase Ezh2. Ezh2 activates the Notch signaling pathway in antigen-activated γδ T cells through epigenetic mechanisms by suppressing the Notch repressors Numb and FbXw7 via trimethylation of histone H3 at lysine 27 (H3K27). Pharmacological inhibition or knockdown of Ezh2 in antigen-activated γδ T cells lead to poor anti-tumor cytotoxic potential against Zoledronate treated tumor targets. Thus Ezh2 controls the Notch signaling and effector functions in γδ T cells through epigenetic mechanisms. Manipulation of this pathway may be useful in designing effective γδ T cell based immunotherapeutic strategies for cancer patients.

In conclusion, the present study suggests that TCR mediated Notch signaling modulates the effector functions of γδ T cells. Interestingly our study shows that

Notch signaling mediates the effector functions of γδ T cells through transcriptional and epigenetic mechanisms. The study also implicates that the HDAC inhibitors along with immune checkpoint blockade antibodies could be developed as a combination immunotherapy for the treatment of different malignancies. These observations will open new avenues in the field of γδ T cell-based immunotherapy.

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20. Kroesen, M., et al., *HDAC inhibitors and immunotherapy; a double edged sword?* Oncotarget, 2014. **5**(16): p. 6558-72.

Publications in Refereed Journal

A. Published manuscripts

1. **Sajad A. Bhat**, Disha Mohan Vedpathak and Shubhada V. Chiplunkar "Checkpoint Blockade Rescues the Repressive Effect of Histone Deacetylases Inhibitors on γδ T Cell Function" *Frontiers in Immunology 2018 Jul;* **doi 10.3389/fimmu.2018.01615**
2. Asif A Dar*, **Sajad A Bhat***, Dimpu Gogoi, Abhiram Gokhale, Shubhada V Chiplunkar "Inhibition of Notch signaling has ability to alter the proximal and distal TCR signaling events in human $CD3^-$ αβ T-cells" *Molecular Immunology 2017 Dec;* **doi 10.1016/j.molimm.2017.10.013** (* **Equal contribution**)
3. Rushikesh S Patil, **Sajad A Bhat**, Asif A Dar, Shubhada V Chiplunkar "The Jekyll and Hyde story of IL17 producing γδT (Tγδ17) cells" *Frontiers in Immunology* 2015 Jan*;* 6:37. **doi: 10.3389/fimmu.2015.00037**

B. Communicated Manuscript

C. Manuscripts in preparation

1. **Sajad A. Bhat**, Disha Mohan Vedpathak, Sanjeev Galande and Shubhada V. Chiplunkar " Involvement of histone methyltransferase Ezh2 in TCR mediated anti-tumor effector functions of γδ T cells"(Manuscript under Preparation)
2. **Sajad A. Bhat**, Sanjeev Galande and Shubhada V. Chiplunkar "Notch signaling modulate the effector functions of human γδ T cells through TCR

and IL-2 driven signaling pathways" (Manuscript under Preparation)

D. Other Publications / Conference Presentations:

Conference Proceedings:

1. **Sajad A**. Bhat, Sanjeev Galande, Shubhada V. Chiplunkar "Notch and TCR signaling modulate the effector functions of human γδ T cells" *European Journal of Cancer* Aug. 2016 **doi 10.1002/eji.201670200**
2. **Sajad Bhat** and Shubhada Vivek Chiplunkar S45 "Notch signaling augments the TCR driven effector functions of human γδ T cells through IL2 signaling pathway" *Journal of Carcinogenesis*. 2015; 14 (Suppl 1): S21–S38. Published online 2015 Feb 10

Presentations:

1. Presented a Poster titled "Epigenetic regulation and anti-tumor effector functions of Gamma Delta (γδ) T-cells" at **"33rd Annual Convention of Indian Association for Cancer Research"** Jointly Organized by Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram & Srinivasa Ramanujan Institute for Basic Sciences, Kottayam (February 2014).
2. Presented a Poster titled "Notch and TCR signaling modulate the effector functions of human γδ T cells through transcriptional and Epigenetic mechanisms" "**at 41st** Annual Conference of Indian Immunology Society **IMMUNOCON-2014** *Madurai* (February 2014)
3. Presented a Poster titled "*Notch signaling augments the TCR driven effector functions of human γδ T cells through IL2 signaling pathway* "**Molecular pathways to therapeutics: Paradigms and challenges in Oncology"** organized by Carcinogenesis foundation, USA and ACTREC (February 2015)
4. Presented a Poster titled "Notch and TCR signaling modulate the effector

functions of human γδ T cells through transcriptional and Epigenetic mechanisms **"New Ideas in cancer challenging the dogmas"** organized by Tata Memorial Centre, India (February 2016)

5. Presented a Poster titled "Notch and TCR signaling modulate the effector functions of human γδ T cells at **16th International Congress of Immunology (ICI) -2016,** held in Melbourne, Australia. (August 2016)
6. Presented a Poster titled "Modulation of anti-tumor effector functions of human γδ T cells by HDAC inhibitors" **at the XVI Annual conference Evidence-Based Management of Cancers in India- EBM 2018** held at Tata Memorial Hospital, Mumbai Feb. (2018)

Signature of the Student

Date

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Abbreviations:

^{3}H-TdR	Tritiated Thymidine
^{51}Cr	^{51}Chromium
ADAM	A disintegrin and metalloproteinase
ADCC	Antibody dependent cellular cytotoxicity
AML	Acute myeloid leukemia
APC	Antigen Presenting Cell
APC	Allophycocyanin
APS	Ammonium Persulphate
ASCT	Allogeneic stem cell transplantation
ATCC	American type culture collection
bp	Base pair
BSA	Bovine serum Albumin
CAR	Chimeric Antigen receptor
CBF1	C protein binding factor 1
CCL	Chemokine Ligand
CCR	Chemokine receptor
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic acid
ChIP	Chromatin Immunoprecipitation
CIITA	Class II transactivator
cpm	Counts per minute
CRT	calreticulin
CSL CBF1	Suppressor of Hairless, Lag-1
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte associated protein 4
DAB2IP	Disabled homolog 2 –interacting protein
DAMP	Damage associated molecular patterns
DAPI	4,6-diamidino-2-phenylindole
DC	Dendritic Cell
DEPC	Diethyl Pyrocarbonate
Dll	Delta Like Ligand
DMSO	Dimethyl sulphoxide
DN	Double negative
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
DTX	Deltex
DZnep	3-deazaadenosine A
E/T	Effector to Target
ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diamine tetra acetic acid
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial to Mesenchymal Transition
Eomes	Eomesdermin
Ezh2	Enhancer of zeste homolog 2
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDA	Food and Drug Administration
FH	Ficoll Hypaque
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead Box P3
FPPS	Farnesyl pyrophosphate synthase
FSC	Forward Scatter
GAM	Goat anti Mouse
GAPDH	Glyceraldehyde diphosphate dehydrogenase
GSI-X	γ-secretase inhibitor-X
GSK-3β	Glycogen synthase kinase 3 beta
H3K27me3	Tri-methylation of Histone 3 Lysine 27
H3K4me3	Tri-methylation of histone 4 at lysine 4
H3K9Ac	Acetylation of Histone 3 at Lysine 9
H3K9me3	Tri-methylation of histone 4 at lysine 9
HAT	Histone acetyl Transferase
HDAC	Histone Deacetylase
HDMAPP	1-Hydroxy-2-methyl-2buten-4-yl 4-diphosphate
HERP	Hes-related repressor protein
HES	Hairy enhancer of split
HEY2	Hairy/enhancer-of-split related with YRPW motif protein 2
HIF	Hypoxia inducing factor
HIs	Healthy Individuals
HMBPP	4-hydroxy-3-methylbut- 2-eneyl pyrophosphate
HMT	Histone Methyl Transferase
hr.	Hours
HRPO	Horse radish peroxidase
HSC	hematopoietic stem cells
HSP	Heat shock proteins
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon gamma
IKK	Inhibitor of kappa B (IκB) kinase
IPP	Iso pentyl pyrophosphate
ITAMs	immunoreceptor tyrosine based activation motifs
Jag	Jagged

JAK	Janus tyrosine kinase
kDa	Kilo Dalton
KIRs	Killer Ig-like receptors
LDH	Lactate Dehydrohydrogenase
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MACS	Magnetically activated cell sorting
MAML	master-mind-like
MAPK	Mitogen Activated Protein Kinase
MDS	Myleoproliferative dysplastic syndrome
MDSCs	myeloid-derived suppressor cells
mg	microgram
MHC	Major histocompatibility complex
MIC	MHC class I chain-related molecules
min	minutes
MIP-1	macrophage inflammatory protein
ml	millilitre
mM	milli moles
MMP	matrix metalloproteinases
mRNA	Messenger Ribonucleic acid
N1ICD	Notch 1 intracellular domain
N2ICD	Notch 2 intracellular domain
NBP	Nitrogen-containing bisphosphonates
NFAT	Nuclear factor of activated T cells
Nf-κB	Nuclear factor kappa B
NICD	Notch intracellular domain
NK	Natural killer
NKG2D	Natural Killer Group 2D
NKT	Natural killer T cells
nM	Nano Moles
NP 40	Nonidet-40
NRARP	Notch regulated ankyrin repeated protein
NSCLC	Non-small cell Lung cancer
OD	Optical Density
PAMP	Pathogen associated molecular patterns
PBLs	Peripheral blood lymphocytes
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffer Saline
PCR	Polymerase chain reaction
PD1	programmed death-1
PDL1/2	programmed death ligand 1/2
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein

PEST	Proline, glutamine, serine, threonine-rich
pg.	Pico gram
PI	Propidium Iodide
PI3K	Phosphatidyl inositol -3- OH kinase
PIC	Protease Inhibitor cocktail
pmoles	Pico moles
PMSF	Para methyl sulphonyl fluoride
PRC2	Polycomb Repressive Complex-2
PRR	Pattern Recognition Receptor
PTK	protein tyrosine kinases
PTMs	Post Translation Modifications
qPCR	Real time PCR
RANTES	Regulated on activation, normal T cell expressed and secreted
RBP-Jk	Recombination signal binding protein-Jk
rIL-2	Recombinant Interleukin 2
RNA	Ribonucleic acid
RNAase	Ribonuclease
rpm	Revolutions per minute
RPMI	Roswell Park memorial Institute
RT	Room temperature
RT-PCR	Real Time-Polymerase chain reaction
SAHA	Suberoylanilide Hydroxamic acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error mean
siRNA	small interfering ribonucleic acid
SSC	Side scatter
STAT	signal transducer and activator of transcription
TAA	Tumor associated antigen
TAD	Trans activation domain
T-ALL	T cell Lymphoblastic Leukemia
TAMs	Tumor-associated macrophages
TAN-1	Notch homolog
TBST	Tris- Buffered Saline with Tween-20
TCR	T-cell receptor
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Th	T helper
TIL	Tumor infiltrating lymphocytes
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand

Tregs	Regulatory T cells
TSA	Trichostatin A
ULBP	UL16 Binding Protein
VPA	Sodium Valproate
ZAP70	Zeta chain associated Protein kinase -70
αβ	Alpha beta
γ-secretase	Gamma secretase
γδ	Gamma delta
μCi	Micro-Curie
μg	Microgram
μL	Microliter
μM	Micromolar

Chapter 1
Introduction

Introduction:

Innate and adaptive immune responses are sentinels of the host against the diverse repertoire of infectious agents (viruses and bacteria) and cancer. Both components of the immune system identify invading microorganisms or damaged tissues as non-self and activate immune responses to eliminate them. The innate system provides an early and immediate response within minutes to hours against different targets and system consists of a diverse set of cells, such as granulocytes, macrophages, dendritic cells, and natural killer cells [1, 2]. The adaptive immune system consists of cells such as B lymphocytes, CD4 and CD8 T lymphocytes and molecules like antibodies. The adaptive immune system is highly specific against different targets. However, Natural Killer T (NKT) cells and Gamma Delta (γδ) T cytotoxic lymphocytes overlap with both innate and adaptive immune systems and show features of both adaptive and innate immune systems. γδ T cells constitute about 5-10% of the circulating T cell population, which express the variant form of TCR heterodimer [3, 4]. TheVγ9Vδ2 T cell subset of γδ T cells predominates in peripheral blood, and these cells play an important role in the defense against microbial pathogens, stressed cells, and tumor cells of various origin [5, 6]. Apart from expressing a variant form of TCR γδ T cells also possess unique characteristics such as MHC independent antigen recognition and ability to establish tissue resident populations [7, 8]. γδ T cells exhibit diverse functional abilities besides their potent cytotoxic responses, they are proficient in antigen presentation, display B cell helper activity and can contribute to immune regulation through the production of different cytokines [9]. The most original attribute of γδ T cells is that they are activated by a class of non-peptide phosphoantigens such as isopentenyl pyrophosphate (IPP) or 4-hydroxy-3-methyl-but-2-eneyl pyrophosphate (HMBPP), which are produced through the mevalonate

pathway in mammalian cells or non-mevalonate/Rohmer pathway in non-mammalian cells, respectively [10].γδ T cells are also activated indirectly by aminobisphosphonates such as Zoledronate. Aminobisphosphonates inhibit the key enzyme of the mevalonate pathway, farnesyl pyrophosphate synthase that leads to accumulation of isopentenyl pyrophosphate (IPP). Tumor cells treated with aminobisphophonates show an increase in the intracellular level of IPP and therefore are easily targeted by γδ T cells [7, 8].

Activated γδ T cells are known to produce large amounts of the pro-inflammatory cytokines IFN-γ (interferon-γ) and TNF-α (tumor necrosis factor-α) as well as the chemokines MiP-1(macrophage inflammatory protein) and RANTES (regulated on activation, normal T cell expressed and secreted) [9]. in addition, cytolytic mediators such as granzyme B and Perforin are produced to induce specific lysis of cells with elevated phosphoantigen levels [10]. Transcription factors like Eomes and T-bet are known to be expressed upon activation by γδ T cells and are essential for anti-tumor effector functions [11].

γδ T cells control the diverse immune responses through their organized antigen recognizing γδ T cell receptor (TCR). For T cells to become activated, myriad of signals that integrate together are required. For the optimum activation and effector responses of T cells, signaling through different receptors such as Notch is necessary. Notch signaling pathway is an evolutionarily conserved pathway in all metazoans that regulates cell-fate determination during development and other physiological processes [12, 13]. The interaction between the Notch receptor with its ligand activates the Notch signaling pathway. in mammals, there are four Notch receptors, Notch1, Notch2, Notch3 and Notch4, and five canonical Notch ligands, Jagged1, Jagged2, Delta-like 1 (DLL1), DLL3 and DLL4 [14, 15]. The interaction between the

Notch receptor with its ligand activates the Notch signaling pathway. The exact mechanism by which Notch ligands activate Notch receptors is not fully elucidated, however widely accepted model shows that after binding of Notch receptor with canonical Notch ligands leads to conformational change that allows cleavage of Notch receptor by ADAM (a disintegrin and metalloproteinase) followed by the second cleavage by gamma-secretase (γ-secretase) resulting in the release of the Notch intracellular domain (NICD) fragment [16]. The final intracellular product NICD is the signaling component of Notch that translocates to the nucleus and modulated the expression of different target genes [17, 18]. In the nucleus the active intra-cellular fragment of Notch interacts with the transcriptional repressor recombination signal binding protein-Jk (RBP-Jk) followed by recruitment of other cofactors like p300 and master-mind-like (MAML), and RBP-Jk is converted to a transcriptional activator leading to the expression of downstream target genes such as HES1 (hairy and enhancer of split-1), HERP (HES related repressor protein) and Nf-κB, etc [19-21]. It has been demonstrated that activation of T cells through Notch receptor leads to the generation of Notch intracellular domain (NICD). Inhibition of Notch activation with γ-secretase inhibitors (GSI-X) inhibits the generation of NICD which decreases CD8 T cell activation as well as proliferation [22]. In $CD4^+$ T cells, Notch regulates the antigen sensitivity through the modulation of the co-stimulatory signal. Notch signaling influences effector functions in fully differentiated $CD4^+$ T cells. Laky et al showed that Notch can also enhance T cell activation during the priming phase by increasing the frequency of $CD4^+$ T cells that respond to low doses of antigen and by improving the quality of the response through the modulation of the co-stimulatory signal [23]. However, this role of Notch signaling in human γδ T cells has not been investigated [24]. It is not known whether Notch signaling is required for controlling

proximal and distal signaling events in γδ T cells and controlling their effector functions.

A well-defined feature of γδ T cell is the ability to show rapid transition from a quiescent state to a highly proliferative, cytolytic population of effector cells upon TCR mediated stimulus. γδ T cells show rapid expression of effector genes upon antigenic stimuli and release effector molecules that eventually lead to the establishment of effector functions [8, 25].

Downstream events of TCR signaling and other co-stimulatory signaling pathways like Notch signaling are regulated by the accessibility of the genetic material DNA [26]. Within eukaryotic cells, genomic DNA is wrapped around a complex of histone proteins that organize to form a structure termed as chromatin. The basic functional unit of chromatin is the nucleosome, where DNA is wrapped around an octameric histone complex, typically containing two each of the core histones H2A, H2B, H3, and H4 [27, 28]. The tails of histone proteins undergo different complex and coordinated post-translational modifications like histone acetylation, methylation, phosphorylation and ubiquitination. According to the histone code hypothesis, these modifications are read by specific factors which ultimately lead to downstream events [29]. Histone modifications are reversible in nature and influence many fundamental biological processes [30]. Histone modifications modulate gene expression either by changing chromatin conformation or provide a platform that allows binding of transcription machinery [31]. For example acetylation of histone H3 at lysine 9 (H3K9Ac) at promoters is associated with the transcriptionally active gene. Because of the negative charge of an acetyl group, acetylation of histones leads to electrostatic repulsion between histones and associated negatively charged DNA, thus promoting the open relaxed transcriptionally active chromatin structure [32]. On the other hand,

methylation of histone H3 at lysine 9 (H3K9me3) or lysine 27 (H3K27me3) does not alter the charge of chromatin and promotes compacted transcriptionally inactive state of chromatin [33, 34]. However, histone methylation is more complicated than acetylation. For example, tri-methylation of histone 3 at lysine 4 (H3K4me3) is associated with almost all actively transcribed genes and has a strong correlation with histone acetylation and recruitment of RNA polymerase ii, indicative of a transcriptionally permissive gene [35, 36]. It has been demonstrated in CD8 T cells that H3K9 acetylation and H3K9 trimethylation is associated with the expression of effector molecules Perforin and Granzyme B [37]. Thus through this epigenetic mechanism CD8 T cells regulates the effector functions. However, there are no reports on how epigenetic mechanisms regulate the effector functions of human γδ T cells.

T cell-based therapy is rapidly emerging as an alternative to conventional anti-cancer therapies. Although chemotherapy, radiation therapy, and surgical intervention remain the main procedures for the treatment of cancer they are often associated with unwanted treatment-related side-effects. in order to make cancer therapy more tumor-specific without having any adverse effect on the non-cancerous components of the body, cell-based cancer immunotherapy by harnessing different immune cells are now explored for the treatment of different tumors [38]. γδ T cell display potent anti-tumor potential against different types of tumors has raised a great interest to explore their use in cell-based immunotherapy. γδ T cells show high reactivity towards tumor cells and do not react with normal cells, which makes them a good candidate for the cell-based immunotherapy [39].

The functional aspects of γδ T cells make them attractive arsenal in the anti-tumor immunity. They have been harnessed in cancer immunotherapy using two strategies

either in vivo activation of γδT cells or Adoptive transfer of ex-vivo expanded γδT cells [40]. Several clinical trials have investigated the potential of γδT cells against tumor after using in-vivo activation. In-vivo activation was achieved either with phosphoantigens (BrHPP) or aminobisphonates (Zoledronate) [41]. In tumors like B cell type non-Hodgkin lymphoma or multiple myeloma, which are highly sensitive to γδT cell-mediated lysis, have showed notable activation and anti-tumor responses. In these patients, Pamidronate + rIL2 was used for in-vivo expansion and γδT cell-based therapy was well tolerated [42]. These successful results have not been reproducible in solid tumors like prostate, breast, renal cell carcinoma and neuroblastoma [43-45]. In breast and prostate cancer patients, though the in-vivo activation was seen the significant clinical response was not observed [46].In neuroblastoma trial, *in-vivo* activation with Zoledronate and IL-2 showed significant expansion of γδT cells and presented better outcomes [47]. The clinical studies utilizing the second approach of ex-vivo expansion followed by adoptive transfer have shown success in solid tumors. In advanced renal cell carcinoma patients, two separate studies have shown that Zoledronate expanded γδT cells when injected into patients were well tolerated. The adoptively transferred γδT cells underwent significant expansion, infiltrated to the tumor site and delayed tumor growth was observed [45]. Ex-vivo expanded γδ T cells have shown tumor reduction in gastric cancer, but in patients with non small cell lung cancer (NSCLC), ex-vivo activated γδT cells showed no significant anti-tumor effect [48-50]. Thus γδT cells based immunotherapies have achieved significant success in stimulating circulating γδT cells, however, this approach is limiting in the sense that their reach to tissue-resident γδ T cells is not known and whether the in-vivo expanded γδ T cells are recruited to tumor site also remains a question. In ex-vivo activation, cases of activation-induced cell anergy have been reported. Hence though

the potential of γδT cell-based immune therapies is immense, they still need to be fine-tuned to achieve optimal activation in order to increase clinical efficacy [40]. Alternative approaches to harness γδT cell in cancer treatments has been speculated. One such approach includes lentiviral-mediated transduction of T cells with chimeric antigen receptors. A study has demonstrated polyclonal γδ T cells with CD19 specific CAR can efficiently target CD19 leukemic cells. Similarly, a combination of γδT cells with small molecule inhibitors which have an immunomodulatory role is also a fascinating area to be explored [51].

Histone acetylation is directed by histone modifying enzymes, histone acetyl transferases (HAT) and histone deacetylases (HDAC) which participate in potential cross-talk between different modifications [52]. Normal physiological functions require a balance between HAT and HDAC. Abrupt alterations that skew this balance can give rise to different pathophysiological conditions like cancer [53, 54].Altered genetic and epigenetic regulation is the main cause of cancer. Aberrant expression of epigenetic modifiers such as HDACs can lead to tumorogenesis [55]. Due to this reason targeting the HDACs can be a potential treatment modality against various tumors and other diseases. HDAC inhibitors are an emerging class of novel epigenetic anti-cancer drugs. HDAC inhibitors utilize varied mechanism to exert their anti-tumor activity which involves regulating gene expression through modification of histone or non-histone proteins that induce aberrant mitosis and apoptosis, eventually leading to cell death or differentiation in tumors [61, 62]. Moreover, HDAC inhibitors interfere with the process of angiogenesis in tumors thereby stunting tumor growth and metastasis [63]. Studies have shown that treatment of tumor cells with HDAC inhibitors enhanced the expression of putative tumor antigens, major histocompatibility complex (MHC) class I and II molecules and associated

machinery, costimulatory molecules and natural killer (NK) cell-activating ligands and enhanced phagocytosis by dendritic cells. This could augment the immunogenicity and antigen-presenting capacity of tumor cells and increase their susceptibility to killing by cytotoxic lymphocytes [64, 65]. Structurally different HDAC inhibitors are at different stages of clinical trials and some of them have shown promising anti-tumor effects [66, 67]. HDAC inhibitor, depsipeptide, first identified as a natural prodrug, induces a complete or partial clinical response in cutaneous T cell lymphoma patients along with the hydroxamic acid-based HDAC inhibitor suberoylanilide hydroxamic acid (SAHA). Both depsipeptide and SAHA have now been approved to manage advanced cutaneous T cell lymphoma patients [56, 57]. VPA is registered for the therapy of epilepsy, bipolar disorders, and migraines, and is now together with other short-chain fatty acids HDAC inhibitors tested in clinical studies as anticancer drugs [58]

Impact of HDAC inhibitors on tumor cells is well studied. Extensive evidence suggests that HDAC inhibitors play a role in anti-tumor immunity [59]. HDAC inhibitors lead to growth arrest, induction of apoptosis and differentiation in tumors. It has been demonstrated that HDAC inhibitors inhibit angiogenesis and increase the tumor cell antigenicity which makes them easily recognized by the immune. Due to an increase in the expression of tumor cells easily recognized by the immune system [60-63]. Because of these promising anti-tumor functions, HDAC inhibitors are now assessed in clinical trials and some of them have been approved for treatment [64, 65].Though HDAC inhibitors have achieved clinical success with hematological malignancies, their efficiency in solid tumors is limited. HDAC inhibitor resistance was speculated to be related to elevated levels of different signaling genes which are necessary for the survival of tumor cells such as MAPK, PI3K or STAT3 [63]. Apart

from acquiring resistance, HDAC inhibitor have not paved way into an efficacious treatment option for solid tumors and clear proof of concept remains to be established for their limited success. One possible reason could be structural diversity and chemistry of different HDAC inhibitors. Moreover, the drug efflux mechanisms and accumulated mutations as an evolutionary consequence of overexposure to HDAC inhibitor can be a contributing factor towards the resistance of cancer cells to HDAC inhibitor [66]. However, HDAC inhibitors have shown synergistic effects with other anti-tumor therapies such as tyrosine kinase inhibitors, radiotherapy, chemotherapy, topoisomerase inhibitors; other epigenetic modifiers, immune checkpoint blockade antibodies [67]. Immune checkpoint inhibitors in combination with HDAC inhibitors have shown promising results in vivo and in vitro. Recent studies reported that HDAC inhibitors augment immune response against melanoma cells, triple negative breast cancer, and lung adenocarcinoma when combined with immune checkpoint blockade antibodies [68-72].

HDAC inhibitor treatment may also have an impact on the immune cells. There are few studies which have analyzed the effect of HDAC inhibitors on immune cells in depth at functional and genomic levels [73]. Most of the studies till this date have focused on investigating the impact of HDAC inhibitors on tumor cell lines and immune cells other than γδ T cells. Report by Suzuki et al demonstrated that treatment with VPA leads to an increase in the expression of MiCA and MiCB which are recognized by NKG2D receptor on γδ T cells [74]. Another study by Kabelitz *et al* reported that HDAC inhibitor VPA induces differential modulation of cell surface markers on γδ T cells compared to αβ T cells [75]. Although these studies show the direct effect of VPA on γδ T cells, they do not address the impact of HDAC inhibitors on functional responses of γδ T cells.

On this background, in the present thesis, we aim to address the following objectives

Aims and Objectives:

1) To study the role of HDAC inhibitors on immune phenotype, proliferation and cytotoxic functions of γδ T cells.
2) To study the mechanism of how Notch regulates cytotoxic effector molecules in γδ T cells
3) To investigate how epigenetic changes modulate effector functions of γδ T cells.

Chapter 2
Review of literature

2.1 γδ T cells

2.1.1 γδ T cells

The immune system can be classified into two types: innate and adaptive system. Innate and adaptive immune responses are sentinels of the host against the diverse repertoire of infectious agents (viruses and bacteria) and cancer. Both components of the immune system identify invading microorganisms or damaged tissues as non-self and activate immune responses to eliminate them. The innate immune system forms the first line of defense of the host and provides a set of disease resistance mechanisms constituting the cellular and molecular components that are non-specific in nature but help in combating the pathogenic invasion in the site of infection (**Figure 2.1**)[76].

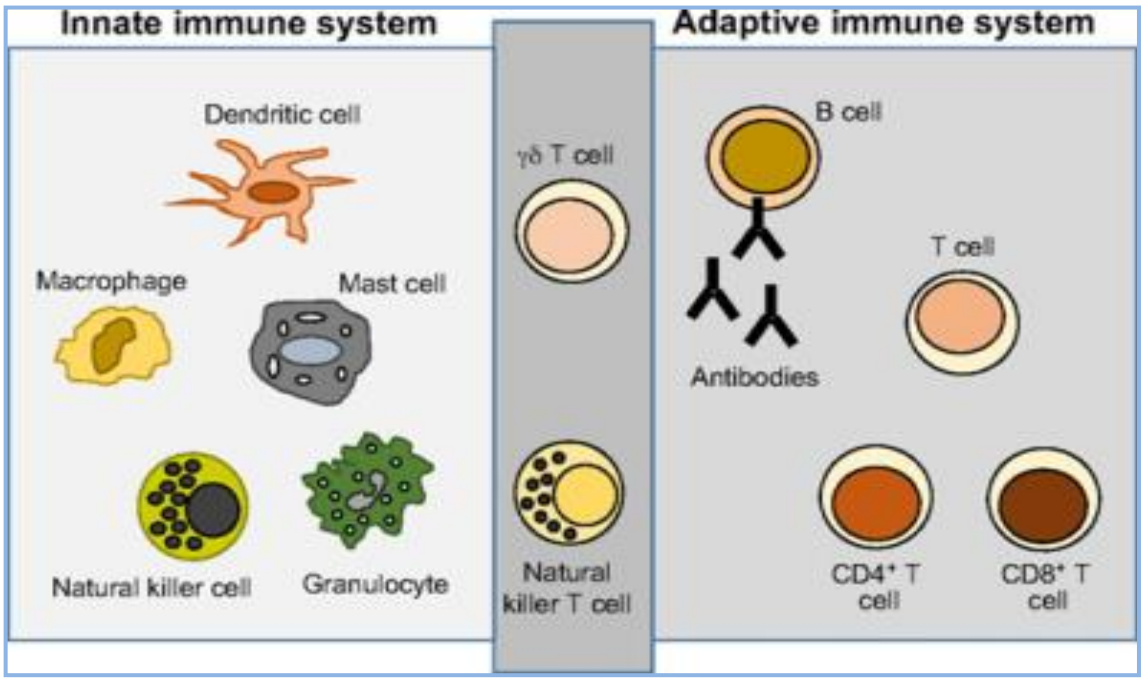

Figure 2.1: Cells of the innate and adaptive immune systems. The innate immune system consists of dendritic cells, mast cells, macrophages and NK cells. The adaptive immune system consists of B and T lymphocytes. Natural killer T cells and γδ T cells are cytotoxic lymphocytes overlap both innate and adaptive immune systems. (**Source:** Michaela Sharpe, and Natalie Mount, **Dis. Model. Mech**. 2015; 8: 337-350)

In contrast, adaptive immunity mounts a highly specific immune response upon antigenic challenge. The cellular components of the adaptive system require an intricate mechanism of activation to eliminate the antigenic source either through the release of soluble effector molecules or cell-mediated lysis of the target; additionally, they also retain the memory of the encounter. In addition to these Innate and adaptive immune systems, some Immune cells such as natural killer T (NKT) cells and gamma delta (γδ) T lymphocytes possess the features of both the systems. These unconventional T cells form a connecting bridge between innate and adaptive immunity [77].

T lymphocytes recognize antigen via the heterodimeric T-cell receptor (TCR) molecule, which is associated with the CD3 molecular complex. Most T cells carry a TCR that is composed of αβ chain heterodimer and are referred to as αβ T cells. But a second TCR chain was identified during cloning the of αβ TCR genes which led to the discovery of a different T cell subset which expressed TCR composed of γδ chain heterodimer [78, 79]. Hence the cells expressing this TCR were called as γδ T cells. Both the αβ and γδ cell lineages arise from common multipotent double negative (DN) precursor in the thymus, where they undergo extensive rearrangement at the β, γ and δ loci during the developmental programs, generating two functionally distinct T cell subsets of the immune system [80].

In human γδ TCR expressing cells constitute 1–5% of total T cells in the peripheral blood but γδ T cells are abundantly present in the epithelia of skin, genital and intestinal tract in the range of (20–50%) [3, 4, 81]. γδ T cells are the first T cells to appear in the thymus during T cell ontogeny in every vertebrate, which suggests that their primary contribution could be neonatal protection because at this point conventional αβ T cell responses are severely functionally impaired and DCs are

immature [82, 83].In humans, there are two major subsets of γδ T cells identified by their Vδ chain. Vδ1 T cells are predominant in the thymus and peripheral tissues and recognize various stress-related antigens mostly uncharacterized and are paired with various Vγ elements. Vδ2 T cells constitute the majority of blood γδ T cells. They always associate with the Vγ9 chain in adults [84]. The predominant subset of γδ T cells in peripheral blood consists of Vγ9Vδ2 T cells [85].

2.1.2 Features and antigen recognition of γδ T lymphocytes

γδ T cells are unconventional $CD3^+$ T cells and differ from the conventional αβ T cells in their biology and function (**Table 2.1**). Although a small fraction of γδ T cells in the intraepithelial lymphocyte compartments of human and mice are $CD8\alpha\alpha^+$ but most of the peripheral blood γδ T cells are predominantly double negative ($CD4^-CD8^-$) T cells [86]. γδ T cells have the ability to recognize a broad range of antigens without the presence of major histocompatibility complex (MHC) molecules [87]. Thus the absence of CD4 or CD8 expression on the majority of the circulating γδ T cells is well in line with the fact that γδ T cells are not MHC restricted (**Figure 2.2**)[88-90]. The crystal structure analysis of the γδ TCR it has been revealed that γδ TCR is highly variable in length and closely resembles the immunoglobulins (Ig) more than the αβ TCR[91]. The antigen recognition property of γδ T cells is fundamentally different from αβ T cells but similar to antigen-antibody binding, which is more likely to occur independently of MHC cross-presentation [91]. However, a critical role of the butyrophilin family member BTN3A1 (CD277) in the phosphoantigen-mediated activation of human Vδ2 cells has been discovered [92-94]. Even though the precise role of BTN3A1 is still under investigation, recent findings support intracellular sensing of prenyl pyrophosphates by BTN3A1 rather than its role on extracellular presentation of exogenous or endogenous phospho- antigens [95].

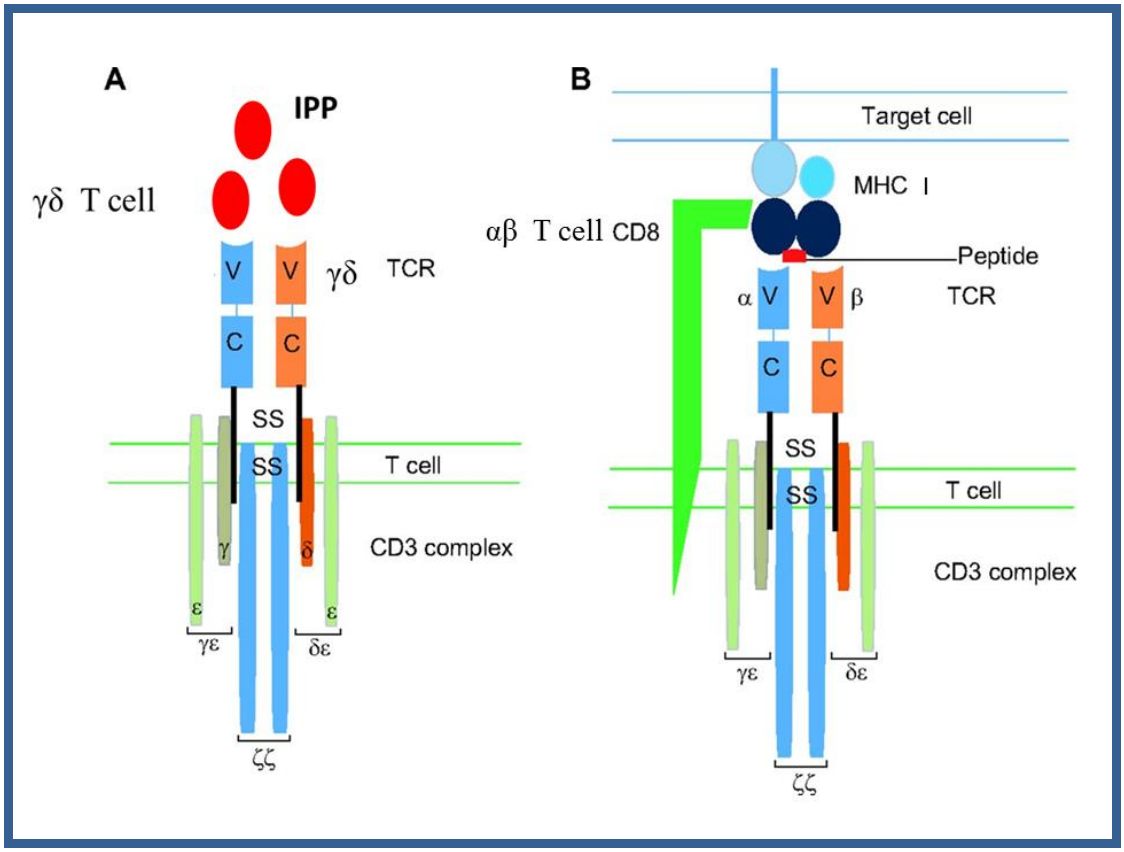

Figure 2.2: Antigen recognition by αβ TCR and γδ TCR: The γδ TC bind to phospho antigens such as Isopentenyl pyrophosphate (IPP) directly without any antigen presenting molecule **(A).**αβ TCR bind to the processed peptide antigen through MHC dependent manner.

The important feature of γδ T cells is their tropism to epithelial tissues. γδ T cells are well represented among peripheral blood mononuclear cells (PBMC) and in afferent and efferent lymph. γδ T cells are not found in lymph node parenchyma, spleen, Peyer's patches and thymus [96]. The diversity of the αβ TCR repertoire is largely due to a large number of Vα and Vβ gene segments in the germline genome which can be used for to generate a large number of rearranged mature TCR during T-cell differentiation in the thymus. In contrast to this, there are only a few Vγ and Vδ gene segments that are used to generate functional mature γδ TCR. In human 6 Vγ gene segments (Vγ2,3,4,5,8,9) are expressed that combine a small number of Vδ gene segments [97]. Although the germline repertoire is very small, γδ T-cells use non-

germline mechanisms such as N-nucleotide insertion to generate tremendous diverse rearranged γδ TCR [98].

S.No.	αβ T Cells	γδ T cells
1	Constitutes about 65% to 70 % of total PBMCs	Constitutes about 1% to 1o% of total PBMCs
2	Recognize the processed peptide antigen with the help antigen presenting molecule MHC1 and MHC II	Do not show MHC restriction but may require the antigen-presenting molecule Butyrophilin 3A1 molecule
3	Express either $CD8^+$ or $CD4^+$	Mostly double negative, murine intestinal IELs may be CD8αα+
4	TCR junctional diversity is very diverse	TCR junctional diversity is small
5	Do not show tissue tropism	Show tissue tropism
6	αβ T Cells response is late	γδ T cells respond earlier
7	Regulatory phenotype is attributed to $CD4^+CD25^+$ T cells	Regulatory phenotype is attributable to various subsets, including murine $V\gamma5^+$ DETCs and human $V\gamma1^+$ peripheral cells

Table 2.1: Comparison between αβ T cells and γδ T cells Table showing the features of γδ T cells and αβ T cells (**Source**: S. Chiplunkar *et al*, **Frontiers in Immunology**, Volume 5, Issue July 2014 Article 366.

2.1.3 Antigen recognition and activation of γδ T lymphocytes

γδ T-cells share features of both innate and adaptive immunity, recognize the conserved nonpeptide antigens through γδ TCR without the requirement of antigen processing and MHC-dependent presentation. Vγ9Vδ2 T-cells show rapid proliferation and activation in the presence of a broad range of bacteria, such as M. tuberculosis [99]. More importantly, a number of γδ T-cells rapidly increases during many bacterial and parasitic infections [100]. The microbial agents which are recognized by the γδ T-cells mainly include phosphoantigens which are the intermediates of non-mevalonate also called “Rohmer”-pathway. One such compound is (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP). In eukaryotes, these phosphoantigens are intermediates of the mevalonate pathway. Isopentenyl pyrophosphate (IPP), an intermediate of the mevalonate pathway activate and

proliferate the peripheral blood γδ T-cells (**Figure 2.3**) [101, 102]. There is now a number of synthetic phosphorylated compounds that are capable of stimulating γδ T cells like bromohydrin pyrophosphate (BrHPP), picostim and mono-ethyl pyrophosphate. Synthetic analogue, BrHPP activate Vγ9Vδ2 T-cells at pico- to nanomolar concentrations. The mevalonate pathway intermediate isopentenyl pyrophosphate (IPP) activates γδ T cells in the range of micromolar concentrations. These phosphorylated compounds are collectively called as phosphoantigens [103, 104]. Another class of molecules that stimulate Vγ9Vδ2 T cells is alkylamines. Alkylamines are present in edible plant products such as tea. They are also secreted by certain commensal bacteria. Alkylamines activate Vγ9Vδ2 T cells by an indirect mechanism similar to aminobisphosphonates through the inhibition of mevalonate pathway [105, 106]. 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 [104, 107].In addition to this, γδ T lymphocytes also recognize the pathogen-associated molecular pattern (PAMP) or danger-associated molecular pattern (DAMP) through pattern recognition receptors (PRR) expressed on γδ T cells [86].

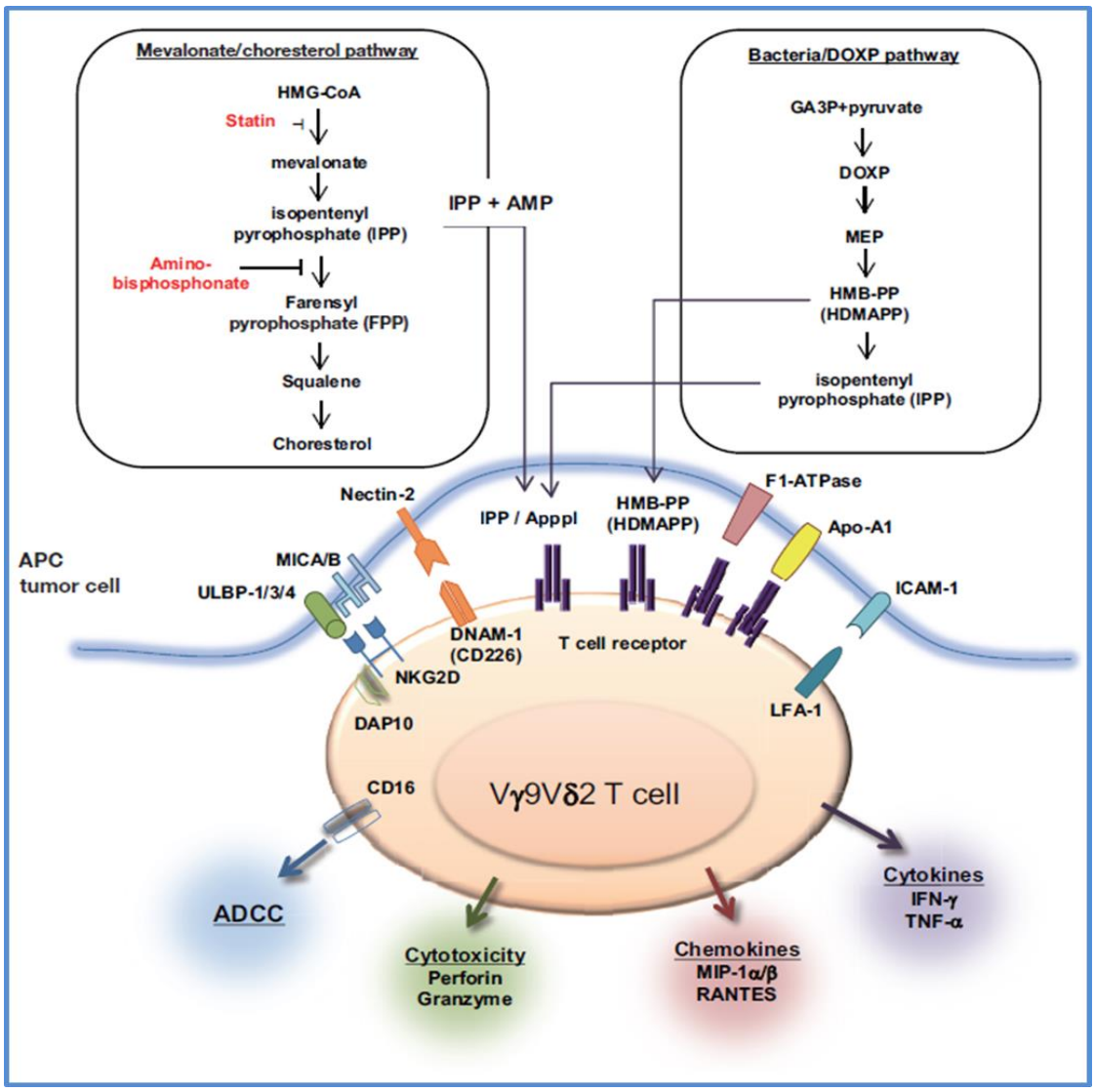

Figure 2.3: Antigen recognized by γδ T cells. γδ T-cells recognize phosphoantigens which are the intermediates of Mevalonate pathway in eukaryotes or non-Mevalonate in bacteria. Blocking of Mevalonate pathway by aminobisphosphonates increases the level of IPP which binds to γδ TCR. (**Source**: Jun Nakajima *et al.*, **Translational Lung Cancer Research**, Volume 3, Issue No 1, February 2014)

There are some minor differences in TCR signaling when γδ T cells are stimulated with anti-CD3 mAb as compared to phosphoantigens in Vγ9Vδ2 T-cells. Activation with anti-CD3 mAb induces rapid but transient phosphorylation of kinases such as ERK1/2 and p38 associated with down-modulation of cell surface TCR expression. However activation with phosphoantigens induce sustained and prolonged protein phosphorylation of kinases such as ERK1/2 and p38 and no TCR downmodulation

[108, 109].In addition to phosphoantigens. γδ T cells can be also activated indirectly by bisphosphonates which inhibit the critical enzyme farnesyl pyrophosphate synthase (FPPS) in the mevalonate pathway. Inhibition of FPPS leads to accumulation of IPP which results in the activation of γδ T cells [110, 111]. Bisphosphonates, especially nitrogen-containing bisphosphonates (NBP) such as pamidronate, alendronate, Zoledronate, etc. are widely used to treat postmenopausal osteoporosis and skeletal malignancies [112, 113]. Thus the recognition of structurally related pyrophosphates generated provides an explanation why the same population of γδ T cells plays a fundamental role in bacterial infection and anti-tumor immunity [114, 115]. Vγ9Vδ2 T-cells from human (and primate) generate a unique and strong response to microbial pyrophosphates. Further, there is no homologous γδ TCR expressed in mice. Apart from the evolutionary aspect, the conventional mouse models cannot be used to investigate the role of pyrophosphate-recognizing γδ T-cells in infection and tumor immunity functional study [88].

2.1.4 Surface receptors present on γδ T lymphocytes

In addition to the TCR, Vγ9Vδ2 T-cells express numerous surface receptors potentially associated with different stages of differentiation, previous antigen exposure, homing pattern and effector functions of Vγ9Vδ2 T-cells. Based on the surface expression of CD45RA and CD27γδ T cells have been used to identify naïve ($CD45RA^{+}CD27^{+}$), TCM ($CD45RA^{-}CD27^{+}$), TEM ($CD45RA^{-}CD27^{-}$), and TEMRA ($CD45RA^{+}CD27^{-}$) subpopulations within circulating Vγ9Vδ2 T-cells [116, 117]. Naïve γδ T cells account for approximately 15 to 20%, TCM for 40 to 60%, $CD45RA^{-}$ TEM for ≈ 30%, and TEMRA for ≈ 7% of the total circulating Vγ9Vδ2 T-cell population. Naïve and TCM Vγ9Vδ2 subsets readily proliferate in response to phosphoantigen as compared to TEM and TEMRA subsets [118]. Alterations in the

balance of different subset are observed in different pathophysiological conditions such as TEM are markedly reduced in pulmonary tuberculosis and HIV infections [119].

In addition to these surface markers, Vγ9Vδ2 T-cells also express NK-cell-related antigens/NK-receptors, Fc-receptors, co-stimulatory/-inhibitory receptors, Toll-like receptors, and others. Natural killer (NK) receptors expressed on γδ T cells play a vital role in mediating the antitumor Effector responses of γδ T cells [120].

Natural killer group2, member D protein (NKG2D), a lectin-like type II membrane receptor expressed on Vγ9Vδ2 T cells is critical for tumor recognition. NKG2D recognizes the tumors through the interaction of tumor expressed MHC class I related molecules A/B (MICA/MICB) and UL-16 binding protein (ULBP) families expressed on tumor cells [121]. This interaction between NKG2D and its ligands on tumor cells activate the γδ T cells [122]. Besides providing a co-stimulatory signal to γδ T-cells NKG2D receptors also directly activate Vγ9Vδ2 T-cells independently of TCR signaling [123].

Vγ9Vδ2 T-cells also express functionally important CD56 and CD16 (FcγRIII) surface receptors which are also expressed on NK cells.CD56 is up-regulated upon phosphoantigen stimulation, and $CD56^{+}$ Vγ9Vδ2 T-cells display more anti-tumor cytotoxic potential against different tumors as compared to $CD56^{-}$ Vγ9Vδ2 T-cell population [124, 125].CD16, the low-affinity type 3 receptor for the Fc portion of IgG (FcγRIIIA) is expressed on the TEMRA subset of γδ T cells. Antigen stimulation of Vγ9Vδ2 T cells up-regulates the expression of CD16. The presence of CD16 receptor onVγ9Vδ2 T cells binds to Fc portion of immunoglobulin G (IgG) and is the potent activator of antibody-dependent cellular cytotoxicity (ADCC) [126, 127]. Through the ADCC process, Vγ9Vδ2 T cells easily lyse the different tumor cells that are bound by

specific antibodies[128, 129]. ADCC is a process in which $CD16^+$ effector cells actively lyse tumor cells that have been bound by specific antibodies. It is well demonstrated that the expression of CD16 confers to Vγ9Vδ2 T-cells potent ADCC activity in the presence of therapeutic anti-tumor monoclonal antibodies (mAbs) like rituximab, trastuzumab, ofatumumab and alemtuzumab [130]. It has been also demonstrated that stimulation of γδ T cells increases the efficacy of trastuzumab in vivo in $Her2^+$ breast cancer patients [131].

Besides these NK receptors, Vγ9Vδ2 T cells also express DNAX accessory molecule-1 (DNAM-1). This accessory molecules binds to its ligands Nectin-like-5 and Nectin-2 present on different tumors and promotes the lysis of tumor targets [132]. γδ T cells also express the inhibitory receptors like KIR2DL2 and KIR2DL3. KIR2DL2/3 are members of the inhibitory killer Ig-like receptors (KIRs), which recognize a defined group of polymorphic HLA-I molecules [133]. Binding KIR2DL2/3 to its ligand HLA-A inhibits the NK cell-mediated anti-tumor effector function. Inhibition of antitumor potential of NK cells by the interaction between KIR2DL2/3 and HLA-I allotypes is through the recruitment of tyrosine phosphatases (SHP-1 and SHP-2) [134, 135]. It has been also observed the $KIR2DL2/3^+$ γδ T cells were less cytotoxic to tumor cells expressing HLA-I molecules than KIR2DL2/3− γδ T cells. These observations suggest that signaling through KIR2DL2/3 regulates the anti-tumor cytotoxic potential of γδ T cells [136].γδ T cells also show expression of programmed death-1 (PD-1) receptor and its ligand programmed death-1 ligand (PDL-1). The binding of PD-L1 or PD-L2 to PD-1 on activated T cells to triggers phosphorylation mediated recruitment of SHP-2 (Src homology 2 containing tyrosine phosphatase). SHP-2 dephosphorylation activity of TCR- associated CD3ζ and zeta chain-associated protein kinase 70 (ZAP70) which leads to inhibition of downstream TCR signaling

through blocking of PI3K- AKT pathway. Interaction of PD-1 and PDL-1 decreases the anti-tumor potential of cytotoxic CD8 T cells and γδ T cells through the recruitment of phosphatases which inhibit the TRC mediated signaling [137, 138]. Blocking antibodies for PD-1 have been approved for the treatment of different malignancies [139].

2.1.5 Functional plasticity of γδ T cells

Gamma delta T cells are being evaluated as immunotherapeutic agents for a variety of malignancies and have so far shown excellent safety profiles. Yet, the γδ T cells show remarkable functional plasticity which depends on the environment in which they find themselves and cytokines and chemokines they secrete. This is an important issue when it comes to the design Immunotherapies based on the γδ T cells[120]. Upon TCR stimulation and co-stimulation γδ T cells secrete high amounts of different cytokines such as IFN-γ, TNF-α, and chemokines like RANTES and MIP-1α. The cytokine spectrum of γδT cells is regulated by the environmental milieu [140-142]. In humans, upon activation with different cytokines, γδT cells can be polarised towards different effector subtypes like Th1, Th2 and Tγδ17. Vγ9Vδ2 T cells under Th1 driving conditions (IL-12 and anti-IL4) polarize towards high IFN-γ and low IL-4 cells, while under Th2 conditions (IL-4 and anti IL-12) polarize towards Th2 phenotype [143, 144]. Similarly, the chemokine spectrum also correlates with the cytokine spectrum. Based on CXCR5 expressionCXCR5^{-} CD27^{-} TEM γδ T cells subset secrete TNF-α and IFN-γ whereas CXCR5^{+} CD27^{+} γδ T-cells secrete IL-2, IL-4 and IL-10 [145]. A portion of Vγ9Vδ2 T cells from adult blood can be differentiated into IL-17 producing cells in presence of a cocktail of cytokines IL-1β, IL-6, TGF-β, and IL-23. Similar to Th17 cells, Tγδ17 cell expresses RORγt as a lineage determination transcriptional factors [146, 147]. In contrast, Vγ9Vδ2 T-cells can be

induced to express FoxP3 regulatory phenotype in the presence of appropriate cytokines (TGF-β and IL-15) while IL-21 induces the development of Tfh like properties in γδ T cells [148]. These subtypes can also function as γδ T regulatory cells and antigen presenting cells (APCs). Up-regulation of APC-associated surface molecules such as HLA class II, CD40, CD80, CD86 occurs rapidly on the majority of phospho antigenstimulatedVγ9Vδ2 T-cells [149-151].

2.1.6 Antitumor effector functions of γδ T cells.

The ability of γδ T lymphocytes to produce abundant pro-inflammatory cytokines like IFN-γ, potent cytotoxic effector function and MHC-independent recognition of antigens makes them an important player of cancer immunotherapy[39]. γδ T cells kill many different types of tumor cell lines and tumors *in vitro*, including leukemia, neuroblastoma, esophageal tumors and various carcinomas [129, 152-155]. The ability of γδ T lymphocytes to produce abundant proinflammatory cytokines like IFN-γ, TNF-α and potent anti-tumor effector function makes them an important player of cancer immunotherapy. The anti-tumor effector functions of γδ T lymphocytes are mainly attributed due to MHC-independent recognition non-peptide antigen.Vγ9Vδ2 T-cells are potent killer cells, making use of both the secretory effector molecules (Perforin and Granzyme B)and other death receptor/ligand pathways when delivering the lethal kiss to bacteria- or virus-infected cells and tumor cells [156, 157]. The ability of γδ T cells to efficiently kill bisphosphonate treated colon cancer stem cells and ovarian cancer stem-like cells have also been reported [155, 158]. Studies carried out in nude mice demonstrated that repeated infusion of γδ T cells leads to tumor growth arrest [159]. Another study carried out in SCID mice showed the anti-tumor effector functions of NK cells and γδ T lymphocytes against autologous melanoma cells [160].

2.1.7 γδ T cell immunotherapy

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 tumors [7, 161]. 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 [42]. 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 [162] and non-small cell lung carcinoma [49] where the 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 phosphoantigen, such as BrHPP or aminobsphosphonates, like Zoledronate or pamidronate or their infusion into the patients. Few reports showed that in vitro co-culture of human regulatory T cells (Tregs) and peripheral blood mononuclear cells from patients with cancer strongly inhibited phosphoantigen induced proliferation of γδ T cells, whereas depletion of Tregs restored phosphoantigen induced γδ T cell proliferation [163]. These results explain the frequently observed γδ T cell proliferative anergy in patients with cancer [164]. Aminobisphosphonates have also been used in clinical trials to treat metastatic prostate cancer [165] and advanced breast cancer [166] 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 [167]. 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 [167]. 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 [131]. In addition, a survival advantage to patients with an increased γδ T cell following allogeneic stem cell transplantation (ASCT) has been reported. Long-term survival advantage in a group of high-risk acute leukemia patients who recovered with an increased number of circulating γδ T cells following partially mismatched related hematopoietic stem cell transplantation was reported [168]. 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 [169]. 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 [170]. The safety and efficacy of γδ T cell-based immunotherapy have been evaluated in several clinical trials [171]. 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(**Figure 2.4**) [172].

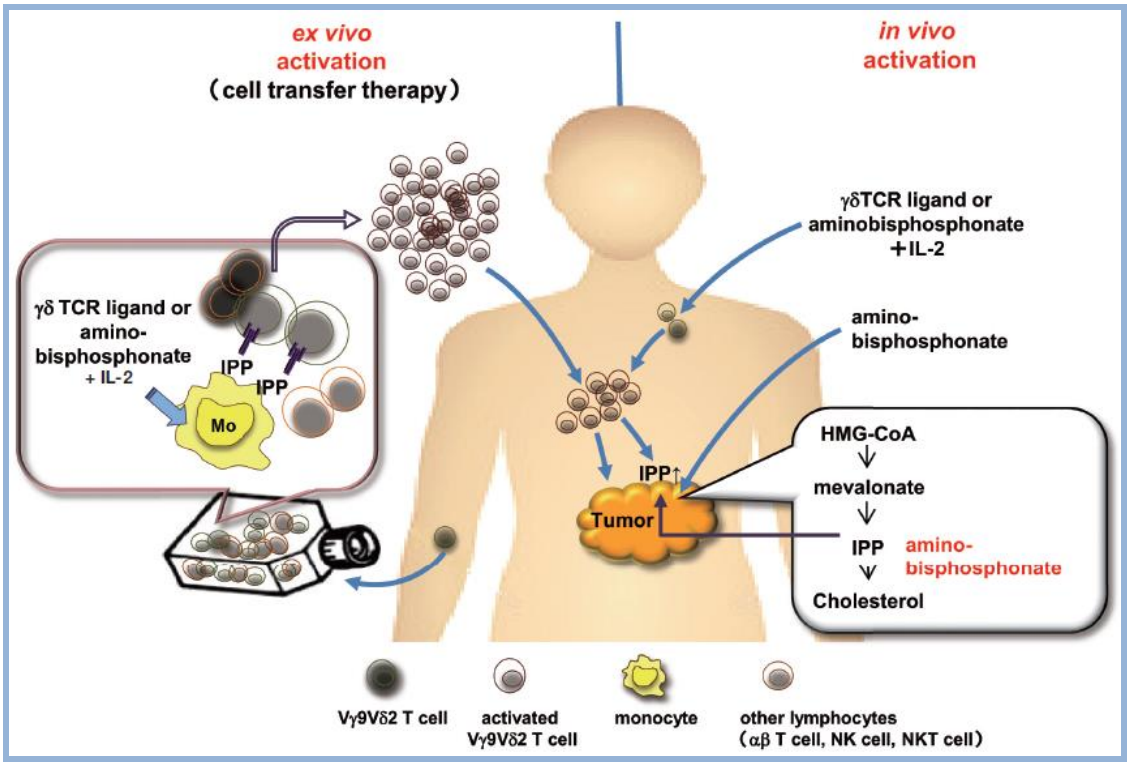

Figure 2.4: Strategies for γδ T cell-based immunotherapy. Left panel, the adoptive cell transfer of in vitro expanded γδ T cells. Right panel, the in vivo activation of γδ T cells by phosphoantigens (e.g., BrHPP) or aminobisphosphonates and low-dose IL-2.(**Source**: Jun Nakajima *et al*, **Translational Lung Cancer Research**, Volume 3, Issue No 1, February 2014)

A new wave of immune therapies based on unique genetically engineered immune cells, called chimeric antigen receptor (CAR) T cells are becoming a huge trend in the treatment of cancer. CAR-T cells have shown remarkable remission rates in patients that did not respond to many other treatments. MHC independent antigen recognition and ex vivo expansion of γδ T cells make them a great candidate for the development of "off-the-shelf" CAR-T cells that are derived from donors instead of from the patient. MHCs vary among individuals and are the basis of donor compatibility in transplantation [173]. The tissue resident γδ T cells have excellent potential for the treatment of solid tumors as they are primed to reside in tissue within a low nutrient and hypoxic environment, where they can carry out tissue surveillance, moving through tissues and recognizing and eliminating transformed cells. Genetic

modification of T cells has clinical applications as adoptive transfer of CAR^+ T cells with specificity for CD19 can lead to antitumor responses in patients with refractory B-cell malignancies. The human application of CAR γδ T cells is appealing given their inherent potential for antitumor effects and their apparent lack of alloreactivity [42, 174]

Besides cell-based therapy, upregulation of the PD-1/PD-L1 axis in the tumor microenvironment allows it to be targeted therapeutically. Immune modulating antibodies have been developed into a new breed of cancer immunotherapy, which aims to enhance T cell function via blockade of PD-1/PD-L1 signaling **(Figure 2.5)**. Studies have shown that blockade of PD-1 or PD-L1 alone leads to increased infiltration of T cells in the tumor compartment and increased IFN-γ as well as the decreased immunosuppressive population of myeloid-derived suppressor cells (MDSC) [175, 176]. Recent reports have demonstrated that tumors associated with PD-1 expressing NK cells show poor survival and blockade of PD1/PD-L1 signaling in NK cells have led to promising responses in cancer patients [177]. PD-1/PD-L1 blocking strategy has led to tumor regression in patients with melanoma, renal cell carcinoma, non-small cell lung cancer and bladder cancer [178-180]. Hence several phase I and II clinical trials have been based on immune checkpoint blockade for the treatment of melanoma, lymphoma and advanced solid tumors [181-187].

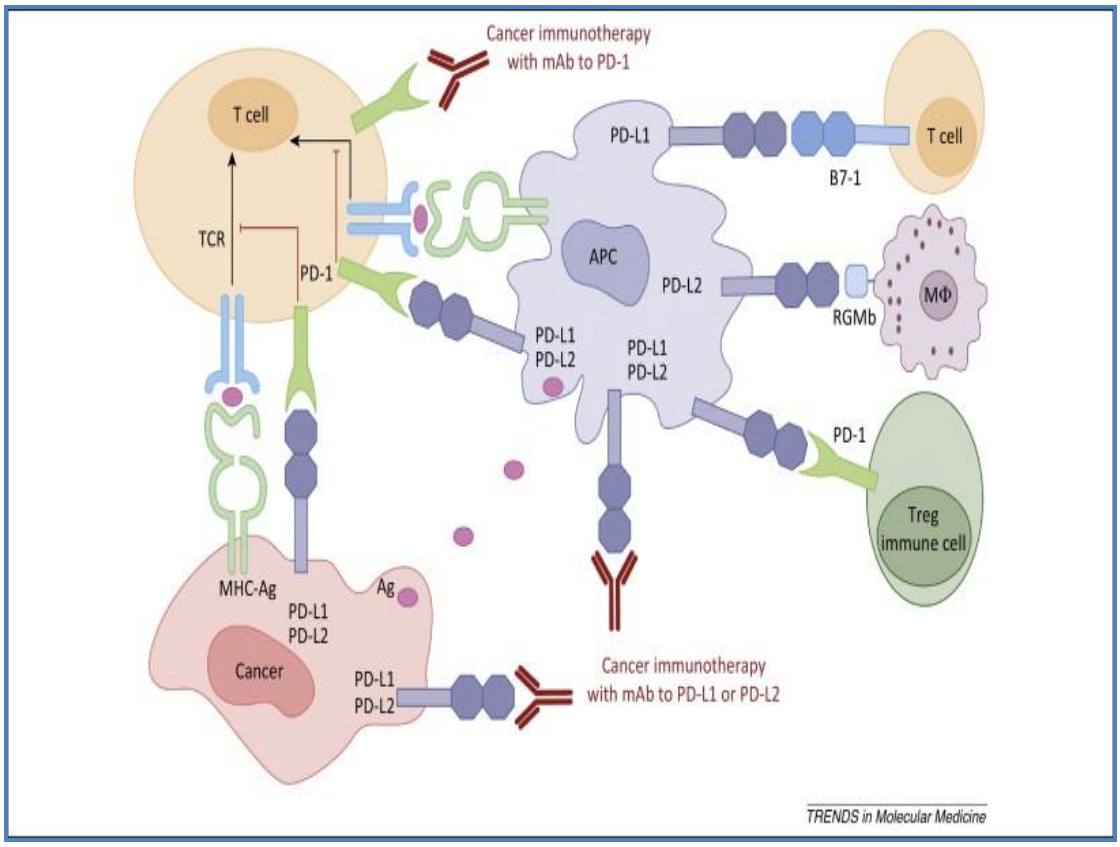

Figure 2.5 Targeting immune checkpoints for cancer immunotherapy. PD-1/PD-L1 interaction leads inhibition of TCR mediated signaling which in tumor microenvironment leads immunosuppression of effector T cells. Therapeutic targeting of PD-1 has huge implication for cancer treatment to enhance T cell function via blockade of PD-1or PD-L1. (**Source**: Ohaegbulam KC *et al*, **Trends Mol Med**. 2015 Jan; 21(1):24-33)

2.2 Notch signaling

2.2.1 Notch Origin

Evolutionally-conserved Notch proteins play an important role in deciding cell fate from insects to mammals [13]. Almost 90 years ago the Notch gene was discovered by T.H Morgan in loss of function studies which led to the wingless phenotype in Drosophila. The name Notch originated from the Notched wing of Drosophila which is as a result of the haploinsufficient Notch expression [13, 188]. In humans, it was found that chromosomal 9 translocation results in T lymphoblastic leukemia (T-ALL). Later it was found that the locus on chromosome 9 contains a gene, Notch1, highly homologous to the Drosophila gene, Notch. Transcripts of the human gene named translocation-associated Notch homologue (TAN-1) and its murine counterpart is ubiquitously expressed both in normal human fetal and adult mouse tissues, but are most abundant in lymphoid tissues [189]. In addition to Notch1, Notch2and 3 Notch 4 have been identified.Notch1, Notch2 has been associated with neoplasia translocation whereas Notch4primarily expressed in endothelial cells. Notch 4 has a specific role in the development of vertebrate endothelium [190]. Notch signaling pathway now is known to be evolutionarily conserved regulating several biological processes, including thymic development of T cells. Malfunctioning in Notch signaling pathway activity has been associated with different human genetic disorders and cancers [12].

2.2.2 Notch receptors and ligands family

In mammals, four Notch receptors (Notch 1 – 4) and five transmembrane Notch ligands, two Jagged ligands (Jag1 and 2) three Delta-like ligands (Dll1,3 and 4) are described so far(**Figure 2.6**) [191]. Notch receptor is 300 kilo Dalton transmembrane receptor, which requires ligands to activate the signaling events in the cell [192]. Notch proteins are synthesized as full-length transmembrane proteins are transported

through the secretory pathway to the trans-Golgi network. During post-translational processing, Notch proteins are cleaved at the S1 cleavage site by a furin-like convertase to generate two subunits. The first subunit is composed of the majority of the extracellular domain while the second consists of the remainder of the extracellular domain and the complete transmembrane and intracellular domains. The mature Notchheterodimer then associates with the plasma membrane, where it becomes available to interact with Notch ligands on a ligand-expressing cell [193, 194].

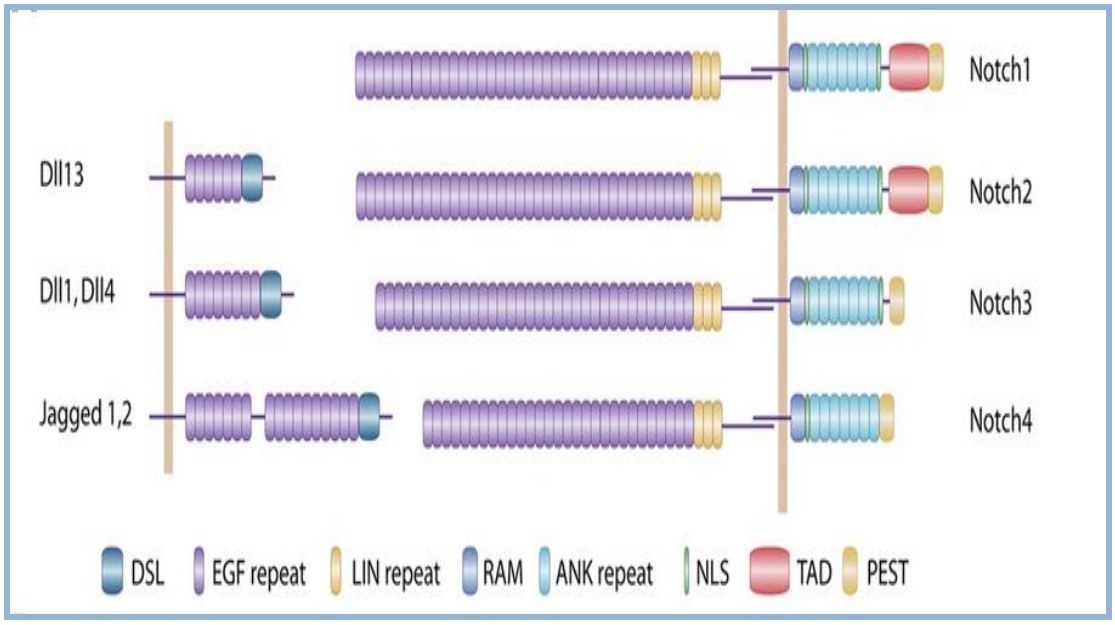

Figure 2.6: Mammalian Notch receptors and Notch ligands. There are 5 Notch ligands in mammals: Jagged1, Jagged2, Delta-like1 (Dll1), Delta-like3 (Dll3), and Delta-like4 (Dll4) (**Source**: Camille Lobry *et al*. **Blood** 2014;123:2451-2459)

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The extracellular domain of Notch receptors comprises epidermal growth factor-like repeats. These epidermal growth factor-like repeats are essential for ligand binding and also prevent ligand-independent activation of Notch receptors. The intracellular domain of Notch proteins possesses a RAM domain, ankyrin repeats, nuclear localization sequences, a proline, glutamine, serine, threonine-rich (PEST) domain,

and a transactivation (TAD) domain. Although Notch receptors are transmembrane proteins yet they show differences in the extracellular and intracellular domains. Notch 1 and Notch2 consists of 36 extracellular epidermal growth factor repeats whereas Notch 2 and Notch 4 consists of 34 and 29 epidermal growth factor repeats respectively. The structural organization of the intracellular domain of Notch receptors also shows differences. Intracellular domain ofNotch1 and Notch2 consists of TAD domain which is absent in Notch 3 and Notch 4. Also, Notch1has a strong TAD region whereas Notch2 has a weak TAD region [195, 196]. Similar to Notch receptors Notch ligands are also transmembrane proteins consists of an extracellular region, a transmembrane region, and an intracellular region. The extracellular region consists of EGF repeats and also insertions within the EGF repeats. As compared to Delta-like ligands jagged ligands contain a greater number of EGF repeats. The extracellular domain of Jagged also consists of a cysteine-rich region which is absent in the Delta ligands of Notch signaling (**Figure 2.6**) [197].

2.2.3 Notch signaling: Canonical and non-canonical

Activation of Notch signaling cascade begins upon interactions between Notch receptors and their ligands (Delta or Jagged) [198]. In the sense of molecular level interaction, Notch receptor when triggered by ligand binding leads to two proteolytic cleavage events at the Notch receptor. The first cleavage is catalyzed by the ADAM-family of metalloproteases. The extracellular domain of Notch receptors constitutes several EGF-like repeats, which upon binding to ligand undergo a conformational change that results in the exposure of an S2 cleavage site within the Notch extracellular domain. Upon the second cleavage catalyzed by a γ-secretase containing complex, the Notch intracellular domain (NICD) is released, which translocates to the nucleus to function as transcriptional co-activator. Since NICD cannot bind directly to

DNA, it heterodimerizes with DNA binding protein RBP-Jk (recombination signal sequence-binding protein Jk) and activates transcription of target genes. However, in the absence of ligand binding, heterodimeric Notch receptors are inactive [18, 199].

The DNA binding protein RBP-J was originally discovered to function as a co-repressor in the absence of nuclear NICD also called C protein-binding factor 1 (CBF1; or CSL for CBF1/Su(H)/Lag1) and repressed Notch target gene expression. Upon active Notch signaling and NCID binding lead to the conversion of CBF1 (RBP-J) from a transcriptional repressor to an activator [199]. Apart from CBF1, Notch-mediated transcription also requires association with other transcriptional co-activators such as Mastermind-like (MAML), a family of transcriptional activator proteins comprising 3 members (MAML1-3). MAML directly interacts with NICD and forming a ternary complex with NICD-CBF1. MAML recruits the histone acetyltransferase p300/CBP that acetylates histones, thereby altering the structure of chromatin to a form amenable to active transcription (**Figure 2.7**) [200-202].

Canonical Notch signaling is well-studied pathway which plays an active role during the development, cell lineage decisions as well as different functions of in cells. For canonical Notch signaling interaction between NICD and RBP-Jk is necessary for the activation of downstream targets. However recent evidence from published data showed that Notch signaling can be also activated by non-canonical RBP-Jk-independent mechanisms. This noncanonical pathway is attributed due to the interaction of NICD with different proteins other than RBP-Jk [203-206]. Similar to the canonical pathway, the non-canonical Notch signaling pathway is important in several cellular processes including oncogenesis and in the immune system [207]

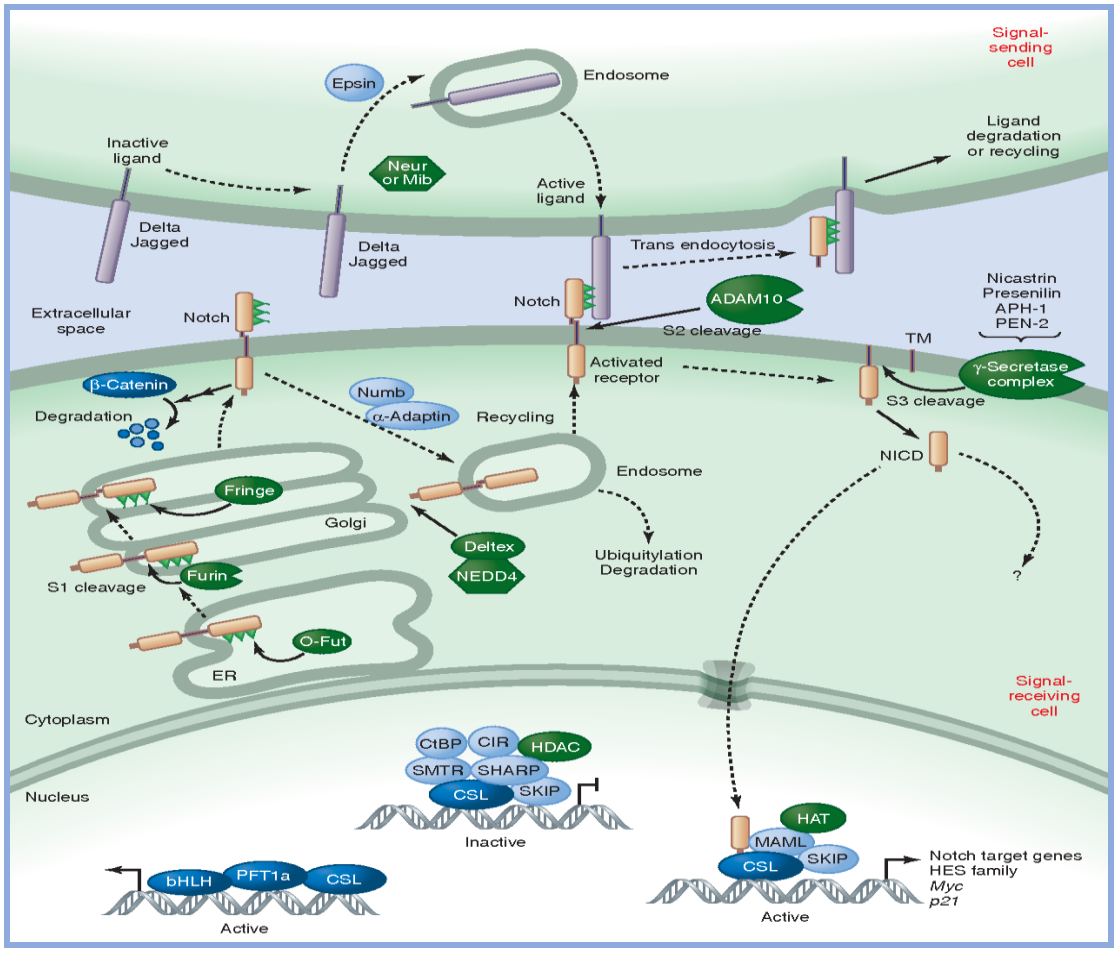


Figure 2.7: **Mechanism of Notch signaling.** Interaction between Notch receptors and ligands activates Notch signaling through the release of Notch intracellular domain which binds different cofactors and modulates the gene expression. (**Source**: **Cold Spring Harb,** Perspect Biol 2012; 4:a011213)

2.2.4 Role of Notch signaling in T cell lineage commitment and differentiation

For the last two decade, the function of Notch during lymphoid development has been extensively studied. Notch signaling plays an essential role in cell fate decision during various stages of T-cell development [191]. Notch signaling is required in lineage decisions in hematopoietic progenitor cells. Signaling through Notch1 is essential for the selection between T and B cell lineage commitment from a common precursor [208]. It has been demonstrated that conditional knockout of Notch1 reduces the T cell population as compared to B cell population in mice[191, 209, 210]. On the other

hand, constitutive activation of Notch1 inhibits B cell development and results in extrathymic T cell development [208]. Further, it has been also shown that interaction between Dll1- or Dll4 and Notch1 are essential T cell development and differentiation of double-negative T cell precursors to double-positive (DP) T cell precursors [211, 212].

Notch signaling besides controlling the B and T cell lineage commitment also determines αβ vs. γδ T cell lineage commitment [208, 213]. However, there are also controversial reports regarding the role of Notch signaling in αβ vs γδ lineage commitment in mice. Notch1 signaling in is mice promotes the αβ T cell development at the expense of γδ T cell development and knock out of one allele of Notch 1 leads to the development of more γδ T cell [208, 213, 214]. In contrast it has been also reported by Kang *et al.* have shown that conditional knockout of Notch1 in mice results in severe impairment in the development αβT cells but does not have any impact on the development and absolute number of γδ T cells suggesting that Notch1 is not essential for αβ vs γδ lineage commitment [215]. However, activation of Notch1signaling in humans is necessary for γδ lineage commitment compared to αβ [216].

When peripheral T cells emigrate from thymus they come to possess attribute differentiation into specialized subsets. In $CD4^+$ T cells, at least four subsets have been identified T helper-type 1 (Th1), T helper-type 2 (Th2) and T helper type 17 (Th17) cells and various subsets of regulatory T cells Although Notch signaling in Th1 and Th2 differentiation has been shown to play an important role, it still remains controversial, because of different experimental approaches used [217]. It has been demonstrated inhibition of Notch signaling by GSI in CD4 T cells in vivo or in vitro or from Notch1 antisense(AS) mice showed defects of IFN-γ production in Th1-

polarizing conditions but does not have any effect on IL-4 production in Th2-polarizing conditions [217, 218]. During Th1 differentiation Notch1 directly bind to the promoter region of Tbx21 (T-bet) and regulates its expression at the transcription level. Increased expression N1ICD in GSI treated $CD4^+$ T cells rescues the expression of IFN-γ and T-bet expression thus suggesting that Notch signaling is necessary for Th1 differentiation [219]. However, a report by Amsen *et al*. and Fang et al. showed that Notch signaling leads to Th2 differentiation. They used both Notch1 and 2 deficient mouse models and showed that these mouse models had impairment in Th2 cell differentiation but had normal Th1 cell differentiation [218, 220]. Hence, it is possible that Notch signaling regulates both Th1 and Th2 cell differentiation through the direct or indirect regulation of Th1 transcription factor T-bet and Th2 transcription factor GATA3. Based on these reports the role Notch signaling in Th1 and Th2 differentiation remains controversial. This unresolved question needs to be further investigated in an appropriate experimental systems.

Besides the role in Th1 and Th2 differentiation, Notch signaling is also implicated in $CD4^+CD25^+$ regulatory T differentiation. It has been shown that Notch3 plays a vital role in $CD4^+CD25^+$ regulatory T cells differentiation, implicating that Notch signaling can be a potential therapeutic target in different autoimmune diseases such as type 1 diabetes [221]. It has been demonstrated by Anastasi *et al*. that Notch3 transgenic mice have increased $CD4^+CD25^+$ T regulatory cells, both in the thymus and in the spleen and these mice have fewer chances to develop streptozotocin-induced autoimmune diabetes due to increased number Treg population in lymphoid organs[221]. Notch ligands Jag1 and Dll1 have been shown to the differentiation of antigen-specific $CD4^+$ T cells into regulatory T cells [222]. Notch and TGF-β signaling have been shown to cooperate to regulate the expression T reg specific

transcription factor Foxp3 and thus helps in maintaining the functionally active Treg population [223]. Other subsets of CD4 lineage such as Th9 are also dependent on Notch signaling for differentiation. In CD4 T cells, stimulation with Jag2 can substitute the requirement for IL-4 for Th-9 induction [224]. Notch signaling has been also implicated in Th17 differentiation [225]. Notch inhibition impedes Th17 differentiation even after stimulation with IL-6 and TGF-β [226]. Thus, Notch can mediate differentiation of different subsets of $CD4^+$ T cells.

2.2.5 Role of Notch signaling in effector functions of T lymphocytes

Notch signaling plays an important role in the fine-tuning of the immune system by regulating the differentiation and effector functions of Immune cells. Notch signaling has been demonstrated to mediate diverse functional outcomes in T cells such as tolerance, T cell proliferation, cytokine production, and T cell-mediated effector responses [227, 228].T cell receptor (TCR) mediated activation of peripheral T cells is a fundamental process of the adaptive immune system. TCR mediated activation of CD4 and CD8 T cells by the antigen bound to MHC molecules and co-stimulatory molecules such CD28 results in the onset of different downstream signaling events. Naïve CD4 and CD8 T cells show low expression of Notch 1 and 2, while upon antigenic activation through TCR signaling CD4 and CD8 increase the expression of Notch receptors [22, 229]. In CD4 T cells, it has been demonstrated that interactions between DLL4 on APCs and Notch on naive T cells are a critical factor in determining the magnitude and quality of primary immune responses [23]. Notch signaling leads to an increased level of activation markers including CD69, CD71, CD98, Glut1, and proliferation. It has been also shown that in CD4 T cells Notch determines the antigen sensitivity of CD^+ T cells. In the presence of Notch signaling, $CD4^+$ T cells need less antigen in order to show full activation. Further, it has been

demonstrated that Notch signaling augments the IL-2 signaling in $CD4^+$ T cells through a positive feedback loop by increasing the expression of high-affinity IL-2 receptor CD25 [23, 229].

Activation-induced Notch signaling is essential for both generation and effector functions of primary naïve CD8 T cells. In CD8 T cells, Notch regulates the activation and effector fates by directly regulating expression of IFN-γ and effector molecules, Perforin and Granzyme B [230]. A study by Maekawa et al showed that Notch 2 deletion in CD8 T cell decreased granzyme B expression and further demonstrated that Notch 2 and RBPJ directly bind to Prf1 and Gzmb promoters to activate their transcription [231]. Maturation of naive $CD8^+$ T cells into effector cytotoxic T lymphocytes (CTLs) is a critical feature CD^+ T cells. Development of CTLs depends, upon the two important transcription factors eomesodermin (EOMES) and T-bet [231]. Notch1 in CD8 T cells regulates the expression of Eomes, and T-bet through direct binding to the promoters of these crucial transcription factors. Inhibition of Notch signaling in $CD8^+$ T cells by chemical inhibition or genetic ablation of Notch signaling decreased the lytic activity of $CD8^+$ T cells [230]. Thus, the Notch pathway in $CD8^+$ T cells has a critical role in antitumor immunity.

In addition to the regulation of effector functions of $CD8^+$ T cells, Notch signaling mediates proximal and distal downstream TCR signaling events [24]. In $CD4^+$ T cells, it has been shown that Notch 1 co-localized with CD4 during the Immune synapse upon activation. CD4 is necessary for the stabilization of initial interaction between TCR complex and MHC class II molecules and enhance TCR effector response by the recruitment of p56Lck [232]. Notch signaling also mediates the Nf-κB activity in T cells which is a crucial transcription factor in regulating the effector functions. Over-expression of cleaved Notch1 (N1ICD) in a mouse model of T-cell acute

lymphoblastic leukemia (T-ALL) and in human T-ALL cells leads to the activation of Nf-κB through the direct interaction with the I kappa B (IκB) kinase (IKK) signalosome and enhancing IKK activity [233]. This suggests that Notch signaling has a potential role in regulating the antitumor immunity and mediating the different effector responses of T cells.

2.3 Epigenetic regulation and immune responses:

2.3.1 Epigenetic modulators

The physiological basis of cell in an organism depends upon the genetic material packed into its nucleus. It bestows upon cell unique physical characteristics and biological functions of specific tissues and organs. However, maintenance of these heritable differences depends on the packaging of DNA and chromatin that dictate the functional distinction of every cell through distinct cellular gene expression programs but do not involve changes in the underlying DNA sequence of the organism. Thus, epigenetics (which literally means 'above genetics') encompasses the heritable changes in the gene expression, without changing the underlying DNA sequences.

The epigenetic state of a cell governs the cellular differentiation and development of an organism mediated by the environmental factors. Thus epigenetic mechanisms have been implicated in multiple diseases such as cancer, inflammation, regenerative medicine, metabolic disease and neuropsychiatric disorders [234]. The molecular mechanisms of epigenetic regulation, include chromatin remodeling, exchange of histone variants, regulation by mi RNAs, methylation of cytokines on DNA, as well as covalent modification of histones.

Histone post-translational modifications (PTMs) are key regulators of changes in chromatin structure. They occur at the lysine residue of the histone N-termini and include acetylation, methylation, and ubiquitination [52]. The protein families that

bring about these changes are called histone modifying enzymes and can be classified into writers, readers, and erasers by the virtue of their function **(Figure 2.8)**. The writers are enzymes that methylate or acetylate either the histone tails directly marking the histone thereby regulating cellular processes. These histone marks are read by a group of regulatory proteins with DNA binding domains that mediate the functional responses to the histone marks hence called 'Readers'. The erasers are enzymes that remove the acetyl and methyl groups.

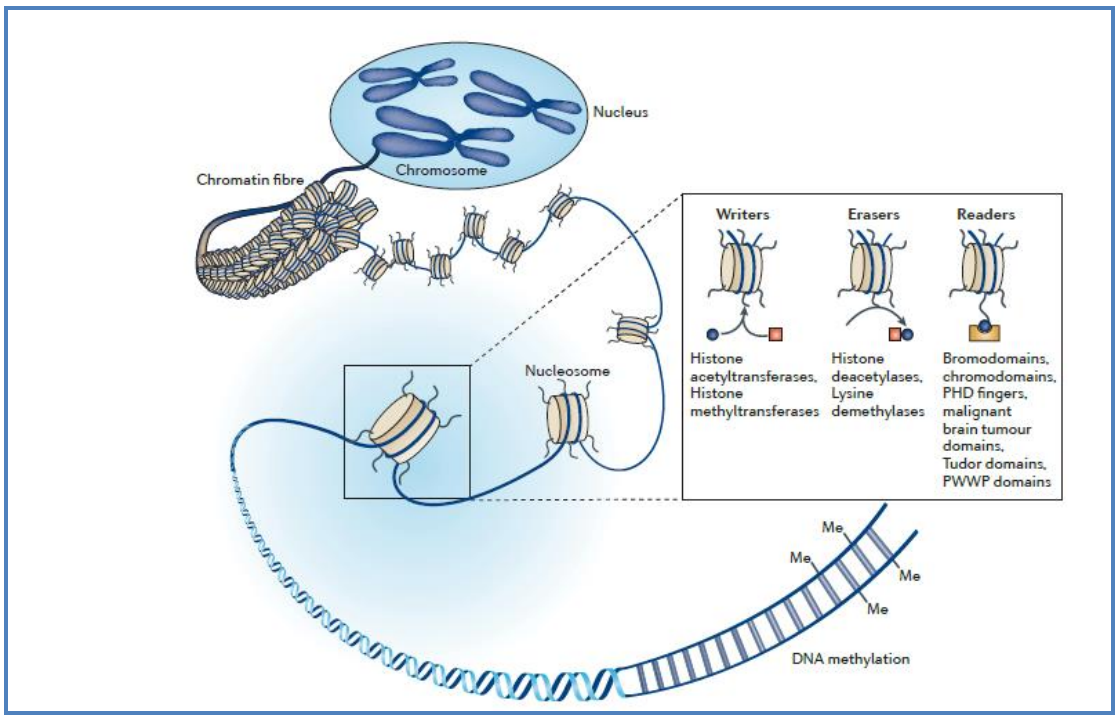


Figure 2.8 Different mediators of epigenetic regulation for gene transcription. The epigenetic regulation of gene expression is mediated by three categories of histone modifying enzyme or protein families which have been designated into writers, erasers and readers. (**Source:** C.H. Arrowsmith *et al* **Nat Rev Drug Discov**. 2012 Apr 13;11(5):384-400)

2.3.2. Histone acetylation

Acetylation status is a common posttranslational modification of both histone and nonhistone proteins hence these enzymes are important in the epigenetic regulation of

gene expression and the control of cellular activities with 1,750 proteins (nuclear and cytosolic) identified as being regulated by posttranscriptional changes in acetylation [235].

Histone acetylation is regulated by two families of enzymes histone acetyltransferases (HATs) and Histone deacetylases (HDACs) that act in an opposing manner. HATs are a group of enzymes that the transfer of an acetyl group to the lysine side chains on the histone protein which neutralizes the positive charge on lysine, thus reducing the affinity of the histone to DNA. Thus, the chromatin undergoes change to acquire more relaxed architecture, which allows the recruitment of the transcriptional elements. HDACs reverse these effects by deacetylation of lysine residues restoring their positive charge. The chromatin structure adopts a condensed state again thereby mediating transcriptional repression [236].

Histone deacetylases (HDACs) family of epigenetic enzymes are not specific in their action but deacetylate a wide range of nonhistone proteins such as transcription factors, DNA-repair proteins, signal transduction molecules, chaperone proteins and are more appropriately termed lysine-specific protein deacetylases [237]. There are 18 HDAC enzymes have been identified in mammalian cells, which are subdivided into four main classes based on their homology to yeast HDACs. Three of the four classes (Classes I, II, and IV) are zinc-dependent enzymes while Class III HDACs called sirtuins and they are NAD^{+}-dependent for their activity [238]. Each of them has a distinct location in the cell. Class I, II and IV are named classical HDACs due to shared sequence similarity and dependence on Zn^{+2} for catalytic activities. Class I HDACs (HDAC 1, 2, 3 and 8) are homologous to yeast RPD3 and are localized to the nucleus. Class II HDACs share similarity to yeast HDA1 and are further classified into Class IIa (HDAC 4,5,7 and 9) and Class IIb (HDAC 6 and 10). They can shuttle

between the nucleus and cytoplasm as they possess nuclear export signals at their C-terminus. HDAC 11 is the sole member of Class IV HDAC family [239]. All three HDACs classes act as transcriptional activators through histone modification; however, they control gene expression by recruiting other proteins (corepressors or coactivators).

HDACs, interact with DNA through multiprotein macromolecular complexes. In mammalian systems, two complexes mSin3A and Mi-2/NuRD (nucleosome remodeling HDAC) have been known to associate with HDACs [240, 241]. These complexes are directed to the desired location on the chromosome by specific transcriptional regulatory factors or by proteins (MeCP2, MBD2, and MBD3) that recognize methylated CpG [242]. Histone lysine acetylation occurs at various sites of Histone 3 and 4 [29]. HDAC enzymes act on an array of non-histone substrates which are involved in several biological processes such as gene expression, proliferation, differentiation, and cell death.

2.3.3 Role of HDACs in immunity

Several members of the HDAC family have a significant role in the regulation of immune responses. They have been implicated in every aspect of the immune response, such as hematopoiesis, lineage specification of distinct immune cells as well as differentiation and acquisition of effector functions [243-245]. HDAC mediate their biological effects through diverse mechanisms either by direct regulation of gene transcription through histone modification or indirectly through modifying non-histone substrates such as transcription factors. Nf-κB, GATA-3, T-bet, and Foxp3 which are crucial transcription factors for the development of immune responses [246-249]. Class I family of HDACs (HDAC1 and 2) have been demonstrated to bind to Nf-κB family corepressor protein p65 and downregulate Nf-κB mediated gene

transcription [250-252]. Thus HDAC enzymes play an important role by in maintaining the balance of inflammatory responses mediated by IL-6, IL-8, IL-1β, and GM-CSF by regulating the histone acetylation status of Nf-κB and AP-1. Studies have shown that HDAC 3 regulates the acetylation of promoters of IκB-α, IL-2, IL-6 and thereby control the activation of Nf-κB [253]. However, another study showed that HDAC1 and HDAC3 directly bind to IκB-α and prevents inactivation of Nf-κB [254]. HDAC also inhibit dendritic cell function by deacetylation of STAT-3 which leads to functional repression of the transcription factors [255]. Furthermore, distinct HDACs are positive as well as negative regulators of TLR pathways [256-258]. Class, I HDAC are also implicated in positive modulation of IFN response [259-261]. Thus HDACs have an integral role in the regulation of innate immunity.

Similarly, HDACs have diverse effects in regulating acquired immunity such as modulating signaling pathways involved in antigen presentation, T helper (Th) cell polarization and lymphocyte development and function. Studies have found that HDAC 11 is required for co-stimulatory molecule expression in macrophages thereby promoting antigen presentation [262]. However, another study showed that HDAC 2 negatively regulates antigen presentation in macrophages through deacetylation and degradation of class II transactivator (CIITA) [263]. Class I HDAC family members HDAC 1 and 2 are essential for early B cell development, as B-cell specific deletion of HDAC1 and HDAC 2 led to almost no maturation of B cells, while in mature cells it led to decreased proliferation and lowered antibody production [264, 265]. T cell development is regulated by HDAC 7 which was supported by a study that demonstrated nuclear transport of HDAC 7 was triggered upon TCR signaling, inducing expression of genes required for T cell selection [266]. In in-vivo experiments, mice deficient in HDAC7 and transgenic mice with a modified form of

HDAC 7 were used to validate the role of HDAC 7 in positive selection in the thymus [267]. Similarly, Treg development requires transcription factor Foxp3, which actually functions as multimolecular complex along with TIP60 (HAT), HDAC7 and HDAC 9[268]. Acetylation of Foxp3 is required for its stability and function [269]. Thus, the role of HDACs in Treg homeostasis was demonstrated in $Hdac9^{-/-}$ mice which showed high expansion of Treg and effective protective responses in inflammatory bowel disease model [270]. Similarly, HDAC 6 was also shown to constrain T reg development by preventing Foxp3 acetylation [271]. In cytotoxic T cells, HDAC 7 was essential for TCR-mediated IFNγ production [272]. In contrast, Class I HDACs (HDAC1 and HDAC 4) negatively regulate the production of IL-2 and IL-5 respectively in T cells [273]. HDACs also modulate Th1 and Th2 differentiation of naïve $CD4^+$ T cells by reversing the hyperacetylation of IFN-γ locus [274].

2.3.4 Histone deacetylase (HDAC) inhibitors

HDAC enzymes are implicated in tumorigenesis. Dysfunctions in both histone acetyltransferases and HDACs are observed in many cancers [275]. Thus many small molecule inhibitors targeting HDAC were intensively investigated in preclinical models as well as clinical studies. HDAC inhibitors consist of natural and synthetic compounds with different specificities and activities and are broadly classified into four main groups based on their structure: hydroxamic acids, cyclic peptides, benzamides, and short-chain fatty acids. HDAC inhibitors act on the HDAC enzyme catalytic sites by blocking the Zn^{2+} binding domain [276].

Pan HDAC inhibitors inhibit HDACs from all classes except Class III. The two widely used pan HDAC inhibitors are Trichostatin A (TSA) and Suberoyl anilide hydroxamic acid (SAHA). Trichostatin A was the first hydroxamate identified to

inhibit HDACs [277]. SAHA also is known as Vorinostat is structurally similar to TSA and approved for clinical use by the Food and Drug Administration [278]. Both are pan-inhibitors of class I and class II HDAC proteins. The mechanisms by which HDAC inhibitors arrest growth and induce cell death of transformed cells are complex. Upon treatment with HDAC inhibitors, histones and many non-histone proteins undergo hyperacetylation that is involved in the regulation of expression, proliferation, cell migration, and cell death. This altered gene expression can lead to cell cycle arrest, terminal cell differentiation and cell death of transformed cells by activating the intrinsic or extrinsic apoptotic pathway, mitotic failure, autophagic cell death, oxidative stress-induced cell death [279-283]. HDAC inhibitor also mediates its antitumor activity by blocking angiogenesis [284].

Several of the HDAC inhibitors are recruited in clinical trials for the treatment of solid tumors and hematological malignancies [58]. However, HDAC inhibitors as single agents have shown limited clinical efficacy which may be due to complicated mechanisms by which HDAC inhibitors modulate tumorigenesis and progression [285-287]. Hence a combination of HDAC inhibitors with other chemotherapeutic agents or anti-tumor therapies has been considered as an effective treatment approach. One such potential therapy that could be combined with HDAC inhibitor treatment is immunotherapy.

The HDAC inhibitors combination with immunotherapy is principally based on the phenomenon of immunogenic cell death, a process in which dying tumor cells can stimulate cellular uptake, activation and cross-present through antigen presenting cells (APCs), thereby inducing antitumor T cell responses [288]. In-vitro studies showed that treatment with pan HDAC inhibitor Vorinostat resulted in efficient uptake of MC38 colon carcinoma cells, by dendritic cells (DCs) [289]. Moreover, another

study stated that SAHA stimulated the release of mediators like HMGB1, calreticulin, and ATP, which serve as important immunologic mediators, by dying tumor cells [290]. Thus HDAC inhibitors induce a form of cell death with immunogenic properties. HDAC inhibitor also led to changes in gene expression within the tumor that result in activation of the immune response. Various studies have demonstrated that treatment with HDAC inhibitor resulted in tumor upregulation of NK ligands that lead to activation of cytotoxic lymphocytes such as natural killer cells [291-294].

Studies have reported synergistic effects of treatments combining HDAC inhibitors and immunotherapy in the treatment of cancer such as pan HDAC inhibitors in combination with the immune cell stimulating antibodies to lead to synergistic inhibition of tumor growth by activating CD8 T cells in immunocompetent models of mammary, renal and colon carcinoma[295].In another study involving CD8 T cells, they have demonstrated that the pan HDAC inhibitors helped to enhance both adoptive transfers of tumor-specific $CD8^+$ T cells in mice bearing B16 melanoma tumors [296].

However to obtain clinically significant results with combinational therapies the effect of HDAC inhibitors on immune cells need to be delineated. Several studies in this arena have presented exciting results; HDAC inhibitors distinctly affect each immune cell subset and have pleiotropic effects on immune cell function **(Figure 2.9)**. Several studies have reported that HDAC inhibitor such as Sodium butyrate, TSA and trapoxin promote tumor eradication by upregulating expression of MHC class I and class II proteins, pCD40, CD80, and CD86 as well as adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) on acute myeloid leukemic (AML) cells, human neuroblastoma tumor cells, and mouse plasmacytoma cells in vitro [297, 298].

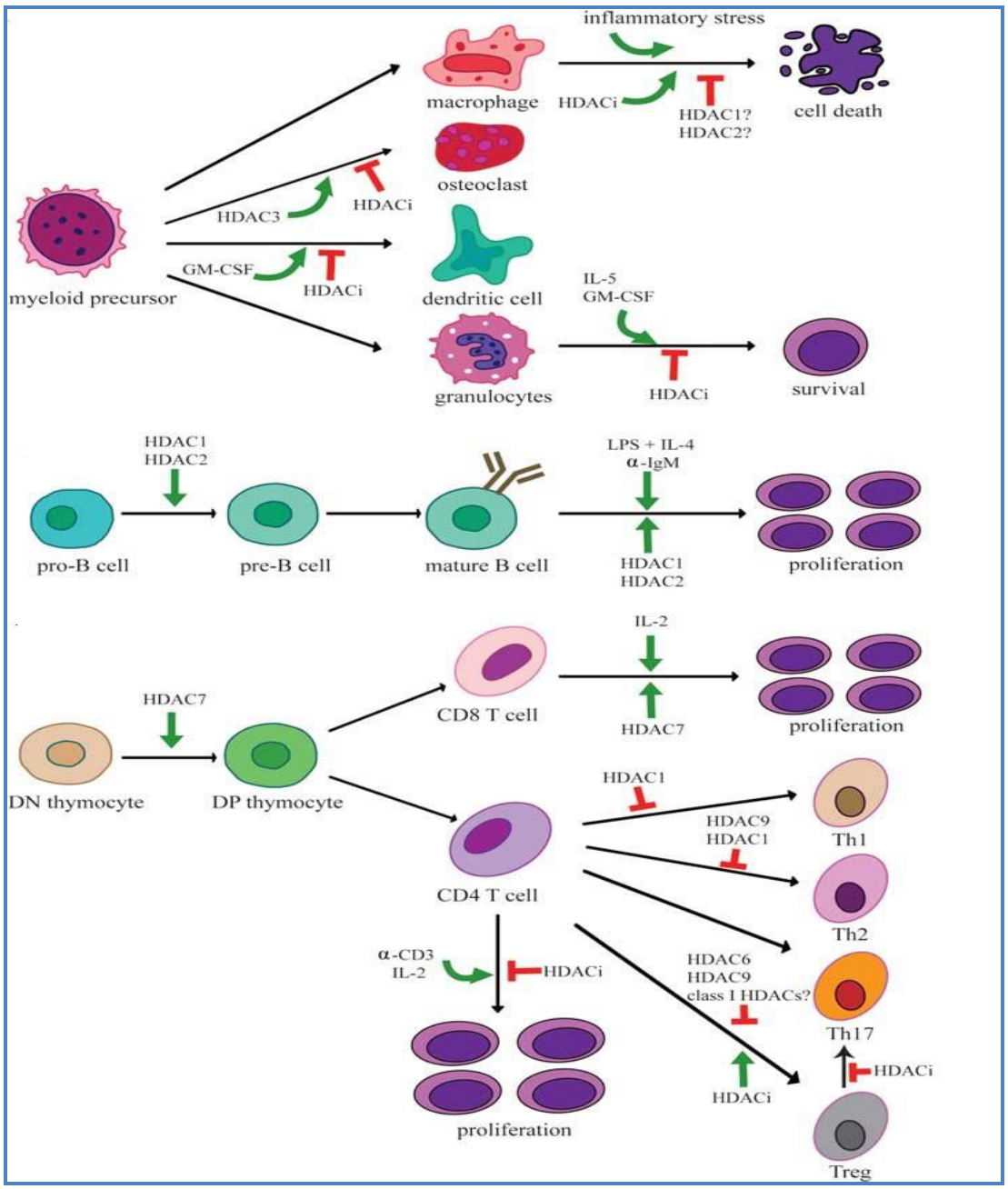


Figure 2.9: Immune modulation by HDAC inhibitors. The effect of HDAC inhibitors on each immune cell is differential. In some cells like B cells and myeloid lineage cells it promotes survival and proliferation, however, in T cells the effects are pleiotropic depending on the cell subsets (**Source**: Sweet, M. J. *et al* (2012), **Immunol Cell Biol**, 90: 14-22.)

Although immunogenicity of the tumor is enhanced by HDAC inhibitor, studies show that they produce anti-inflammatory effects in immune cells. Specifically, HDAC inhibitors have been shown to induce the regulatory T cell (Tregs) generation or

stabilization of Tregs in the inflammatory microenvironment[299]. However, a contradictory study reports that HDAC inhibitor treatment reduces Treg expansion [300]. Furthermore, HDAC 4 was shown to repress IL-2 mediated expansion of Tregs while HDAC inhibitor LAQ824 represses IL-10 production and also hinders Treg function.

HDAC inhibitors have dual effects in the modulation of immune cells. In-vitro as well as in-vivo studies have shown that HDAC inhibitors enhance the function of CD8 T cells by upregulating pro-inflammatory cytokines IFNγ and TNF α [301, 302]. However, in the tumor microenvironment, these effector T cells do not encounter the pro-inflammatory environment necessary for their anti-tumor effector functions as HDAC inhibitor repress the expression of pro-inflammatory cytokines and co-stimulatory molecules which are required for tumor cell growth and survival. Although in case NK cells HDAC inhibitors are known to upregulate NKG2D ligands thereby leading to efficient recognition by activated NK cells, but studies recently have shown that HDAC inhibitors suppress the viability and function of NK cells [303-307]. Similar studies were also reported on CD4 T cells, wherein one study demonstrated HDAC inhibitor treatment suppressed cytokine production and induced apoptosis while in another study HDAC inhibitors led to activation and increased functional responses inCD4 T cells [308-310]. Valproic acid treatment to bladder carcinoma cells was demonstrated to increase γδ T cell cytotoxicity against tumor cells [74]. Sodium valproate treatment, however, showed decreased proliferation of antigen expanded γδ T cell population [75]. Thus it appears that the effect of HDAC inhibitors on immune cells is like a double-edged sword and more studies are needed to elucidate the mechanism of HDAC inhibitor-mediated immune modulation.

2.3.5 Histone methylation

Histone methylation can occur at either lysine or arginine residues on histones. These residues can be monomethylated, dimethylated or trimethylated. Methylation is carried out by S-adenosylmethionine (SAM)-dependent methyltransferases and these epigenetic marks are removed by lysine-specific histone demethylases [311].

Histone lysine methylation brings about both transcriptional activation and repression. For example, H3K4me3 is a hallmark of transcriptionally active genes, whereas H3K9me3 and H3K27me3 are associated with silenced genes [299]. The repressive histone marks are mediated by multi-protein complexes, one such evolutionarily conserved complex is the Polycomb repressive complex (PRC). PRC2 complex is responsible for the methylation (di- and tri-) of lysine 27 of histone H3 (H3K27me2/3) via its enzymatic subunits Ezh1 and Ezh2 [312]. The enhancer of zeste homolog 2 (Ezh2), contains a SET domain promoting the methyltransferase activity, while the three other protein components of PRC2, namely EED, SUZ12, and RpAp46/48, induce compaction of the chromatin permitting the enzymatic activity of Ezh2 **(Figure 2.10)** [313]. Several studies have demonstrated Ezh2 to be essential for cell cycle progression, stem cell pluripotency as well as has been implicated in oncogenesis [314-318].

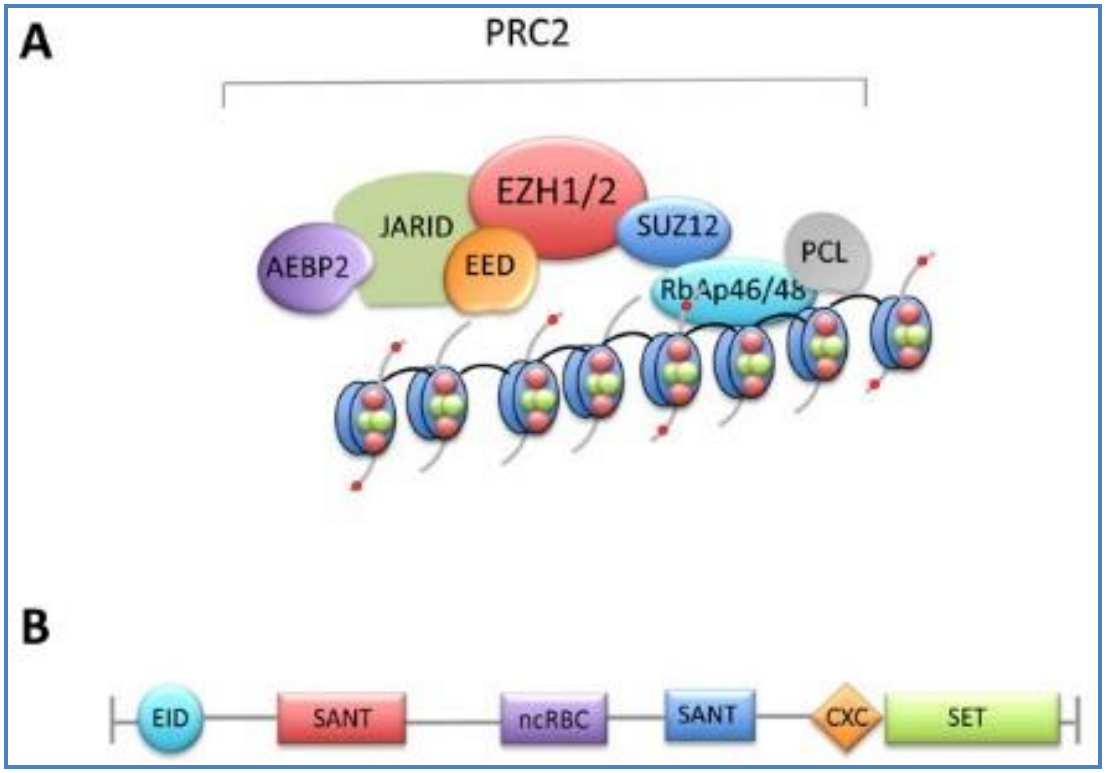


Figure 2.10: Ezh2 methyltransferase in PRC2 complex. (A) PRC2 complex is composed of either of the two methyltransferase domains Ezh2 or Ezh1, EED, Suz12, and RbAp46/48, which are required for its enzymatic activity. The other components AEBP2, PCL, and JARID2 also form the part of PRC2 but there are still uncharacterized. **(B)** Characterized domains of Ezh2 are EID, EED interaction domain; SANT, SWI3, ADA2, N-CoR, and TFIIIB DNA-binding domain; ncRBD, non-coding-RNA-binding domain; CXC, cysteine-rich domain; SET, Su(var)3-9, enhancer of zeste, trithorax domain which together forms the complete and functional Ezh2. (**Source**: Christofides A *et al* **Oncotarget**. 2016 Dec 20;7(51):85624-85640)

2.3.6. Role of methyltransferase Ezh2 in oncogenesis

The methyltransferase Ezh2 modulates the expression of important genes which are involved in fundamental cellular processes such as cell cycle progression, cell proliferation, cell differentiation, and apoptosis. Mutations in Ezh2 have been reported in various malignancies and detailed studies have evolved Ezh2 to be a prognostic biomarker for cancer progression and development [319]. It has been demonstrated that elevated expression of Ezh2 was associated with advanced stages of prostate cancer and led to poor prognosis [320]. Further detailed studies have

elucidated Ezh2 is upregulated in solid tumors such as lung, hepatocellular, colorectal, breast and pancreatic cancers [321-325]. In blood malignancies like myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), both overexpression and loss-of-function mutations have been reported [326-328]. Hence Ezh2 has been shown to play the role of both oncogenes as well as a tumor suppressor to drive oncogenesis. The dysregulated expression of Ezh2 in cancer can be attributed to other oncogenic signaling pathways as well. In Ewing's sarcoma, the fusion protein EWS-FLI1 induces Ezh2 overexpression [329]. Similarly, gain-of-function mutations in ras oncogene have also been known to promote Ezh2 expression in pancreatic and non-small cell lung cancer through PI3K/AKT or MEK/ERK signaling [330]. Studies have shown that dysregulated Rb-E2F signaling in the bladder and small lung cell cancer activate Ezh2 expression through direct binding of E2F to Ezh2 promoter [331, 332]. Another tumor-driven factor, Hypoxia can promote Ezh2 overexpression in solid tumors is mediated by binding of HIF-1α to Ezh2 promoters and have shown to be associated with poor prognosis in breast cancers [333]. Besides transcriptional activation and overexpression, posttranscriptional modification of Ezh2 has been implicated to drive tumorogenesis. Micro-RNAs that bind and modulate the stability and integrity of Ezh2 have a role to play in regulating Ezh2 expression during oncogenesis. Downregulation of miR-138 in osteosarcoma targets Ezh2 to degradation and thereby increases cisplatin sensitivity of tumor cells [334]. Similarly, downregulation of miR-101 leads to proliferation and invasion due to Ezh2 mediated overexpression of oncogenes in glioblastoma [335]. Direct covalent modifications such as AKT1 mediated phosphorylation activates Ezh2 to directly interact and induce the expression of androgen receptor target genes in prostate cancer leading to disease progression [336].

The mechanisms underpinning Ezh2 in driving oncogenesis are diverse and tumor specific. Direct chromatin modulation through PRC2 mediated suppression of transcriptional programs specifies differentiation and apoptosis such as GSK-3β and p53 in cervical cancer while p21 in ovarian cancer [337-339]. Ezh2 also leads to activation of oncogenic signaling pathways such as Wnt, Notch and Stat3 in colorectal cancer, prostate cancer, and glioblastoma respectively [339-341]. Activation of these oncogenic pathways promotes survival and proliferation of cancer stem cells. Ezh2 may also promote oncogenesis through disruption of cell cycle progression. Studies have demonstrated that dysregulated Ezh2 leads to cancer development in melanoma, colorectal cancer and breast cancer [342-344]. Ezh2 mediated tumorogenesis could also be promoted through invasion and epithelial to mesenchymal transition (EMT). Studies have reported that Ezh2 interacts with SNAIL1 and downregulated E-Cadherin expression and also silence disabled homolog 2 –interacting protein (DAB2IP) thereby regulating EMT and promote metastasis in colorectal cancer [345-347]. Ezh2 even acts independently of PRC2 to activate oncogenic pathways to induce tumorogenesis. Individual studies have shown that Ezh2 induces expression of androgen receptor target genes in prostate cancer and promote estrogen receptor and Wnt target gene expression in breast cancer while functioning as transcriptional co-activator [336, 348]. Ezh2 can posttranscriptionally modify non-histone substrates that contribute to tumorigenesis. Studies have reported that methylation of STAT3 and androgen receptor in glioblastoma and prostate cancer respectively by Ezh2 led to the expression of downstream oncogenic targets that drive tumor development [336, 339].

Methyltransferase Ezh2 has been extensively implicated to trigger oncogenesis and thus therapeutic targeting of Ezh2 with small molecule inhibitors have been an arena

of immense potential. The most studied nonspecific Ezh2 inhibitor 3-deazaadenosine A (DZNep) have shown significant efficacy in various malignancies but also showcased toxicity in animal models [349, 350]. Other small molecule inhibitors similar to Dznep such as ECGC, PL3, and CDF also pose the problem of toxicity [351-353]. Recently potent and specific small molecule inhibitors of Ezh2, GSK126, EPZ00587, and GSK926 have been developed that have shown significant anti-tumor activity in in-vitro as well as in xenograft mice models [354-356]. Hence phase 1/2 clinical trials have been pursued with these agents for B cell lymphomas and advanced solid tumors [354, 357, 358]. Ezh2 inhibitors have to overcome the barriers of specificity and toxicity to establish clinical efficacy to be used for cancer therapy. The tumor suppressive role of Ezh2 poses further complications in therapeutic targeting of ezh2 in cancer cells. Thus alternative ways of targeting Ezh2 for cancer therapy are been currently explored.

2.3.7. Role of methyltransferase Ezh2 in T cell differentiation and function

The development and maintenance of T cells are regulated by several factors such as cytokines, transcription factors, and epigenetic regulators. Ezh2 is one such epigenetic modifier that regulates various aspects of T cell immunity **(Figure 2.11)**. Earlier studies have shown that mature peripheral T cells express Ezh2 and are critical for Th-1 and Th-2 polarisation of naïve $CD4^+$ T cells elucidated with T cell-specific Ezh2 deletion [359, 360]. Ezh2 was also shown to suppress lineage-specific cytokines in CD4 T cells [361]. Detailed studies in the role of Ezh2 in differentiation of Th1 subset, showed that Ezh2 mediates a particular histone methylation signature for genes associated with Th1 differentiation such as Ifng, Tbx21, and Stat4.Ezh2 increases the stability of T-bet, thereby implicating Ezh2 as an inducer of Th-1 differentiation[362]. Interestingly, Ezh2 was also demonstrated to have a novel

cytosolic function of mediating TCR induced actin polymerization and T cell differentiation apart from the methyltransferase activity [363]. In effector CD4 T cells, Ezh2 promotes maintenance of effector function by promoting the survival of T cells via inhibiting expression of apoptotic proteins [364]. In regulatory T cells, Ezh2 was shown to co-localize and upregulate Foxp3 expression [365].

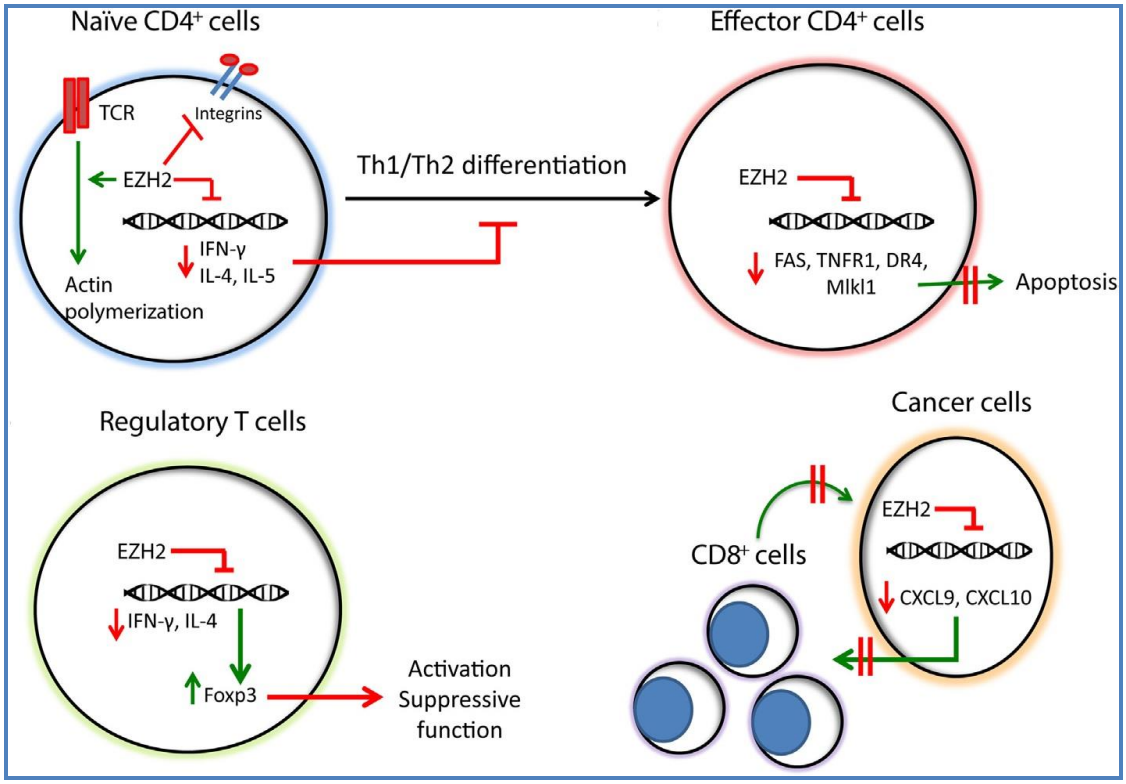


Figure 2.11: Ezh2 regulates immune responses in different subsets of T cells. Ezh2 mediates regulation of T cell responses in various aspects, either by downregulation of cytokines such as IFN-γ and IL-4/IL-5 of naïve $CD4^+$ T cells or inhibiting the expression of apoptotic molecules, such as FAS, TNFR1, DR4, and Mlkl1 in effector CD4 T cells. Ezh2 also promotes the expression of Foxp3 and the activation of T regulatory cells. Ezh2 prevents the mobilization of cytotoxic $CD8^+$ T to tumor sites through inhibition of chemokines CXCL9 and CXCL10 produced by cancer cells required for T cell recruitment[366]. (**Source**: Karantanos T *et al*, 2016 **Front. Immunol**. 7:172.)

Ezh2 was implicated to have a role in regulating anti-tumor immunity. In an ovarian cancer mouse model, inhibitors of Ezh2 and DNMT1 led to increasing expression of Th-1 type chemokines and infiltration effector T cells to tumor site [367]. Thus targeting Ezh2 was shown to augment anti-tumor CD8 T cells and better prognosis. A

similar effect was observed in colorectal cancer as well, wherein ezh2 inhibition increased the production of CXCL9 and CXCL10 by cancer cells which led to CD8 T cell recruitment [368]. In melanoma mice model, IL-2 or anti- CTLA-4 immunotherapy in conjunction with Ezh2 inhibition led to the accumulation of CD8 T effector cells and suppressed melanoma growth. Ezh2 also directly regulates anti-tumor immune responses, a study has reported that in NK cells Ezh2 inhibition leads to enhanced cytotoxicity against tumor cells and increased expression of IL-15R and NKG2D receptor [369]. Ezh2 also modulates the functional responses of T cells. In a recent study, genetic and pharmacological depletion of Ezh2 in T cells led to a functional alteration in Tregs and enhanced cytotoxic effector T cells. Further, the group also demonstrated that Ezh2 combined with anti-CTLA-4 therapy improved the anti-tumor response of T cells in the murine model [370]. Ezh2 regulates the immune responses in tumor microenvironment through metabolic reprogramming, which was demonstrated in ovarian cancer by Zhao *et al*. The study showed that ovarian cancer cells induce miRNA expression in intratumoral CD4 and CD8 T cells and suppress Ezh2 expression via competitive glucose consumption which leads to decreased survival and function of T cells [371]. Thus, Ezh2 plays a significant role in regulating tumor-specific effector T cells which sparks interesting speculation about whether Ezh2 could be therapeutically targeted into effective immunotherapy. However, it is necessary to evaluate the role of Ezh2 in anti-tumor functions of effector T cells.

2.3.8. Epigenetic regulation in T cells

Epigenetic mechanisms in conjunction with transcription factors play a critical role in orchestrating the transcriptional changes associated with T cell differentiation. T cells undergo a circular cycle of differentiation; wherein upon antigen encounter naïve T

cells differentiate into effector T cells. When the infection or antigen load clears off, majority of effector cells undergo apoptosis and a selected few further differentiate into memory T cells. This cycle again begins when the second encounter with the same antigen occurs [372]. This cyclic nature of differentiation requires an on-off pattern of transcriptional and epigenetic modification (**Figure 2. 12**) [373]. The two major epigenetic modifications that govern T cell differentiation are DNA methylation and histone post-translational modifications. DNA methylation majorly occurs on the CG nucleotide pattern called CpG islands. These CpG islands that are subject to methylation are generally located at transcription start sites [374]. DNA methylation is associated with transcriptional repression as well as activation. During T cell differentiation, methylation decreases at promoter sites of differentiation associated genes such as those required for effector function acquisition. While methylation marks are gained at genes required to maintain the naïve state. In the case of histone modifications such as acetylation and methylation also play a significant role in the regulation of T cell differentiation [375]. Histone acetylation marks (H3K9ac) are associated active chromatin poised for transcription while histone methylation marks have differential control of transcription such H3K9me3 and H3K27me3 are repressive but H3K4me3 are permissive.

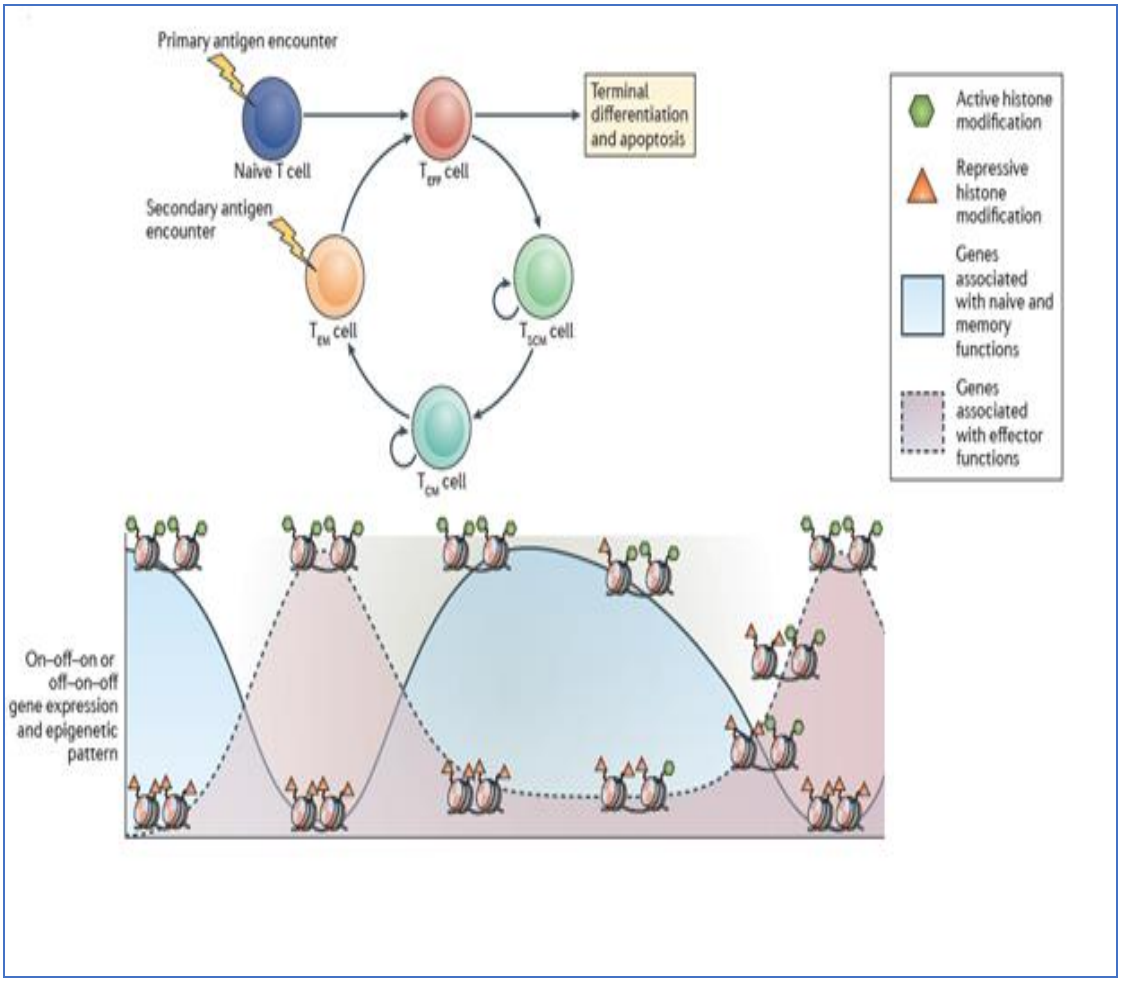


Figure 2.12: Epigenetic regulation associated with phases of T cell differentiation. In the differentiation model of T cells, upon antigen encounter, naïve T cells undergo a progressive cycle of differentiation to acquire effector functions. This differentiation cycle is dictated by the specific transcriptional program which follows the on-off-on pattern. These transcriptional changes are accompanied by similar changes in the epigenetic landscape, which are illustrated by gain or loss of activating and repressive histone modifications. TCM, central memory T; TEM, effector memory T; TSCM, stem cell memory T [376].(**Source**: A. N. Henning *et al* 2018 **Nat Rev Immunol**. 2018 May;18(5):340-356.)

During differentiation of naïve T cells into effector fates, studies have found that permissive epigenetic patterns (H3K9ac and H3K9me) are lost while repressive marks are increased at gene loci whose expression is the notion of required for the acquisition of effector functions. These gene loci include transcription factors FOXO1, KLF2, LEF1, and TCF7, also gene expressed in memory subsets like

IL2RA, CD27, TNF and CCR7.[377]. On the other hand, effector function linked transcription factors (Eomes, TBX21, and PRDM1) and functional genes (GZMB, PRF1, IFNG and KLRG1) have decreased repressive marks and present an open chromatin state [37]. Thus the epigenetic state of T cell during differentiation also follows specific patterns during each phase of differentiation. The epigenetic marks and the modulators that mediate these states in effector T cells could be targeted therapeutically to achieve effective functional responses in conditions of immune dysfunction such as cancer or autoimmune diseases. Hence further studies to delineate the epigenetic regulation of effector functions of T cells are needed for each subset of T cells.

Chapter 3
Material and methods

3.1 Cell culture media

Culture media were used for growth and maintenance of mammalian cell lines used in this study. Plain media was prepared by dissolving powdered RPMI-1640 medium (Invitrogen Life-Technologies, Grand Island, N.Y.) or IMDM (Invitrogen Life - Technologies, USA.) 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 filtered with 0.45mM membrane filter (Millipore Co, USA) and after sterility check was stored at -20ºC until use.

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

3.2 Maintenance of mammalian cells

Mammalian tumor cell lines as listed below were cultured in the specified medium and were maintained at 37^0C and 5% CO_2.

3.2.1 Passaging suspension cultures

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

3.2.2. Passaging Adherent Cultures

The adherent cell lines were sub-cultured at 65-75% confluency. The cells were removed from tissue culture flasks or plates by dissociation reagent PBS-trypsin (Sigma, USA; 0.3% trypsin in 0.01M PBS pH 7.5, containing 0.02% EDTA, sterilized by Millipore filtration). Briefly, Cells were washed using plain medium gently without disturbing the cell layer. Wash solution was removed from the culture vessel and pre-warmed dissociation reagent was added to it. When 90% of cells have detached from the flask, cells were collected and washed with the plain medium before use. Cells were seeded at a density of$0.5x10^6$cells per 5 ml of complete medium (containing 10% FCS) in $25cm^2$ flasks or 60mm plates.

Table 3.1 List of cell lines:

Cell line	Type	Tumor	Media used
AW13516	Adherent	Squamous oral carcinoma	IMDM + 10% FCS
COLO 205	Mixed	Colorectal adenocarcinoma	RPMI + 10% FCS
Raji	Suspension	Burkitt's lymphoma	RPMI + 10% FCS

3.2.3 Cryopreservation of cell lines

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

3.2.4 Reviving of cell lines

Cryovials containing the frozen cells were removed from liquid nitrogen and immediately placed in a water bath at 37ºC. Cells were quickly thawed by gently swirling the vial in a 37ºC water bath. Cells were transferred to a centrifuge tube containing pre-warmed complete growth medium was added drop-wise into the centrifuge tube containing thawed cells. Cells suspension was washed twice and cell viability was checked using the vital dye trypan blue (Filter sterilized 0.4% trypan blue (Fluke AG, Buchs SG, Switzerland).

3.3 Antibodies and Conjugates

Table 3.2: List of Purified Antibodies:

Sr No.	Purified antibody	Clone/ Catalog No.	Source
1	CD3	555329	BD Pharmingen, USA
2	PD-1	329926	Bio legend, USA
3	T-bet	ab91190	Abcam, USA
4	Eomes	Ab23345	Abcam, USA
5	Nf-κB	8242S	Cell signaling technology, USA
6	STAT5	25656S	Cell signaling technology, USA
7	phospho-STAT5	9314S	Cell signaling technology, USA
8	Notch-1	ab52627	Abcam, USA
9	Notch-2	ab8926	Abcam, USA
10	Notch-3	ab23426	Abcam, USA
11	Notch-4	ab184742	Abcam, USA
12	Jagged-1	2620 S	Cell signaling technology, USA
13	Jagged-2	ab109627	Abcam, USA
14	Dll1	ab84620	Abcam, USA
15	Dll-4	ab7280	Abcam, USA
16	p53	sc-55478	Santa Cruz Biotechnology, USA
17	p21	ab80633	Abcam, USA
18	Notch 1 intracellular	D1E11	Cell signaling technology, USA

	domain		
19	Notch 2 intracellular domain	D76A6	Cell signaling technology, USA
20	GSK-3β	12456 S	Cell signaling technology, USA
21	Phospho- GSK-3β	9323 S	Cell signaling technology, USA
22	Total Histone H3	ab46765	Abcam, USA
23	Acetyl Histone H3	ab441	Abcam, USA
24	Total Histone H4	ab10158	Abcam, USA
25	Acetyl Histone H4	06-598	Millipore Sigma,USA
26	c-Jun	9165 S	Cell signaling technology, USA
27	Phospho c-Jun	3270 S	R & D Systems, USA
28	Total Akt	4691 S	Cell signaling technology, USA
29	Phospho-Akt	9611 S	Cell signaling technology, USA
30	Ezh2	4905 S	Santa Cruz Biotechnology, USA
31	H3K9 ac	07-593	Millipore Sigma,USA
32	H3K9me3	07-442	Millipore Sigma,USA
33	H3K27me3	07-449	Millipore Sigma,USA
34	β-actin	A5316	Millipore Sigma,USA
35	Normal Rabbit IgG	12-370	Millipore Sigma,USA

Table 3.3: List of conjugated Antibodies:

Sr No.	Conjugated antibody	Clone/catalog No.	Source
1	CD3-PE	UCHT1	B D Biosciences, USA
2	CD3-FITC	HIT3a	B D Biosciences, USA
3	CD3-PECF594	UCHT1	B D Biosciences, USA
4	CD3-PerCp5.5	SK7	B D Biosciences, USA
5	CD3-PE Cy7	SK7	B D Biosciences, USA
6	CD14-APC	HIT3a	B D Biosciences, USA
7	CD3-BV421	SK7	B D Biosciences, USA
8	CD3-APC	RPA-T_4	B D Biosciences, USA

9	CD3-PerCP	SK7	B D Biosciences, USA
10	CD3-BV786	SK7	B D Biosciences, USA
11	CD19-FITC	4G7	B D Biosciences, USA
12	CD14-PerCp	M5E2	B D Biosciences, USA
13	CD56-PerCPCy5.5	B159	B D Biosciences, USA
14	Vδ2-PE	B6	B D Biosciences, USA
15	CD25-PECy7	M-A251	B D Biosciences, USA
16	CD69-BV786	FN 50	B D Biosciences, USA
17	CD71- PECy7	C2F2	B D Biosciences, USA
18	Phospho-Tyrosine PE	WM53	B D Biosciences, USA
19	CD132-PE	581	B D Biosciences, USA
20	CD122-FITC	HIT2	B D Biosciences, USA
21	CD68- APC	Y1/82A	B D Biosciences, USA
22	Perforin BV421	G10F5	B D Biosciences, USA
23	Granzyme B APC	1610A1	B D Biosciences, USA
24	PD-1 PerCPCy5.5	1610A1	B D Biosciences, USA
25	PD-L1 PECF594	hIL4R-M57	B D Biosciences, USA
26	CD16-BV421	GH1/61	B D Biosciences, USA
27	NKG2D-APC	HI100	B D Biosciences, USA
28	CD107A-APC	M-T271	B D Biosciences, USA
29	KIR2DL2/3-PE	B1	B D Biosciences, USA
30	Notch-1 PE	B1	B D Biosciences, USA
31	Notch-2 APC	6B10.2	SantaCruz Biotechnology, USA
32	CD62L-APC	DREG-56	B D Biosciences, USA

FITC: Fluorescein isothiocyanate; **PE**: Phycoerythrin;
PerCP: Peridinin Chlorophyll Protein; **APC**: Allophycocyanin;
AF488: Alexa Fluor 488; **AF647**: Alexa Fluor 647,
APC-Cy7: Tandem conjugate that combines APC and a cyanine dye (Cy7):
PE-Cy5: Tandem conjugate that combines phycoerythrin and a cyanine dye.
PerCP-Cy™5.5: Tandem conjugate that combines PerCP with a cyanine dye;
PE-Cy7: Tandem conjugate that combines phycoerythrin and a cyanine dye (Cy7):
APC-H7: APC-cyanine tandem fluorochrome, which uses an analog of Cy7 and has similar spectral properties to APC-Cy7; **BV421:** Brilliant violet 421; **BV 786:** Brilliant violet 421

PE-CF594: Tandem conjugate of PE and CF594, **PB:** Pacific Blue

Table 3.4 List of Secondary Antibodies:

Sr No.	Secondary antibody	Catalog No.	Source
1	Goat anti-Mouse HRPO	A-4416	Millipore Sigma, USA
2	Goat anti-Rabbit HRPO	HPO-3	Millipore Sigma, USA

Table 3.5 List of recombinant proteins used:

Sr No.	Recombinant proteins used	Catalog No.	Source
1	Recombinant IL-2	200-02	Peprotech

3.4 Antigens used in the study

Table 3.6 List of Antigens used:

Sr No.	Antigens used	Source
1	Bromohydrin pyrophosphate (BrHPP/IPH1101)	Innate Pharma, Marseille, France
2	1-Hydroxy-2-methyl-2-buten-4-yl 4-diphosphate (HDMAPP)	Echelon Bioscience, Salt Lake City, USA
3	Zoledranate (Zoldonat)	Natco, India

3.5 Inhibitors used in this study

Table 3.7 List of Inhibitors used:

Sr No.	Inhibitors used	Catalog No.	Function of Inhibitor	Source
1	Gamma Secretase Inhibitor (GSI-X)	565771	Notch signaling Inhibitor	Millipore Sigma,USA
2	3-Deazaneplanocin-A hydrochloride	SML-0305	Ezh2 inhibitor	Millipore Sigma,USA
3	Sodium Valproate	P4643	HDAC inhibitor	Millipore Sigma,USA
4	Trichostatin-A	T-8552	HDAC inhibitor	Millipore Sigma,USA
5	Suberoylanilide	SML-0061	HDAC inhibitor	Millipore Sigma,USA

	hrdroxamic acid (SAHA)			
6	CHIR 99021	SML-1046	GSK-3β inhibitor	Millipore Sigma,USA
7	SB415286	SP-3567	GSK-3β inhibitor	Millipore Sigma,USA

3.6 Study group

The study was approved by the Institutional Ethics Committee (ACTREC-IEC). Peripheral blood was collected from healthy individuals. The study was carried out upon the approval of the Institutional Ethics Committee (TMC-IECIII Project no. 166) and written informed consent was obtained from the donors prior to collection of blood samples. The experimental conditions and procedures for handling blood samples were performed as per the biosafety guidelines of the Institute Biosafety Committee.

3.7 Separation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood samples 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 an equal volume of normal saline (0.82% NaCl in double distilled water). The diluted blood sample (8ml) was carefully overlaid 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) in Beckman centrifuge. The lymphocytes form a ring at the interface between Ficoll – Hypaque and plasma due to their specific buoyant density. These cells were collected in fresh culture tubes washed twice with saline and viability was checked with trypan blue dye.

3.8 Immunomagnetic isolation of γδ T cells

γδ T cells were isolated from peripheral blood lymphocytes (PBLs) using TCR-γδ Microbead Kit (Miltenyi Biotech, Germany). Freshly isolated PBLs were washed with MACS buffer (degassed PBS with 0.5% BSA and 2 mM EDTA) and cells were suspended in 40µl of buffer per total 10^7 cells. The PBLs were incubated with 10 µl of Anti-TCR-γδ hapten mAb per 10^7 total cells for 10 min at 4ºC. Following incubation, cells were washed with 30µl of MACS buffer and incubated with 20µl of MACS anti-FITC micro-beads per 10^7 cells for 15 min at 4ºC. Cells were then washed by adding 10-20 times the labeling volume of buffer and centrifuged at 1000 rpm for 10 min. Next, cells were resuspended in 500 µl MACS buffer and the positively selected cells were separated using LS MACS column (10^7 to $5x10^7$ cells). The LS MACS column was placed in the magnetic field of MACS separator and was washed thrice with 500µl of MACS buffer. 500µl of cell suspension was applied onto the column and negative fraction was washed out and collected separately. The column was then removed from the separator and positively selected cells were collected in fresh centrifuge tubes in 1mL of MACS buffer. The FITC labeled positive fraction was checked for the expression of γδ TCR by flow cytometry.

3.9 Viability of γδ T cells by MTT assay

The viability of γδ T cells upon treatment with HDAC inhibitors was evaluated with MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. Briefly, 0.1×10^6 γδ T cells, seeded in 96-well flat bottom plates (Nunc), were treated with the HDAC inhibitors with the given concentration: VPA (4–0.25 mM; Sigma-Aldrich), TSA (200–25 nM; Sigma-Aldrich), and SAHA (4–0.25 µM; Sigma-Aldrich) along with HDMAPP (1 nM; Echelon) and rIL-2 (50 IU/ml; Peprotech) for 72 h. γδ T cells activated only with HDMAPP (1 nM) and rIL-2 (50 IU/ml) were used as control.

MTT (5 mg/ml) was added and incubated for 4 h at 37°C. After incubation, the spent medium was discarded, the formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm by a microplate reader (TECAN, Switzerland). Untreated γδ T cells were used as a reference for calculating the viability. Concentrations of HDAC inhibitors, which had no impact on the viability of γδ T cells, were used for all the further experimental procedures.

3.10 Flow Cytometry

3.10.1 Purity of immune-magnetically isolated γδ T cells

Magnetically isolated γδ T cells were stained for cell surface markers Vδ2 TCR, CD19, CD14, and CD56 to check the purity of isolated γδ T cells. Briefly, γδ T cells were washed with ice-cold PBS at 1000 rpm for 10mins. The cells were fixed with 1% paraformaldehyde at 4^0C for 15min, labeled with fluorophore-tagged antibodies Vδ2 –PE, CD3- PECy7, CD19-FITC, CD14- PerCP and CD56-PerCPCy5.5 and incubated for 45min on ice. Cells were washed with FACS buffer (0.01M PBS pH-7.4, 1%FCS, 0.01% Sodium azide) at 1000rpm and resuspended in 200µL buffer. γδ T cells stained with IgG conjugated with the corresponding fluorophore were used as isocontrol and CD3 tagged with the corresponding fluorophore were used for compensation. Cell was acquired on FACS Aria (BD Biosciences, USA), 50,000-100,000 events were collected and analyzed with FlowJo software (Tree Star, USA).

3.10.2 Detection of apoptosis by Annexin V/PI staining

γδ T cells ($1x10^5$) were stimulated with HDMAPP (1 nM; Echelon) and rIL-2 (50 IU/ml; Peprotech) and were treated with VPA (2mM, 1mM and 0.5mM), TSA (100nM, 50nM and 25nM) and SAHA(1µM, 0.5 µM and 0.25 µM) or left untreated for 72h at 37°C in round-bottomed 96 well plate (Nunc). After 72h cells were harvested and washed with cold PBS and resuspended in 1X annexin binding buffer

(10mM HEPES pH 7.4, 150mM NaCl, 0.25mM CaCl2*)*. Cells were then stained with FITC conjugated Annexin V and propidium iodide (PI) for 15 mins at room temperature in the dark. After incubation, 400µl of annexin binding buffer was added and acquired on FACS Calibur (BD Biosciences, USA).

3.10.3 Detection of activation markers

Expression of activation markers CD25, CD69, CD71, and phospho-Tyrosine was detected by multi-color flow cytometry.γδ T cells were treated with rIL2 and HDMAPP or anti-CD3 mAb for 72 h with or without GSI-X (15µM) or Dznep (15µM). After 72h of treatment cells were harvested and washed with ice-cold PBS at 1000rpm for 10min. The cells were fixed with 1% paraformaldehyde at 4^0C for 15min, labeled with fluorophore-tagged CD25- PECy5, CD69- BV421, CD71- FITC and phospho-Tyrosine- PE incubated for 45min on ice. Cells were washed with FACS buffer (0.01M PBS pH-7.4,1%FCS, 0.01% Sodium azide) at 1000rpm and resuspended in 200µL buffer. Similarly, γδ T cells, activated with rIL2 and HDMAPP, were treated in the presence or absence of HDAC inhibitors VPA (2mM, 1mM, and 0.5mM), TSA (100nM, 50nM and 25nM) and SAHA (1µM, 0.5 µM and 0.25 µM) for 72h and stained for activation markers as described above. γδ T cells stained with IgG conjugated with the corresponding fluorophore was used as isocontrol and CD3 tagged with the corresponding fluorophore were used for compensation. Cells were acquired on FACS Aria (BD Biosciences, USA), 50,000-100,000 events were collected and analyzed with FlowJo software (Tree Star, USA).

3.10.4 Expression of Notch receptors and ligands on γδ T cells

For analyzing expression of Notch receptors and ligands, magnetically isolated γδ T cells were fixed with 1% paraformaldehyde at 4^0C for 15min, stained with PE-

conjugated Notch-1, APC conjugated Notch-2 and purified mAb for Notch-3, Notch-4, Dll-1, Dll-3, Dll-4, Jag-1 and Jag-2 for 45min on ice. Upon incubation, cell was washed with FACS buffer at 1000rpm for 10min and cells stained with purified antibodies were incubated with secondary antibody PE-conjugated GAM for 45min on ice. Cells were again washed with FACS buffer (0.01M PBS pH-7.4, 1%FCS, 0.01% Sodium azide) at 1000rpm and resuspended in 200µL buffer. Cells were acquired on FACS Aria (BD Biosciences, USA) and analyzed with FlowJo software (Tree Star, USA). Similarly, expression of Notch receptors (Notch-1 and Notch-2) and ligands (Dll-1 and Jag-1) was analyzed by immunostaining. γδ T cells were treated with rIL2 and HDMAPP or anti-CD3 mAb for 72 h with or without GSI-X (15µM). After 72h of treatment cells were harvested and washed with ice-cold PBS at 1000rpm for 10min. Cells were stained as described above and γδ T cells stained with IgG conjugated with the corresponding fluorophore was used as isocontrol.

3.10.5 Expression of IL-2 signaling receptors on γδ T cells

The expression of proximal and distal components of IL-2 signaling (CD122, CD132, and CD25) on γδ T cells was evaluated. γδ T cells were treated with rIL2 and HDMAPP or anti-CD3 mAb for 72 h with or without GSI-X (15µM). After 72h of treatment cells were harvested and washed with ice-cold PBS at 1000rpm for 10min. The cells were fixed with 1% paraformaldehyde at 4^0C for 15min, stained with fluorophore-tagged CD25- PECy5, CD122- FITC and CD132- PE for 45min on ice. Cells were washed with FACS buffer (0.01M PBS pH-7.4, 1%FCS, 0.01% sodium azide) at 1000rpm and resuspended in 200µL buffer. Similarly γδ T cells, activated with rIL2 and HDMAPP, were treated in the presence or absence of HDAC inhibitors VPA (2mM, 1mM, and 0.5mM), TSA (100nM, 50nM and 25nM) and SAHA(1µM, 0.5 µM and 0.25 µM) for 72h and stained for IL-2 signaling components as described

above. γδ T cells stained with IgG conjugated with the corresponding fluorophore was used as isocontrol and CD3 tagged with the corresponding fluorophore were used for compensation. Cells were acquired on FACS Aria (BD Biosciences, USA), 50,000-100,000 events were collected and analyzed with FlowJo software (Tree Star, USA).

3.10.6 Expression of activating receptors on γδ T cells

γδ T cells, activated with rIL2 and HDMAPP, were treated in the presence or absence of HDAC inhibitors VPA (2mM, 1mM, and 0.5mM), TSA (100nM, 50nM and 25nM) and SAHA(1µM, 0.5 µM and 0.25 µM) for 72h. After 72h, cells were fixed with 1% paraformaldehyde at 4^0C for 15min, stained with fluorophore-tagged Vδ2-PE, NKG2D- PerCPCy5.5 and CD16- BV421 for 45min on ice. Cells were washed with FACS buffer at 1000rpm and resuspended in 200µL buffer. γδ T cells stained with IgG conjugated with the corresponding fluorophore was used as isocontrol and CD3 tagged with the corresponding fluorophore were used for compensation. Cells were acquired on FACS Aria (BD Biosciences, USA), 50,000-100,000 events were collected and analyzed with FlowJo software (Tree Star, USA).

3.10.7 Expression of immune checkpoints and Inhibitory receptors on γδ T cells

For analyzing expression of inhibitory receptors PD-1, PD-L1 and KIR2DL2/3, γδ T cells, activated with rIL2 and HDMAPP, were treated in the presence or absence of HDAC inhibitors VPA (2mM, 1mM, and 0.5mM), TSA (100nM, 50nM and 25nM) and SAHA(1µM, 0.5 µM and 0.25 µM) for 72h. Cells were harvested and washed with ice-cold PBS at 1000rpm for 10min. The cells were fixed with 1% paraformaldehyde at 4^0C for 15min, stained with fluorophore-tagged Vδ2-PE, PD-1–PECF594, PD-L1 PerCPCy5.5, and KIR2DL2/3- PE for 45min on ice. Cells were washed with FACS buffer (0.01M PBS pH-7.4, 1%FCS, 0.01% Sodium azide) at 1000rpm and re-suspended in the 200µL buffer. γδ T cells stained with IgG

conjugated with the corresponding fluorophore were used as isocontrol and CD3 tagged with the corresponding fluorophore were used for compensation. Cells were acquired on FACS Aria (BD Biosciences, USA), 50,000-100,000 events were collected and analyzed with FlowJo software (Tree Star, USA).

3.10.8 Expression of effector molecules Perforin and Granzyme B in γδ T cells

Expression of effector molecules Perforin and Granzyme were detected by multi-color flow cytometry. γδ T cells were treated with rIL2 and HDMAPP or anti-CD3 mAb for 72 h with or without GSI-X (15μM) or Dznep (15μM). After 72 hrs γδ cells were fixed with paraformaldehyde and permeabilised with 1% saponin. Cells were washed and stained with γδ TCR-PE, Perforin- BV421 and Granzyme B-PECF594.Additionally for HDAC inhibitor-treated γδ T cell experiment γδT cells treated with or without HDAC inhibitors for 72hrs as described earlier were stained with live-dead fixable dead cell stain kit (Thermo Fischer) as per manufacturer's protocol. After staining with LD dye the cells were fixed with paraformaldehyde and permeabilised with 1% saponin. Cells were washed and stained with γδ TCR-PE, Perforin- BV421 and Granzyme B (BD Biosciences, USA). For small interfering RNA (siRNA) knockdown experiments γδ T cells were transfected with small interfering RNA (siRNA) specific for NOTCH1, NOTCH2, and Ezh2 genes and fluorescent oligonucleotide siGLO (transfection indicator) (transfection indicator) (Cell Signaling Technology, USA), Walton, USA). siRNA oligos were transfected at a concentration of 100 nM using X-tremeGENE HP transfection reagent (Roche Diagnostics, Indianapolis, IN). After 40 hours post-transfection cells were stimulated with HDMAPP or anti-CD3 mAb in the presence of rIL-2. After 72 hours, cells were harvested and expression of effector molecules Perforin and Granzyme were checked by flow cytometry as described above.

3.10.9 Measurement of degranulation marker Lamp-1

For determination of degranulation marker, Lamp-1 (CD107a) purified γδ T cells were activated with rIL2 (50 IU/ml) and HDMAPP(1nM) in the presence and absence of TSA (100nM), SAHA (1μM) and VPA (2mM) for 72 hours at 37^0C. Additionally, for PD-1 blockade anti-PD1 antibody (3μg/mL; Biolegend San Diego, CA) was added along with HDAC inhibitors. These effectors were then cocultured with Zoledronate treated tumor targets (AW13516, COLO-205, and Raji) for 4 hours in polypropylene tubes (BD Biosciences, USA) at effector-target ratio of 4:1 in presence of monensin (5mg/ml; Sigma-Aldrich) as described previously [378]. Anti CD107a APC Ab (Biolegend San Diego, CA) was added at the start of coculture assay. After 4 hours, cells were washed, fixed, permeabilized with (1%) saponin and stained with anti-human TCR γδ PE and Granzyme B-PECF-594 (BD Biosciences, USA). Cells were acquired on FACS Aria (BD Biosciences, USA) and analysis was done by using FlowJo software (Tree Star, Ashland OR).

3.10.10 Cell cycle analysis

For cell cycle analysis, $1x10^6$ γδ T cells were activated with CD3 (2.5μg/ml, BD Biosciences) or HDMAPP and recombinant IL-2 (rIL2, 10U/ml, PeproTech, Rocky Hill, NJ) for 72 hrs. For Notch inhibition experiments, both HDMAPP and anti-CD3 mAb activated γδ T cells were treated with GSI-X. Similarly, for HDAC inhibition, 1 x 10^6 of HDMAPP activated γδ T cells were treated in the presence or absence of VPA (2mM, 1mM, and 0.5mM), TSA (100nM, 50nM and 25nM) and SAHA (1μM, 0.5 μM and 0.25 μM). γδ T cells were left in the medium alone served as an unstimulated control. After 72 hrs, cells were harvested and fixed by adding chilled 70% ethanol dropwise under constant vortexing. Cells were washed and resuspended

in 500 µl of PBS followed by addition of propidium iodide (PI) and RNase at a concentration of 40µg/ml and 10µg/ml respectively. Cells were incubated at room temperature for 30 mins and the cell cycle was assessed on FACS Calibur flow cytometer and analyzed using Modfit software.

3.10.11 Intracellular Calcium release assay

Intracellular calcium release was estimated by flow cytometry using the fluorescent dye Fluo-3-AM. It is an acetoxymethyl (AM) ester derivative, which is cleaved by non-specific intracellular esterases, thus trapping Fluo-3 inside the cell. Fluo-3-AM is a non-fluorescent compound, but upon binding to Ca^{2+} its fluorescence increases sharply and can be detected by flow cytometry.γδ T cells were activated with CD3 (2.5µg/ml, BD Biosciences) or HDMAPP and recombinant IL-2 (rIL2, 10U/ml, PeproTech, Rocky Hill, NJ) treated with or without GSI-X for 72 hrs.After GSI-X treatment, cell was incubated with 5µM fluo-3-AM for 30 mins at 37ºC with 5% CO_2. Cells were washed with calcium estimation buffer (137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 5 mM Glucose, 0.5 mM $MgCl_2$, 10 mM HEPES, 1 mM $CaCl_2$, 1 g/L BSA) and acquired on the flow cytometer (FACSAria, BD Biosciences) for 30 s to determine the baseline fluorescence intensity in GSI-X or untreated cells. Soluble α-CD3 mAb (5µg/ml) and HDMAPP (1nM) was used as stimulants and Fluo-3 fluorescence intensity was measured immediately for up to 5 min in a continuous manner. Changes in Fluo-3 intensity were analyzed by FlowJo software (Tree Star, Ashland, OR).

3.11 Small interfering RNA

Magnetically isolated γδ T cells were transfected with small interfering RNA (siRNA) specific for NOTCH1, NOTCH2, and Ezh2 genes and fluorescent oligonucleotide

siGLO (transfection indicator) (transfection indicator) (Cell Signaling Technology, USA), Walton, USA). siRNA oligos were transfected at a concentration of 100 nM using X-tremeGENE HP transfection reagent (Roche Diagnostics, Indianapolis, IN). The inhibition of NOTCH1, NOTCH2 and EZH2 expression was assessed at 60 hrs post transfection. Briefly, $2x10^5$ cells per well were seeded in 24 well plates in 400µL media supplemented with 10% 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 (3 µl corresponding to 100nM). 6 µL of transfection reagent was gently mixed with 50µL of transfection media (Opti-MEM) and incubated for 5 mins. After 5 mins both the solutions were combined and incubated at room temperature for 30 mins to allow the complex to form. Then mixed gently and overlaid the mixture onto the cells. Cells were incubated for 48hrs and monitored for siGLO by flow-cytometry for transfection efficiency (70±5%).

3.12 Blockade of PD-1 signaling

To study the role of PD-1 signaling in HDAC inhibitor-mediated alteration γδ T cell effector functions PD-1 blocking experiments were carried out. The PD-1 receptor was blocked using 3µg/ml PD-1 or normal IgG isotype control antibody was added to γδ T cell stimulated with HDMAPP and rIL-2 in presence or absence VPA (2mM, 1mM and 0.5mM), TSA (100nM, 50nM and 25nM) and SAHA(1µM, 0.5 µM and 0.25 µM) for 72 hrs. After 72hrs functional readout of was observed in the expression of Granzyme B, Lamp-1 using flow cytometry and cytolytic ability was evaluated by cytotoxicity assay.

3.13 Gene expression by Real-time Polymerase chain reaction (RT-PCR)

3.13.1 Extraction of RNA

γδ T cells ($1x10^6$) were stimulated with rIL-2 (100U/ml, PeproTech) and HDMAPP or Anti CD3 mAb. These cells were treated with 15 μM GSI-X (Calbiochem, La Jolla, CA) or CHIR(15 μM) or left untreated, along with antigen stimulation for 72h. Similarly, γδ T cells ($1x10^6$) were stimulated with rIL-2 (100U/ml, PeproTech) and HDMAPP or Anti CD3 mAb were treated with DZnep (15 μM) for 72hrs. Alternatively, for HDAC inhibition γδ T cells were stimulated with rIL-2 (100U/ml, PeproTech) and HDMAPP in the presence or absence of VPA (2mM, 1mM and 0.5mM), TSA (100nM, 50nM and 25nM) and SAHA (1μM, 0.5 μM and 0.25 μM) for 72 hrs. After the treatment, γδ T cells were harvested in ice-cold 1x PBS and stored at –80 ºC TRIzol (Invitrogen Life-Technologies, N.Y.) in a ratio of $1X10^6$ cells/500 μl TRIzol solution until further use. At the time of RNA extraction, chloroform was added to make 1:5 ratio of chloroform: TRIzol (eg. 100μl chloroform in 500 μl TRIzol) mixed and centrifuged at 12,000 rpm for 15 min at 4 ºC. The aqueous phase was collected in fresh DEPC treated tubes, to which equal volume of chilled isopropyl alcohol (Qualigens, India) and centrifuged at 14,000 rpm for 15min at 4 ºC. After centrifuging, the RNA pellet was washed with 75% ethanol at 12,000 rpm for 10min at 4 ºC and air dried. The RNA dissolved in an appropriate volume of DEPC (Sigma)-treated water and RNA concentration was determined by NanoDrop spectrophotometer (Thermo Scientific, DE). The RNA was run on a 1.5% agarose gel containing ethidium bromide to confirm its purity and integrity.

3.13.2 Complementary DNA (cDNA) synthesis by reverse transcription PCR

Total RNA from the γδ T cells was converted into cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) as per manufacturer's protocol. Briefly, 1- 2µg of RNA (10 µl volume with nuclease free water) was reverse transcribed with following PCR reaction mixture. The final reaction volume was 20 µl

Sr. No.	Component	Volume in µl
1	RT PCR buffer	2 µl
2	Random primers	2 µl
3	RNase inhibitor	2 µl
4	Reverse Transcriptase	1 µl
5	dNTPs	0.8 µl
6	Nuclease-free water	1.2 µl
7	RNA (1-2 µg)	10 µl

This PCR reaction mixture was then run on the thermal cycler Veriti (Applied Biosystems, Foster City, CA) and the quality of the cDNA products was checked with GAPDH/ 18SrRNA primers.

3.13.3 Real-time PCR

Quantitative q-PCR for different Notch receptor isoforms (Notch1-4), ligands (Dll1, Dll3, Dll4, Jag1 and Jag2), its target genes (Hes1, Hey2, DELTEX, NRARP) , effector molecules (Perforin, Granzyme B), cytokines (IFN-γ and TNF- α) and transcription factor (T-bet, Eomes and Nf-κB) was performed with Quantstudio 15k Flex system (Applied Biosystems). Samples were analyzed using TaqMan primer sets purchased from Applied Biosystems The PCR reaction mixture was prepared as follows

Sr. No.	Component	Volume in µl
1	TaqMan Master mix	2.5 µl
2	Primer probe mixture	0.25 µl
3	cDNA(10ng)+ DEPC water	2.25µl
	Total volume	5 µl

Table 3.8 List of sequence-specific TaqMan primers probes

Sr No.	Target Gene	Assay ID/primer sequences	Source
1	Notch-1	Hs01062014_m1	Applied Biosystems
2	Notch-2	Hs01050702_m1	Applied Biosystems
3	Notch-3	Hs01128537_m1	Applied Biosystems
4	Notch-4	Hs00965889_m1	Applied Biosystems
5	Dll-1	Hs00194509_m1	Applied Biosystems
6	Dll-3	Hs01085096_m1	Applied Biosystems
7	Dll-4	Hs00184092_m1	Applied Biosystems
8	Jag-1	Hs01070032_m1	Applied Biosystems
9	Jag-2	Hs00171432_m1	Applied Biosystems
10	Hes-1	Hs00172878_m1	Applied Biosystems
11	Hey-2	Hs00232622_ml	Applied Biosystems
12	DELTEX	Hs00269995_m1	Applied Biosystems
13	NRARP	Hs01104102_m1	Applied Biosystems
14	T-bet	Hs00894392_m1	Applied Biosystems
15	Eomes	Hs00172872_m1	Applied Biosystems
16	Nf-κB	Hs00765730_m1	Applied Biosystems
17	Perforin	Hs00169473_m1	Applied Biosystems
18	Granzyme-B	Hs00188051_m1	Applied Biosystems
19	18srRNA	Hs99999901_s1	Applied Biosystems
20	TNF-α	Hs00174128_m1	Applied Biosystems
21	IFN-γ	Hs00989291-m1	Applied Biosystems

All values were normalized to the expression of the endogenous control 18S rRNA. The relative gene expression was quantified by $\Delta\Delta C_T$ method using the following formulas:

$\Delta C_T = C_T$ (reference control) - C_T (endogenous control)

$\Delta\Delta C_T = \Delta\Delta C_T$ (Target) - $\Delta\Delta C_T$ (reference control)

Relative gene expression = $2^{-\Delta\Delta CT \times 100}$

3.14 Proliferation assay

The proliferation of cells was assayed by ^{3}H-thymidine (^{3}H TdR) incorporation assay. A total of $5x10^4$ γδT cells were stimulated with rIL-2 (1IU/ml, PeproTech) and HDMAPP for 72 h in round-bottom 96-well tissue culture plates. For experiments using HDAC inhibitors, cells were treated VPA, TSA, and SAHA as described above. Alternatively, γδ T cells were stimulated with rIL-2 (100U/ml, PeproTech) and HDMAPP or Anti CD3 mA, were treated with either 15 μM GSI-X (Calbiochem, La Jolla, CA) or DZnep (15 μM) for 72hrs. The cultures were pulsed with 1 μCi [^{3}H] thymidine/well (Board of Radiation and Isotope Technology, Mumbai) during the last 18 h of the assay. Following incubation, cells were harvested onto glass-wool filter paper using a cell harvester (Perkin Elmer, UK). 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).

3.15 Estimation of cytokines

Levels of IFN-γ and TNF-α were estimated in supernatant collected from γδT cells stimulated with rIL-2 (50IU/ml, PeproTech) and HDMAPP or anti-CD3 mAb, treated or untreated with GSI-X or DZnep for 72h. Similarly, supernatants were also

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

3.16 Cytotoxicity assay

^{51}Chromium release assay and Lactate dehydrogenase (LDH) assay was used to measure the cytotoxicity of γδ T cells (effector) against a panel of tumor cell lines AW13516 (Oral cancer), Raji (B cell lymphoma) and COLO 205 (Colon cancer) were used as targets. γδ T cells were left alone or activated with rIL2 (50IU/mL, PeproTech) and anti-CD3 mAb or HDMAPP and treated with or without GSI-X (15µM) and DZnep (15µM) for 72h. Alternatively, Ezh2 inhibition was mediated by

siRNA knockdown, wherein γδ T cells were left untransfected or transfected with 100nM of control Siglo siRNA or Ezh2 siRNA duplex. After 40h of transfection, the EZH2 depletion was untransfected or transfected γδ T cells were activated with rIL2 (50IU/mL, PeproTech) and HDMAPP for 48h. These EZH2 silenced γδ T cells were also used as effectors to assess their cytotoxic potential against Zoledronate treated tumor targets. For HDAC inhibition, γδ T cells activated with rIL2 (50IU/mL, PeproTech) and HDMAPP were treated with or without VPA (2mM, 1mM and 0.5mM), TSA (100nM, 50nM and 25nM) and SAHA(1μM, 0.5 μM and 0.25 μM) for 72h. The tumor targets (AW13516, Raji, and COLO 205) cells were treated for 18 h with Zoledronate (100μM) (Panacea Biotech Ltd.). Tumor targets were labeled with ^{51}Chromium for 90 min at 37ºC. Labeled target cells were co-cultured 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 HDMAPP activated 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.

Alternatively, we also assessed the cytotoxic potential of γδ T cells with the non-radioactive based method using Lactate Dehydrogenase based assay (LDH). γδ T cells treated as described above were co-cultured with Zoledronate treated tumor targets at 40:1 effector-target (E:T) ratio for 4 h at 37°C in 96-well plates (Nunc, Denmark). At

the end of the co-culture, an aliquot of 50 µl of media was used in LDH cytotoxic assay using the LDH cytotoxic assay kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. γδ T cell cytotoxicity was defined as % specific lysis = Experimental value – Effector cells spontaneous control − Target cells spontaneous control/Target Cell Maximum Control − Target cells spontaneous control.

Additionally, for PD-1 blockade experiments, anti-PD1 antibody (3 µg/ ml) was added to HDAC inhibitor-treated γδ T cells for 72 h and were also used as effectors. Normal IgG added separately was used as control. After the standard 4h co-culture at 37°C the γδ T cell cytotoxicity against the three tumor targets was evaluated by 51Chromium release or LDH based assays as described above.

3.17 Western Blotting

$1x10^6$ γδ T cells were stimulated with rIL-2 (100U/ml, PeproTech) and HDMAPP or Anti CD3 mAb for 72h. These cells were treated GSI-X (Calbiochem, La Jolla, CA) at a concentration of 15 µM, or with DZnep (15 µM). Alternatively, for HDAC inhibition γδ T cells were stimulated with rIL-2 (100U/ml, PeproTech) and HDMAPP in the presence or absence of VPA (2mM, 1mM and 0.5mM), TSA (100nM, 50nM and 25nM) and SAHA(1µM, 0.5 µM and 0.25 µM) for 72 hrs. Upon treatment for 72h, the cells were harvested and probed for the expression of different proteins using western blotting. β-actin, tubulin, histone H3, and histone H4 were used as a loading control for all western blotting experiments

3.17.1 Key reagents for Western blotting

Composition of 2X SDS buffer (10 ml)

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

SDS Lysis Buffer (1 ml)

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

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

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

25mM Tris	**3.0285 gms**
190mM Glycine	14.26gms
0.1%SDS	1gm

Transfer Buffer: 1000 ml

25mM Tris	**3.0285 gms**
190mM Glycine	14.26gms
20% methanol	200 ml

Ponceau S Staining Buffer (100 ml)

0.2% (W/V) Ponceau S	200 mgs
5% glacial acetic acid	5 ml

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

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

Blocking buffer

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

Nitrocellulose Membrane

Stripping Buffer

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

Table 3.9: Resolving and Stacking gel components

	10% Resolving gel 8ml	18% Resolving gel 8ml	5% Stacking gel 5ml
Double distilled water	3.2 ml	1ml	2.6 ml
30% Bis-acrylamide mixture	2.67 ml	4.8 ml	1 ml
1.5M Tris-HCl (pH 8.8)	2 ml	2 ml	-
0.5M Tris-HCl (pH6.8)	-	-	1.25 ml
10% SDS	80 µl	80 µl	50 µl
10% ammonium per sulfate	80 µl	80 µl	50 µl
TEMED	08 µl	08 µl	05 µl

3.17.2 Cell lysate preparation

Cells were washed with ice-cold 0.01M PBS (PH 7.5) and lysed in SDS lysis buffer. Briefly, to the cell pellet of 1x 10^6 cells, 30 µl of SDS lysis buffer was added; cells were resuspended by vortexing and boiled for 10 mins at 100ºC. The lysates were loaded immediately or stored at80ºC. In the case of frozen lysate, the samples were boiled once again at 100ºC for 5 mins and then loaded.

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

Equal amounts of protein lysate were separated on SDS PAGE and the gel was run at 120V for 1-2 hrs. The selection of gel percentage was dependent on the size of the protein of interest. 8-18% gels were used to separate the protein of interests.

3.17.4 Transfer of proteins from the gel to the membrane

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

3.18 Chromatin Immunoprecipitation (ChIP) q-PCR assay

Chromatin Immunoprecipitation assays were performed using to find the occupancy of various histone modification on the promoter regions of different genes.

3.18.1. Chromatin preparation

γδ T cells ($1x10^6$) were stimulated with rIL-2 (50IU/ml) and HDMAPP (1 nM/ ml) or anti CD3 mAb (5 μg/ml) in presence and absence of 15μM of GSI-X) for 72 hrs. Similarly, γδ T cells ($1x10^6$) were stimulated with rIL-2 (50IU/ml) and HDMAPP (1 nM/ ml) or anti CD3 mAb(5 μg/ml) were treated with DZnep (15 μM) for 72hrs. For HDAC inhibition, ($1x10^{6)}$ γδ T cells were stimulated with rIL-2 (50IU/ml) and

HDMAPP (1 nM/ ml) in the presence or absence of VPA (2mM, 1mM and 0.5mM), TSA (100nM, 50nM and 25nM) and SAHA (1µM, 0.5 µM and 0.25 µM) for 72 hrs. After 72 hrs of culture γδ T cells were cross-linked with methanol-free formaldehyde at room temperature for 10 minutes on a constant shaker. Methanol-free formaldehyde directly in the culture media drop wise in the chemical wood at a final concentration of 1%. Glycine was added to a final concentration of 125mM to the media containing cells and incubated with shaking for 5 minutes at room temperature. Cells were washed 2 times with cold 1xPBS and centrifuged at 4°C at 2000 rpm for 4 min. The pellet of γδ T cells was resuspended in swelling buffer. 6 times swelling buffer was added to the packed pellet and incubated on ice for 10 minutes. After 10 minutes cells were dounced 25 times using loose piston dounce homogenizer. γδ T cell lysate was again centrifuged for 5min at 5000 rpm at 4 ^{0}C in eppendorf centrifuge. After centrifugation supernatant was discarded and 8 times sonication buffer to the packed pellet. Chromatin was sheared by using Biorupter (Diagenode) 25 cycles 30 seconds on off at 4 ^{0}C. After sonication chromatin lysate was centrifuged at 15,000 rpm for 15 minutes at 4 ^{0}C. Pellet was discarded and supernatant was used for Immunoprecipitation. The fragment of the sonicated chromatin was in between 200 to 500 bps as checked by isolation of DNA from 20 µl of supernatant (Chromatin). The concentration of Chromatin was also checked by calculating the DNA eluted which gives the idea of total chromatin.

3.18.2. Immunoprecipitation

Based on the DNA amount, 35-40 ug of chromatin was from each test in a separate eppendrof tube and diluted up to 1 ml with chromatin dilution buffer. 1 % of chromatin was stored as input DNA (positive control) which will be processed for DNA extraction at the end, The diluted chromatin was pre-cleared with non-saturated

beads for 2 hours at 4 ºC (80 µl of bead slurry washed with chromatin dilution buffer). After the pre-clear supernatant was again taken in separate tube and antibody was added to the supernatant (chromatin), incubated with specific antibodies anti-acetyl histone H3 (Abcam) or anti-acetyl histone H4 (Abcam) or anti H3K27me3 or anti H3K9ac and anti-H3K9me3 overnight 4 ºC. Normal rabbit IgG was used as a negative control.

3.18.3. Preparation of saturated protein G beads

Protein G beads were washed three times in chromatin dilution buffer. Single-stranded herring sperm DNA to a final concentration of 10 mg/ml beads and BSA to a final concentration of 1% of the total volume of beads was added along with chromatin dilution buffer to twice the bead volume and incubated for in cold room for 2-3 hrs on the rotatory platform. The saturated beads were washed once with chromatin dilution buffer and chromatin dilution buffer to twice the bead volume was added.

3.18.4. Addition of saturated protein G beads to chromatin antibody complex

60 µl of Salmon Sperm DNA/Protein G bead slurry was added into each test and incubated at 4°C, with rotating, for 4 hrs. After the 4 hrs incubation, the supernatant was removed carefully from protein G magnetic beads by using a strong magnet. Separated Protein G beads were washed with the following buffer.

1. Three washes of low salt buffer wash (10 minutes each)
2. Three washes of High salt buffer wash (10 minutes each)
3. Three washes of LiCl buffer wash (10 minutes each)
4. Three washes of TE buffer wash (10 minutes each)

DNA protein-bead complexes were resuspended in 150 µl fresh elution buffer (1% SDS, 0.1M $NaHCO_3$) and incubated at 100 ^{0}C on dry heat shaker for 15 min at

100rpm. This step was repeated again with 150 µl fresh elution buffer. The beads were separated and the supernatant was pooled in one tube. Reverse-cross-linking of DNA was performed by adding 20 µl 5 M NaCl (8 µl for 1% µl input control) and heating at 65°C for 4 hr. The de-cross-linked DNA was eluted by gel extraction columns (Quiaquic Spin Kit, Qiagen), according to the manufacturer's protocol.

3.18.5. Real-time quantitative PCR

The DNA eluates were detected by real-time PCR with primers specific for each promoter. Following primers were used for real-time PCR: Perforin primer set1 forward 5′-GATGAGGGCTGAGGACAG-3′, reverse: 5′-TCTTCACCGAGGCTCCTG-3; Perforin primer set 2 forward 5′-CTGCTGGCCTGTTCATCAAC-3′, Reverse: 5′-CTGTCCTCAGCCCTCATC-3′; Granzyme B primer set1 forward: 5′-GGGTGGGCAGCATTTACAG-3′, reverse: 5′-TTCTCAGGAAGGCTGCCC-3; Granzyme B primer set2 forward:5′-CACTTCA TAGGCTTGGGTTCC-3′, reverse: 5′-CCTCTGGTTTTGTGGTGTCTC-3′; Fbwx7 forward 5′-CCGGGAGAAGTGGCCCTGGA-3′, Fbwx7 reverse 5′-GAAGCGGTGCTCGTGTCGCT-3′; Numb forward 5′-GCGTCTGCCCCTCCTTTGCT-3′, Numb reverse 5′-TACCCGCCCGTC ATCGCTCT-3′. The occupancy of different histone modifications on the promoter region was calculated by percent Input method by using formula 100*2^(Adjusted input-CtIP). Where the adjusted input =Ct value of input-6.644 (1% input used then dilution factor is 100 or 6.644 cycles i-e log2 of 100)

3.18.6 Key solutions for Chromatin Immunoprecipitation (ChIP) assay

1XPBS/PIC/PMSF

1x PBS, 1xPIC and 0.5 mM PMSF

Swelling buffer

25 mM Tris ph 7.9

1.5 mM MgCl2

10mM KCL

0.1% NP40

1mM DTT

0.5 mM PMSF

1x PIC

Sonication buffer

50 mM Tris-Cl ph 7.9

140 mM NaCl

1mM EDTA

1% Triton X-100

0.1% Sodium deoxycholate

1.0% SDS

0.5 mM PMSF

1x PIC

Chip dilution buffer

0.01% SDS

1.1% Triton X-100

1.2 mM EDTA

16.7mM Tris-Cl ph 8.0

167 mM NaCl

Low salt wash buffer

0.1% SDS

1% Triton X-100

2 mM EDTA

20 mM Tris-Cl ph 8.0

150 mM NaCl

High salt wash buffer

0.1% SDS

1% Triton X-100

2 mM EDTA

20 mM Tris-Cl ph 8.0

500 mM NaCl

LiCl wash Buffer

0.25 M LiCl

1% NP40

1% Sodium deoxycholate

1 mM EDTA

10 mM Tris-Cl ph 8.0

TE buffer -50 ml

250 ul of 2.0 M Tris-Cl ph 7.5 and 100 ul of EDTA (0.5M**)**

Elution buffer

1% SDS

0.1 M NaHCo3

Saturate Bead

Protein –G-sepharose – 1.0 ml (50 % slurry)

tRNA (10 mg/ml) -40 ul

Fish skin gelatin -1 %

3.19 Statistical analysis

Data analysis was done by Student's t-test using GraphPad Prism software (GraphPad Software Inc., CA, and the USA). The comparative CT method was applied in the quantitative real-time RT-PCR according to $2^{-(\Delta\Delta Ct)}$ method. Results were indicated as means $\pm$ standard error (SE) and considered significant at $p < 0.05$.

Chapter 4

To study the role of HDAC inhibitors on immune phenotype, proliferation and cytotoxic functions of γδ T cells

4.1 Introduction:

Initially, cancer was viewed as diseases that are mainly driven by the accumulation of genetic mutations [379]. However, this paradigm has been now changed with the discovery of the role of epigenetic modifiers. Besides the genetic factors, Aberrant expression of epigenetic modifiers are also prevalent in cancer and Histone deacetylases (HDACs) have been implicated in tumorogenesis [55]. Due to this reason targeting the HDACs has become one of the important fields to treat the different tumor and other diseases. In recent decades, HDAC inhibitors have received great attention as emerging anti-neoplastic treatment [380]. HDAC inhibitors mediate their anti-tumor effects through diverse mechanisms which eventually culminate into selective of growth arrest, induction of apoptosis and differentiation in tumors [60, 61]. Moreover, HDAC inhibitors inhibit angiogenesis and increase the tumor cell antigenicity [381]. HDAC inhibitors mediate increase the expression of antigens on tumor cells so that they can be easily targeted by immune cells [382, 383]. HDAC inhibitors are thus popular epigenetic therapy for cancer treatment and several clinical studies are underway to evaluate its efficacy [64, 65]. Due to their promising anti-tumor activity FDA approval has been given to HDAC inhibitors for the treatment of different malignancies such as depsipeptide and SAHA are used for the treatment of advanced cutaneous T cell lymphoma [56, 57]. The short chain fatty acid HDAC inhibitors, valproic acid (VPA), butyric acid and phenyl butyric acid, are pan inhibitors of Class I and Class II HDACs.VPA is registered for the therapy of epilepsy, bipolar disorders, and migraines, and is now together with other short-chain fatty acids HDAC inhibitors tested in clinical studies as anticancer drugs [58].

The effect of HDAC inhibitors on tumors is well known. Interestingly, they also have an effect on immune cells It has been observed that cytotoxic functions of NK cells

are repressed in patients with cutaneous T cell lymphoma treated with HDAC inhibitor vorinostat [384]. Suzuki et.al. have shown that HDAC inhibitors treated tumors cells are easily targeted by γδ T cells [74]. However, the role played by HDAC inhibitors in modulating immune response is still emerging and their impact on the functional response of human γδ T cells is not clear. The present chapter focuses on the direct impact of HDAC inhibitors on human γδ T cells. Human γδ T cells represent the small subset of T cell constituting 5 to 10 % in peripheral blood[385] and differ from αβ T cells by their TCR gene usage, tissue tropism, and MHC-independent antigen recognition [88, 171]. γδ T cells display broad functional plasticity, like regulatory potential, antigen-presenting capacity, B-cell helper activity, and have the potential for diverse cytokine production[386]. γδ T cells recognize non peptide phosphoantigens such as isopentenyl pyrophosphate (IPP) or 4-hydroxy-3-methyl but-2-eneyl pyrophosphate (HMBPP) [387].

We have studied the effect of three different HDAC inhibitors TSA, SAHA, and Sodium valproate (VPA) on on the phenotype, proliferation and anti-tumor effector function of γδ T -cells. Based on this in the present chapter we aimed to address the following:

1. Effect of HDAC inhibitors on proliferation and phenotype of γδ T cells
2. Effect of HDAC inhibitors on the expression of key transcription factors and effector molecules
3. Impact of HDAC inhibitors on the anti-tumor effector functions of γδ T cells.

4.2 Results:

4.2.1 Effect of HDAC Inhibitors on Viability of γδ T Cells

In order to check the effect of HDAC inhibitors on the viability of γδ T cells, we sorted the γδ T Cells from the peripheral blood of healthy individuals using immune-magnetic columns. The sorted population showed more than 90% positivity for γδ TCR. To further validate that the sorted population was enriched in γδ T cells, we did the flow staining for different surface markers, γδ TCR, αβ TCR, CD19, CD14, and CD56. γδ T cells were positive for γδ TCR (90%), CD56 (53%) and negative for αβ TCR, CD19, and CD14 **(Figure 4.1A)**. The sorted γδ T cells were used in all further experiments.

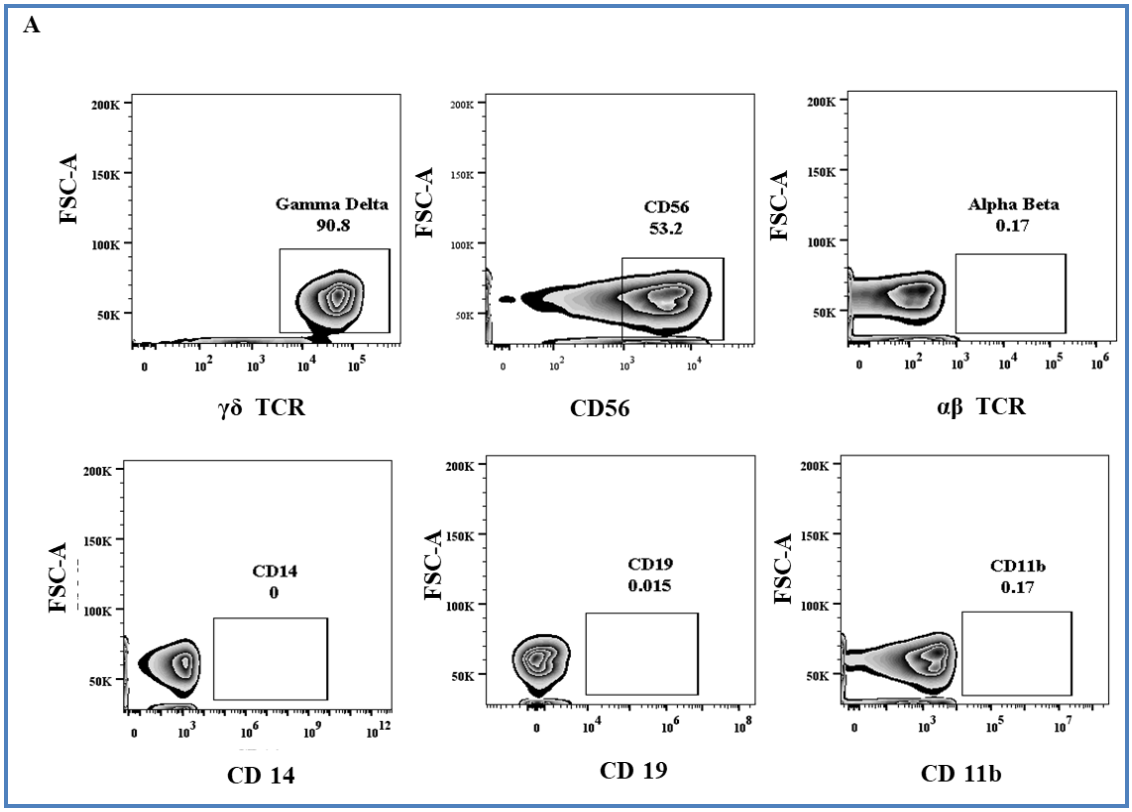


Figure 4.1: Purity of sorted γδ T cells. γδ T cells were purified from PBMCs using immunomagnetic MicroBeads (Miltenyi Biotech, Bergish Gladbach, Germany) by positive selection. These γδ T cells were positive for γδ TCR (90.8%), CD56 (53.2%) and negative for αβTCR, CD14, CD19. The purity of separated γδ T cells was >90% as confirmed by flow cytometry (FACS Aria, BD Biosciences, USA).

We first studied effects of HDAC inhibitors trichostatin-A (TSA), suberoylanilide hydroxamic acid (SAHA) and sodium valproate (VPA) at different concentrations (0.25mM–4mM for VPA, 0.25uM–4µM for SAHA and 25nM-200nM for TSA) on the viability of γδ T cells by MTT assay **(Figure 4.2 A-C).** γδ T cells activated with HDMAPP (1nM) and rIL2 (30IU) treated with the above mentioned HDAC inhibitor concentrations for 72 hours. HDMAPP is the synthetic analog of phospho antigen isopentenyl pyrophosphate (IPP). From the viability experiment, it was observed that higher concentrations of HDAC inhibitors are toxic to γδ T cells. γδ T cells showed the least viability at 150 and 200 nM TSA, 2 and 4 uM SAHA, and 3 and 4 mM VPA. At lower concentrations, these HDAC inhibitors were not toxic and γδ T cell was viable (>90%). Based on these results concentrations of HDAC inhibitors which show the minimum effect on γδ T cell viability were used for further experiments.

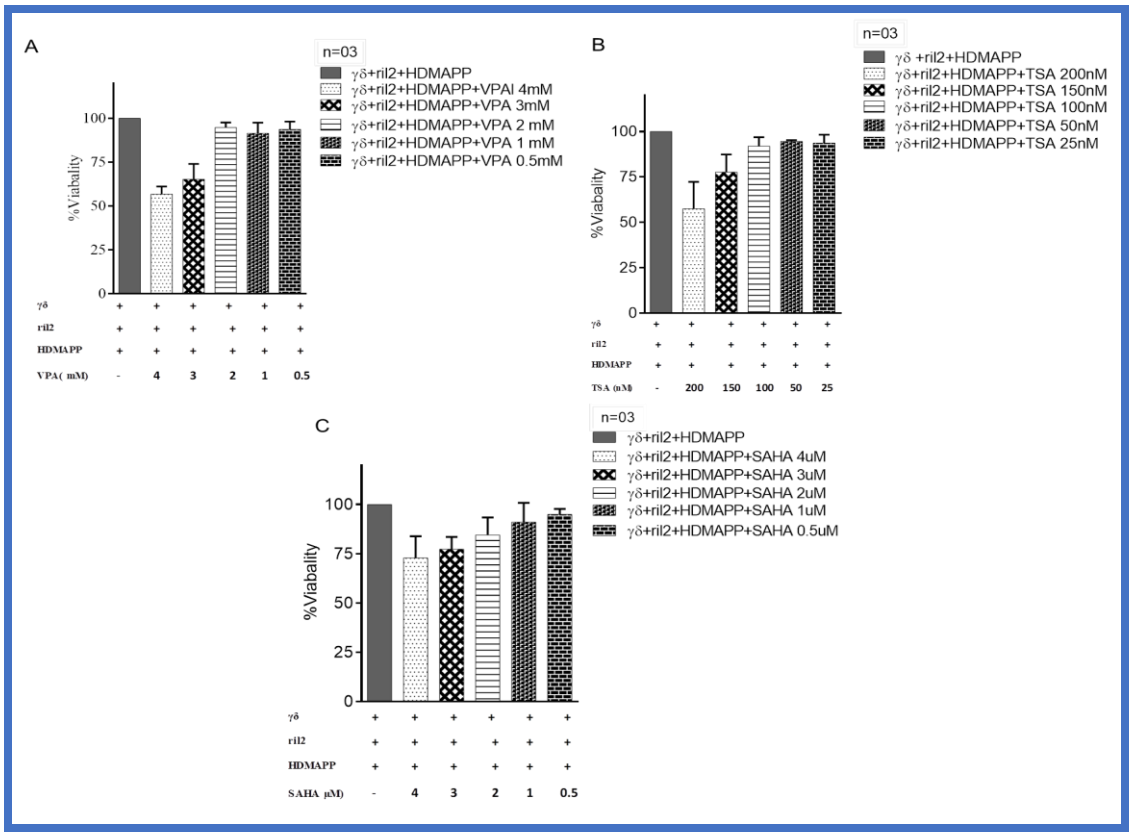
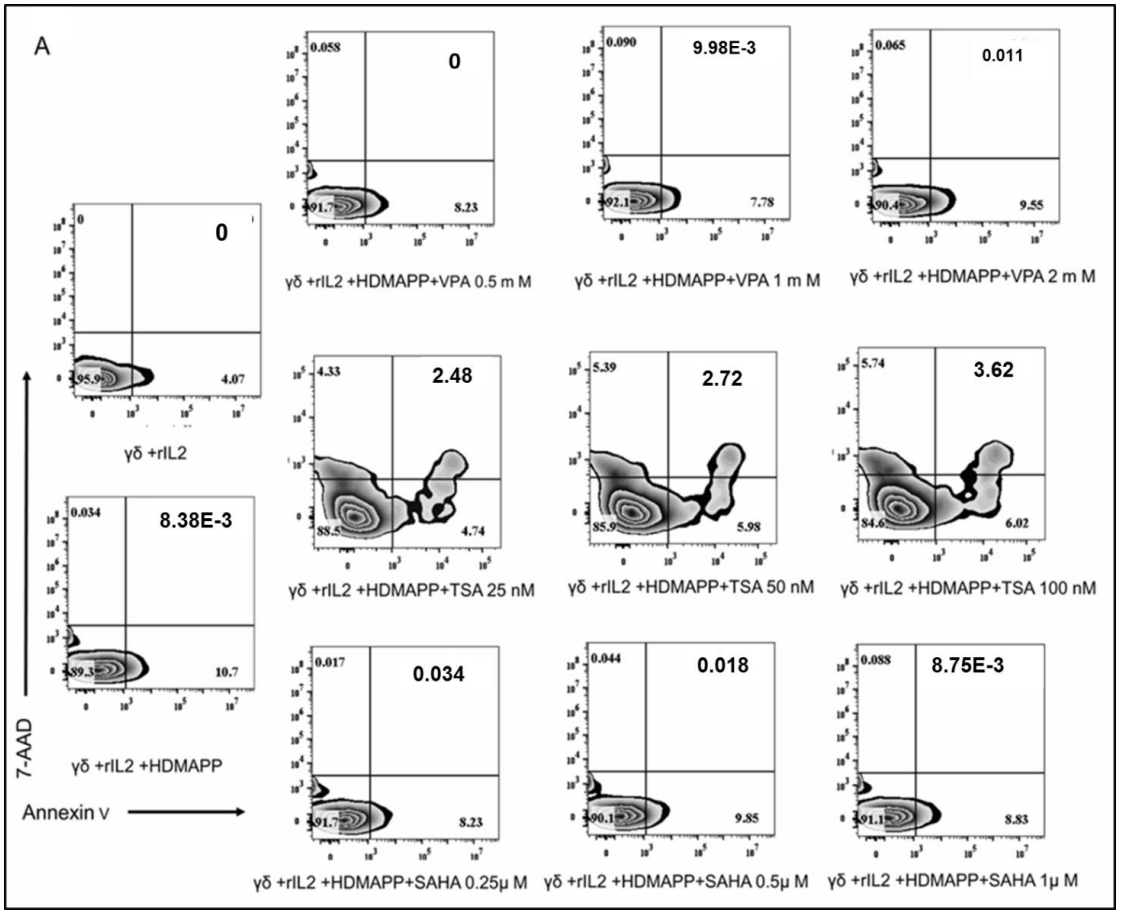


Figure 4.2: **HDAC inhibitors and viability of γδ T cells.** HDAC inhibitors affect the viability of γδ T cells only beyond specific concentrations. γδ T cells stimulated

with HDMAPP in the presence of rIL-2 were treated with HDAC inhibitors **(A)**VPA (4mM, 3mM, 2mM,1mM and 0.5mM), **(B)**TSA (250nM, 150nM, 100nM, 50nM and 25nM) and **(C)** SAHA (4μM, 3 μM, 2 μM, 1 μM, 0.5 μM) for 72 hours. The viability of γδ T cells was assessed by MTT assay. The results indicated are mean $^{\pm}$ SE percent viability of γδ T cells and are representative of three experiments.

For further validation of the effect of HDAC inhibitors on the viability, γδ T cell was activated with phosphoantigen HDMAPP and rIL2 in presence and absence of HDAC inhibitors VPA (0.5-2mM), TSA (25nM-100nM) and SAHA (0.25μM-1 μM) for 72 hours. After 72 hours of treatment, γδ T cells were harvested and stained with Annexin V and 7-AAD to rule out the possibility of cell death and apoptosis in HDAC inhibitor-treated γδ T cells. We observed that at these concentrations HDAC inhibitors did not induce any significant apoptosis. Since HDAC inhibitor concentrations, VPA (0.5–2 mM), TSA (25–100 nM), and SAHA (0.25–1 μM) showed the least effect on the viability of γδ T cells these were selected in further experiments **(Figure 4.3 A-D).** The concentrations of HDAC inhibitors selected were in the therapeutic range as used in other studies for in vivo and ex-vivo experiments.


A
0
95.9
4.07
γδ +rIL2
0.034
8.38E-3
89.3
10.7
γδ +rIL2 +HDMAPP
7-AAD
Annexin v
0.058
0
91.7
8.23
γδ +rIL2 +HDMAPP+VPA 0.5 m M
0.090
9.98E-3
92.1
7.78
γδ +rIL2 +HDMAPP+VPA 1 m M
0.065
0.011
90.4
9.55
γδ +rIL2 +HDMAPP+VPA 2 m M
4.33
2.48
88.5
4.74
γδ +rIL2 +HDMAPP+TSA 25 nM
5.39
2.72
85.9
5.98
γδ +rIL2 +HDMAPP+TSA 50 nM
5.74
3.62
84.6
6.02
γδ +rIL2 +HDMAPP+TSA 100 nM
0.017
0.034
91.7
8.23
γδ +rIL2 +HDMAPP+SAHA 0.25μ M
0.044
0.018
90.1
9.85
γδ +rIL2 +HDMAPP+SAHA 0.5μ M
0.088
8.75E-3
91.1
8.83
γδ +rIL2 +HDMAPP+SAHA 1μ M

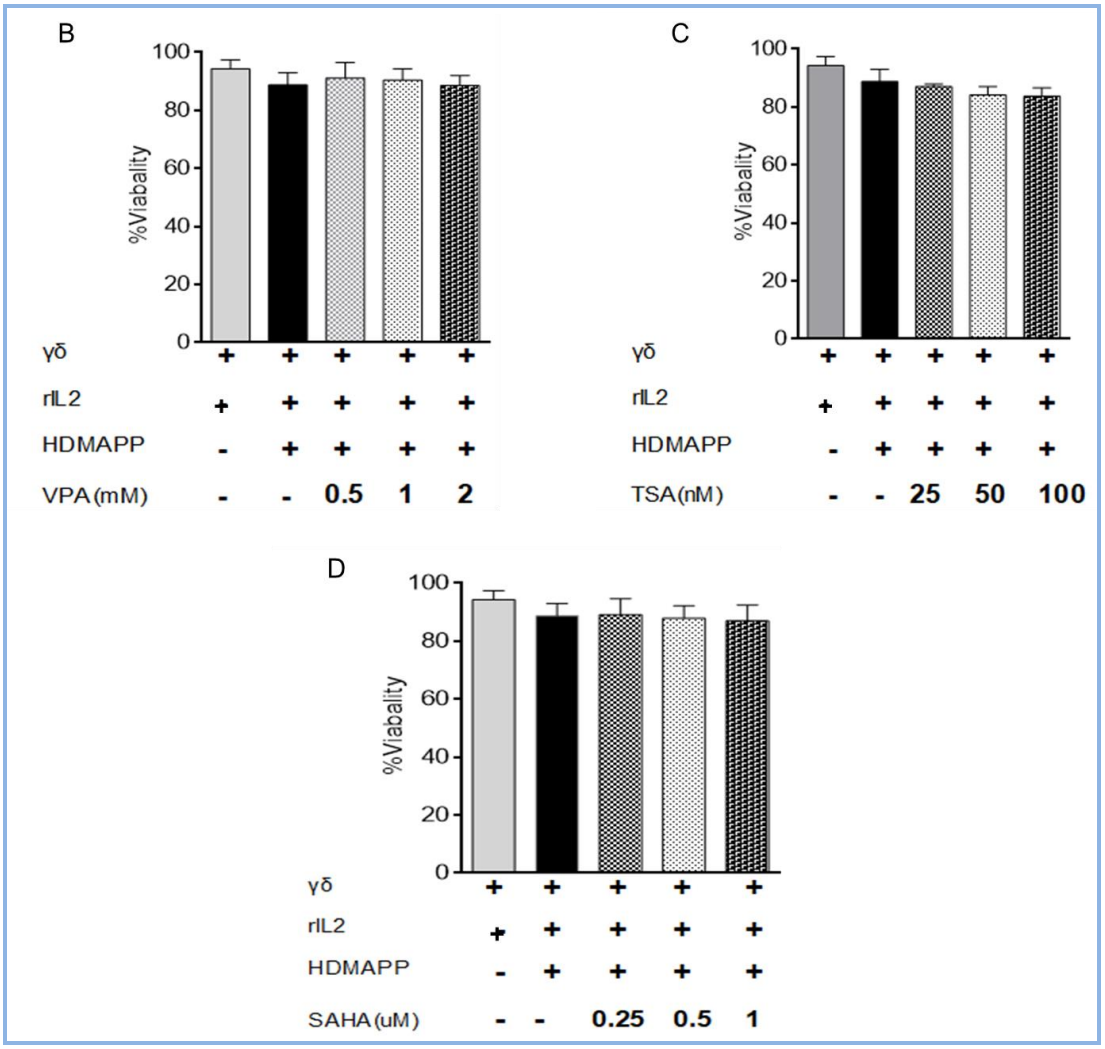


Figure 4.3: The effect of HDAC inhibitors on γδ T cell apoptosis. **(A)** Effect of HDAC inhibitor treatment on apoptosis of γδ T cells measured by Annexin V and 7-AAD staining. γδ T cells were activated with HDMAPP and rIL-2. HDAC inhibitors VPA (2 mM,1 mM, 0.5 mM), TSA (100nM, 50nM, 25nM) and SAHA (1μM, 0.5 μM, 0.25 μM) were added to the culture and apoptosis was measured after 72 hours with Annexin V and 7-AAD staining. Data shown is a representative figure of three independent experiments. **(B-D)** The bar diagrams show consolidated γδ T cell viability post HDAC inhibitor treatment (n=3). Data represent mean $^{\pm}$ SE.

4.2 .2 HDAC inhibitors inhibit the antigen-driven proliferation of γδ T cells

γδ T cells show robust proliferation when activated with the antigen in the presence of co-stimulation. Antigens recognized by the γδ T cells differ from the antigens recognized by the classical αβ T cells. γδ T cells recognize the phosphoantigens and show robust proliferation when stimulated with the synthetic analogs of these phosphoantigens like HDMAPP. In order to investigate the effect of HDAC inhibitors

VPA,TSA and SAHA on the proliferation of γδ T cells, γδ T cells were activated with phosphoantigen HDMAPP and rIL2 in presence or absence of HDAC inhibitors (VPA; 0.5–2 mM, TSA; 25–100 nM, and SAHA; 0.25–1μM) and proliferation was monitored using ^{3}H thymidine incorporation assay. γδ T cells showed robust proliferative responses to phosphoantigen HDMAPP in the presence of rIL-2, compared to unstimulated γδ T cells. However, in the presence of various concentrations of VPA, TSA, and SAHA, the proliferative responses of γδ T cells were significantly reduced in a concentration-dependent manner with maximum decrease in proliferation of γδ T cells observed at higher concentration of HDAC inhibitors, VPA 2 mM, TSA 100 nM, and SAHA 1 μM, respectively. Thus HDAC inhibitors inhibit the antigen-specific proliferative response of γδ T cells **(Figure 4.4 A-C).**

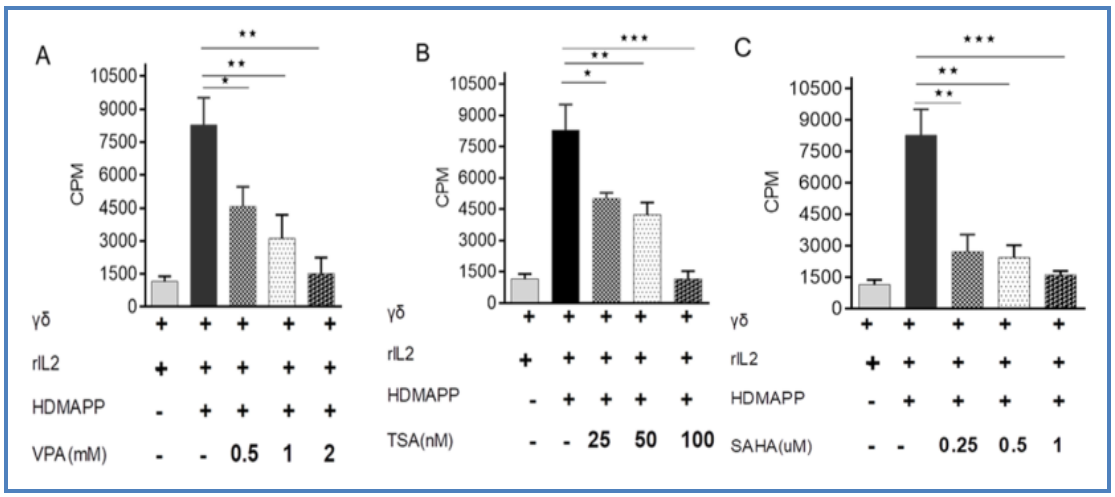
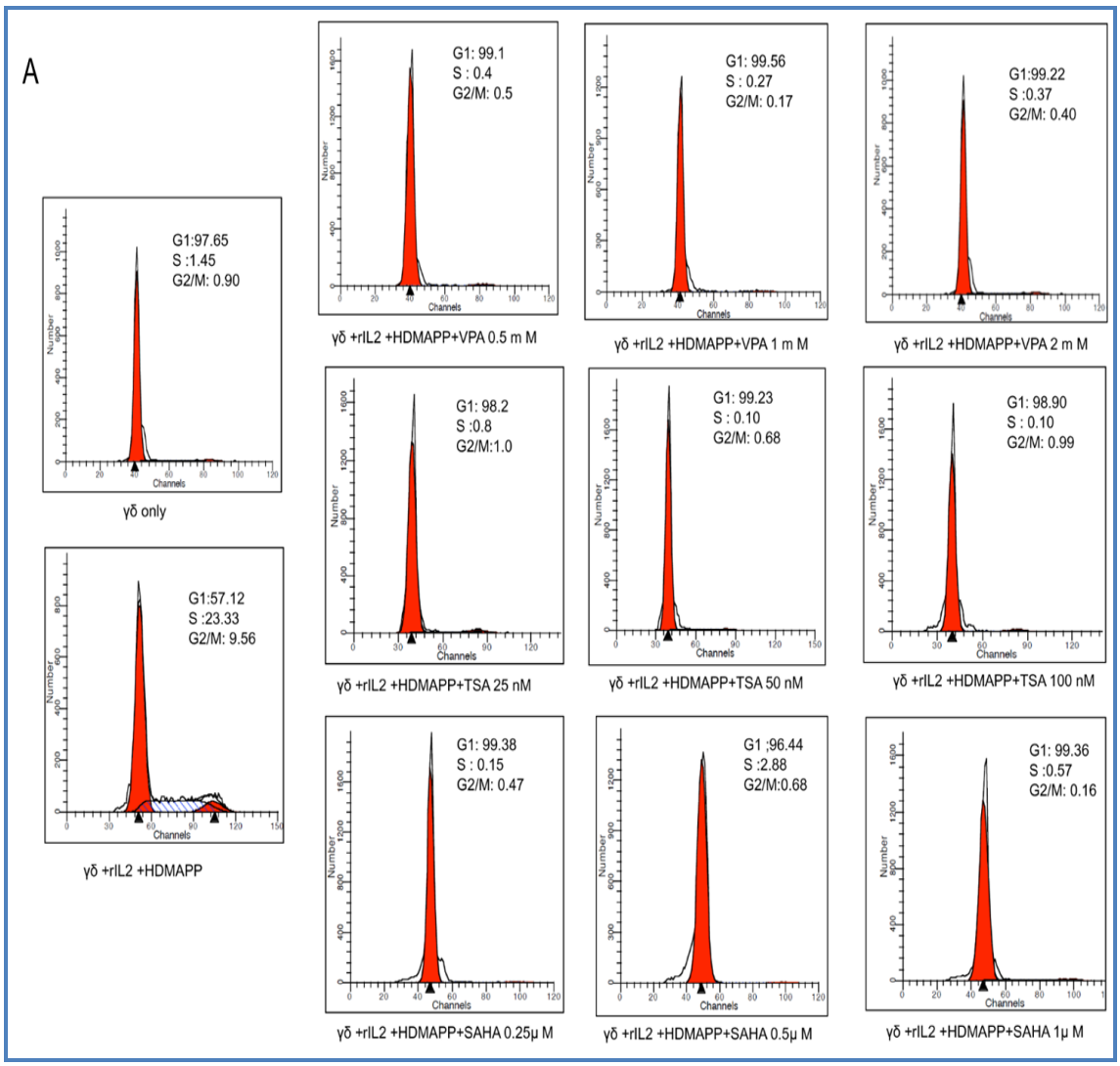


Figure 4.4: **HDAC inhibitors impede the proliferation of γδ T cells in a dose-dependent manner**. The proliferative response of γδ T cells was assessed by thymidine incorporation assay. Sorted γδ T cells were stimulated with phosphoantigen HDMAPP, with or without treatment with HDAC inhibitors VPA **(A)**, TSA **(B)** and SAHA **(C)** at different concentrations for 72 hours.The graphs illustrate the cumulative mean cpm and are representative of three independent experiments where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$

4.2.3 Effect of HDAC inhibitors on the cell cycle progression of γδ T cells

We evaluated the effect of HDAC inhibitors on the cell cycle progression of γδ T cells. γδ T cells were sorted from peripheral blood of healthy individuals. γδ T cells were left untreated or treated with HDMAPP and rIL2 in the presence and absence of different concentrations of HDAC inhibitors for 72 hours. After 72 hours γδ T cells were harvested and fixed with 70% ethanol. The γδ T cells were analyzed for DNA content using propidium iodide (PI), by flow cytometry and data analyzed by ModFit software. In the absence of strong phosphoantigen (HDMAPP) based TCR stimulus, γδ T cells failed to proliferate**.** .However in the presence of strong HDMAPP phosphoantigen and rIL2 stimulation for 72hrs a significant increase in a number of γδ T cells in S phase and G2/M phase was observed. The addition of HDAC inhibitors (VPA; 0.5–2 mM, TSA; 25–100 nM, and SAHA; 0.25–1µM) prevented the HDMAPP and IL2 driven the proliferation of γδ T cells and arrested them at G0/G1

phase **(Figure 4.5 A-D)**. Further, we observed that inhibition of cell cycle progression in γδ T cells upon HDAC inhibitor treatment was reflected in the increased expression of cell cycle and cell proliferation regulators, p53 and its downstream target p21, suggesting that HDAC inhibitors impede the G0/G1-S phase transition in γδ T cells in a p53-dependent manner. Thus HDAC inhibitors arrest the cell cycle in γδ T cells at G0/G1 phase which leads to an increase in the expression of cell cycle regulator p53 and its downstream target p21 **(Figure 4.6 A-I).**



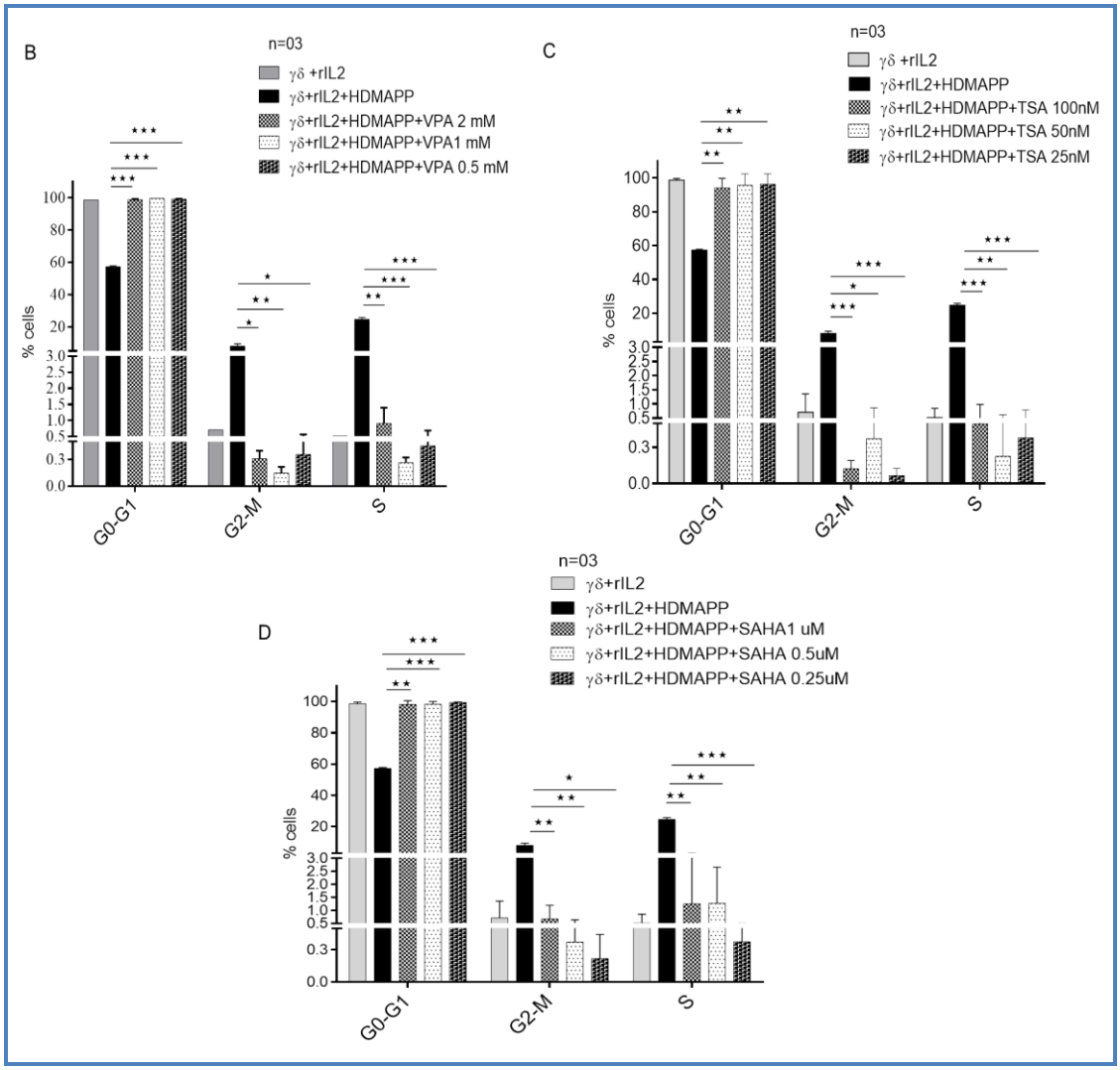


Figure 4.5: **HDAC inhibitors impede the proliferation of γδ T cells in a dose-dependent manner and leads to cell cycle arrest in G0-G1phase**. Representative figure of cell cycle analysis of γδ T cells upon HDAC inhibitor treatment **(A)**. Freshly isolated γδ T cells were activated with HDMAPP with or without HDAC inhibitors for 72 hours and cell cycle progression was analyzed with Propidium iodide (PI) staining using FACS Calibur. The graphs are representative of three independent experiments. **(B-D)** The graphs indicate the cumulative mean percentage of PI-positive cells in each phase of the cell cycle. Each graph is representative of three independent experiments where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$

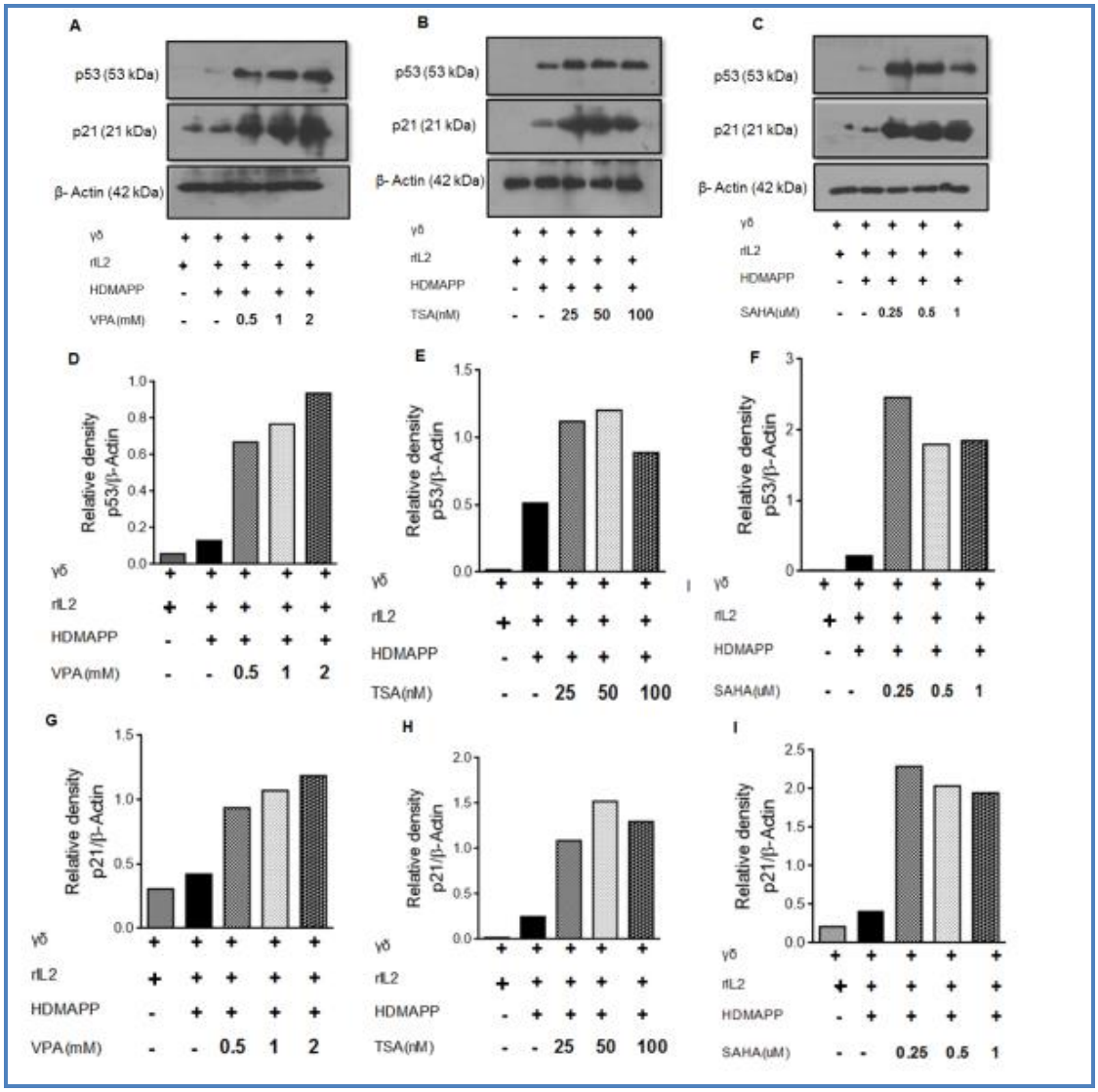


Figure 4.6: HDAC inhibitors increase the expression of cell cycle checkpoint proteins p53 and p21. Protein expression of p53 and p21 by γδ T cells upon treatment with **(A)** VPA, **(B)** TSA and **(C)** SAHA as detected by western blotting. Cell lysates of γδ T cells, stimulated with HDMAPP after treatment with HDAC inhibitors at different concentrations for 72 hours were prepared and p53, p21 proteins were detected, β-actin was used as a loading control. Densitometry quantification of p53 **(D-F)** and p21 **(G-I)** expression in γδ T cells upon treatment with VPA, TSA and SAHA, relative to β-actin.

4.2.4 HDAC inhibitors regulate cytokine production in γδ T Cells

γδ T cells upon activation by phosphoantigens, secrete copious amount of cytokines such as IFN- γ and TNF- α [378]. γδ T cells are known to be the earliest source of IFN- γ during different pathological conditions and are responsible for activating other immune cells like dendritic cells. Since we observed that HDAC inhibitor treatment in γδ T cells abrogates the antigen-specific proliferative capacity and causes cell cycle arrest, it prompted us to check the effect of HDAC inhibitors on the expression of IFN- γ and TNF- α. γδ T cells were isolated from the peripheral blood of healthy individuals and were activated with the HDMAPP and rIL2 in presence or absence of different concentrations of HDAC inhibitors (VPA; 0.5–2 mM, TSA; 25–100 nM, and SAHA; 0.25–1μM) for 72 hours. After 72 hours supernatants and cells were collected for checking the expression of IFN- γ and TNF- α at the protein level by Sandwich ELISA and at mRNA level by quantitative real-time PCR. A marked increase in the expression of cytokines IFN- γ and TNF- α was observed upon stimulation of γδ T cells with HDMAPP and rIL2 compared to un-stimulated γδ T cells. Expression of IFN-γ and TNF-α was decreased significantly when treated with HDAC inhibitors TSA, SAHA, and VPA. This inhibition was observed both at mRNA and protein levels. It was observed that inhibition of cytokine expression was concentration dependent for HDAC inhibitors **(Figure 4.7 A-D).**

Functionally active γδ T cells secrete copies amount of IFN- γ and TNF- α. Our data demonstrated that a decrease in the expression of IFN- γ and TNF- α in HDAC inhibitor-treated γδ T cells suggests HDAC inhibitors may be controlling the anti-tumor potential of γδ T cells.

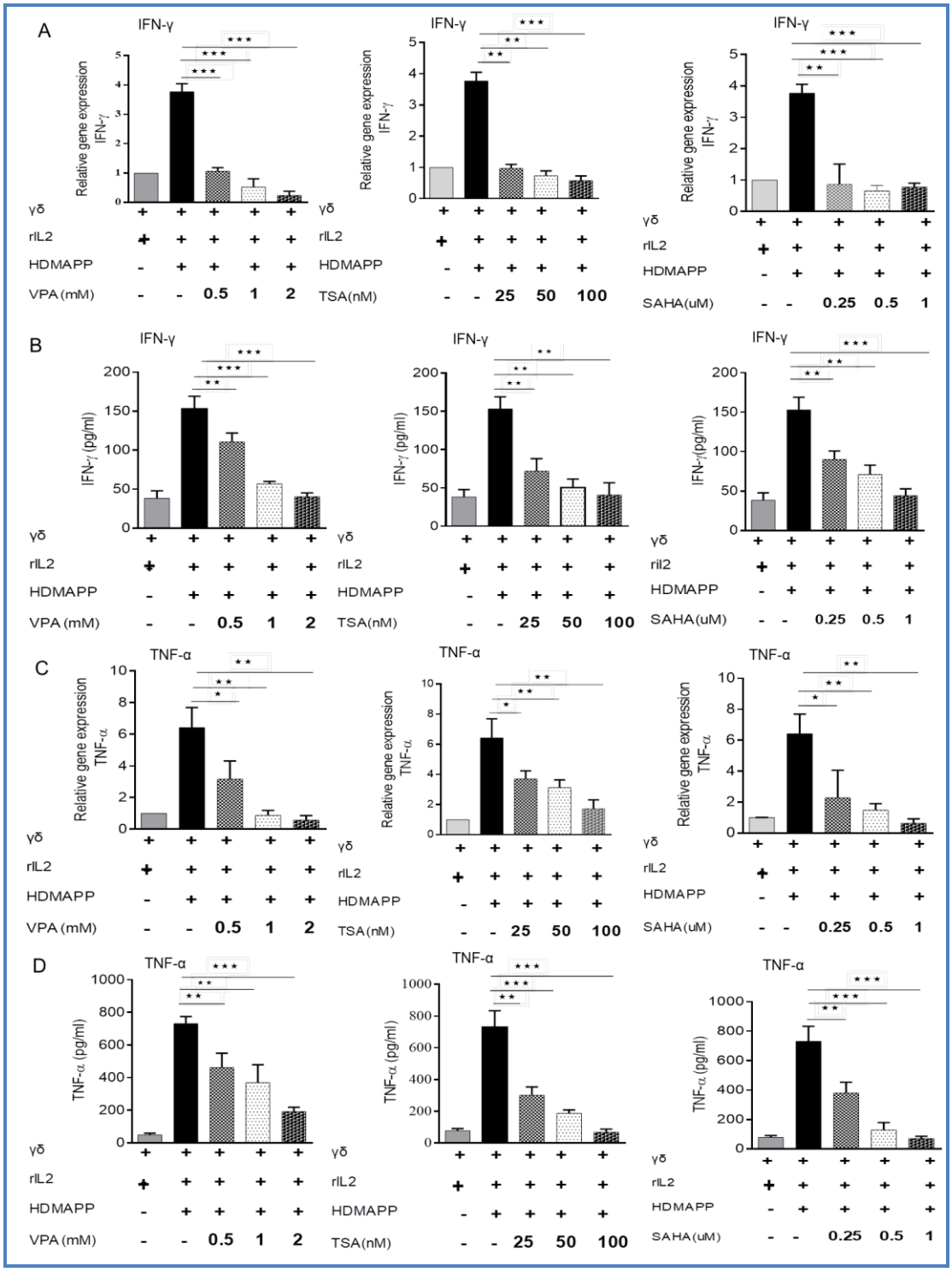


Figure 4.7: HDAC inhibitors regulate cytokine production. Expressions of IFN-γ and TNF-α were detected by quantitative real-time PCR and sandwich ELISA. Panel **(A)** and panel **(B)** indicate IFN-γ expression by γδ T cells stimulated with HDMAPP, treated with or without HDAC inhibitors VPA, TSA, and SAHA at different concentrations at mRNA and protein levels respectively. Panel **(C)** and panel **(D)** represent an expression of TNF-α in the supernatants collected from HDMAPP stimulated γδ T cells in the presence or absence of HDAC inhibitors VPA, TSA, and SAHA at different concentrations at mRNA and protein levels respectively. The

expression of different mRNA transcripts was normalized to 18S rRNA. All the results indicated are mean ± SEM of three independent experiments, where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$

4.2.5 HDAC inhibitors decrease the expression of activation markers in γδ T Cells and increase inhibitory receptor KIR2DL2 and KIR2DL3

γδ T cells upon activation show an increase in the expression of different markers. We evaluated the effect of HDAC inhibitors on the expression of early activation marker CD69 and late activation marker CD25 on γδ T cells. Treatment of γδ T cells with HDAC inhibitors led to a decrease in the expression of early activation and late activation marker on γδ T cells. The expression of these activation markers on γδ T cells were significantly reduced in a concentration-dependent manner, with a maximum decrease at VPA 2 mM, TSA 100 nM, and SAHA 1 μM, respectively **(Figure 4.8 A-B and Figure 4.9 A-B).** Percentage of γδ T cells positive for these markers was also less in HDAC inhibitor-treated γδ T cells as compared to untreated γδ T cells. CD25 is the high-affinity IL-2 receptor subunit and IL-2 signaling is necessary for the proliferation and co-stimulation of T cells. The other subunits of the IL-2 receptor include CD132 and CD122. We further investigated the effect of HDAC inhibitors on the expression of other subunits of the IL-2 receptor. Antigen-activated γδ T cells showed an increase in the expression of CD132 and CD122, however upon HDAC inhibitor treatment expression of these receptors also decreased **(Figure 4.10 A-B)**. IL-2 signaling is necessary for the γδ T cell co-stimulation along with TCR signaling, decrease in the expression of IL-2 signaling receptors due to HDAC inhibitor treatment further confirms that HDAC inhibitors also modulate the co-stimulatory signaling in γδ T cells. Stimulation of the T cell antigen receptor (TCR) induces the formation of a phosphorylation-dependent signaling network via multiprotein complexes. Antigen activation in T cells leads to an increase in phospho

tyrosine level. γδ T cells activated with HDMAPP show an increase in the level of phospho-tyrosine level. The level of a phospho-tyrosine level decreases in antigen-activated γδ T cells in the presence of HDAC inhibitor. We also evaluated the role of HDAC inhibitors on the expression of other activating receptors like NKG2D, CD71, and CD16. Activated and proliferating cells respond to the demand for intracellular iron by upregulating membrane expression of the transferrin receptor (CD71) which necessary for the optimum activation of T cells. We investigated the effect of HDAC inhibitors on the expression of CD71 in γδ T cells. We observed a remarkable decrease in the expression of CD71 in γδ T cells activated with HDMAPP in the presence of HDAC inhibitors **(Figure 4.10 C-D)**.

NKG2D, an activating receptor for γδ cells, has been described as a potent co-stimulatory receptor in the antigen specific activation of γδ T cells, NK cells and CD8 T cells [123]. In humans, seven ligands of NKG2D have been identified to date; the MHC class I-related proteins A and B (MICA/B) and members of the UL16-binding protein family (ULBP1–4, RAET1G). The NKG2D ligands MICA and MICB are often expressed on tumors. Interaction between NKG2D and its ligands is necessary in tumor surveillance. In order to investigate the role of HDAC inhibitors on the expression of NKG2D, γδ T cells were treated with HDAC inhibitors VPA 2 mM, TSA 100 nM, and SAHA 1 μM for 72 h. We observed that HDAC inhibitor-treated γδ T cells showed decreased expression of NKG2D as compared to untreated γδ T cells **(Figure 4.10 E)**.

Beside the activating receptors γδ T cells also express the inhibitory receptors like Killer cell Immunoglobulin-Like Receptors KIR2DL2 and KIR2DL3. It was reported, γδ T cells which are positive for KIR2DL2 and KIR2DL3 receptors have less anti-tumor cytotoxic potential as compared to γδ T cells which are negative for these

receptors [136]. In order to investigate whether the HDAC inhibitor treatment has any impact on the expression of KIR2DL2 and KIR2DL3, We analyzed the expression of these receptors on γδ T cells after the treatment of HDAC inhibitors VPA (2 mM), TSA (100 nm), and SAHA (1 μM). We observed that γδ T cells show an increase in the expression of these receptors after HDAC inhibitor treatment. However, we did not observe any change in CD16 expression **(Figure 4.10 E)** after HDAC inhibitor treatment. Collectively, the data advocate the role of HDAC inhibitors in abating the expression of activation markers CD69, CD25, NKG2D, CD71, phospho-tyrosine and increasing expression of inhibitory receptors KIR2DL2 and KIR2DL3 in γδ T cells. The results suggest that HDAC inhibitors influence the functional responses of γδ T cells and may play a role in regulating the anti-tumor cytotoxic potential of γδ T cells.

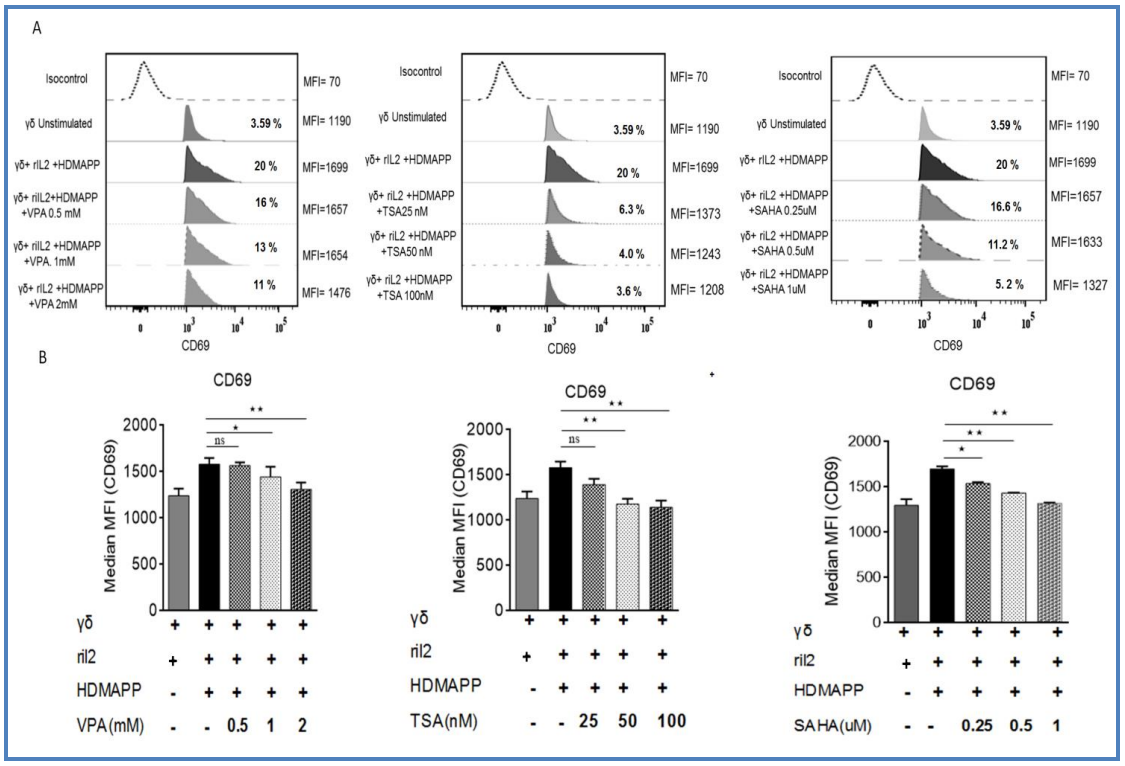


Figure 4.8: HDAC inhibitors affect the activation of γδ T cells (Early activation). **(A)** The expression of early activation marker (CD69) on unstimulated γδ T cells, HDMAPP and rIL-2 stimulated γδ T cells with or without HDAC inhibitor treatment was analyzed by multi-color flow cytometry. Values on the right side indicate the median fluorescence intensity (MFI) of CD69, while the values inside the histogram represent the percent CD69 positive γδ T cells. The histograms shown are

representative of three independent experiments. **(B)** The cumulative MFI of CD69 expression on γδ T cells is represented as bar graphs. Data shown is representative of three independent experiments where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$. . ns= Non significant

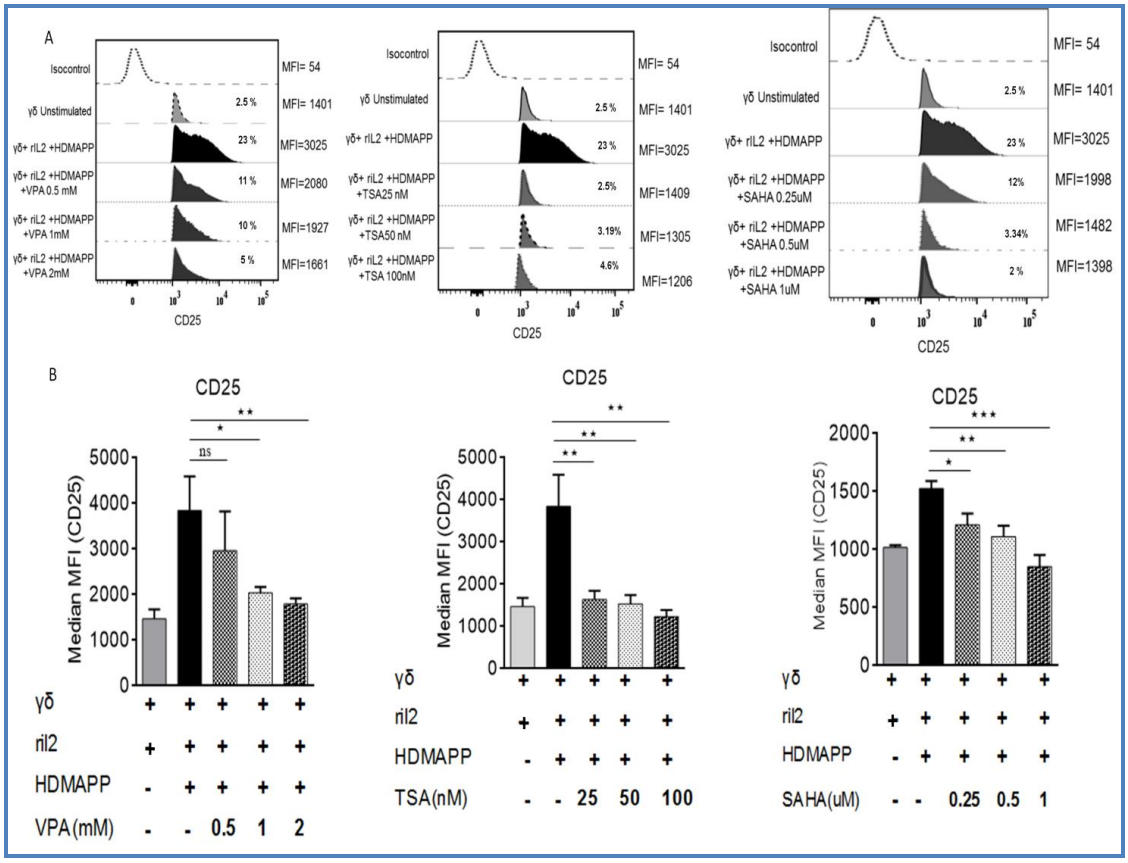
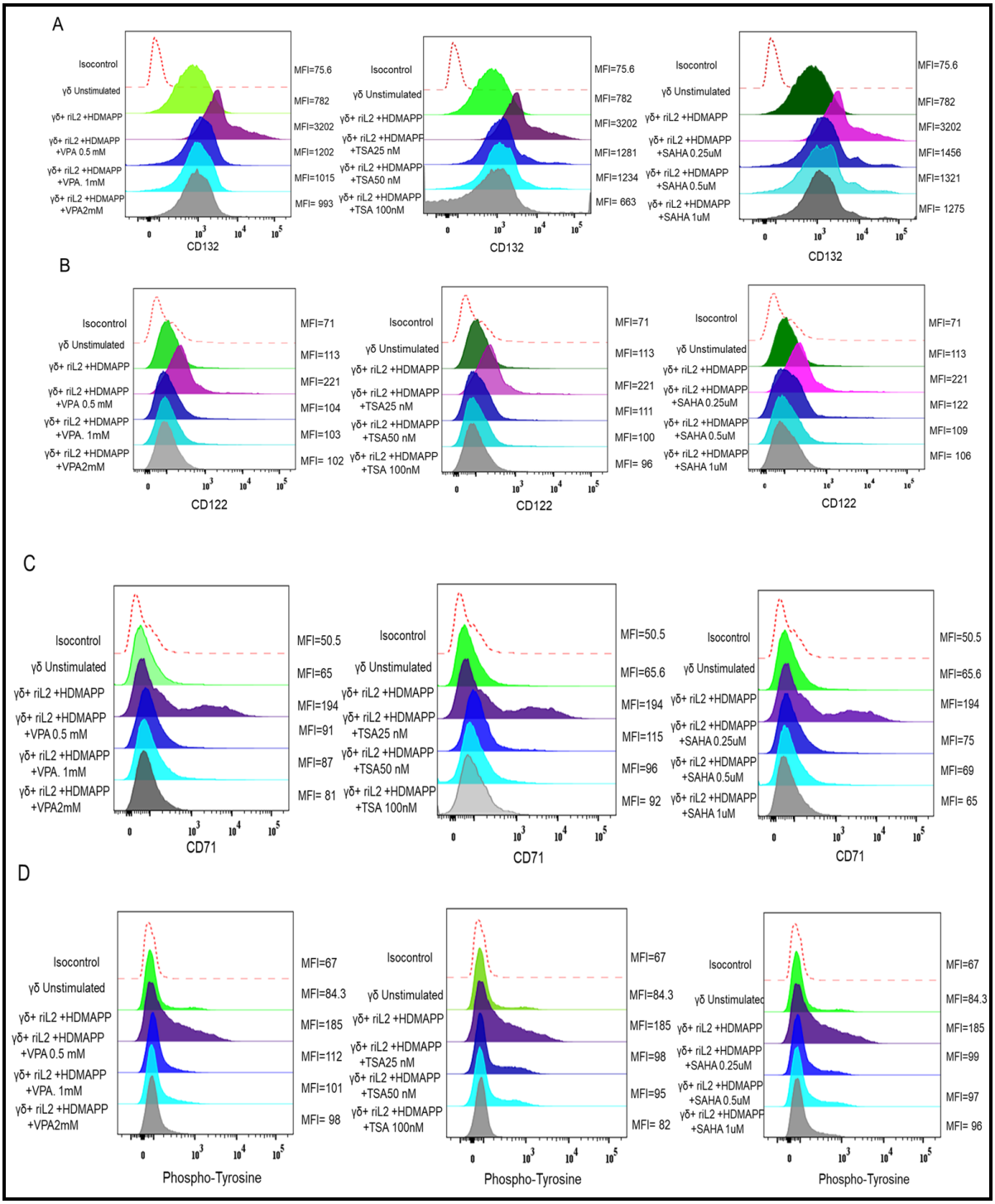


Figure 4.9: HDAC inhibitors affect the activation of γδ T cells (Late activation). **(A)** The expression of early activation marker (CD25) on unstimulated γδ T cells, HDMAPP and rIL-2 stimulated γδ T cells with or without HDAC inhibitor treatment was analyzed by multi-color flow cytometry. Values on the right side indicate the median fluorescence intensity (MFI) of CD69, while the values inside the histogram represent the percent CD25 positive γδ T cells. The histograms shown are representative of three independent experiments. **(B)** The cumulative MFI of CD69 expression on γδ T cells is represented as bar graphs. Data shown is representative of three independent experiments where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$, ns= Non significant


A
Isocontrol
γδ Unstimulated
γδ+ riL2 +HDMAPP
γδ+ riL2 +HDMAPP +VPA 0.5 mM
γδ+ riL2 +HDMAPP +VPA. 1mM
γδ+ riL2 +HDMAPP +VPA2mM
MFI=75.6
MFI=782
MFI=3202
MFI=1202
MFI=1015
MFI= 993
γδ+ riL2 +HDMAPP +TSA25 nM
γδ+ riL2 +HDMAPP +TSA50 nM
γδ+ riL2 +HDMAPP +TSA 100nM
MFI=1281
MFI=1234
MFI= 663
γδ+ riL2 +HDMAPP +SAHA 0.25uM
γδ+ riL2 +HDMAPP +SAHA 0.5uM
γδ+ riL2 +HDMAPP +SAHA 1uM
MFI=1456
MFI=1321
MFI= 1275
CD132
B
MFI=71
MFI=113
MFI=221
MFI=104
MFI=103
MFI= 102
MFI=111
MFI=100
MFI= 96
MFI=122
MFI=109
MFI= 106
CD122
C
MFI=50.5
MFI=65
MFI=194
MFI=91
MFI=87
MFI= 81
MFI=65.6
MFI=115
MFI=96
MFI= 92
MFI=75
MFI=69
MFI= 65
CD71
D
MFI=67
MFI=84.3
MFI=185
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Phospho-Tyrosine

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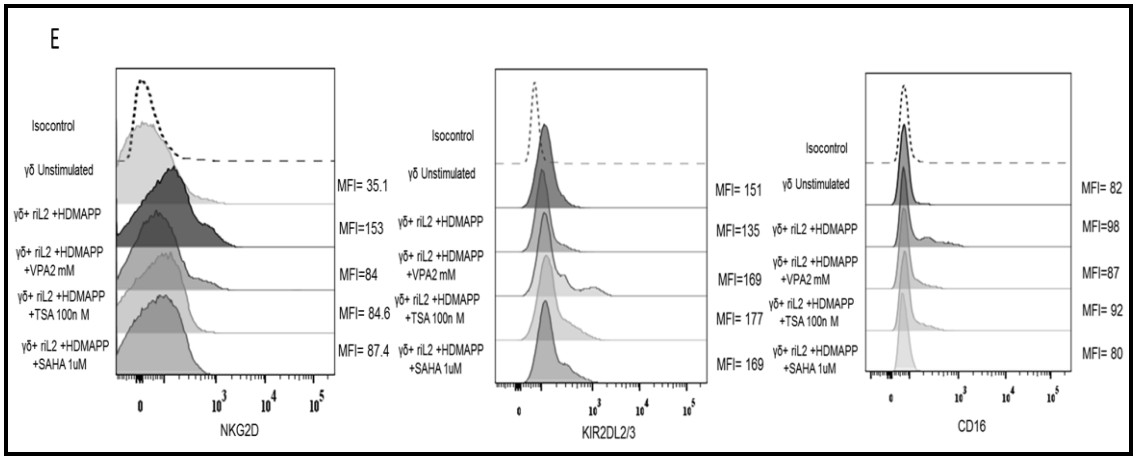

Figure 4.10: HDAC inhibitors affect the proximal IL-2 signaling receptors, activation markers and inhibitory receptors of γδ T cells. The expression of proximal IL-2 signaling receptors CD132 **(A),** CD122 **(B)**, CD71**(C)** and phospho-tyrosine (**D**) on unstimulated γδ T cells, HDMAPP and rIL-2 stimulated γδ T cells with or without HDAC inhibitor treatment was analyzed by multi-color flow cytometry. **(E)** Expression of activating receptor NKG2Dand CD16; inhibitory receptors KIR2DL2/3 on unstimulated γδ T cells, HDMAPP and rIL-2 stimulated γδ T cells with or without HDAC inhibitor treatment was analyzed by multi-color flow cytometry.Values on the right side indicate the median fluorescence intensity (MFI) of NKG2D, KIR2DL2/3, and CD16 on γδ T cells. The histograms depicted are representative of three independent experiments.

4.2.6 HDAC inhibitors suppress the expression of transcription factors Eomesodermin (Eomes), T-bet and Nf-κB in γδ T Cells

Eomesodermin (Eomes) and T-bet transcription factors are the members of the T-box family regulating the function and development of cytotoxic lymphocytes such as NK, $CD8^+$ and γδ T cells. They are the main transcription factors, which regulate the effector functions of CD8 T cells through the expression of effector genes Perforin and Granzyme B [230, 388]. Besides CD8 T cells, γδ T cells also express Eomes and T-bet [389]. Upon activation with phosphoantigen (HDMAPP) and rIL2, γδ T cells show increased expression of these two transcription factors. We hypothesized that HDAC inhibitors may have an impact on the expression of these two transcription factors in γδ T cells. Therefore, the role of HDAC inhibitors in regulating expression

of Eomes and T-bet in γδ T cells activated with phosphoantigen (HDMAPP) and rIL2 was analyzed. γδ T cells treated with HDAC inhibitors VPA; 0.5–2 mM, TSA; 25–100 nM, and SAHA; 0.25–1μM) for 72 hours showed a decrease in the expression of Eomes and T-bet **(Figure 4.11 A-C)**. This decrease in the expression of Eomes and T-bet was observed both at the mRNA and protein level. We also validated the effect of HDAC inhibitors on the expression of Nf-κB in antigen-activated γδ T cells. Nf-κB is one of the main transcription factors besides Eomes and T-bet that has been shown to regulate the effector functions of cytotoxic cells like NK cells, $CD8^+$ T cells, and γδ T cells. γδ T cells activated with non-peptide antigen HDMAPP showed increased expression of Nf-κB transcription factor. However, γδ T cells activated with HDMAPP and rIL2 in presence or absence of HDAC inhibitors showed decrease in the expression of NF- κB. This decrease was observed at the protein level **(Figure 4.11 D)**. Inhibition of Eomes, T-bet, and Nf-κB by HDAC inhibitors clearly demonstrates that HDAC inhibitors regulate the effector functions of γδ T cells.

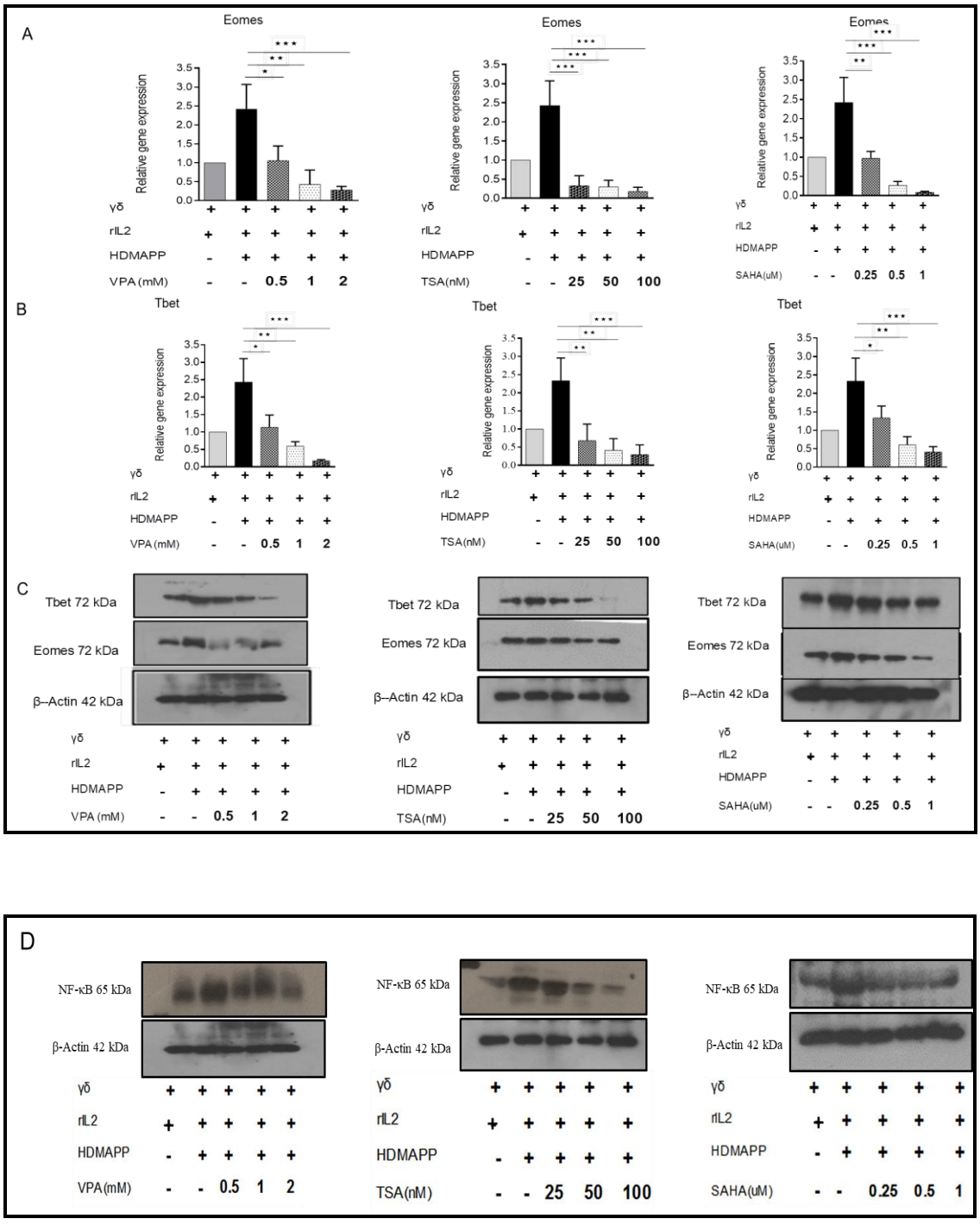

Figure 4.11: **HDAC inhibition abrogates expression of transcription factors regulating effector functions of γδ T cells**. The m-RNA expression of Eomes **(A)** and T bet **(B)** in γδ T cells activated with HDMAPP, in the presence or absence of HDAC inhibitors VPA, TSA and SAHA at different concentrations were quantified by quantitative real-time PCR. The results indicated are cumulative mean of relative gene expression normalized to 18s rRNA where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$, compared with γδ T cells activated with HDMAPP. The data shown are representative of three independent experiments. **(C)** The protein level expression of

T bet and Eomes was detected by western blotting. HDAC inhibitor treatment decreases the expression of T-bet and Eomes. β- Actin was maintained as a loading control. The blots shown are representative of three experiments. HDAC inhibitors reduce NFκB expression in γδ T cells. Protein expression of Nf-κB by γδ T cells upon treatment with VPA, TSA, and SAHA as detected by western blotting **(D).** Cell lysates of γδ T cells, stimulated with HDMAPP after treatment with HDAC inhibitors at different concentrations for 72 hours were probed with NFκB antibody, β-actin was used as a loading control. The blots shown are representative of three experiments.

4.2.7. HDAC inhibitors inhibit the expression of effector molecules Perforin and Granzyme B

Cytotoxic lymphocytes utilize the Perforin/Granzyme pathway for target-cell killing. γδ T lymphocytes and other cytotoxic cells contain specialized cytotoxic granules, which degranulate upon contact with a target-cell. These granules contain effector molecules Perforin, a membrane-disrupting protein that facilitates the delivery of Granzymes, granule proteases that initiate apoptotic death in target-cells. The molecular mechanism(s) whereby γδ lymphocytes destroy susceptible target cells is through granule-mediated cytotoxicity and requires the combination of both Perforin and Granzyme B. Perforin polymerizes to form trans-membrane channels and presumably allows Granzyme B access to target cell substrates.

To evaluate the role of HDAC inhibitors in the modulation of the antitumor potential of γδ T cells, we analyzed the expression of effector molecules Perforin and Granzyme B in γδ T cells at mRNA and protein level. Freshly isolated γδ cells activated with phosphoantigen HDMAPP and rIL2 for 72 hours showed increased expression of Perforin and Granzyme B effector genes both at mRNA and protein level. However, γδ T cells activated in presence of HDAC inhibitors showed a decrease in the expression of Perforin and Granzyme B. Maximum effect on the expression of Perforin and Granzyme B was observed with VPA 2 mM, TSA 100 nM, and SAHA 1 μM **(Figure 4.12 A-C)** and **(Figure 4.13 A-C)**

HDAC inhibitors are known to increase the acetylation of histones. We investigated the effect of HDAC inhibitors on the level of acetylation of histone H3 and histone H4 in γδ T cells treated with HDAC inhibitors VPA 2 mM, TSA 100 nM, and SAHA 1 μM using western blotting. We observed that γδ T cells treated with HDAC inhibitors show an increase in the level of total histone H3 and histone H4 acetylation as compared to untreated γδ T cells **(Figure 4.14 A)**. In order to further validate the effect of HDAC inhibitors on the level of histone H3 and H4 acetylation level on the gene-specific promoter regions of Perforin and Granzyme B, we did the chromatin immunoprecipitation (ChIP) assay followed by the quantitative real-time PCR (qPCR). We observed that HDAC inhibitor-treated γδ T cells showed less histone H3 acetylation and H4 acetylation on promoters of effector molecules Perforin and Granzyme B compared to untreated γδ T cells as determined by ChIP qPCR assay **(Figure 4.14 B-C)**. Histone acetylation positively correlated with transcriptional activity. Thus, our data showed that epigenetic changes on promoters of effector molecules Perforin and Granzyme B control the expression of these molecules in HDAC inhibitor-treated γδ T cells and hence the effector functions of γδ T cells are epigenetically regulated.

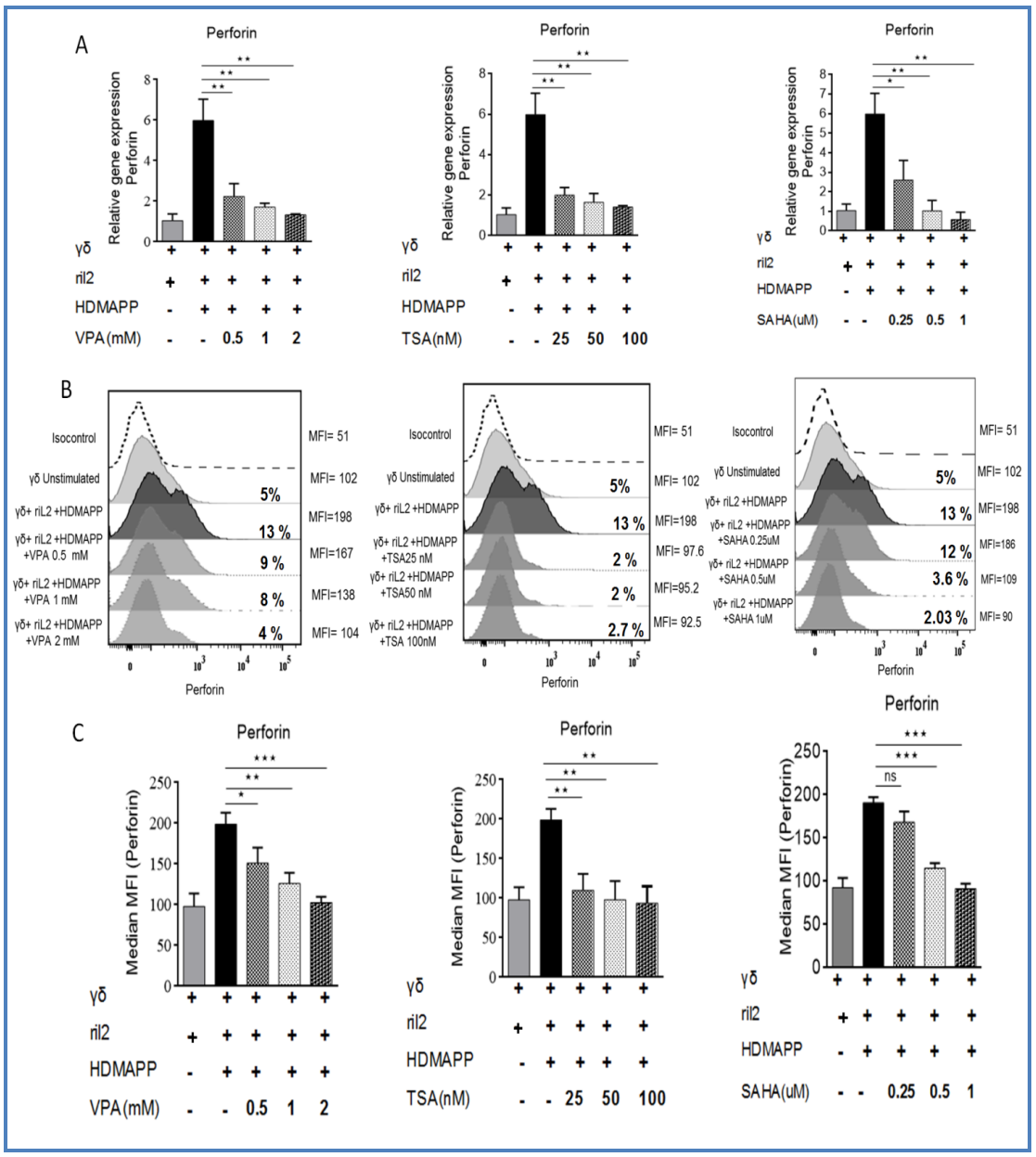

Figure 4.12: HDAC inhibitor treatment abrogates Perforin expression in γδ T cells. Expression of Perforin was studied at mRNA and protein levels using quantitative real-time PCR and flow cytometry respectively. **(A)** The gene expression of Perforin by HDMAPP stimulated γδ T cells with or without HDAC inhibitor treatment was quantified by quantitative real-time PCR. The results shown are cumulative means of relative gene expression, normalized to 18S rRNA and representative of three independent experiments. **(B)** γδ T cells activated by HDMAPP with or without VPA, TSA, and SAHA at different concentrations were stained after 72 hours with the corresponding fluorophore tagged antibody and expression of Perforin was analyzed by flow cytometry. The values on the right side of histograms indicate median fluorescence intensity of Perforin, while the values inside the histogram represent the percent positive γδ T cells for Perforin .**(C)** Bar graphs represent cumulative MFI values where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$, compared with γδ T cells activated with HDMAPP.

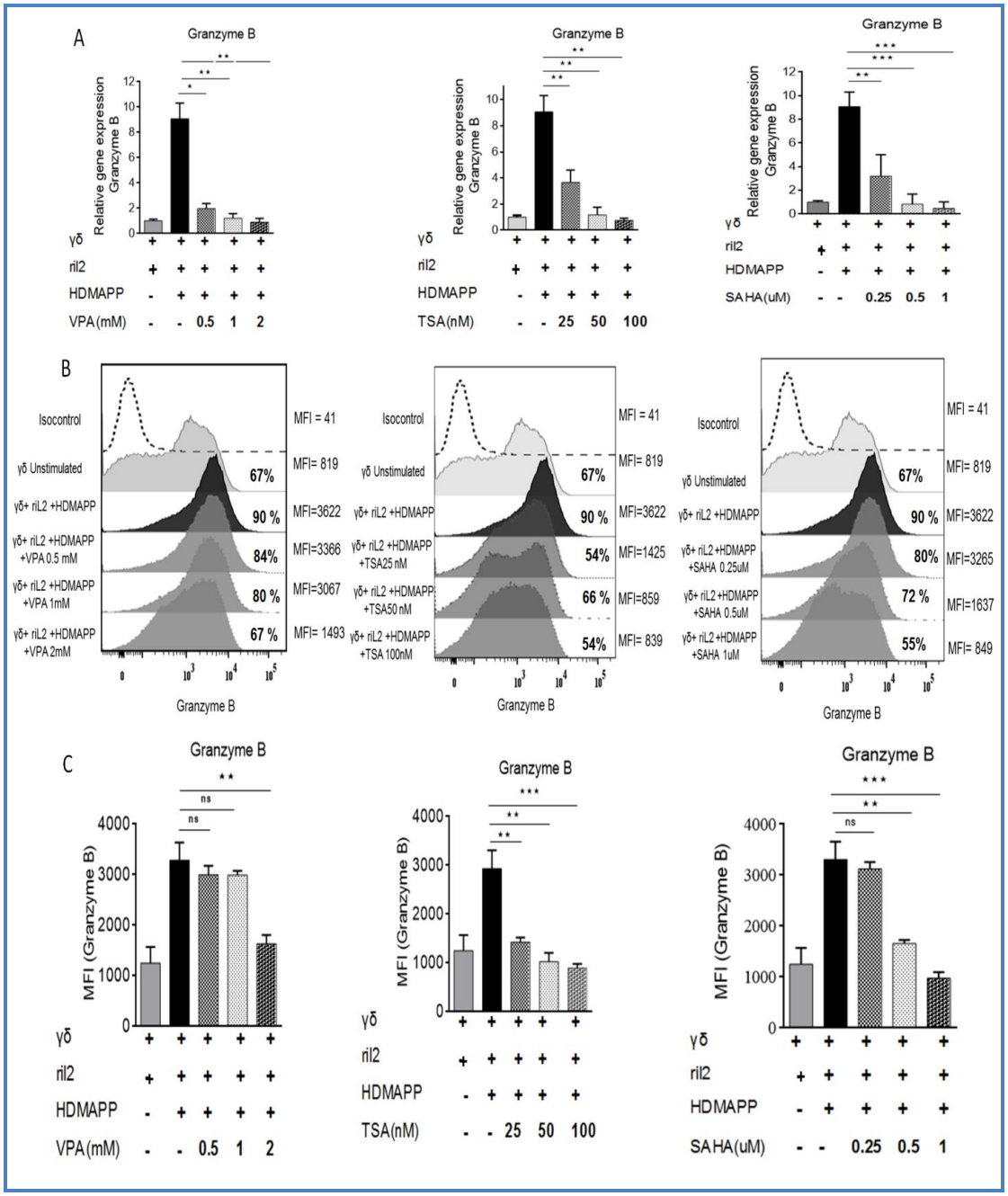

Figure 4.13: Granzyme B expression diminished in γδ T cells upon HDAC inhibition. Expression of Granzyme B was studied at mRNA and protein levels using quantitative real-time PCR and flow cytometry respectively. **(A)** The gene expression of B by HDMAPP stimulated γδ T cells with or without HDAC inhibitor treatment was quantified by quantitative real-time PCR. The results shown are cumulative means of relative gene expression, normalized to 18S rRNA and representative of three independent experiments. **(B)** γδ T cells activated by HDMAPP with or without VPA, TSA, and SAHA at different concentrations were stained after 72 hours with the corresponding fluorophore tagged antibody and expression of Granzyme B was analyzed by flow cytometry. The values on the right side of histograms indicate median fluorescence intensity of Granzyme B, while the values inside the histogram

represent the percent positive γδ T cells for Granzyme B. **(C)** Bar graphs represent cumulative MFI values of Granzyme B expression on γδ T cells where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$, compared with γδ T cells activated with HDMAPP.

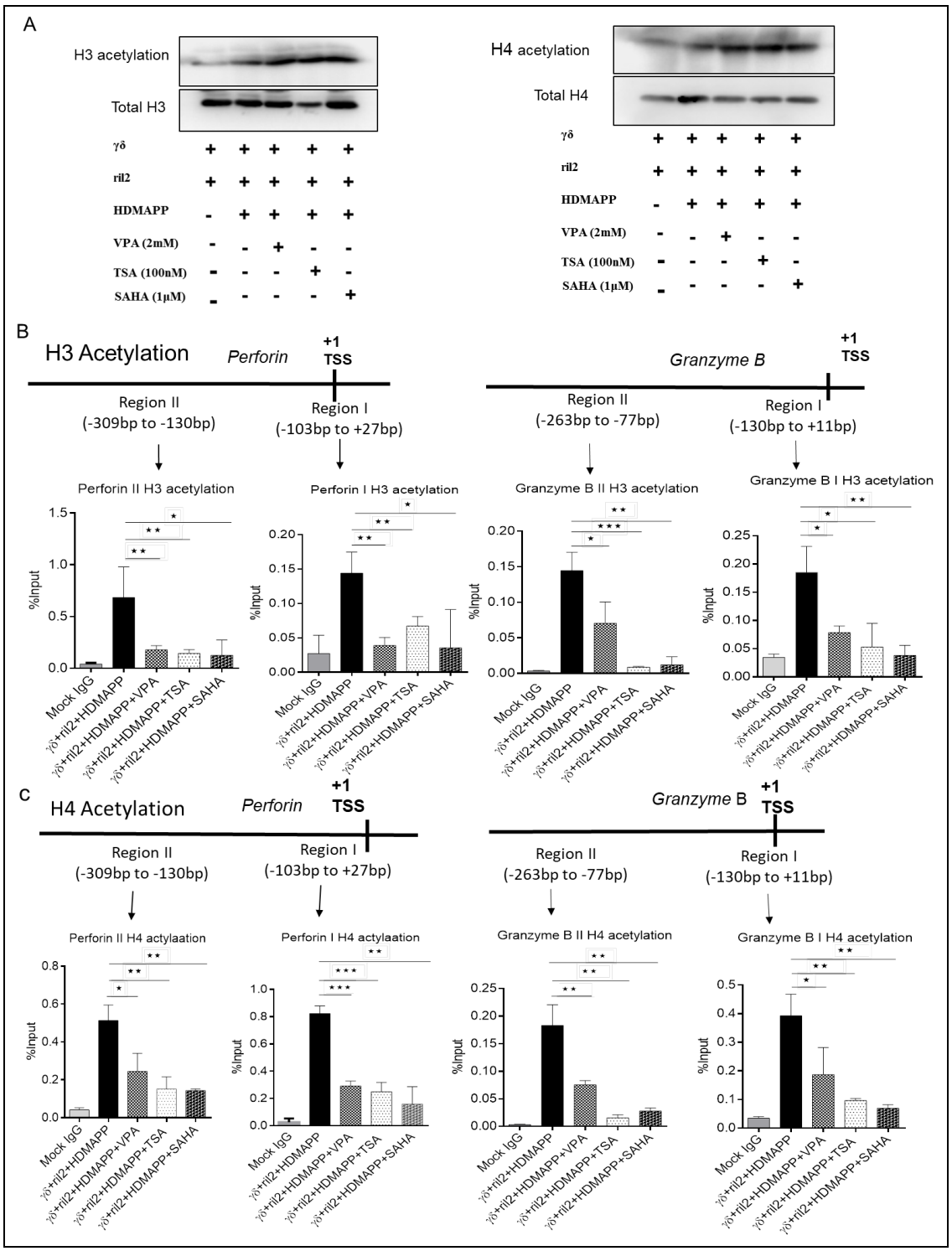

Figure 4.14: **HDAC inhibitors decrease the acetylation on promoters of Perforin and Granzyme B in γδ T cells (A)** γδ T cells treated with HDAC inhibitors VPA, TSA and SAHA show increased acetylation of H3 and H4. Expression of acetyl Histone 3 and acetyl histone 4 in γδ T cells was detected by western blotting and Total H3 and total H4 were used as a loading control. The blots shown are

representative of three experiments. Chromatin immunoprecipitation qPCR was used to analyze the histone H3 acetylation **(B)** and histone H4 acetylation **(C)** on Perforin region I (-103bp to +27bp), Perforin region II (-309bp to -103bp); Granzyme B region I (-130bp to + 11bp) and Granzyme B region II (-263bp to -77bp) from the transcription start site (TSS) of Perforin and Granzyme B.in γδ T cells treated with or without HDAC inhibitors VPA, TSA and SAHA for 72hrs. Enrichment of promoter regions of Perforin and Granzyme B was quantified by qPCR and expressed as a percentage of input DNA. The images above the graphs are a schematic representation of Perforin and Granzyme B promoter regions including the transcription start site (TSS). All results indicated are mean + SEM where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$

4.2.8 HDAC inhibitors abrogate the anti-tumor effector functions of γδ T Cells

γδ T cells perform potent immune surveillance by exerting direct cytotoxicity, strong cytokine production, and antitumor immune responses. The cytotoxic potential of γδ T cells against tumor targets was analyzed by lactate dehydrogenase release assay as described in material and methods. γδ T cells were treated with HDAC inhibitors for 72 hours and used as effectors against tumor targets. The cytotoxic potential of HDAC inhibitor-treated γδ T cells was evaluated against a panel of Zoledronate-treated tumor cells lines AW13516 (oral cancer), COLO-205 (colon carcinoma), and Raji (B cell lymphoma). At different E/T ratios starting from 5:1 to 40:1, HDMAPP-activated γδ T cells in the presence of IL-2, efficiently lysed Zoledronate-treated tumor cells lines (AW13516, COLO- 205, and Raji). Maximum cytotoxicity of γδ T cells was observed at E/T ratio of 40:1 **(Figure 4.15 A-C)**. This ratio of E: T was used in further experiments, to assess the effect of HDAC inhibitors TSA, VPA, and SAHA on the cytolytic ability of γδ T cells. γδ T cells stimulated with HDMAPP and rIL2 in the presence of HDAC inhibitors VPA (2 mM), TSA (100 nM), and SAHA (1 μM) for 72 h were used as effector against Zoledronate-treated tumor cell lines (AW13516, COLO205, and Raji) as targets at E/T ratio of 40:1. Zoledronate, an aminobisphosphonate drug, inhibits the enzyme farnesyl pyrophosphate synthase in the mevalonate pathway leading to accumulation of IPP, which stimulates γδ T cell

activation via TCR signaling. γδ T cells treated with different HDAC inhibitors showed a significant decrease in their cytotoxic potential against Zoledronate treated tumor targets (AW13516, COLO 205, and Raji). It was observed that all three HDAC inhibitors (VPA, TSA, and SAHA) significantly inhibited the ability of γδ T cells to kill Zoledronate treated tumor cell lines **(Figure 4.15 D-F)**. This further confirms that HDAC inhibitors modulate the anti-tumor effector functions of γδ T cells.

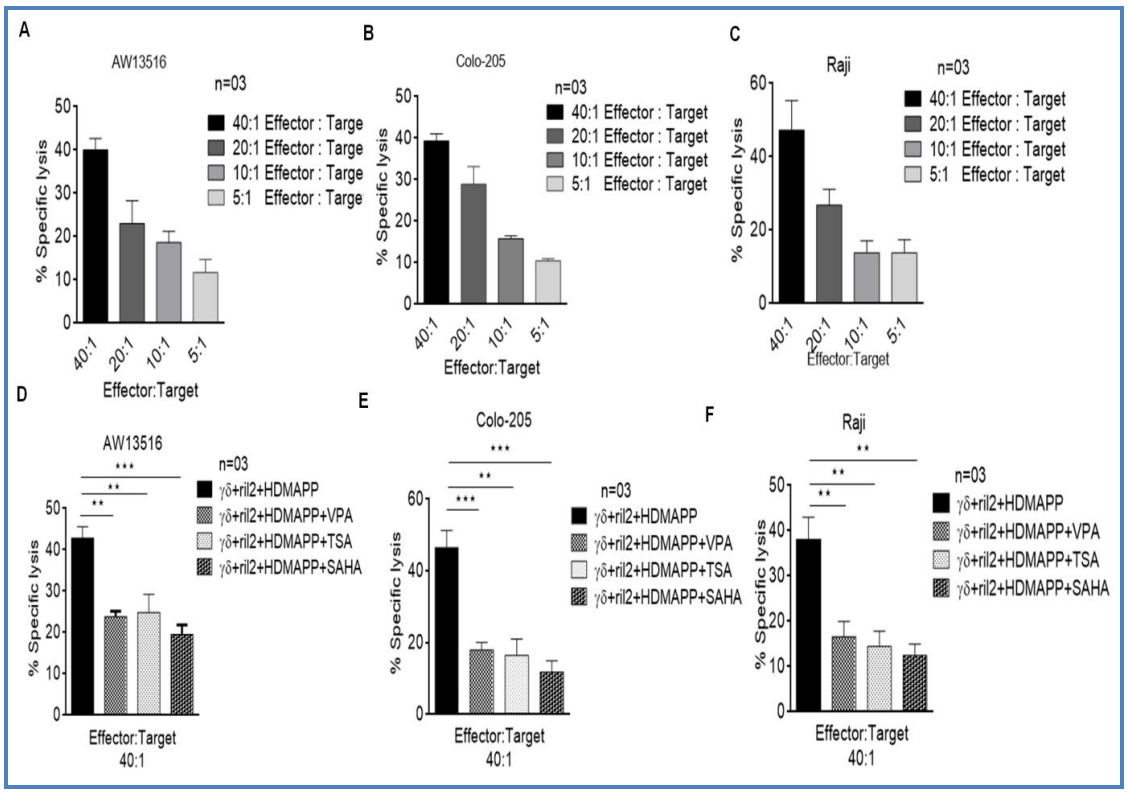

Figure 4.15: HDAC inhibitors decrease the cytotoxic effector functions of γδ T cells. Cytotoxic effector function of γδ T cells was assessed against three tumor cell lines **(A)** AW13516, **(B)** COLO205 and **(C)** Raji. Zoledronate treated tumor targets were co-cultured with γδ T cells for 4hrs and cytotoxicity was assessed by LDH assay. The results indicated are mean percent specific lysis at different effector: target ratio. Data is representative of three individual experiments. Cytotoxic potential of γδ T cells activated with HDMAPP in the presence of rIL-2 with or without treatment with VPA (2mM), SAHA (1μM) and TSA (100nM) was assessed against three Zoledronate treated tumor targets AW13516 **(D)**, COLO-205 **(E)** and Raji **(F)** by LDH cytotoxicity assay. The results indicated are percent specific lysis at the effector to target ratio of 40:1 where **: $p < 0.005$ and ***: $p < 0.0005$ when compared with γδ T cells activated with HDMAPP in the presence of rIL-2 (n=3).

4.2.9 HDAC inhibitors up-regulate the expression of immune checkpoint proteins PD-1, PD-L1 on γδ T cells

Besides activating receptors γδ T cells also express inhibitory receptors. The balance between the activation signals and inhibitory signals is necessary for the proper T cell functioning. The different ligand/receptor interactions at immunological synapse determine the outcome of the immune response. The main inhibitory receptors in such interactions include Programmed death-1 receptor (PD-1) and its ligands. The programmed death 1 (PD-1) receptor and its ligands programmed death ligand 1 (PD-L1) and PD-L2, are members of the CD28 and B7 families. PD-1 is expressed on a variety of immune cells, such as T cells, B cells, and dendritic cells. However, PD-L1 is mainly expressed on tumor cells and antigen presenting cells (APCs). Upon activation, T cells show enhanced expression of immune checkpoint PD-1. Interaction between PD-1 and PD-L1 halt the T cell activation, thus maintaining the immune homeostasis. Tumor cells exploit this pathway to evade immune response **(Figure 2.5 Chapter 2).** Overexpression of PD-L1 and PD-1 on tumor cells and tumor-infiltrating lymphocytes is responsible for highly aggressive disease.

We studied the effect of HDAC inhibitors on the expression of PD-1 and PD-L1 on γδ T cells. γδ T cells were treated with different concentrations of HDAC inhibitors and expression of PD-1 and PD-L1 was analyzed by flow cytometry. Upon activation with antigen HDMAPP and rIL-2, expression of PD-1 and PD-L1 increases on γδ T cells as compared to unstimulated γδ T cells. However, the expression of PD-1 and PD-L1 on γδ T cells substantially increased upon treatment with HDAC inhibitors. The maximum increase in the expression of both PD-1 and PD-L1 on HDMAPP and rIL-2 activated γδ T cells was observed after treatment with VPA (2 mM), TSA (100 nm), and SAHA (1 μM) **(Figure 4.16 A-D)**. Altogether the results indicated that the

decrease in the anti-tumor cytotoxic potential of HDAC inhibitor-treated γδ T cells **(Figure 4.15 D-F)** correlated with the enhanced expression of inhibitory receptors. The high expression of PD-1 and PD-L1 on γδ T cells, (at highest concentration HDAC inhibitor treatment) correlates with the maximum decrease in the cytotoxic potential observed. The interaction between PD-1 and PD-L1 abrogates the antigen-mediated TCR signaling in T cells. Thus our result demonstrates that HDAC inhibitor treatment abrogates the TCR mediated effector functions of γδ T cells through PD-1and PD-L1 signaling axis.

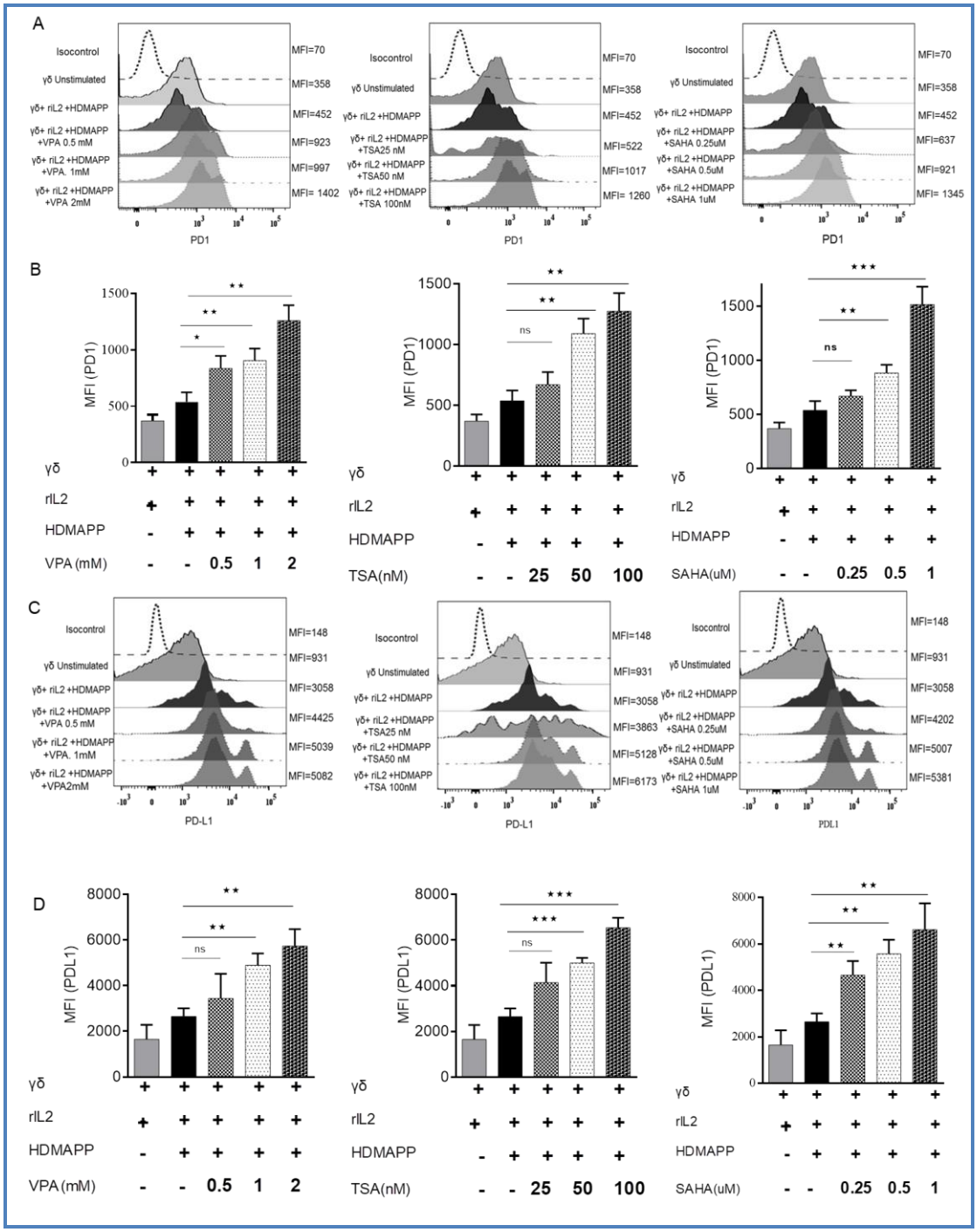

Figure 4.16: HDAC inhibitors upregulate the expression of immune checkpoints on γδ T cells. (A) The expression of PD-1 by HDAC inhibitor-treated γδ T cells at their respective concentration. Histograms are representative of three individual experiments. The values on the right side of histograms indicate the median fluorescence intensity of PD1. Panel **(B)** indicate MFI of PD1 expression as bar graphs where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$ and ns: not significant when compared with γδ T cells activated with HDMAPP in the presence of rIL-2. **(C)** The expression of PDL-1 by γδ T cells treated with HDAC inhibitors VPA, TSA and SAHA at their respective concentration was analyzed by immunostaining. Histograms shown are representative of three individual experiments. The values on the right side

of histograms indicate the median fluorescence intensity of PDL-1. The results indicated in **(D)** are MFI of PDL-1 expression where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$ and ns: not significant when compared with γδ T cells activated with HDMAPP.

4.2.10 Immune checkpoint blockade rescues the anti-tumor effector functions of HDAC inhibitor-treated γδ T cells

γδ T cell responses are essential for effective anti-tumor immunotherapy. Tumors use different mechanisms to evade the immune system. It is well known that tumors show enhanced expression of PD-L1 which is one of the strategies adopted by the tumor to hide and escape from immune attack. Abrogation of interaction between the inhibitory receptors improves the anti-tumor T cell response. We have observed that HDAC inhibitor-treated γδ T cells show increased expression of PD1 and PD-L1. To further investigate the role of PD1/PD-L1 signaling in HDAC inhibitor-treated γδ T cells we carried out the blocking experiments. γδ T cells were activated with HDMAPP and rIL2, treated with HDAC inhibitors for 72 h. PD-1 blocking antibody or normal IgG was added at the start of culture. Normal IgG was used as a control. After 72 h, these γδ T cells were cultured with Zoledronate-treated tumor cell lines AW13516, COLO-205, and Raji for 4 h at E/T ratio of 4:1. After co-culture γδ T cells were analyzed for expression of Lamp-1 (CD107a) and Granzyme B by flow cytometry. During degranulation of cytotoxic T cells, CD107a (lysosome-associated membrane protein-1) is expressed on the extracellular cell membrane of the lymphocytes. Blockade of PD-1 in HDAC inhibitor-treated HDMAPP activated γδ T cells rescued the expression of effector molecules Lamp-1 (CD107a) **(Figure 4.17 A-C and Figure 4.19 A)** and Granzyme B (**Figure 4.18 A-C and Figure 4.19 B)** as compared to only HDAC inhibitor-treated γδ T cells. To further evaluate the role of HDAC inhibitors on the PD1/PD-L1 signaling axis in anti-tumor potential of γδ T cells, we did the

similar experiment by co-culturing the HDAC treated effectors and Zoledronate-treated tumor cell lines AW13516, COLO-205, and Raji,(E:T 40:1) to analyze the cytotoxic potential by LDH release assay . It was observed that blocking of PD-1 in HDMAPP-activated γδ T cells treated with HDAC inhibitors VPA (2 mM), TSA (100 nM), and SAHA (1 μM) improves the cytotoxic potential of γδ T cells as compared to γδ T cells treated with HDAC inhibitor only **(Figure 4.19 C)**. Thus, the results show that blockade of PD-1 and PD-L1 signaling axis in HDAC inhibitor-treated γδ T cells rescued their effector functions.

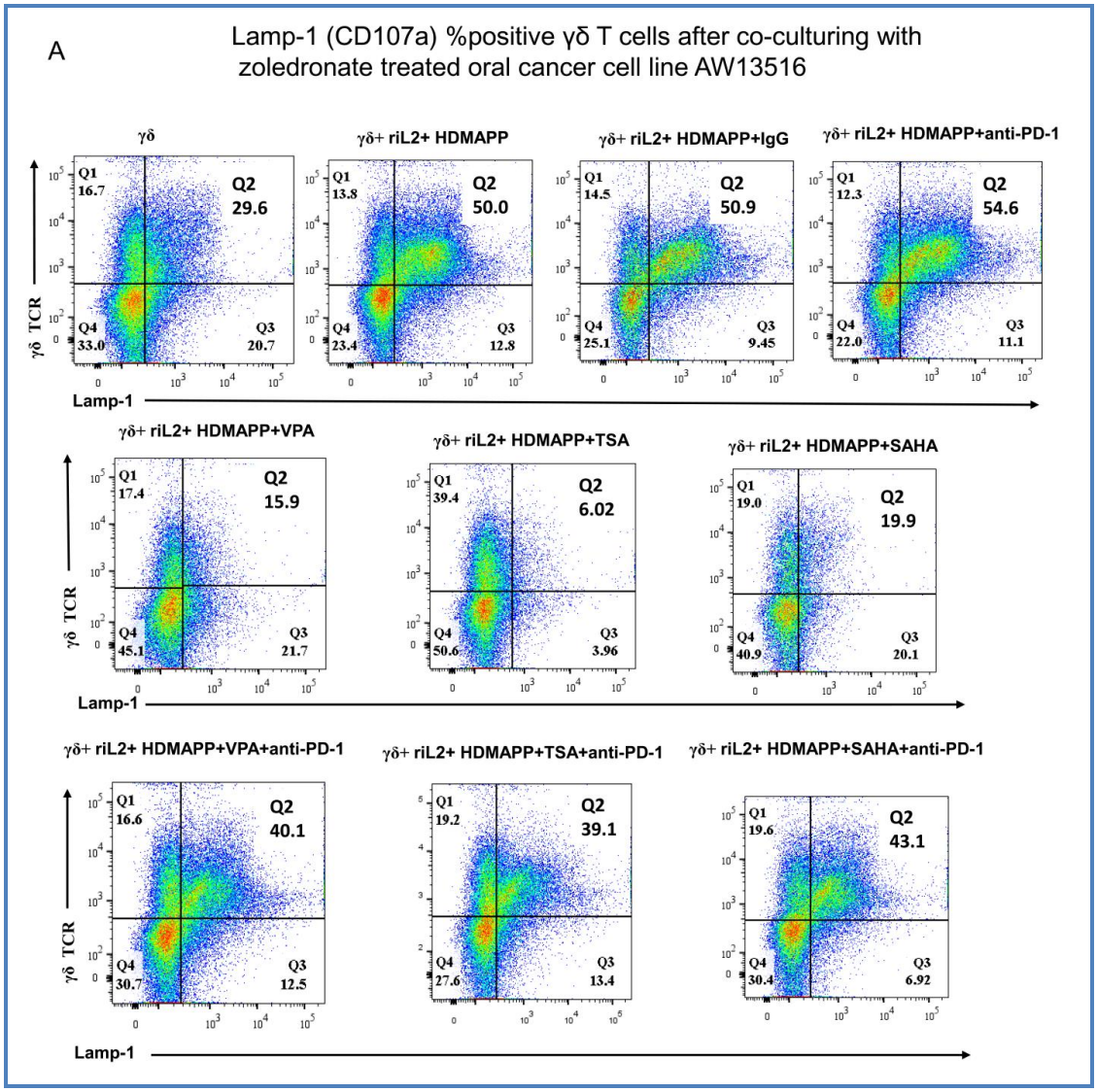

Figure 4.17 (A):-HDAC inhibitors abrogate effector functions of γδ T cells via PD-1 upregulation and inhibition of Lamp-1. Expression of degranulation marker CD107a by γδ T cells was assessed by flow cytometric analysis. HDMAPP activated

γδ T cells upon treatment with TSA (100nM), SAHA (1μM) and VPA (2mM) in presence or absence of PD1 blocking antibody were co-cultured with three Zoledronate treated Oral tumor targets (AW 13516)) cells for 4 hours at an effector to target ratio of 4:1. Normal IgG was used as control.

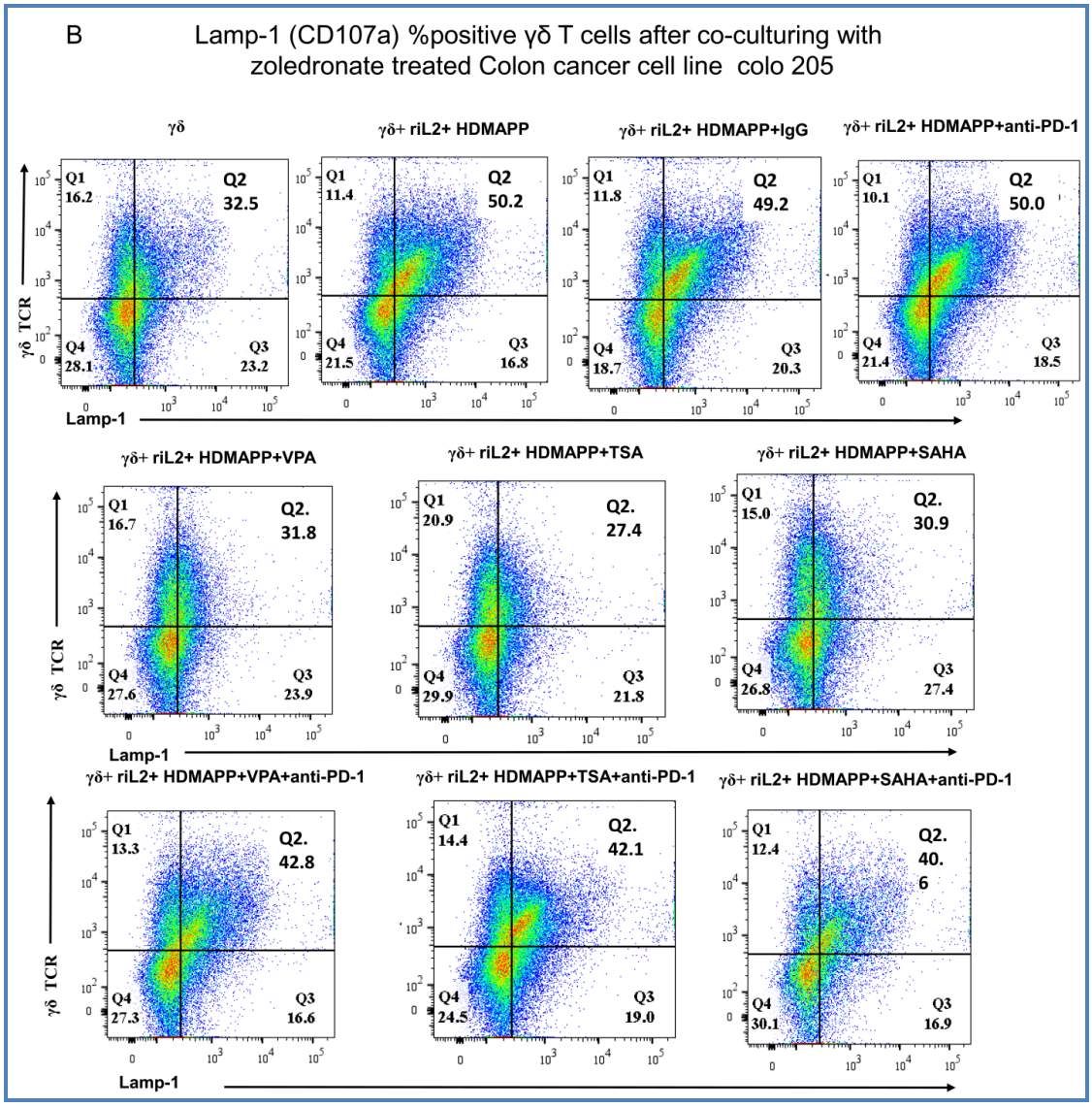

Figure 4.17 (B): HDAC inhibitors abrogate effector functions of γδ T cells via PD-1 upregulation and inhibition of Lamp-1. Expression of degranulation marker CD107a by γδ T cells was assessed by flow cytometric analysis. HDMAPP activated γδ T cells upon treatment with TSA (100nM), SAHA (1μM) and VPA (2mM) in presence or absence of PD1 blocking antibody were co-cultured with three Zoledronate treated Colon tumor targets (Colo-205)) cells for 4 hours at an effector to target ratio of 4:1. Normal IgG was used as control.

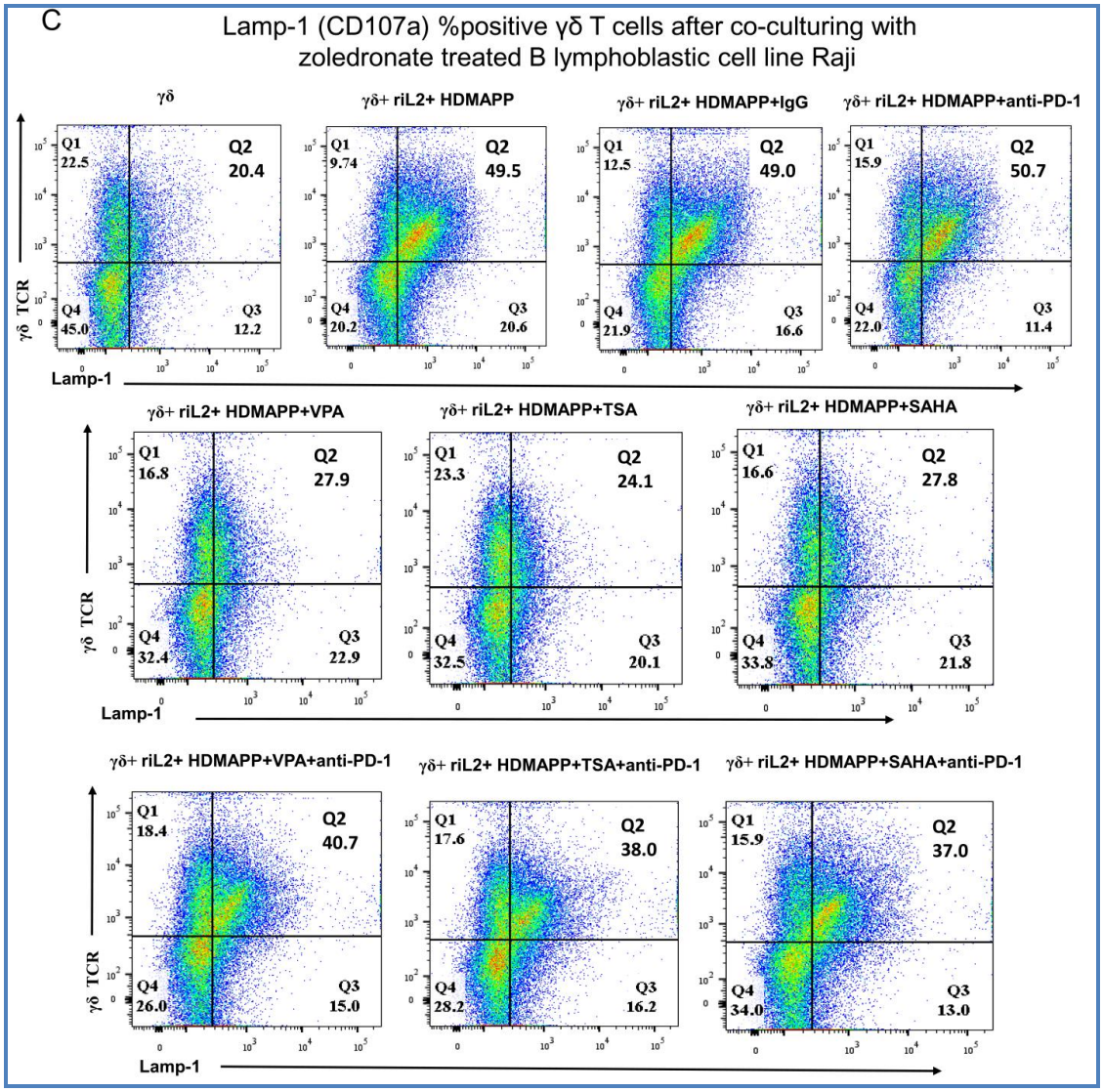

Figure 4.17 (C): HDAC inhibitors abrogate effector functions of γδ T cells via PD-1 upregulation and inhibition of Lamp-1. Expression of degranulation marker CD107a by γδ T cells was assessed by flow cytometric analysis. HDMAPP activated γδ T cells upon treatment with TSA (100nM), SAHA (1μM) and VPA (2mM) in presence or absence of PD1 blocking antibody were co-cultured with three Zoledronate treated B-lymphoblastic cell line (Raji)) cells for 4 hours at an effector to target ratio of 4:1. Normal IgG was used as control

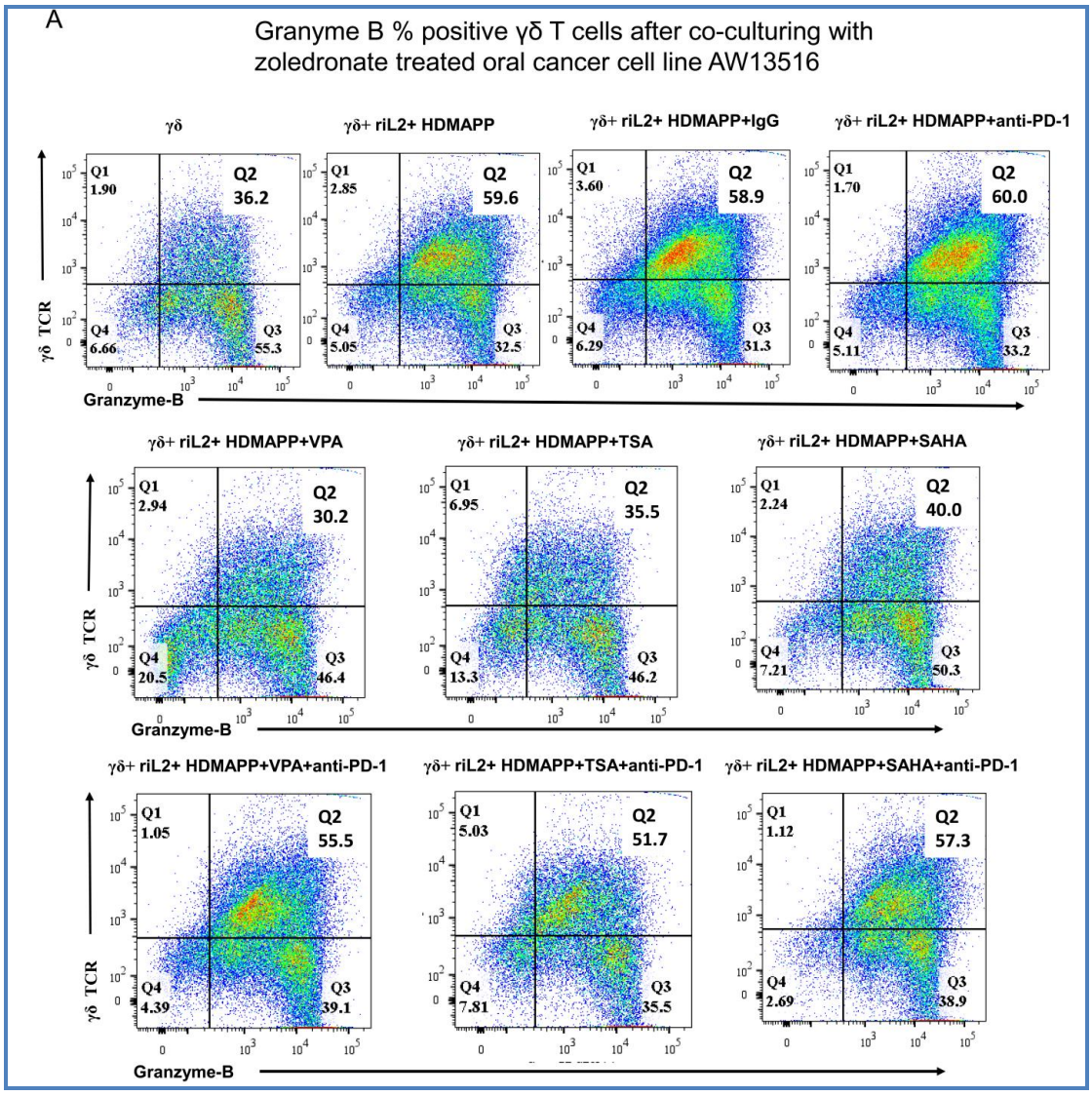

Figure 4.18(A): HDAC inhibitors abrogate effector functions of γδ T cells via PD-1 upregulation and inhibition of Granzyme B. Expression of effector molecule Granzyme B by γδ T cells was assessed by flow cytometric analysis. HDMAPP activated γδ T cells upon treatment with TSA (100nM), SAHA (1μM) and VPA (2mM) in presence or absence of PD1 blocking antibody were co-cultured with three Zoledronate treated Oral tumor targets (AW 13516)) cells for 4 hours at an effector to target ratio of 4:1. Normal IgG was used as control.

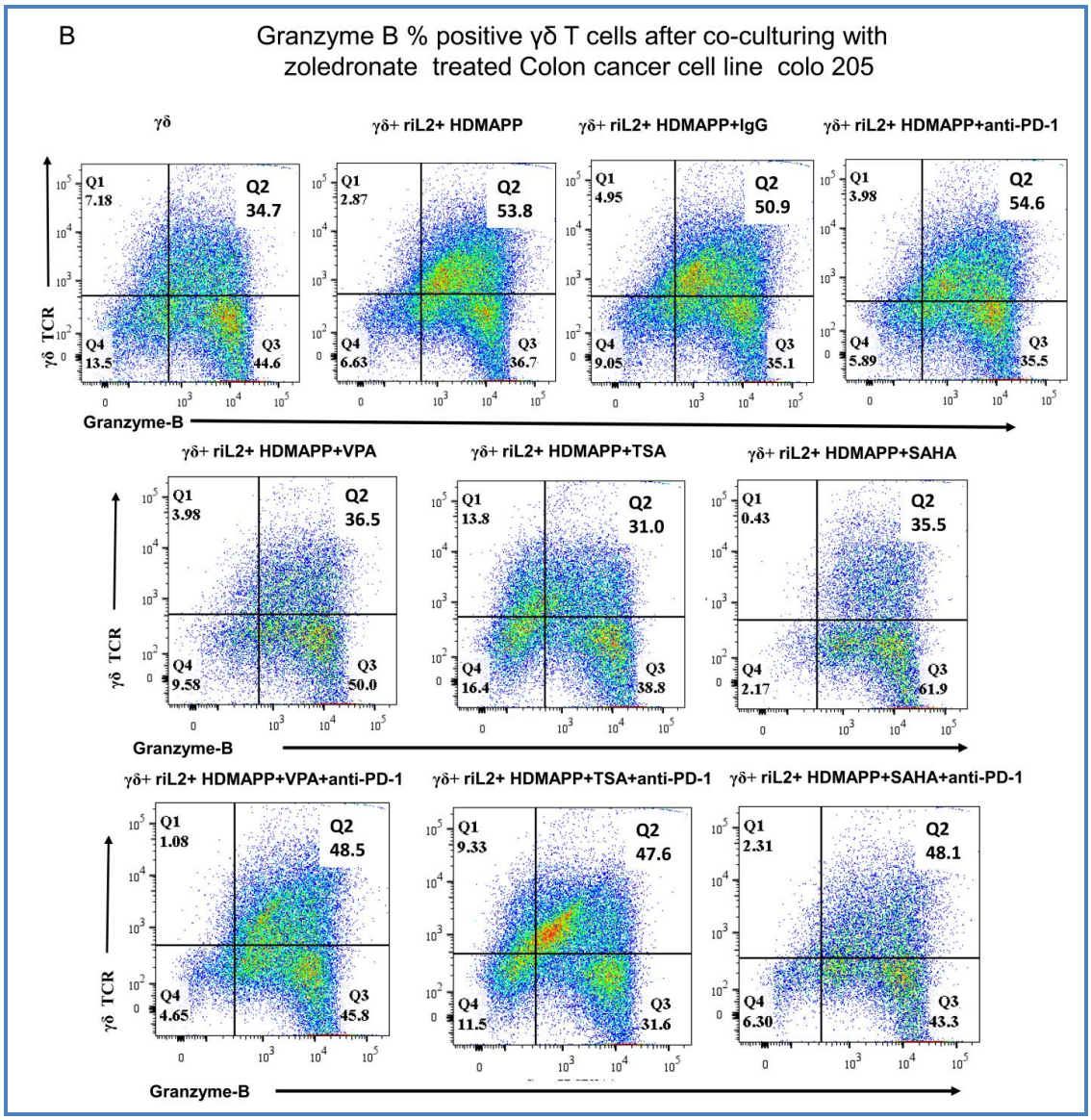

Figure 4.18 (B): HDAC inhibitors abrogate effector functions of γδ T cells via PD-1 upregulation and inhibition of Granzyme B. Expression of effector molecule Granzyme B by γδ T cells was assessed by flow cytometric analysis. HDMAPP activated γδ T cells upon treatment with TSA (100nM), SAHA (1μM) and VPA (2mM) in presence or absence of PD1 blocking antibody were co-cultured with three Zoledronate treated Colon tumor targets (Colo 205)) cells for 4 hours at an effector to target ratio of 4:1. Normal IgG was used as control.

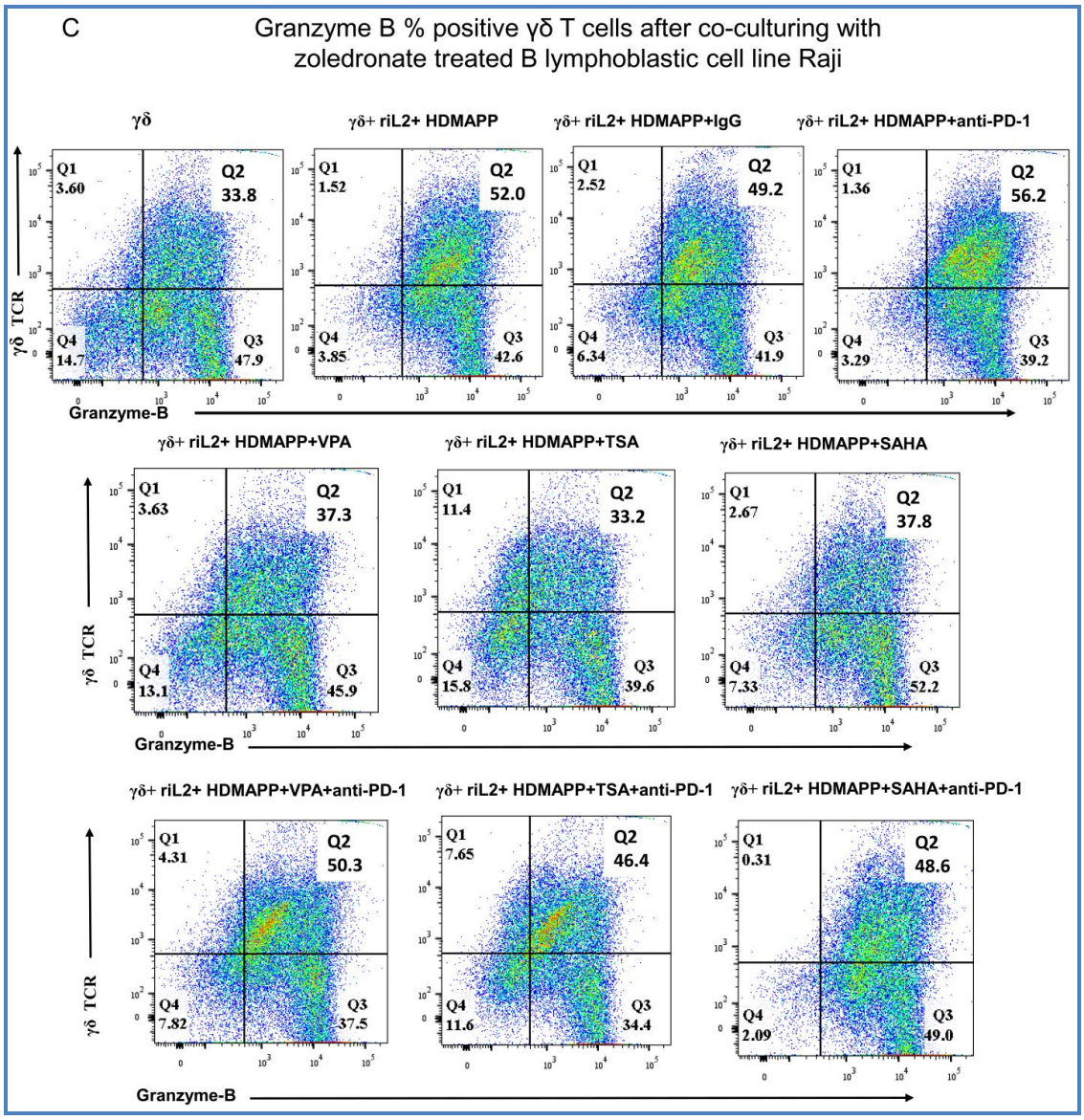

Figure 4.18 (C): HDAC inhibitors abrogate effector functions of γδ T cells via PD-1 upregulation and inhibition of Granzyme B. Expression of effector molecule Granzyme B by γδ T cells was assessed by flow cytometric analysis. HDMAPP activated γδ T cells upon treatment with TSA (100nM), SAHA (1μM) and VPA (2mM) in presence or absence of PD1 blocking antibody were co-cultured with three Zoledronate treated B-lymphoblastic tumor targets (Raji)) cells for 4 hours at an effector to target ratio of 4:1. Normal IgG was used as control.

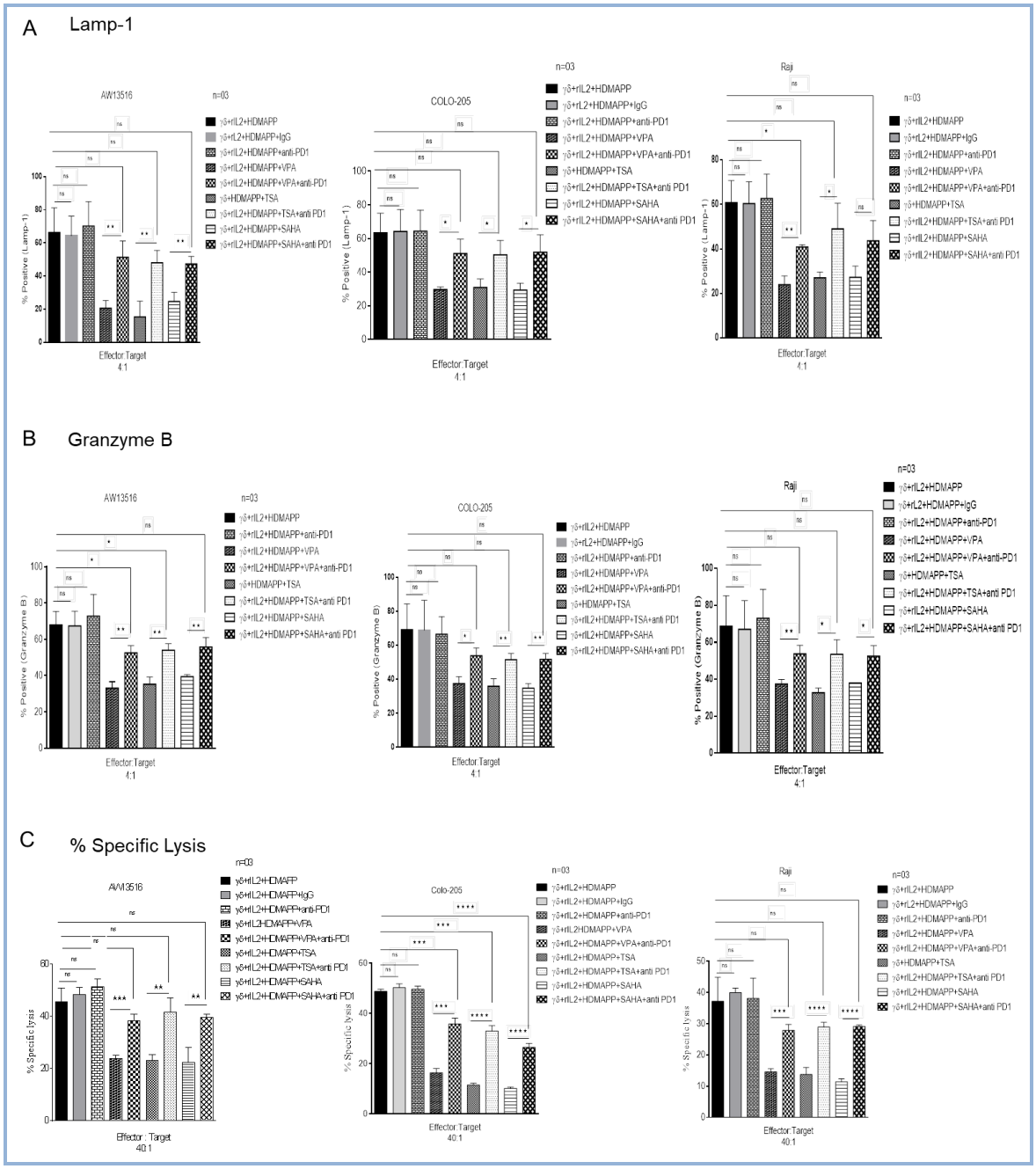

Figure 4.19: HDAC inhibitors abrogate effector functions of γδ T cells via PD-1 upregulation. Expression of (**A)** degranulation marker CD107a and **(B)** Granzyme B by γδ T cells was assessed by flow cytometric analysis. HDMAPP activated γδ T cells upon treatment with TSA (100nM), SAHA (1μM) and VPA (2mM) in presence or absence of PD1 blocking antibody were co-cultured with three Zoledronate treated tumor targets (AW 13516, Raji, COLO205) cells for 4 hours at an effector to target ratio of 4:1. The data represents consolidated MFI values of Granzyme B and CD 107a expressing cells, indicative of three independent experiments (** represents $p < 0.005$, * represents $p < 0.05$ and ns represents not significant). **(C)**The cytotoxic ability of γδ T cells treated with HDAC inhibitors TSA, SAHA, and VPA in the presence or absence of PD-1 blocking antibody was assessed against three Zoledronate treated tumor targets (AW 13516, Raji, COLO205) by LDH cytotoxicity

assay. HDAC inhibitor-treated γδ T cells show increased cytotoxic potential in the presence of PD-1 blocking antibody. The results indicate percent cytotoxicity where **: $p < 0.005$, ***: $p < 0.0005$ and ns: not significant, when compared with HDMAPP- activated γδ T cells treated with the respective HDAC inhibitor. Data represent three independent experiments.

Chapter 5
To study the mechanism of how Notch regulates cytotoxic effector molecules in γδ T cells

5.1 Introduction:

The Notch signaling pathway is an evolutionarily conserved pathway in all metazoans that regulates cell-fate determination during development and other physiological processes [12, 13]. The highly conserved Notch signaling pathway maintains juxtacrine signaling thus allows communication between two neighboring cells and elicits different physiological responses. Notch itself is a cell-surface receptor that transduces short-range signals by interacting with transmembrane ligands [390, 391]. In mammals, there are four mammalian Notch receptors, Notch1, Notch2, Notch3 and Notch4, and five canonical Notch ligands, Jagged1, Jagged2, Delta-like 1 (DLL1), DLL3 and DLL4 [14, 15]. The interaction between the Notch receptor with its ligand activates the Notch signaling pathway.

T cell receptor (TCR) mediated signaling along with co-stimulatory signal is the fundamental process of activation of T cells. These two signals in T cells stimulate the onset of multiple downstream signaling events which ultimately determine the T cell activation, proliferation and other functional responses[392]. It has been demonstrated that activation of T cells through these signals lead to activation of Notch signaling by generation intracellular Notch (NICD) and inhibition of Notch activation with γ-secretase inhibitors (GSI) decreases CD8 T cell activation as well as proliferation [22]. Notch signaling is necessary for the acquisition of cytotoxic effector functions by $CD8^+$ T cells and thus crucial for the control of intracellular infection and tumor invasion [230]. Notch signaling regulates the essential cytokines like IFN-γ, effector molecule like Granzyme B and transcription factors like T-bet in cytotoxic CD8 T cells by directly binding of NICD to promoters of these genes[231]. It has been demonstrated that Notch signaling also determines the antigen sensitivity of CD4 T cells. It has been shown that in the absence of Notch signaling CD4 T cells require

more concentration of antigen to get activated [23]. From the above discussion, it seems that Notch signaling interacts with TCR and co-stimulatory signaling events.

We were interested in understanding whether Notch signaling is required for controlling TCR mediated signaling events both proximal and distal signaling events in γδ T that are a critical regulator of TCR signal strength. Besides this, it is not fully understood whether inhibition of Notch signaling has any impact of co-stimulatory that is IL-2 signaling events in γδ T cells. The previous study from our lab has shown that inhibition of Notch signaling decreases the anti-tumor cytotoxic potential of human γδ T cells against tumor targets [378]. Therefore, the focus of this study was to find how Notch signaling modulates the effector functions of γδ T through TCR signaling and co-stimulatory signaling cascade.

The aim of the present chapter is to investigate:

1. Impact of Notch signaling on the effector functions of γδ T cells by modulation of TCR driven signaling cascade in γδ T cells
2. Impact of Notch signaling on co-stimulatory IL-2 signaling in γδ T cells
3. Impact of Notch Signaling on expression of effector molecules in γδ T cells

5.2 Results:

5.2.1 Expression of Notch receptors and ligands on human γδ T cells after activation

Different T cells subtype show difference in the expression of Notch receptors and ligands. It is well investigated that CD8 T cells express Notch receptors and ligands. This has been well documented in αβ T cells (CD4 and CD8).In the present chapter, we investigated the expression of Notch receptors and ligands on freshly isolated γδ T cells from peripheral blood of healthy individuals. Freshly isolated γδ T cells showed prominent expression of Notch1, Notch 2, Dll1, and Jag1 as analyzed by flow cytometry. The expression of Notch 3, Notch 4, Dll3, Dll4 and Jag2 was either very low or undetectable **(Figure 5.1 A-C)**. To verify the effect of antigenic stimulation on the expression of Notch receptors and ligands, γδ T cells were stimulated with phospho-antigen HDAMPP or with anti-CD3 mAb and rIL2 for 72 hours. The expression at protein and mRNA level for receptors and ligands was quantified by using flow cytometry and real-time PCR. On stimulation with HDAMPP or with anti-CD3 mAb and rIL2, γδ T cells show increased expression of Notch 1, Notch 2 along with ligands Dll1 and Jag1 as compared to unstimulated γδ T cells. However the expression of Notch3, Notch4 Dll2, Dll4 and Jag2 was almost undetectable. We further investigated the effect of γ-secretase inhibitor (GSI-X) that inhibits the Notch signaling on the expression of Notch receptors and ligands in activated γδ T cells. Freshly isolated γδ T cells were stimulated with phospho- antigen HDMAPP or anti-CD3 mAb and rIL2 in presence and absence of GSI-X. Expression of Notch receptors and ligands was checked at protein by flowcytometry and mRNA level by real-time PCR. Inhibition of Notch signaling by GSI-X led to decrease in the expression of Notch 1, Notch 2 along with ligands Dll1 and Jag1 both at mRNA and protein level

(Figure 5.2 A-B and Figure 5.3 A-B). Thus the results indicate that TCR-driven activation of γδ T cells induce expression notch receptors and ligands in γδ T cells and suggesting that TCR and Notch signaling may crosstalk with each other in determining the immune responses of γδ T cells.

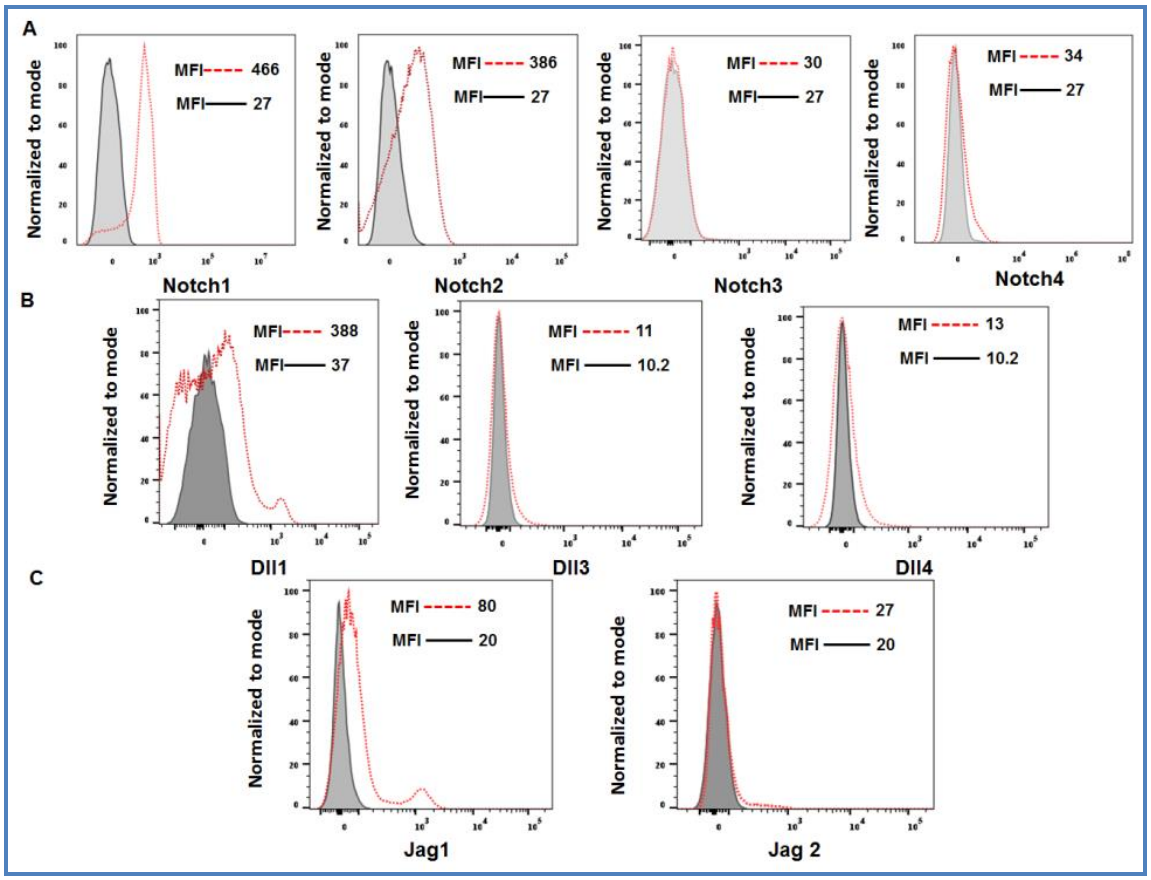

Figure 5.1: Expression of Notch receptor and ligands on human γδ T cells. Human γδ T cells were MACS-purified from the peripheral blood and stained with fluorochrome-conjugated antibodies against different Notch receptors and ligands. **(A)** Flow Cytometric analysis shows the expression of Notch1, Notch 2, Notch3, and Notch4 in human γδ T cells. The data is represented as the median fluorescence intensity (MFI). **(B-C)** Flow Cytometric analysis showing the expression of Notch signaling ligands γδ T cells. The data is represented as the median fluorescence intensity (MFI).

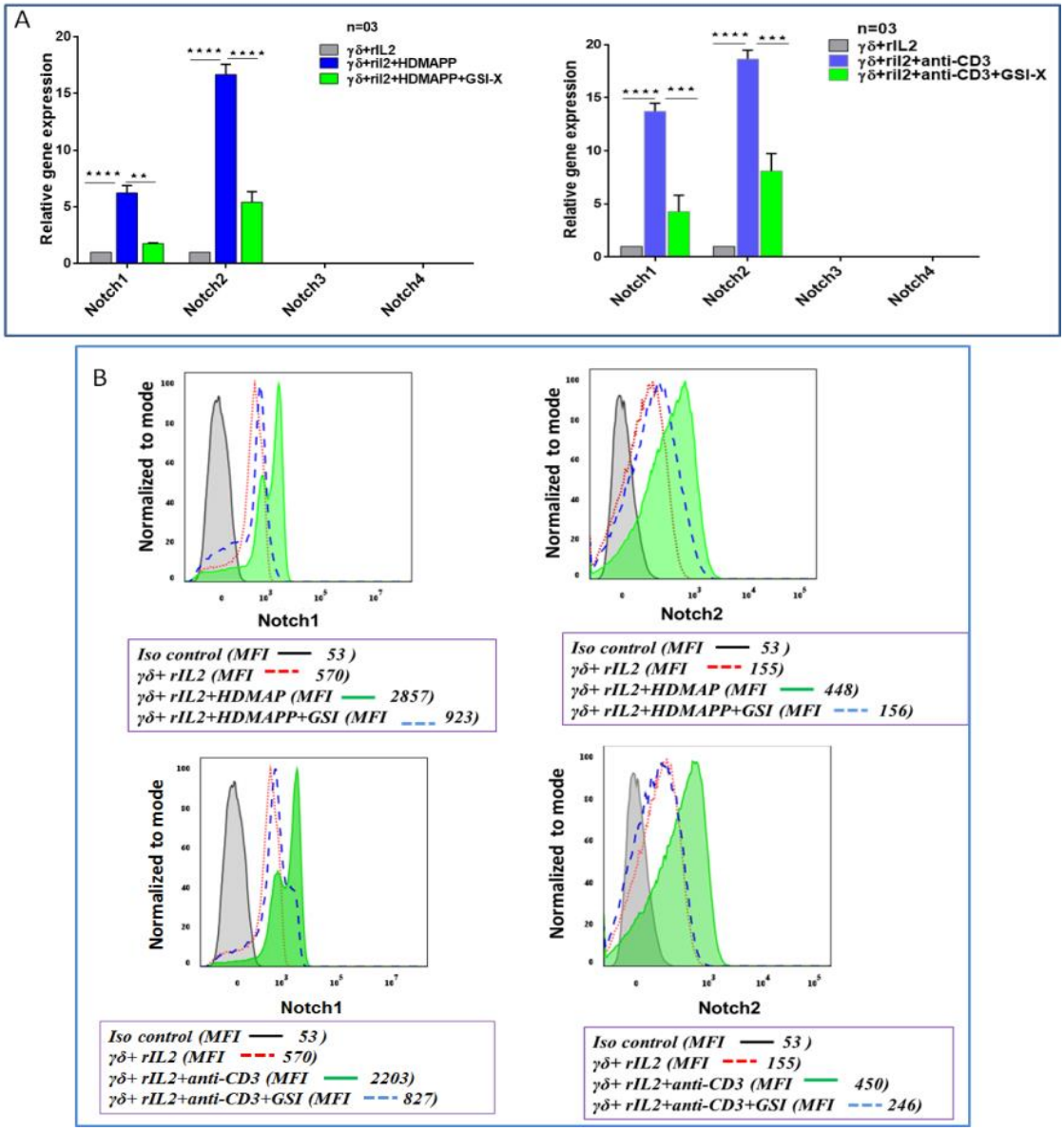

Figure 5.2: Effect of Notch signaling inhibition on the expression of Notch receptors in stimulated γδ T cells. (A) The expression of Notch receptors on unstimulated or γδ T cells stimulated with HDMAPP or anti-CD3 mAb with or without GSI-X was analyzed by quantitative real-time PCR. γδ T cells show increased expression of Notch 1 and Notch 2 upon TCR mediated activation.The results indicated are cumulative mean of relative gene expression normalized to 18S rRNA where *p < 0.05, **p < 0.005, ***p < 0.0005, compared with unstimulated γδ T cells. **(B)** Purified γδ T cells activated by HDMAPP or anti-CD3 mAb in presence and absence of GSI-X were stained after 72 h with the corresponding fluorophore tagged antibody and expression was analyzed by flow cytometry. The values below the histograms indicate median fluorescence intensity (MFI) of Notch receptors. The histograms are representative of three independent experiments.

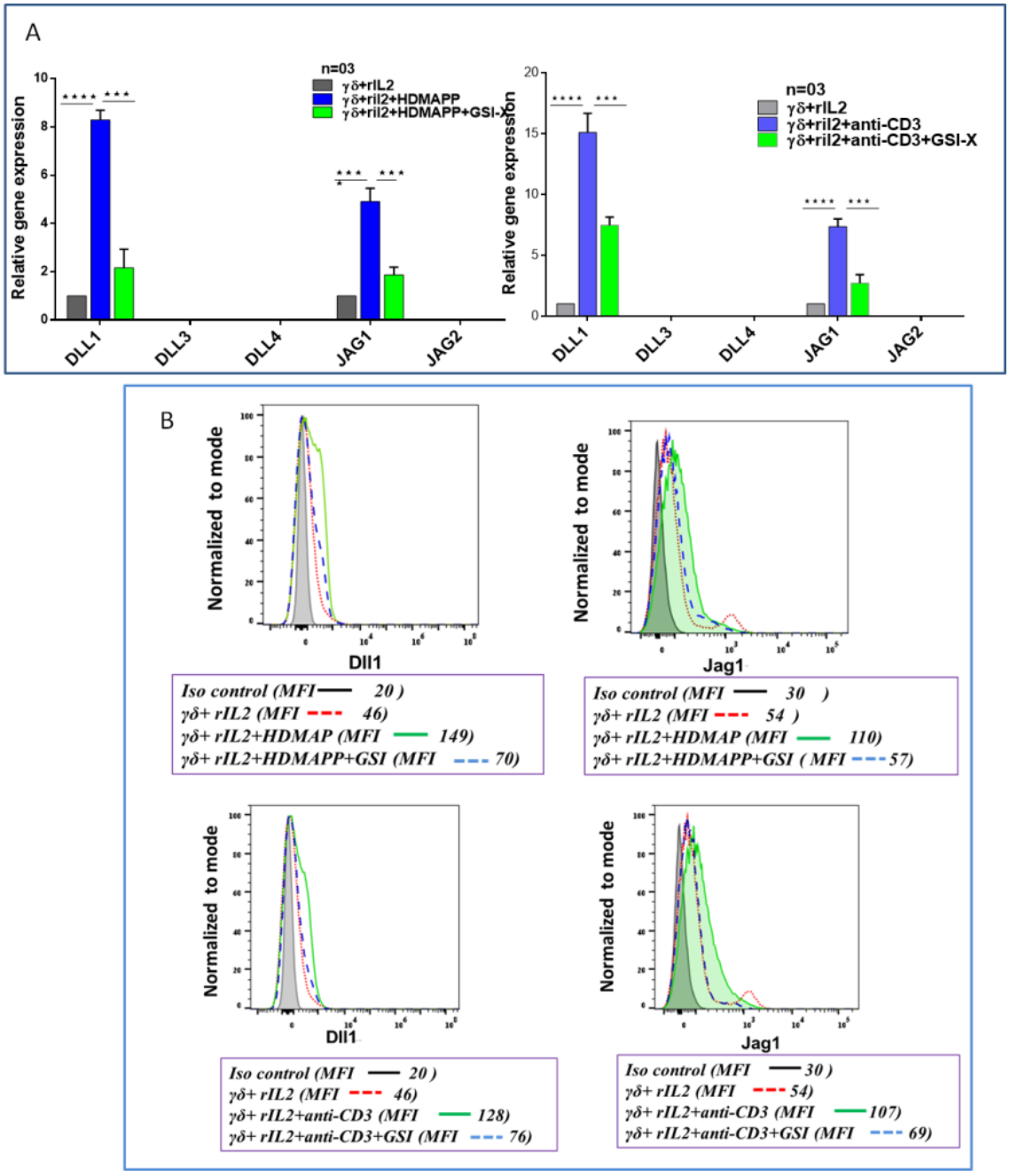

Figure 5.3: Effect of inhibition of Notch signaling on the expression of Notch ligands in stimulated γδ T cells. (A) The expression of Notch receptors on unstimulated or γδ T cells stimulated with HDMAPP or anti-CD3 mAb with or without GSI-X was analyzed by quantitative real-time PCR. γδ T cells show increased expression of Dll1 and Jag 1upon TCR mediated activation. The results indicated are cumulative mean of relative gene expression normalized to 18S rRNA where *p < 0.05, **p < 0.005, ***p < 0.0005, compared with unstimulated γδ T cells. **(B)** Purified γδ T cells activated by HDMAPP or anti-CD3 mAb in presence and absence of GSI-X were stained after 72 h with the corresponding fluorophore tagged antibody against different Notch ligands and expression was analyzed by flow cytometry. The

values below the histograms indicate median fluorescence intensity (MFI) of Notch receptors. The histograms are representative of three independent experiments.

5.2.2: Notch signaling and expression of its downstream targets in γδ T cells.

Activation of T cells via the TCR accompanied by co-stimulation leads to the production of active Notch intracellular domain (NICD) which translocates into the nucleus and interacts with different cofactors like CSL to control the expression of different target genes. In order to further investigate whether Notch signaling is active in γδ T cells, we checked the expression of different downstream targets of Notch signaling in γδ T cells that were activated with HDMAPP and IL2 and increases in the expression of Notch target genes HES1 HEY2 and NRARP (Notch-regulated ankyrin repeat protein) and Deltex was observed. However, the expression of these Notch target genes was abrogated on pharmacological inhibition of Notch signaling using gamma-secretase inhibitor (GSI-X). γδ T cells activated with plate-bound anti-CD3 mAb and rIL2 also showed the same expression of Notch target genes (HES1 HEY2, NRARP, and Deltex), which were abrogated upon inhibition of Notch signaling **(Figure 5.4 A-B)**. These results prove that activation of Notch signaling is a result of antigen-specific activation induced upregulation of Notch ligands and receptors on γδ T cells.

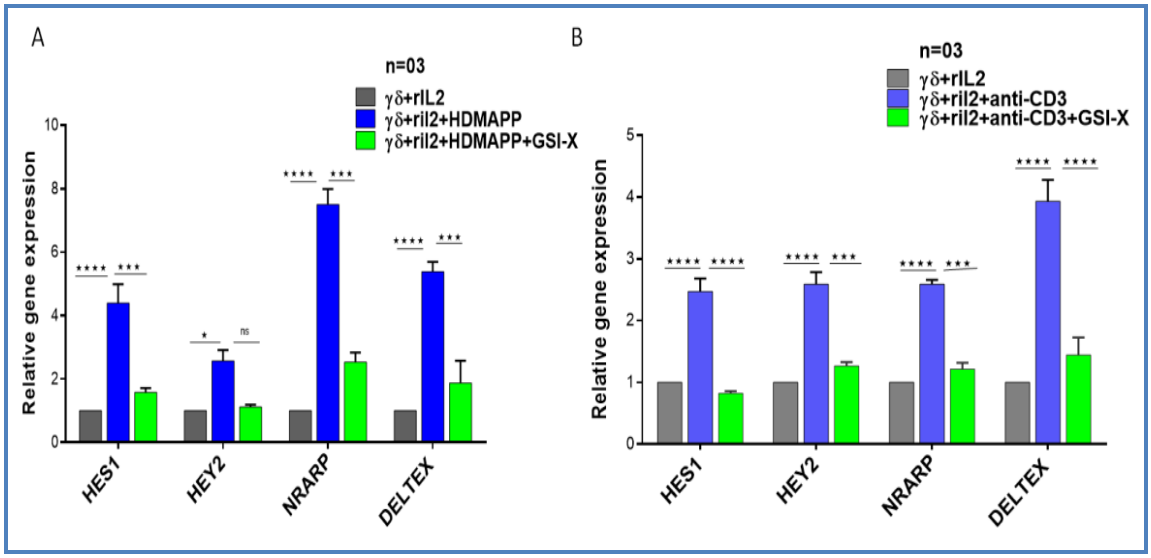

Figure 5.4: Expression of Notch target genes on human γδ T cells. Human γδ T cells were analyzed for Notch signaling target gene expression (Hes1, Hey2, Deltex, and NRARP). γδ T cells activated with HDMAPP **(A)** or anti-CD3 mAb **(B)** in presence and absence of GSI-X for 72 hours Expression of Notch target genes were induced by TCR-driven activation and inhibited by inhibition of Notch signaling. The expression of Notch target genes was quantified by quantitative real-time PCR. The results indicated are cumulative mean of relative gene expression normalized to 18S rRNA where *p < 0.05, **p < 0.005, ***p < 0.0005, ****p< 0.00005 compared with γδ T cells activated with HDMAPP. The data shown are representative of three independent experiments.

5.2.3 Notch signaling is involved in proliferation and cell cycle progression of γδ T cells

As shown in the earlier chapter and previously published word from our lab, that γδ T cells show robust proliferation when stimulated with phosphoantigen (HDMAPP) or plate-bound anti-CD3 mAb in the presence of rIL2. It was observed that TCR mediated Notch signaling led to the induction of Notch signaling in γδ T cells suggesting that Notch signaling may also control the proliferation of antigen-stimulated γδ T cells. In order to evaluate the effect of Notch signaling on the proliferation of freshly isolated γδ T cells, these cells were stimulated with phosphoantigen (HDMAPP) and rIL2; or plate-bound anti-CD3 mAb and rIL2 in

presence and absence of γ-secretase inhibitor (GSI-X) for 72 hours. γδ T cell proliferation was monitored using ^{3}H thymidine (^{3}H-TdR) incorporation assay. Stimulated γδ T cells show an increase in the proliferation rate as compared to unstimulated γδ T cells. However, inhibition of Notch signaling by pharmacological inhibitor γ-secretase inhibitor lead to a significant decrease in the antigen-specific proliferation rate of γδ T cells **(Figure 5.5 A)**

Further, we also investigated the role Notch signaling on the cell cycle progression of γδ T cells. Upon stimulation with HDMAPP or plate-bound anti-CD3 mAb and rIL2, a significant number of γδ T cells were found to be in S phase and G2/M phase. However the pharmacological inhibition of Notch signaling using γ-secretase inhibitor (GSI-X) majority of antigen-activated γδ T cells were seen in the G0/G1 phase **(Figure 5.5 B-C)**. This was also reflected in the increased expression of p53 its downstream target p21 in γδ T cells treated with GSI-X suggesting that inhibition of Notch signaling prevents G0/G1- S phase transition in a p53 dependent manner **(Figure 5.6 A-B)**.

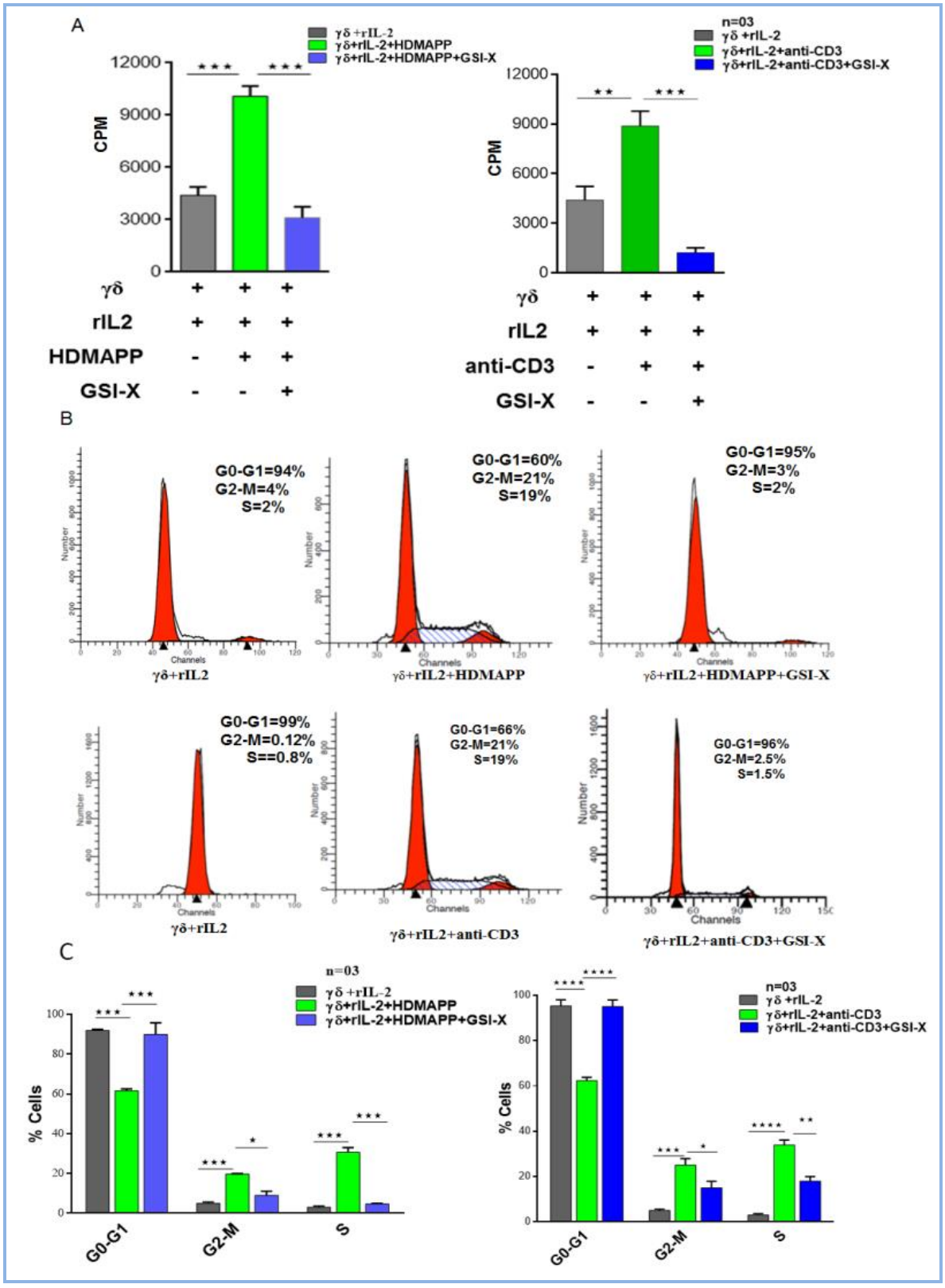

Figure 5.5: **Inhibition of Notch signaling by γ-secretase inhibitor impedes the proliferation of γδ T cells and leads to cell cycle arrest in the G0–G1 phase. (A)** The proliferative response of γδ T cells activated with HDMAPP or anti-CD3 mAb was assessed by thymidine incorporation assay. Sorted γδ T cells were stimulated

with phosphoantigen HDMAPP or with plate-bound anti-CD3 mAb with or without treatment of Notch signaling inhibitor GSI-X for 72 h. The graphs illustrate the cumulative mean cpm and are representative of three independent experiments where *p < 0.05, **p < 0.005, ***p < 0.0005. **(B)** Cell cycle analysis of γδ T cells upon GSI-X treatment. Freshly isolated γδ T cells were activated with HDMAPP or with anti-CD3 mAb with or without Notch inhibitor for 72 h, and cell cycle progression was analyzed with propidium iodide (PI) staining using FACS Calibur. The histograms are representative of three independent experiments. **(C)** The graphs indicate the cumulative mean percentage of PI-positive cells in each phase of the cell cycle. The graphs are representative of three independent experiments where *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005.

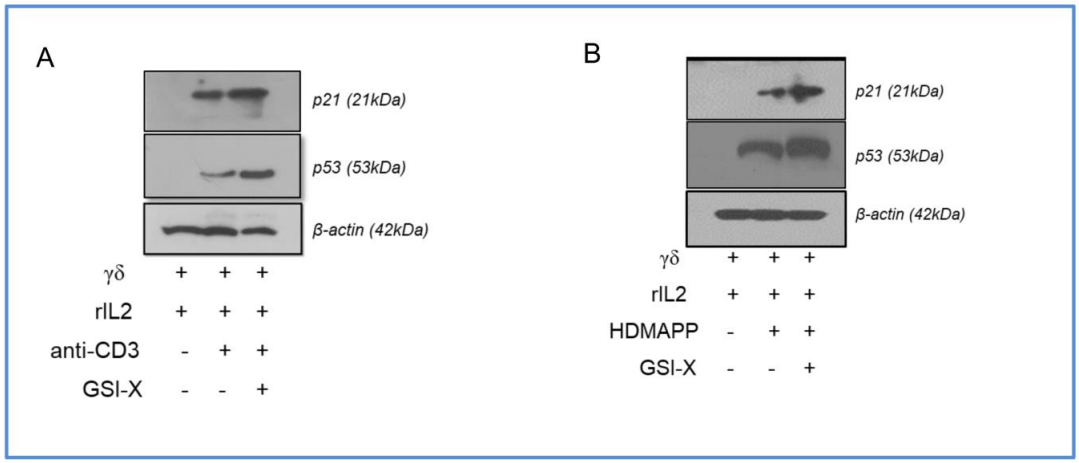

Figure 5.6: Inhibition of Notch signaling increase the expression of cell cycle checkpoint proteins p53 and p21. Immuno-blot showing the expression of p53 and p21 in γδ T cells activated with anti-CD3 mAb **(A)** or with phosphoantigen HDMAPP **(B)** in presence and absence of Notch inhibitor GSI-X. After 72 hrs protein extracts prepared from γδ T cells were loaded into the SDS-PAGE and then transferred to the nitrocellulose membrane. The results showed that inhibition of Notch signaling using GSI-X induced higher levels of p53 and its downstream target gene p21, thereby preventing cell cycle progression even after TCR-driven stimulation. β-actin was used as a loading control.

5.2.4 Inhibition of Notch signaling impairs calcium flux in γδ T cells

Calcium (Ca^{2+}) acts as a second messenger in many cell types, including lymphocytes. Calcium influx is one of the key signaling events triggered upon stimulation of T cell receptors (TCR) with antigen and plays an important role in T cell activation, proliferation, and differentiation. Resting T cells maintain a low

concentration of calcium (Ca^{2+}). However, upon stimulation of T cell with antigen, Ca^{2+} levels in T cells increases rapidly which activates downstream signaling kinases necessary for T cell activation. We reported discussed earlier that inhibition of Notch signaling in γδ T cells decreases the proliferative capacity of γδ T cells **(Figure 5.5 A).** This incited us to investigate the effect of Notch signaling on the Ca^{2+} in human γδ T cells. γδ T cells were activated with plate-bound anti-CD3 mAb or HDMAPP for 72 hours in the presence and absence of γ-secretase inhibitors. After 72 hours. γδ T cells were labeled with 5μM of FLuo-3AM at 37°C for 30 mins. Cells were washed with calcium buffer and acquired on FACS Aria for 5 mins. Initially, a 30-second baseline was collected and HDMAPP or anti-CD3 mAb was added either alone or in the presence of GSI-X. γδ T cells show a rapid increase in the Ca^{2+} flux when activated with HDMAPP or plate-bound anti-CD3 mAb as compared to unstimulated γδ T cells. However, upon inhibition of Notch signaling with GSI-X, we observe a decrease in the flux of calcium in γδ T cells **(Figure 5.7 A-B)**. Thus our results suggest that Notch signaling modulate the TCR mediated signaling in γδ T cells.

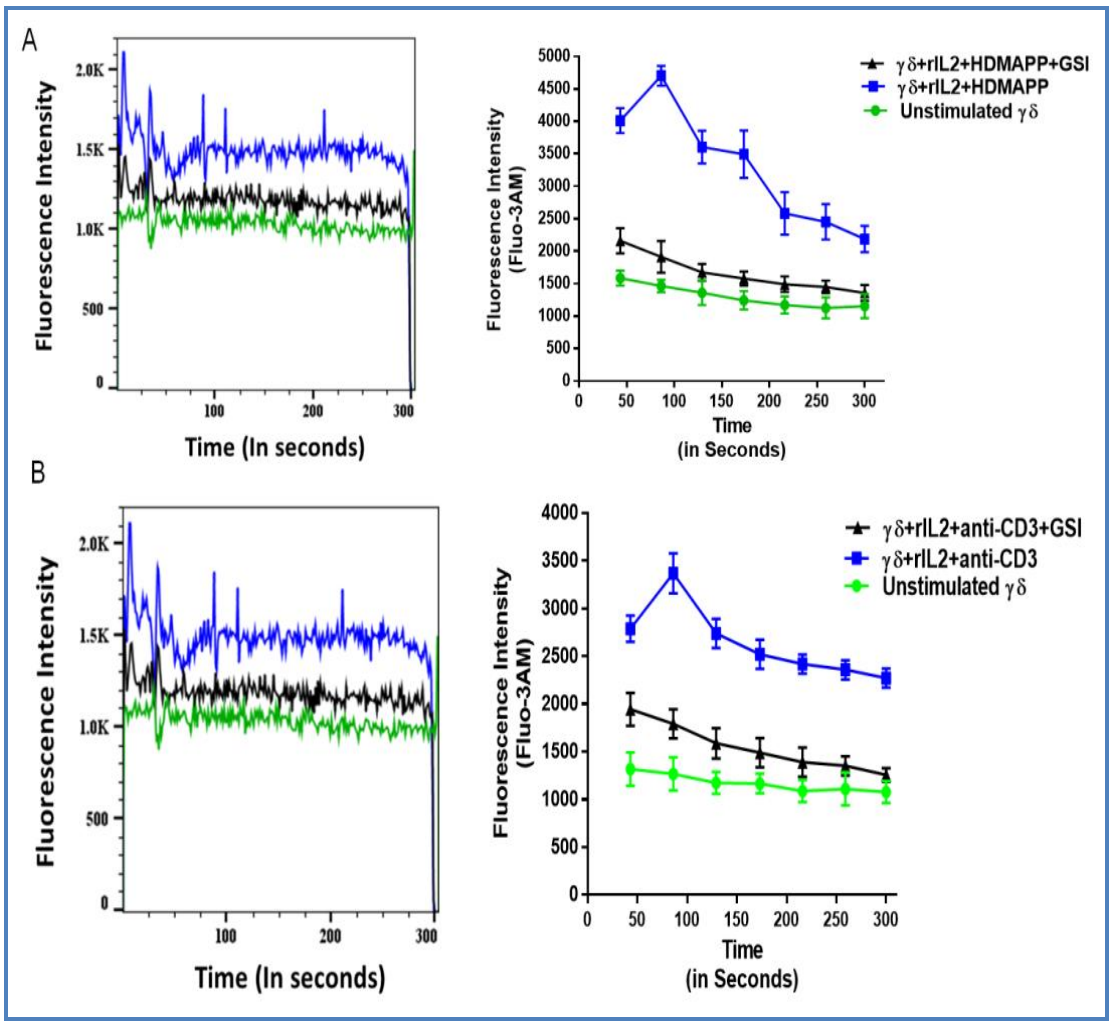

Figure 5.7: Inhibition of Notch signaling impedes the calcium flux in γδ T cells. The γδ T cells were loaded with the 5μM of FLuo-3AM in presence or absence of GSI-X at 37°C for 30 mins. Cells were washed with calcium buffer and acquired on FACSAria for 5 mins. Initially, a 10-second baseline was collected and cells were again stimulation was added either alone or in the presence of GSI-X. The graph shows the kinetic flux of the intracellular calcium in the T cells stimulated with (**A)** HDMAPP or with **(B)** anti-CD3 mAb in the presence or absence of GSI-X. Each point on the graph represents the average fluorescence intensity over a range of 43 seconds each. The data represented is the mean of three independent experiments

5.2.5 Notch signaling regulates activation and cytokine production in γδ T cells

T cell receptor (TCR) mediated activation of peripheral T cells is a fundamental process of the adaptive immune system. The distal events of TCR signaling led to the expression of different activation markers on γδ T cells. The observation that Notch

signaling in γδ T cells is mediated by TCR incited us to look at the expression of different activation markers such as CD69, CD25, CD71, phospho-tyrosine (P-Tyrosine) on γδ T cells upon inhibition of Notch signaling. γδ T cells activated with HDMAPP and rIL2 showed increased expression of activation markers such as CD69, CD25, CD71, phospho-tyrosine (P-Tyrosine). However inhibition of Notch signaling in γδ T cells by GSI-X resulted in a decrease in the expression of activation markers such as CD69, CD25, CD71, phospho-tyrosine (P-Tyrosine) **(Figure 5.8 A-D)**.CD25 is the high-affinity IL-2 receptor subunit and CD71 is the transferrin receptor. IL-2 signaling is necessary for co-stimulation and CD71 is important for the transport of nutrients. Both CD25 and CD71 are important for proliferation and optimum activation of γδ T cells.

One of the initial effector responses upon of γδ T cells is secretion of cytokines IFN-γ and TNF-α. Inhibition of Notch signaling by GSI-X significantly decreased distal TCR signaling events such as the expression of activation markers CD25 and CD69. This effect of Notch inhibition in γδ T cells leads to investigate the effect of Notch signaling on the expression of two main cytokines IFN-γ and TNF-α which determine the effector response of γδ T cells. A marked increase in the expression of cytokines IFN- γ and TNF-α was observed upon stimulation of γδ T cells with HDMAPP or with plate-bound anti-CD3 mAb in the presence of rIL2 both at mRNA and protein level. However, expression of IFN-γ **(Figure 5.9 A and C)** and TNF-α **(Figure 5.9 B and D)** was decreased significantly when γδ T cells were activated in the presence of γ-secretase inhibitor GSI-X. The data further confirms the role of Notch signaling in effector functions of γδ T cells.

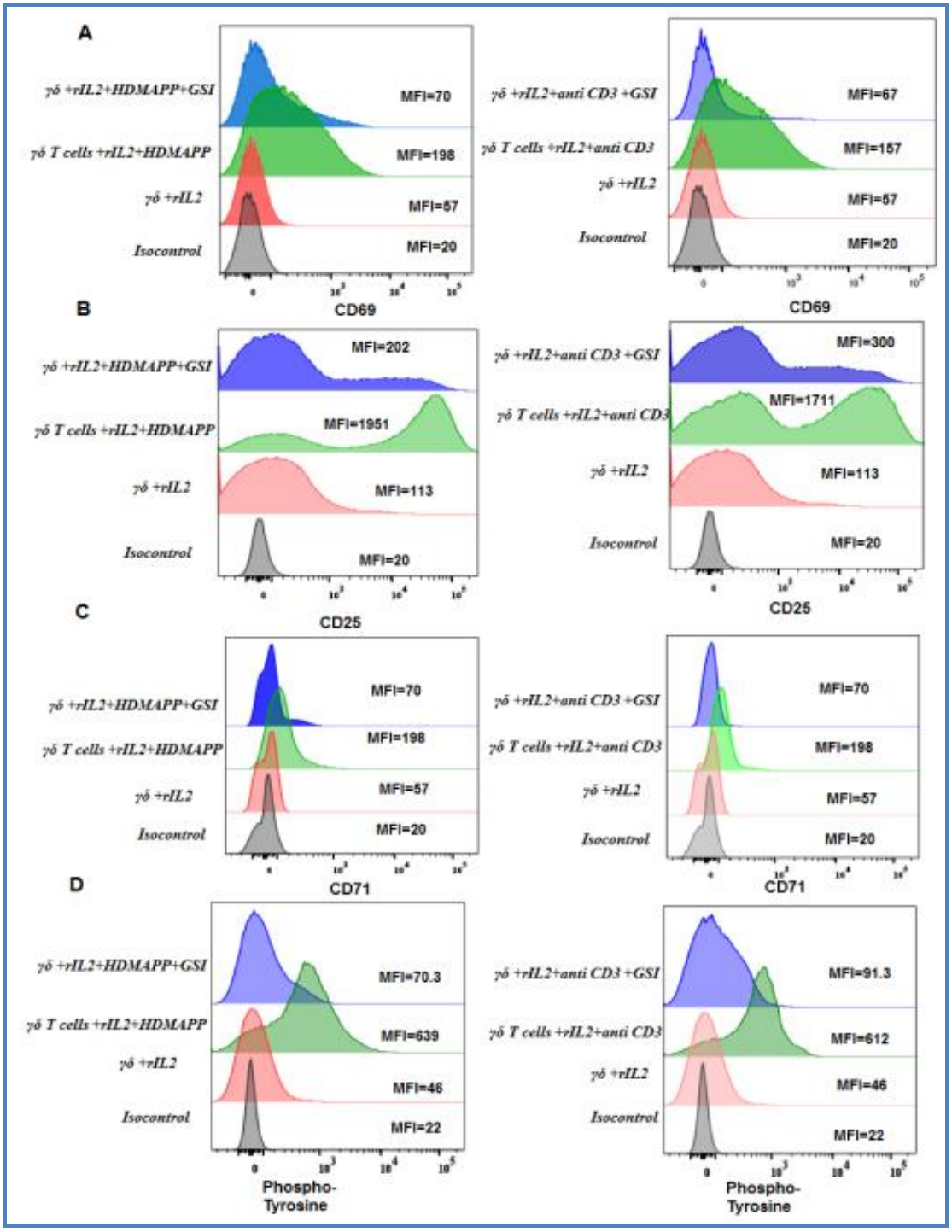

Figure 5.8: Notch signaling inhibition affects the activation of γδ T cells. (A) The expression of early activation marker (CD69) on unstimulated γδ T cells, HDMAPP or anti CD3 and rIL-2 stimulated γδ T cells with or without GSI-X treatment was analyzed by multi-color flow cytometry. Values inside the histograms indicate the median fluorescence intensity (MFI) of CD69. The histograms shown are representative of three independent experiments. **(C)** The effect of Notch inhibition on the expression of late activation marker CD25 was assessed by flow cytometry. Unstimulated γδ T cells and HDMAPP or anti-CD3 mAb stimulated γδ T cells with or

without Notch signaling inhibition after 72 hours were stained with the fluorophore tagged antibody and acquired on FACS Aria. Values inside indicate the median fluorescence intensity (MFI) of CD25. The histograms depicted are representative of three independent experiments. **(C-D)** Expression of CD71 and p-Tyrosine on γδ T cells activated with HDMAPP and plate-bound anti-CD3 mAb in the presence and absence of Notch signaling inhibitor GSI-X for 72 hours. Values inside the histograms indicate the median fluorescence intensity (MFI) of CD71 and p-Tyrosine. The histograms shown are representative of three independent experiments.

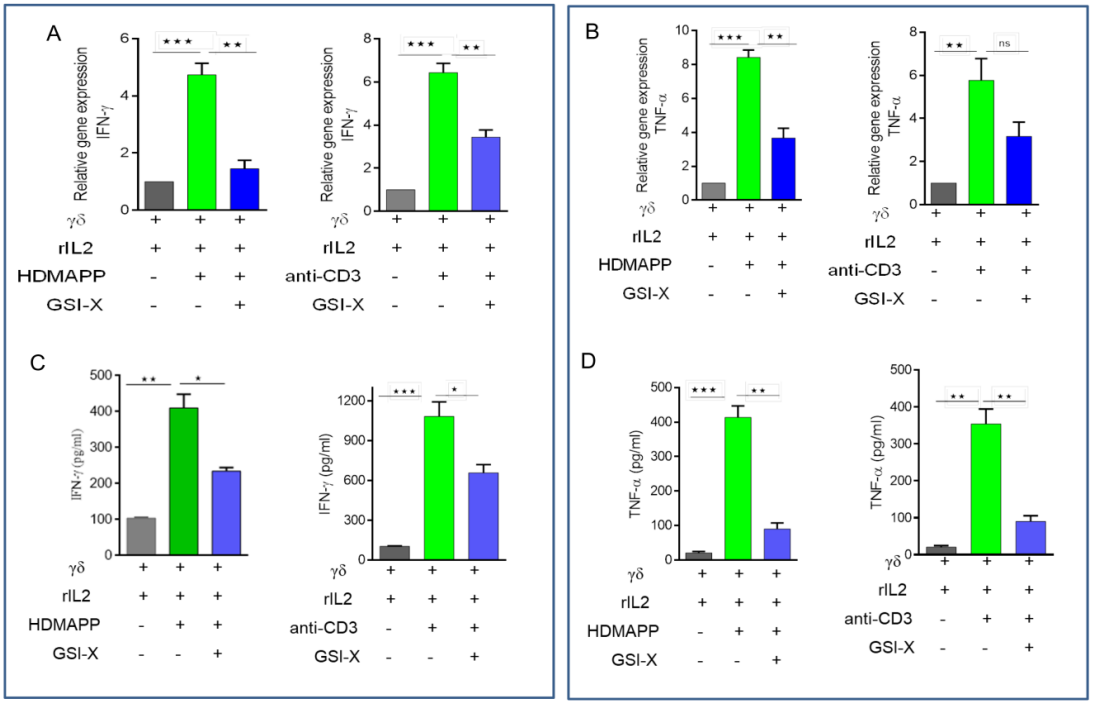

Figure 5.9: Notch signaling regulates cytokine production in γδ T cells. Expression of IFN-γ and TNF-α by quantitative real-time PCR and sandwich ELISA. Panel **(A)** and panel **(C)** indicate IFN-γ expression by γδ T cells stimulated with HDMAPP, treated with or without γ-Secretase inhibitor (GSI-X) at mRNA and protein levels respectively. Panel **(B)** and panel **(D)** represent an expression of TNF-α by γδ T cells at mRNA and protein level respectively. Expression at protein level from supernatants was analyzed by sandwich ELISA. The expression of different mRNA transcripts was normalized to 18S rRNA. All the results indicated are mean ± SEM of three independent experiments, where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$

5.2.6: Effect of Notch signaling inhibition on proximal and distal IL-2 Receptor Signaling

The observations that Notch inhibition leads to cell cycle arrest in antigen-activated γδ T cells and decrease in the expression of late activation marker CD25 which is high-affinity IL-2 receptor subunit prompted us to look at the effect of Notch signaling on proximal and distal IL-2 signaling pathway in γδ T cells. For the functional responses of T cells co-stimulatory signal mediated by IL-2 is necessary. However, less is known about the impact of Notch signaling on the IL-2 receptor-mediated pathway. The IL-2R complex is composed of CD25 (α), CD122 (β), and CD132 (γc) molecules. In order to investigate the effect of Notch signaling on the proximal and distal signaling molecules of the IL-2 signaling pathway, γδ T cells were sorted from peripheral blood of healthy individuals. γδ T cells were activated with HDMAPP or anti-CD3 mAb and rIL2 in the presence or absence of Notch signaling inhibitor GSI-X. Activated γδ T cells show an increase in the expression of proximal IL-2 signaling molecules CD132 and CD122. However inhibition of Notch signaling in antigen-activated γδ T cells led to a decrease in the expression of these proximal IL-2 signaling molecules CD132 and CD122 **(Figure 5.10 A-B)**

To better understand the mechanism how Notch signaling affects the IL-2 signaling pathway in γδ T cells we investigated the expression of different downstream distal signaling molecules of IL-2 mediated signaling pathway. The JAK-STAT pathway represents the principal signaling mode used by cytokines. Downstream of IL-2 signaling, PI-3K-AKT pathway is activated which leads to increase in the expression of active transcription factors c-Myc, STAT5 and c-Jun which translocates into the nucleus and binds to different target genes and regulates their expression. γδ T cells activated with specific phospho-antigen HDMAPP and rIL-2 for 72 hours

showed increase in the level of Akt and p-Akt as compared to unstimulated γδ T cells. However pharmacological inhibition of Notch signaling by γ-secretase inhibitor GSI-X in antigen-activated γδ T cells lead to a decrease in the level of both total Akt and p-Akt.

We also observed the increase in the level of total Akt and p-Akt in γδ T cells activated with plate-bound anti-CD3 mAb and rIL-2 for 72 hours. Inhibition of Notch signaling in anti-CD3 mAb and rIL2 activated γδ T cells led to a decrease in the level of both total Akt and p-Akt **(Figure 5.11 A-B)**

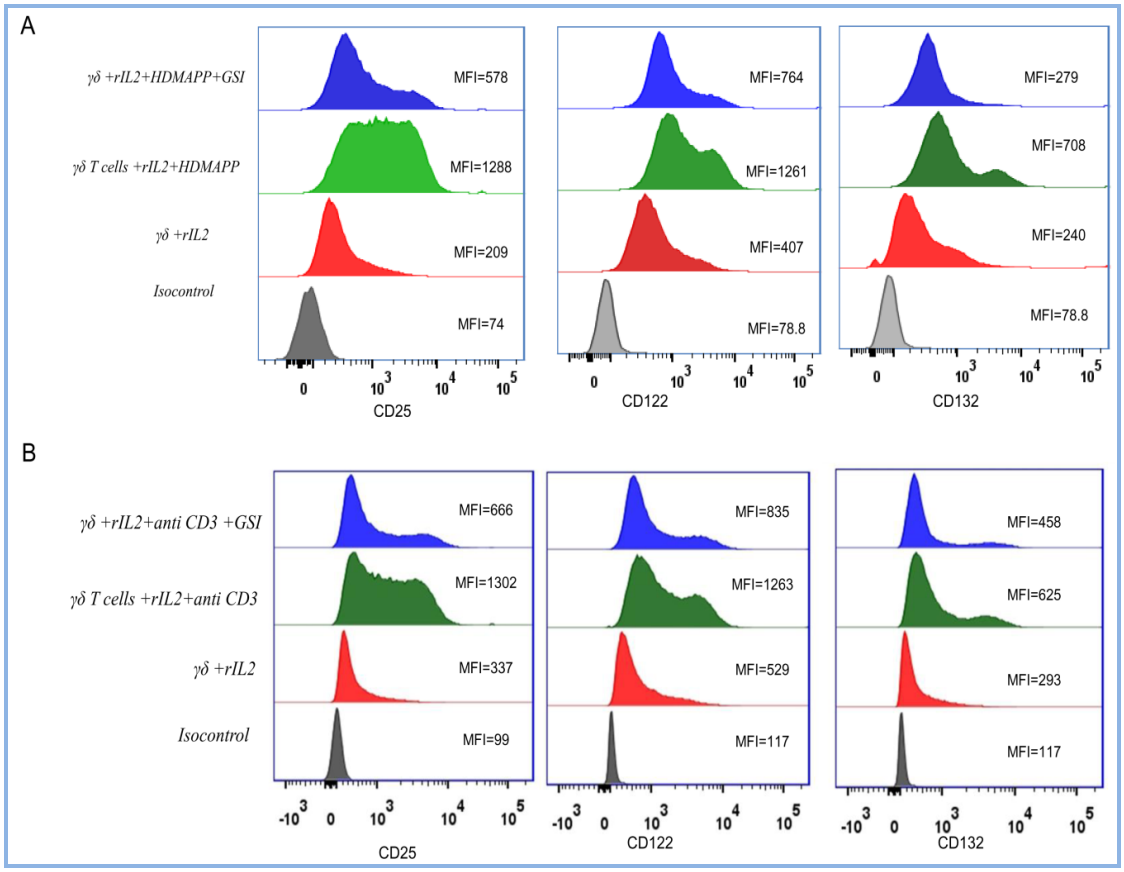

Figure 5.10: Notch signaling modulates proximal IL-2 signaling. The expression of proximal IL-2 signaling receptors CD25, CD122 and CD132 on unstimulated γδ T cells, HDMAPP **(A)** or anti-CD3 mAb **(B)** and rIL-2 stimulated γδ T cells with or without Notch inhibitor GSI-X was analyzed by multi-color flow cytometry. Values inside the histogram indicate the median fluorescence intensity (MFI) CD25, CD122,

and CD132 on γδ T cells. The histograms depicted are representative of three independent experiments.

Further, the principal downstream transcription factors c-Myc, STAT5, and c-Jun mediate the effector response of IL-2 receptor-mediated signaling. γδ T cells upon activation with phosphoantigen (HDMAPP) and rIL2; or with anti-CD3 mAb and rIL-2 for 72 hours show increased level of p-STAT5 and p-jun, the activated form of these transcription factors as compared to unstimulated γδ T cells. We explored the effect of Notch inhibitor GSI-X on level expression p-STAT5 and p-jun in activated γδ T cells. Notch signaling inhibition in activated γδ T cells decreases the expression of p-STAT5 and phospho c-Jun. However, we did not observe any change in the expression of total STAT5 and total c-Jun. Further, we observed that activation of γδ T cells led to increases expression of c-Myc in which upon inhibition of Notch signaling γδ T cells showed less expression of c-Myc **(Figure 5.11 A-B).** Thus our results demonstrate that Notch signaling inhibition in γδ T cells abrogates the co-stimulatory IL-2 signaling pathway. Inhibition of Notch signaling decreases both proximal and distal IL-2 signaling molecules. Our results also suggest that Notch signaling modulates the proliferation and effector functions of γδ T cells through co-stimulatory IL-2 pathway.

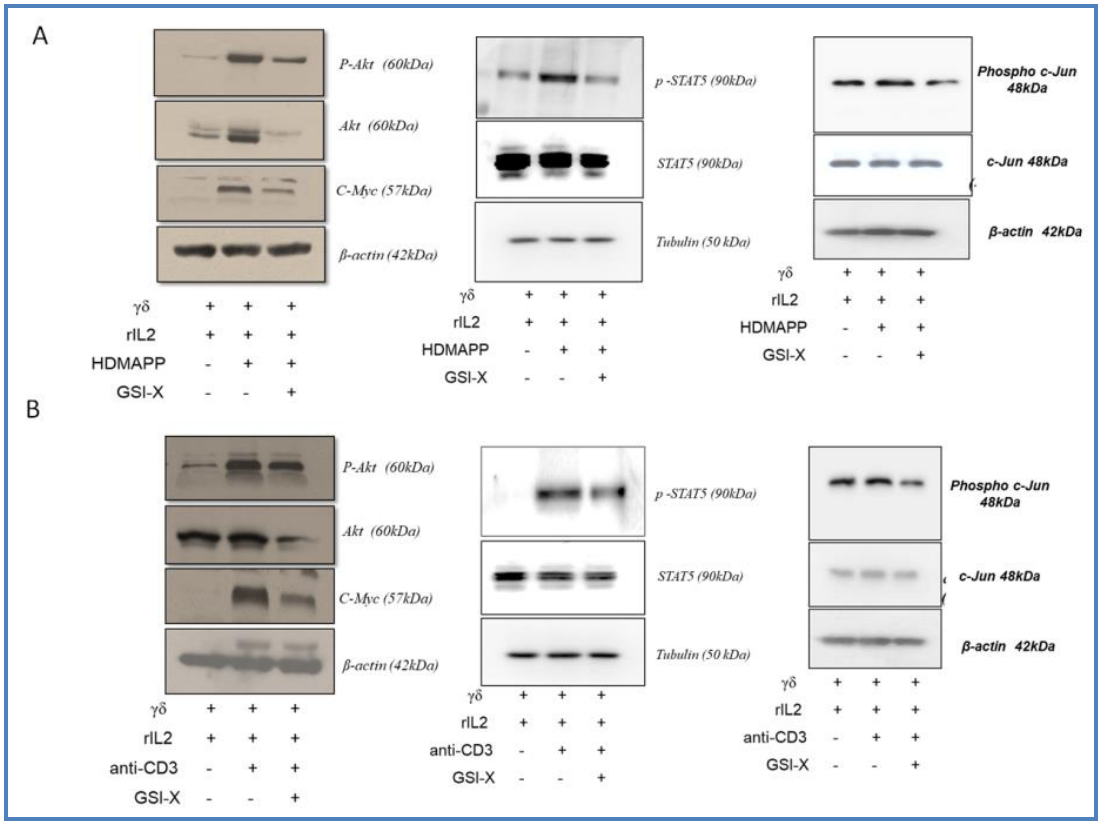

Figure 5.11: Notch signaling modules the distal IL-2 signaling. Inhibition of Notch signaling abrogates expression of distal signaling molecules and transcription factors in human γδ T cells **(A)** MACS purified γδ T cells activated with HDMAPP and rIL2 in presence and absence of Notch inhibitor GSI-X for 72 hours. The protein level expression of STAT5,p-STAT5, phospho c-Jun, p-Akt, Akt, c-Myc, tubulin, and β-actin was detected by western blotting. β- actin, and tubulin were maintained as a loading control. The blots shown are representative of three experiments. **(B).** γδ T cells activated with plate-bound anti-CD3 mAb and rIL2 in presence and absence of Notch inhibitor GSI-X for 72 hours. The protein level expression of STAT5,p-STAT5, phospho c-Jun, p-Akt, Akt, c-Myc, tubulin and β-actin was detected by western blotting. β-actin and tubulin were used as a loading control. Inhibition of Notch signaling decreases the level of p-STAT5 and phospho c-Jun without affecting total STAT5 and c-Jun. GSI-X treatment also decreases the expression of p-Akt and total Akt.

5.2.7: Effect of Notch signaling on expression of transcription factors Eomes, T-bet and Nf-κB in γδ T cells

Antigenic stimulation in γδ T cells triggers a series of diverse TCR mediated intracellular events, including the activation of protein kinases. The initial signaling is amplified through the generation of second messengers, which ultimately lead to

activation of downstream transcription factors Eomes, T-bet, and Nf-κB. These transcriptional factors deliver the signal to the nucleus and leading to regulation of target gene expression, which is required for γδ T cell proliferation and activation. γδ T cells activated with HDMAPP or anti-CD3 mAb in the presence of rIL2 for 72 hours show increased expression of transcription factors Eomes, T-bet , Nf-κB both at mRNA and protein level as compared to unstimulated γδ T cells. In order to investigate the effect Notch signaling on the expression of these key transcription factors γδ T cells were activated with HDMAPP and rIL2 or anti-CD3 mAb and rIL2 in presence of GSI-X for 72 hours. Inhibition of Notch signaling in activated γδ T cells leads to a decrease in the expression of transcription factors Eomes, T-bet and Nf-κB **(Figure 5.12 A-B) (Figure 5.13 A-B)**. The decrease in the expression of Eomes, T-bet, and Nf-κB in γδ T cells upon GSI-X treatment is the direct evidence that Notch signaling controls the effector functions of γδ T cells.

Pharmacological inhibition of the γ-secretase complex may have non-specific effects, we, therefore, assessed the contribution of the Notch pathway in regulating the expression of Eomes, T-bet and Nf- κB by using siRNA mediated knockdown of individual Notch receptors. The synthetic siRNA sequences targeting the Notch-1 were transfected into sorted γδ T cells. γδ T cells were transfected with 100 nM of Notch-1, and control siRNA SiGLO duplexes or left untransfected. Transfection efficiency as checked by flow cytometry was 70% **(5.14 A)**. After 40 hours post-transfection, cells were stimulated with HDMAPP or anti-CD3 mAb in the presence of rIL-2 after 72 hours. Cells were harvested and lysates were prepared. Western blot analysis for N1ICD showed that siRNA transfection led to the reduced expression of N1ICD compared with fluorescent oligonucleotide SiGLO. Next, we also evaluated the expression of Eomes, T-bet, and Nf-κB in siRNA transfected γδ T cells.

Knockdown of Notch1 in γδ T cells showed a decrease in the expression Eomes, T-bet and Nf-κB. Thus, Notch signaling modulates the TCR driven expression of transcription factors Eomes, T-bet and Nf-κB in human γδ T cells and controls the effector functions of γδ T cells **(Figure 5.14 B)**.

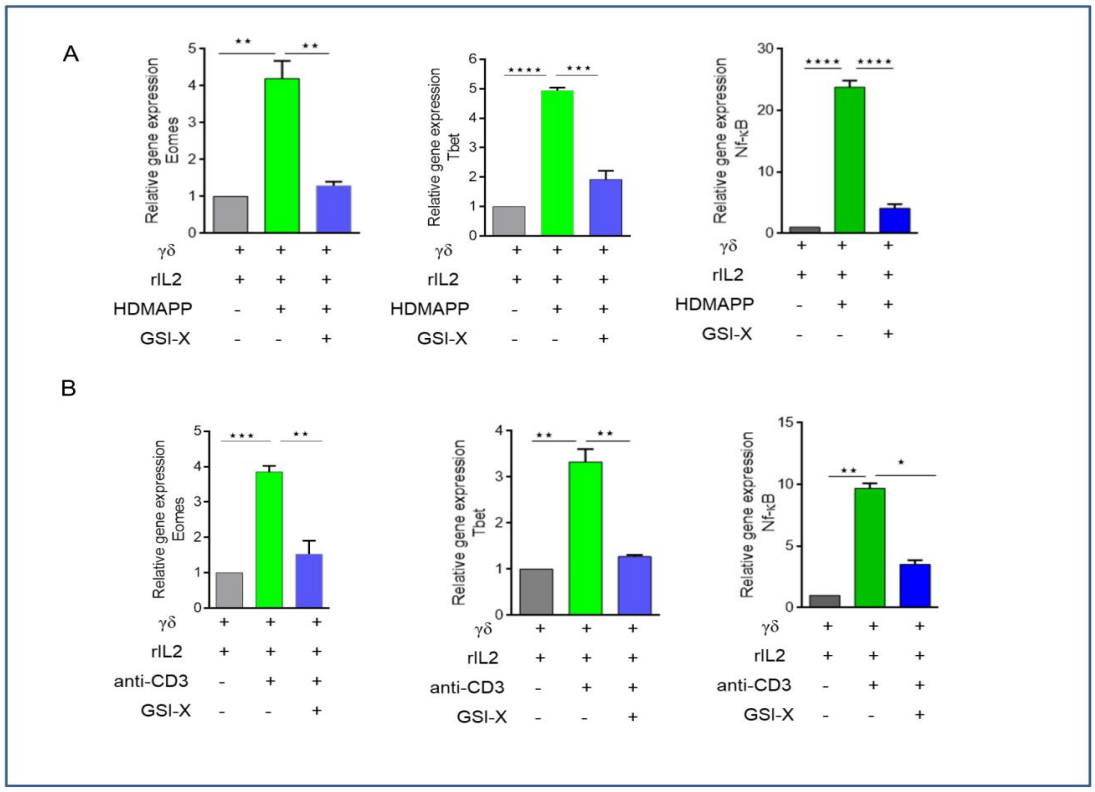

Figure 5.12: **Notch signaling inhibition abrogates expression of transcription factors regulating effector functions of γδ T cells**. The m-RNA expression of Eomes, T-bet, and Nf-κB in γδ T cells activated with HDMAPP and rIL2 **(A) or** anti-CD3 mAb and rIL2 **(B)** in the presence or absence of Notch inhibitor GSI-X was quantified by quantitative real-time PCR. The results indicated are cumulative mean of relative gene expression normalized to 18s rRNA where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$, compared with γδ T cells activated with HDMAPP and anti-CD3 mAb. The data shown are representative of three independent experiments.

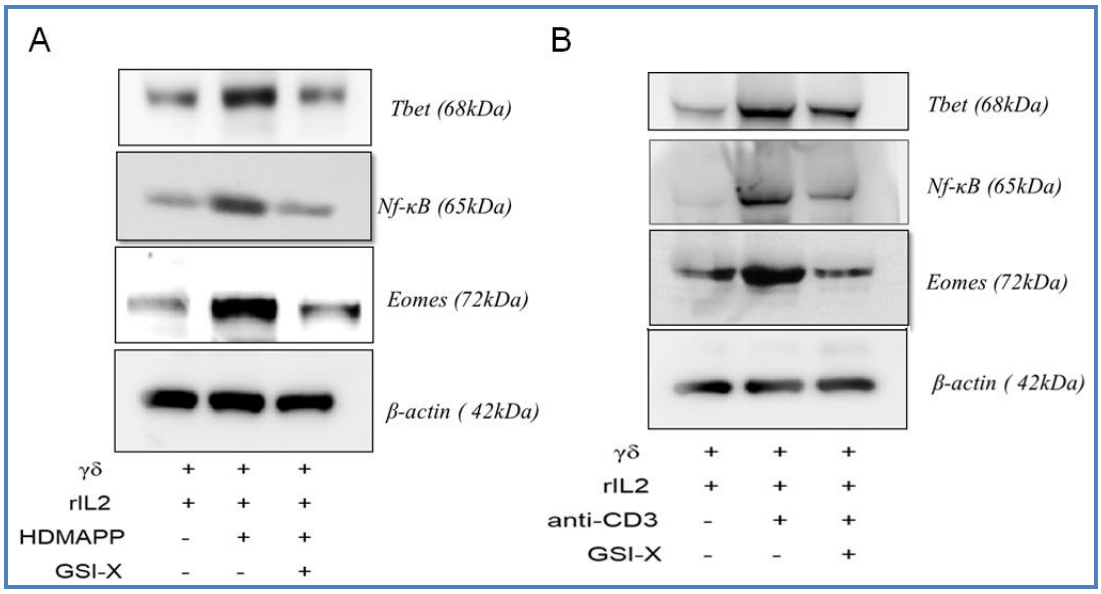

Figure 5.13: Notch signaling regulates expression of transcription factors regulating effector functions of γδ T cells. The protein level expression of c-myc, T-bet, Eomes and Nf-κB was detected by western blotting. Cell lysates of γδ T cells, stimulated with **(A)** HDMAPP or **(B)** anti-CD3 mAb after GSI-X treatment for 72 hours were probed with, Eomes, T-bet, and NFκB antibody; β-actin was used as a loading control. Decreases the expression of T-bet Eomes and Nf-κB was observed upon Notch signaling inhibition by GSI-X in γδ T cells. β- Actin was maintained as a loading control. The blots shown are representative of three experiments.

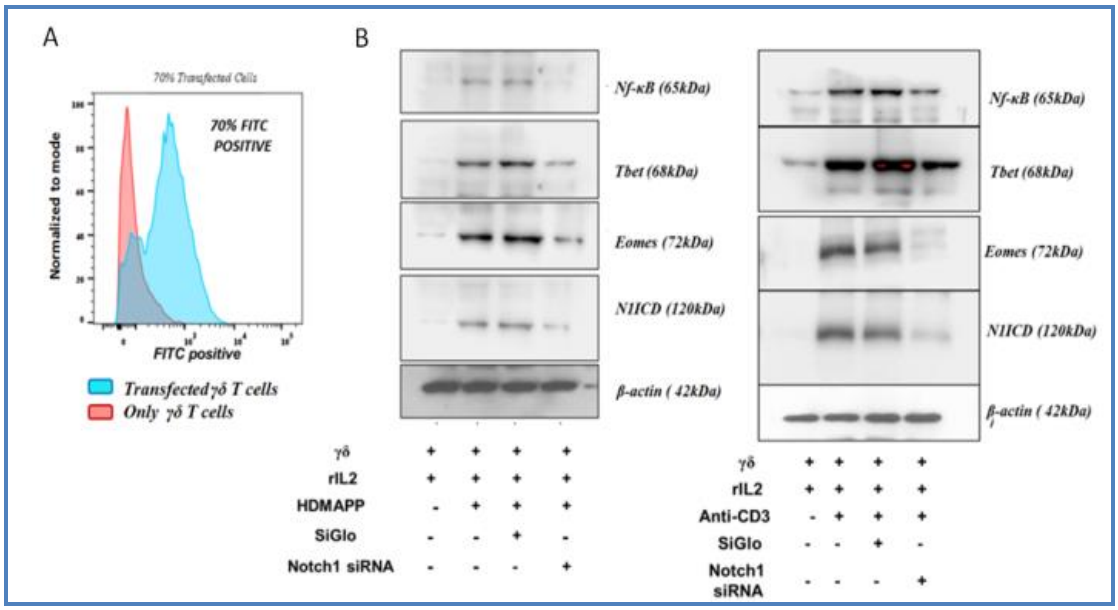

Figure 5.14: *si*RNA mediated downregulation of Notch1 decreases the expression of transcription factors γδ T cells were transfected with 100 nM of control siRNA siGLO or Notch1 siRNA duplexes or left untransfected. **(A)** The transfection efficiency was checked by flow cytometry by calculation the percentage of γδ T cells

positive for siGLO. **(B)** After 40 hrs post transfection cells of Notch1 siRNA, γδ T cells were stimulated with HDMAPP or anti-CD3 mAb for 48 hours. Cell lysates of γδ T cells, stimulated with HDMAPP or anti-CD3 mAb after knockdown of Notch1 were probed with, N1ICD, Eomes, T-bet, and NFκB antibody; β-actin was used as the loading control. Decreased expression of N1ICD, T-bet Eomes, and Nf-κB was observed upon transfection of Notch1 specific siRNAs. β-Actin was maintained as a loading control. The blots shown are representative of three experiments.

5.2.8: Notch signaling plays an intrinsic role in anti-tumor effector functions of γδ T cells through the regulation of Perforin and Granzyme B

γδ T cells possess potent cytotoxic potential against different tumors. Previously our lab has shown that inhibition of Notch signaling in γδ T cells abrogates the anti-tumor potential of γδ T cells against Zoledronate treated oral tumor targets. To further confirm the role of Notch signaling in anti –tumor potential of γδ T cells we used a panel of Zoledronate treated tumor cell lines (AW13516, COLO-205, and Raji) as targets. At different E/T ratios starting from 5:1 to 40:1, HDMAPP-activated γδ T cells in the presence of IL-2 efficiently lysed Zoledronate-treated tumor cells lines (AW13516, COLO- 205, and Raji).Maximum cytotoxicity of γδ T cells was observed at E/T ratio of 40:1 **(Figure 5.15 A-C)**.Inhibition of Notch signaling in γδ T cells using GSI-X significantly decreased the anti-tumor potential of γδ T cells against Zoledronate treated tumor cells lines (AW13516, COLO-205, and Raji) **(Figure 5.15 D)**. However, the mechanism how anti-tumor cytotoxic potential of γδ T cells is regulated by Notch signaling remains undetermined. Upon TCR activation γδ T cells rapidly expand and express effector molecules Perforin and Granzyme B. To investigate the effect of Notch signaling on expression of these effector molecules γδ T cells were activated with HDMAPP or anti-CD3 mAb in presence and absence of γ-secretase inhibitor GSI-X for 72 hours. Stimulation of γδ T cells with HDMAPP or anti-CD3 mAb results in the upregulation of Perforin and Granzyme B both at mRNA

and protein level as compared to unstimulated γδ T cells. In contrast, in vitro treatment with γ-secretase inhibitor markedly diminished mRNA transcripts of Perforin and Granzyme B at mRNA and protein level in activated γδ T cells **(Figure 5.16 A-C)**. Thus, using pharmacological means to block Notch activation in γδ T cells, we showed that inhibiting Notch signaling decreases expression of the principal mediators of cytolytic activity in γδ T cells, Perforin and Granzyme B, both at the transcript and protein levels.

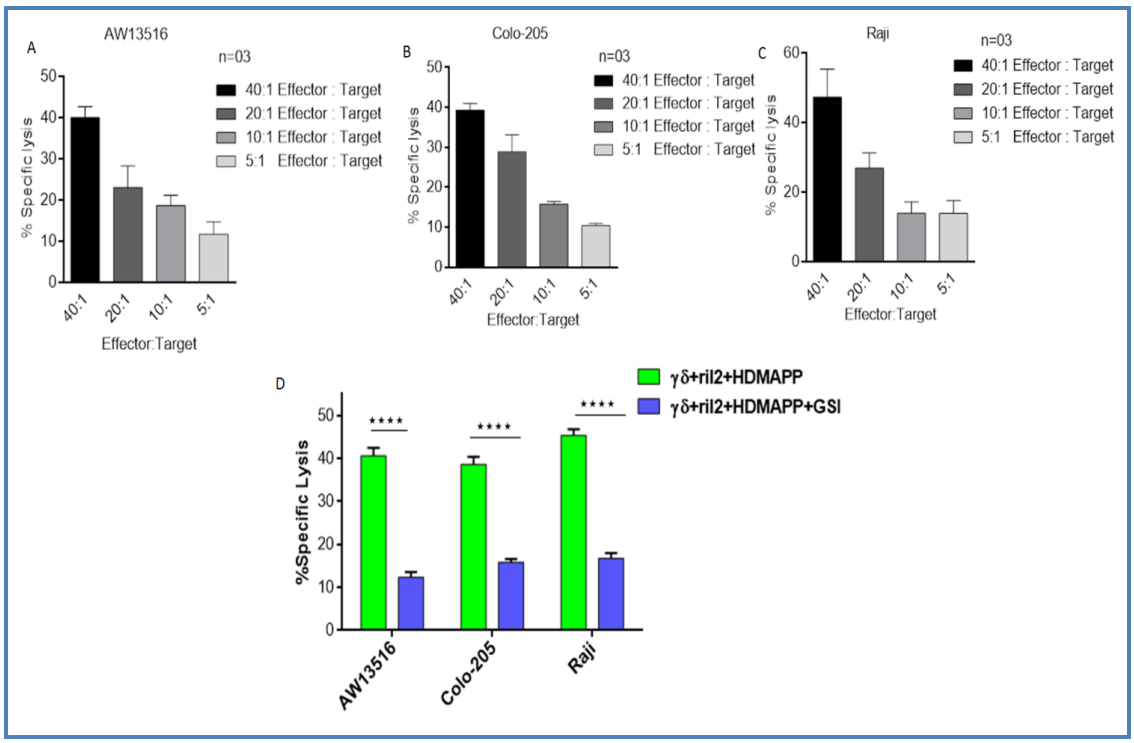

Figure 5.15: **Inhibition of Notch signaling decrease the cytotoxic effector functions of γδ T cells**. Cytotoxic effector function of γδ T cells was assessed against three tumor cell lines **(A)** AW13516, **(B)** COLO205 and **(C)** Raji . Zoledronate treated tumor targets were co-cultured with γδ T cells for 4hrs and cytotoxicity was assessed by LDH assay. The results indicated are mean percent specific lysis at different effector: target ratio. Data is representative of three individual experiments. **(D)**Cytotoxic potential of γδ T cells activated with HDMAPP in the presence of rIL-2 with or without treatment with GSI-X was assessed against three Zoledronate treated tumor targets AW13516, COLO-205, and Raji by LDH cytotoxicity assay. The results indicated are percent specific lysis at the effector to target ratio of 40:1 where **: $p < 0.005$ and ***: $p < 0.0005$ when compared with γδ T cells activated with HDMAPP in the presence of rIL-2 (n=3).

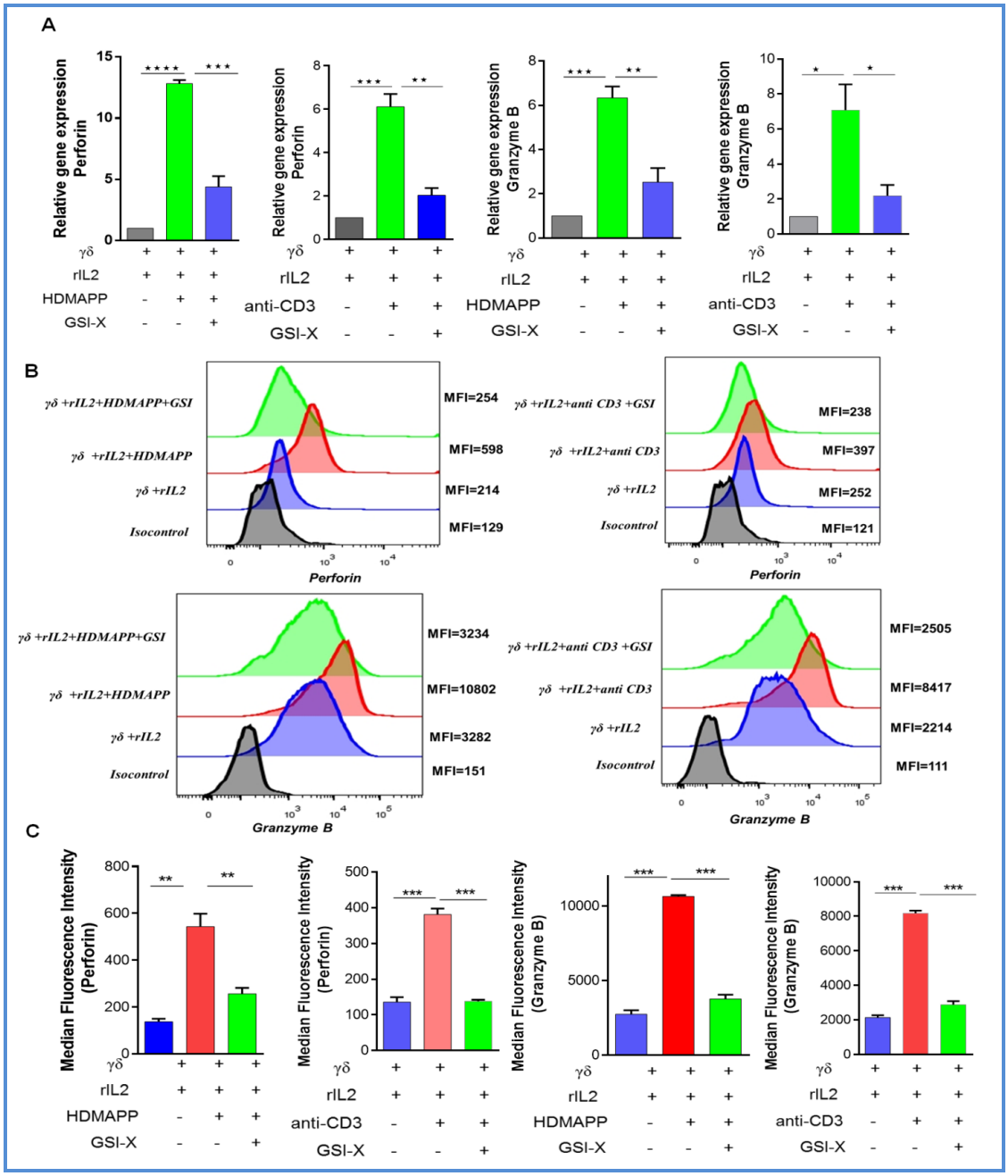

Figure 5.16: Notch signaling inhibition decreases the expression of effector molecules Perforin and Granzyme B in γδ T cells. Expression of Perforin and Granzyme was studied at mRNA and protein levels using quantitative real-time PCR and flow cytometry respectively. The gene expression of Perforin and Granzyme B **(A)** by HDMAPP or anti-CD3 mAb stimulated γδ T cells with or without Notch signaling inhibitor treatment GSI-X was quantified by quantitative real-time PCR. The results shown are cumulative means of relative gene expression, normalized to 18S rRNA and representative of three independent experiments. **(B)** γδ T cells activated by HDMAPP or anti-CD3 mAb with or without Notch signaling inhibitor treatment GSI-X were stained after 72 hours with the corresponding fluorophore tagged antibody and expression of Perforin and Granzyme B was analyzed by flow

cytometry. The values inside of histograms indicate the median fluorescence intensity of Perforin. The histograms are representative of three independent experiments. **(C)** Bar graphs represent cumulative MFI values of Perforin and Granzyme B where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$, compared with γδ T cells activated with HDMAPP or anti-CD3 mAb. The histograms are representative of three independent experiments.

To further validate the mechanism that inhibition of cytotoxic potential of γδ T cells with defective Notch signaling is due to decrease in the expression of effector molecules Perforin and Granzyme B; we used siRNA knockdown approaches of Notch1 and Notch2. The synthetic siRNA sequences targeting the Notch-1 and Notch-2 were transfected into sorted γδ T cells. γδ T cells were transfected with 100 nM of Notch-1, Notch-2, and control siRNA SiGLO duplexes or left untransfected. Transfection efficiency was checked by flow cytometry. γδ T cells were 70% transfected **(Figure 5.17 A)**. After 40 hours post-transfection cells were stimulated with HDMAPP or anti-CD3 mAb in the presence of rIL-2 for 72 hours cells. γδ T cells showed a decrease in the expression of N1ICD and N2ICD thus confirming the knockdown of Notch1 and Notch2 expression in γδ T cells by siRNAs **(Figure 5.17 B).** Using intracellular staining and flow cytometric analysis we assessed the level of Perforin and Granzyme B in Notch1 and Notch2 knockdown γδ T cells. Knockdown of Notch1 and Notch2 in γδ T cells show a decrease in the expression effector molecules Perforin and gramzyme B **(Figure 5.17 C-D)**. Thus, our data confirm that Notch signaling modulates the anti-tumor potential of γδ T cells through the regulation of effector molecules Perforin and Granzyme B.

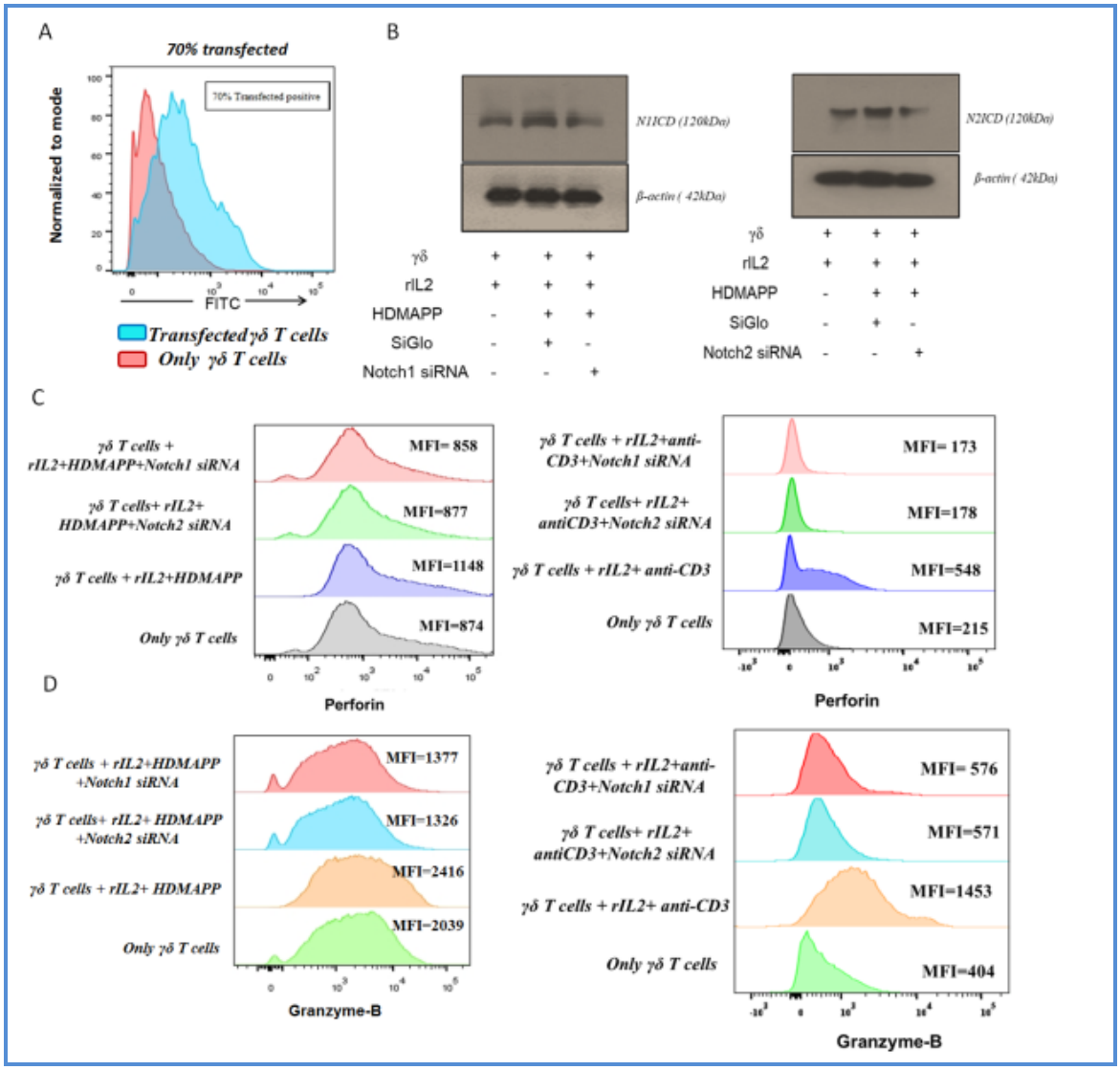

Figure: 5.17: *si*RNA mediated downregulation of Notch1 and Notch2 decreases the expression of effector molecules Perforin and Granzyme B. γδ T cells were transfected with 100 nM of control siRNA siGLO or Notch1 or Notch 2 siRNA duplexes or left untransfected. **(A)**The transfection efficiency was checked by flow cytometry by calculating the percentage of γδ T cells positive for siGLO using FlowJO software**. (B)**To check the expression of N1ICD and N2ICD after transfection of siRNAs γδ T cells were activated with HDMAPP and riL2 and cell lysates were probed with antibodies against N1ICD and N2ICD. Expression of **(C)** Perforin and **(D)**Granzyme was studied at the protein level by flow cytometry. γδ T cells activated by HDMAPP or anti-CD3 mAb after siRNA knockdown of Notch1 and Notch 2 were stained after 72 hours with the corresponding fluorophore tagged antibody and expression of Perforin and Granzyme B was analyzed by flow cytometry. The values inside of histograms indicate the median fluorescence intensity of Perforin. The histograms are representative of three independent experiments.

5.2.9 Inhibition of Notch signaling in γδ T cells leads to decrease in the expression of p-GSK3β

Antigenic activation of T cells through the binding of antigen to the TCR complex results in the expression of different kinases such as tyrosine kinases p56lck which phosphorylates TCR complex ZAP-70. These phosphorylated sites result in the recruitment of different adapter molecules which are necessary for the transmission of TCR signal to the nucleus. Unlike serine/ threonine kinases, it has been shown that resting T cells show high expression of glycogen synthase kinase 3 beta (GSK-3β). GSK-3β regulates multiple signaling pathways. TCR mediated signaling inactivates GSK-3β through phosphorylation. Constitutively active GSK-3β inhibits T cell proliferation [393]. It has been also shown that GSK-3β negatively regulates Notch signaling [394]. This incites us to look at the role of GSK-3β in γδ T cells.

Upon activation with phosphoantigens, bromohydrin pyrophosphate (BrHPP), HDMAPP or with anti-CD3 mAb and rIL2 γδ T cells showed an increased level of p-GSK3β. When γδ T cells were treated with Notch inhibitor GSI-X it led to a decrease in the level of p-GSK3β without a change in the total level of GSK3β **(Figure 5.18 A)**. As Notch regulates the TCR mediated effector functions these results demonstrate that GSK-3β is involved in TCR mediated signaling. Activation of γδ T cells with phospho- antigen (HDMAPP) or anti-CD3 mAb led to an increase in the expression of Granzyme B at mRNA and protein level as compared to γδ T cells. Inhibition of Notch signaling by GSI-X led to a decrease in the expression of Granzyme B in antigen-activated γδ T cells. Further when we inhibited the GSK-3β by CHIR in γδ T cells treated with Notch inhibitor GSI-X it led to rescue in the expression of effector molecules Granzyme B. CHIR is the potent inhibitor of GSK-3β.This rescue of

effector molecules was observed both at the mRNA level **(Figure 5.18 B and C)** and protein level **(Figure 5.18 D and E)**. Our results demonstrate that GSK-3β has an important role in TCR mediated effector functions of human γδ T cells.

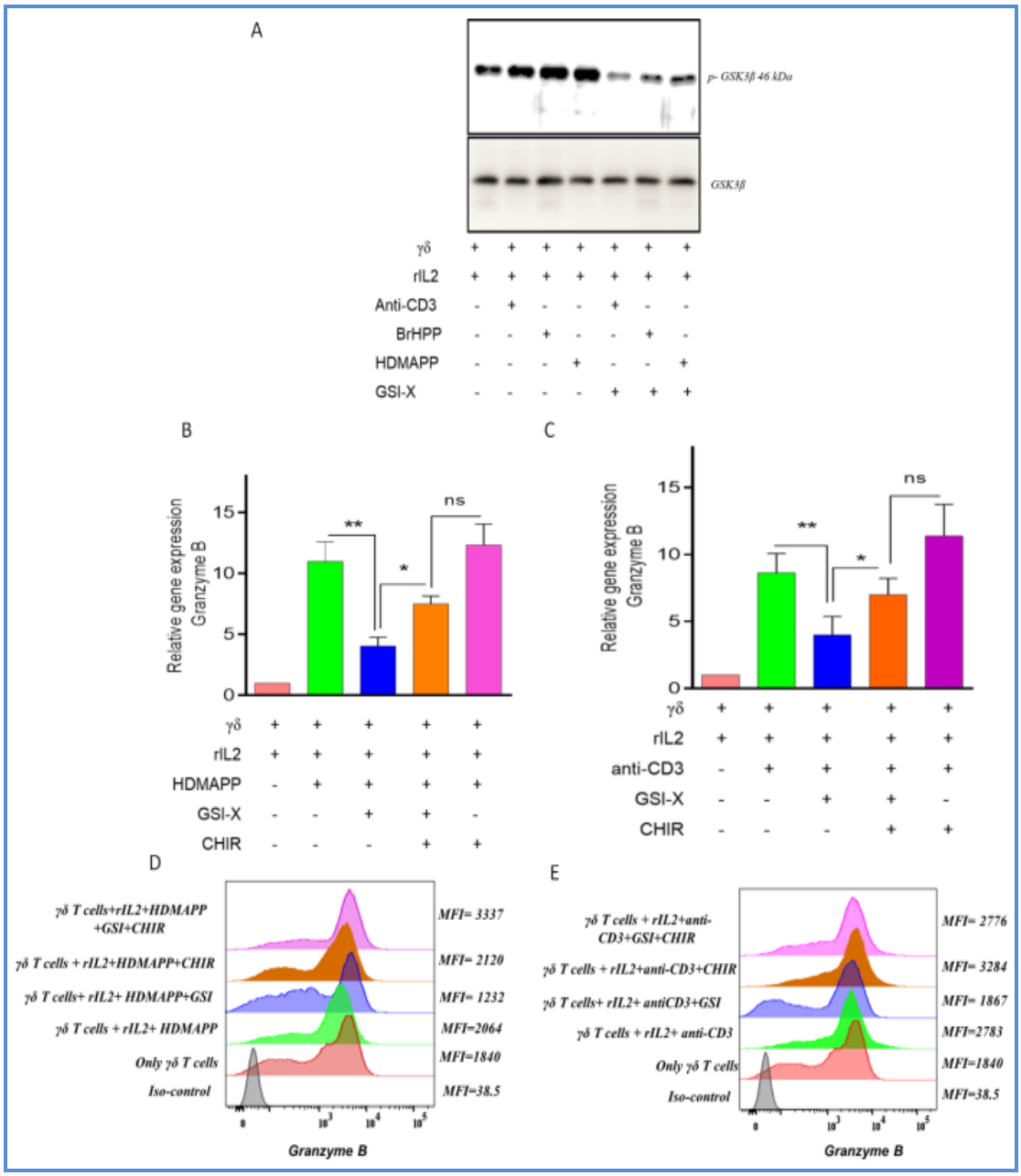

Figure 5.18: Inhibition of GSK3β rescued the expression of effector molecules in γδ T cells. Inhibition of Notch signaling leads to a decrease in the level of p-GSK3β in TCR activated γδ T cells. **(A)** Expression of p-GSK3β and total GSK3β in γδ T cells activated with phosphoantigens BrHPP, HDMAPP or with anti-CD3 mAb by western blotting. Expression of p-GSK3β and total GSK3β in γδ T cells were

activated with HDMAPP, BrHPP or with anti-CD3 mAb in presence and absence of GSI-X for 72 hours and probed with antibodies against p-GSK3β and GSK3β. The m-RNA expression of Granzyme B in γδ T cells activated with HDMAPP and rIL2 **(B) or** anti-CD3 mAb and rIL2 **(C)** in the presence or absence of Notch inhibitor GSI-X and GSK-3β inhibitor CHIR was quantified by quantitative real-time PCR. The results indicated are cumulative mean of relative gene expression normalized to 18s rRNA where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$, and ns=non-significant. The data shown are representative of three independent experiments**.** γδ T cells activated by HDMAPP **(D)** or anti-CD3 mAb **(E)** with or without Notch signaling inhibitor treatment GSI-X and GSK3β inhibitor CHIR were stained after 72 hours with the corresponding fluorophore tagged antibody and expression of Granzyme B was analyzed by flow cytometry. The values on the right side of histograms indicate the median fluorescence intensity of Granzyme B. Histograms are representative of three independent experiments.

Chapter 6
To investigate how epigenetic changes modulate effector functions of γδ T cells

Introduction:

A well-defined feature of γδ T cell is the ability to show rapid transition from a quiescent state to a highly proliferative, cytolytic population of effector cells upon TCR mediated stimulus .γδ T cells to show rapid expression of effector genes upon antigenic stimuli and produce effector molecules that eventually lead to of effector responses [8, 25]. Downstream events of TCR signaling and other co-stimulatory signaling pathways like Notch signaling are regulated by the accessibility of the genetic material DNA [26].

As already described in previous chapter 4 and 5, γδ T cells upon activation show rapid expression of effector molecules Perforin and Granzyme B [395]. It well understood in CD8 cytotoxic T lymphocytes that antigen-activated $CD8^+$ T cells show high levels of transcriptionally active histone modification, histone H3 lysine 9 acetylation (H3K9Ac) as compared to naïve CD8 T cells on promoter regions of Perforin and Granzyme B[37]. This is the main reason why memory CD8 T cells show rapid expression of different effector molecules.

In the case of human γδ T cells, this information is lacking. We have shown that Notch regulates the effector functions of γδ T cells through the regulation of effector molecules Perforin and Granzyme B (**Chapter 5**). However, it is not well understood how TCR mediated Notch signaling in γδ T cells regulates the expression of effector molecules Perforin and Granzyme B through epigenetic mechanisms.

Epigenetic regulation of different signaling pathways is well investigated in different pathological conditions. For example, Notch signaling in different types of cancers is upregulated through the transcription repression of negative regulators of Notch signaling by repressive histone modification H3K27me3. The main methyltransferase responsible for this modification is Enhancer of zesta homolog 2 (Ezh2) [396]. The

methyltransferase Ezh2 is the catalytic subunit of the polycomb-group family that trimethylates histone H3 on Lys27 (H3K27me3) which is the transcriptionally repressive histone modification [397]. Emerging data indicate that methyltransferase Ezh2 has a master regulatory function in controlling key signaling pathways in cancers by transcriptional repression of signaling molecules [398]. Published studies suggest that methyltransferase Ezh2 is involved in the differentiation of type 1 and type 2 helper T cells (TH1 and TH2 cells) in mice [362, 364]. In human CD8 T cells methyltransferase Ezh2 controls the effector functions by regulating the Notch signaling [371]. However, how methyltransferase Ezh2 controls the effector functions of γδ T cells is not well understood. Thus based on this, the key questions addressed are

1. How methyltransferase Ezh2 modulates the TCR mediated Notch signaling in γδ T cells
2. How methyltransferase Ezh2 regulates the expression of effector functions in γδ T cells
3. How epigenetic changes regulate the expression of effector molecules in γδ T cells

6.2 Results:

6.2.1 TCR mediated activation leads to expression of methyltransferase Ezh2 in human γδ T cells

Histone methylation, which is catalyzed by histone methyltransferase such as methyltransferase Ezh2, has been correlated with the expression of genes associated with proliferation, differentiation, and survival of antigen-activated T cells. It has shown that cytotoxic T lymphocytes like CD8 T cells express methyltransferase Ezh2 upon activation [371]. Methyltransferase Ezh2 can influence proliferation and differentiation lymphocytes during a developmental stage in the thymus. This prompted us to look at the expression of methyltransferase Ezh2 in human γδ T cells. To verify the expression of methyltransferase Ezh2 upon antigenic stimuli freshly isolated γδ T cells were activated with phospho-antigen HDMAPP or with anti-CD3 mAb and rIL2 for 72 hours. We observed an increase in expression of methyltransferase Ezh2 mRNA and protein in γδ T cells upon activation with HDMAPP and rIL2 (**Figure 6.1 A-B**) and with anti-CD3 mAb and rIL2 (**Figure 6.1 D-E**) as compared to unstimulated γδ T cells. In HDMAPP and anti-CD3 mAb activated γδ T cells this increase in the expression of methyltransferase Ezh2 was also reflected in the increase in the level of histone 3 lysine 27 trimethylation (H3K27me3). Downstream effector response of methyltransferase Ezh2 is histone methyltransferase activity (HMT) that catalyzes the addition of H3K27me3 (**Figure 6.1 C and Figure 6.1 F**). The results show that TCR mediated signaling in γδ T cells led to the expression of methyltransferase Ezh2, both at mRNA and protein level.

Notably, biochemical inhibition of methyltransferase Ezh2 with histone methyltransferase inhibitor 3-Deazaneplanocin A (DZnep) decreased the expression of methyltransferase Ezh2 in activated γδ T cells. Inhibition of methyltransferase Ezh2 by DZnep was confirmed by the decrease in the level of H3K27me3 levels in γδ

T cells activated with HDMAPP+ rIL2 **(Figure 6.1 C)** and anti-CD3 mAb+ rIL2 (**Figure 6.1 F).** as compared to the only rIL2 activated antigen γδ T cells which act as a control.

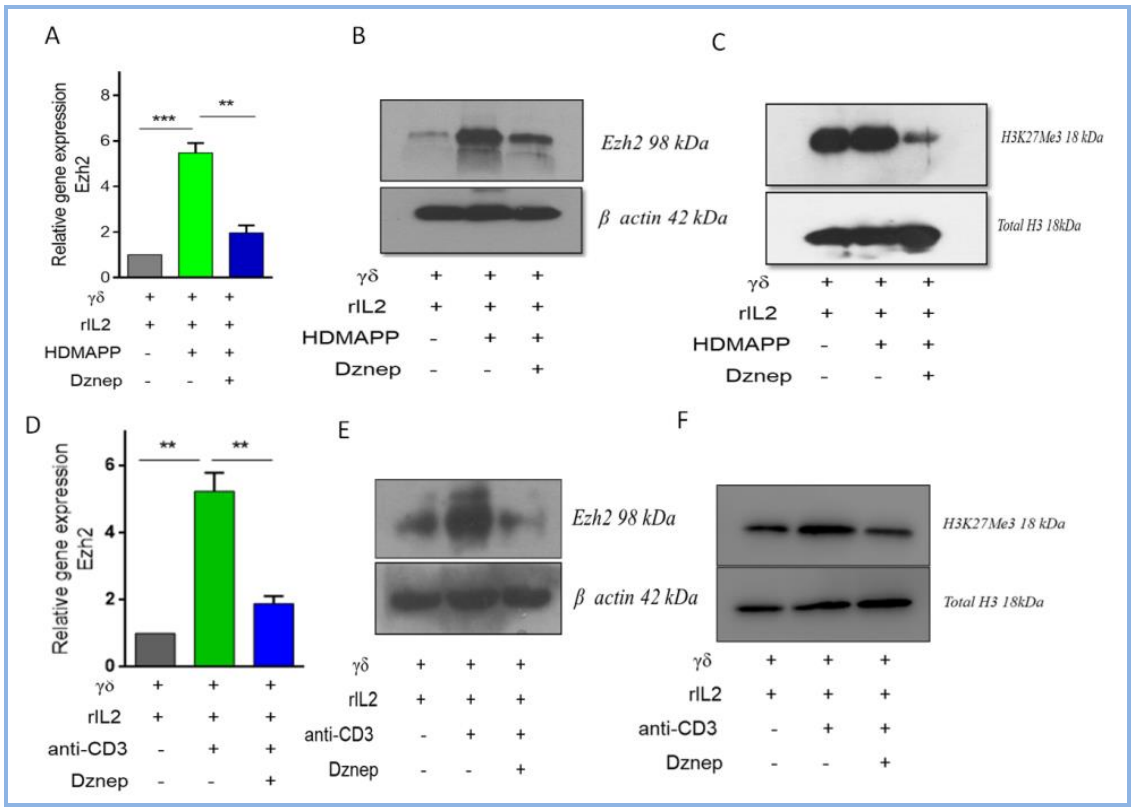

Figure 6.1: TCR signaling in activated γδ T cells mediates expression of methyltransferase Ezh2. γδ T cells activated HDMAPP **(A)** or anti-CD3 mAb **(D)** in the presence of rIL2 were treated with DZnep for 72h. γδ T cells+rIL2 served as control. Expression of methyltransferase Ezh2 was quantified by real-time PCR in activated γδ T cells in the presence or absence of DZnep. The results indicated are cumulative mean of relative gene expression normalized to 18S rRNA where **p < 0.005, ***p < 0.0005, compared with control (γδ T cells+rIL2). Protein expression of methyltransferase Ezh2 was detected in γδ T cells stimulated with HDMAPP **(B)** and anti-CD3 mAb **(E)** with or without DZnep treatment by western blotting. β-Actin was used as a loading control. H3K27me3 expression in HDMAPP **(C)** or anti-CD3 mAb **(F)** activated γδ T cells upon DZnep treatment was detected by western blotting. Total histone H3 was used as a loading control

6.2.2 Inhibition of methyltransferase Ezh2 abrogates the antigen-activated Notch signaling in γδ T cells

The Notch signaling pathway regulates effector T cell survival and effector functions in cytotoxic T lymphocytes including γδ T cells. As described in previous chapter 5 that TCR mediated activation of γδ T cells lead to activation of Notch receptors and ligands. It is also shown in CD8 cytotoxic T cells that Notch and methyltransferase Ezh2 are expressed simultaneously and methyltransferase Ezh2 is an essential positive regulator of Notch signaling. Based on this we hypothesized that methyltransferase Ezh2 modulates the TCR mediated Notch signaling in γδ T cells. To investigate the role of methyltransferase Ezh2, human γδ T cells were directly isolated from peripheral blood of healthy individuals and were activated with phospho antigen HDMAPP+rIL2 or plate-bound anti-CD3 mAb + rIL2 in the presence and absence of pharmacological inhibitor of methyltransferase Ezh2 DZnep for 72 hours. Activated γδ T cells showed an increase in the expression Notch 1 and Notch 2 in γδ T cells. However, upon inhibition of methyltransferase Ezh2 by using inhibitor DZnep, activated γδ T cells show a decrease in the expression of Notch1 and Notch2 at mRNA level **(Figure 6.2 A-D).**

Inhibition of methyltransferase Ezh2 in activated γδ T cells not only decreases the mRNA level of Notch1 and Notch2 receptors but also the functionally active intracellular domains of Notch1 (N1ICD) and the intracellular domain of Notch2 (N2ICD) as observed at the protein level. Functionally active intracellular domains are released when there is active Notch signaling. Thus upon activation of γδ T cells, methyltransferase Ezh2 and the intracellular domain of Notch1 and Notch2 (N1ICD and N2ICD) were synchronically induced **(Figure 6.3 A-B)**.

γδ T cells showed an increase in the expression of Nf-κB when stimulated with HDMAPP or anti-CD3 mAb in the presence of rIL2. Nf-κB is the downstream target of Notch signaling which regulates the different effector responses in γδ T cells. However inhibition methyltransferase Ezh2 in antigen-activated γδ T cells decreased the expression of Nf-κB both at protein **(Figure 6.3 A and B)** and mRNA **(Figure 6.3 C and D)**

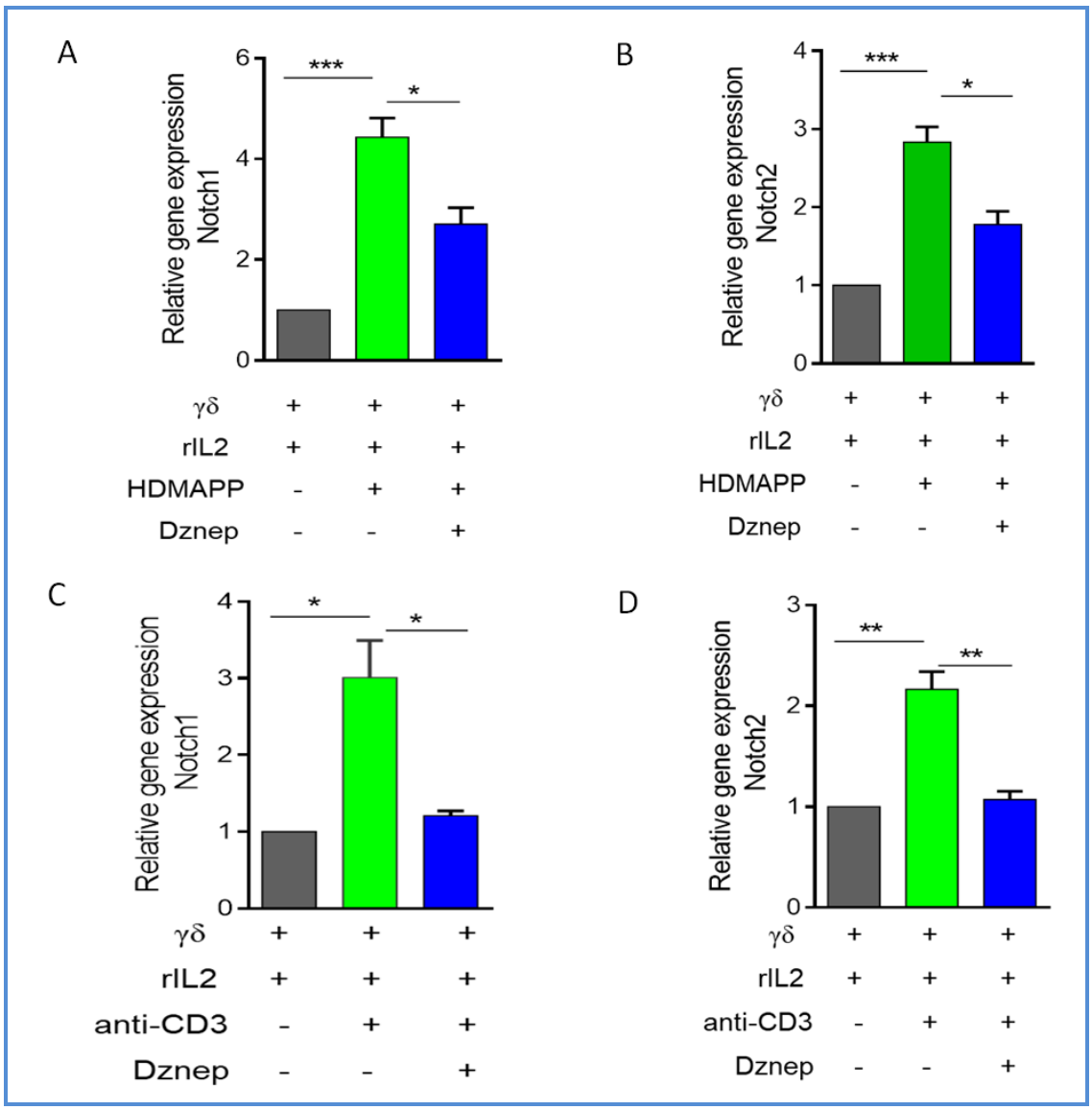

Figure 6.2: Inhibition of methyltransferase Ezh2 reduces Notch receptor gene expression. γδ T cells activated with rIL-2 and HDMAPP were treated with DZnep for 72h. Relative gene expression of **(A)** Notch-1, **(B)** Notch-2 and was quantitated by real-time PCR. Similarly, relative gene expression of **(C)** Notch1, **(D)** Notch2 and in γδ T cells activated with rIL-2 and anti-CD3 mAb and treated with or without DZnep was measured by real-time PCR. The results indicated are cumulative mean of

relative gene expression normalized to 18S rRNA where *p < 0.05**p < 0.005, ***p < 0.0005, compared with unstimulated γδ T cells.

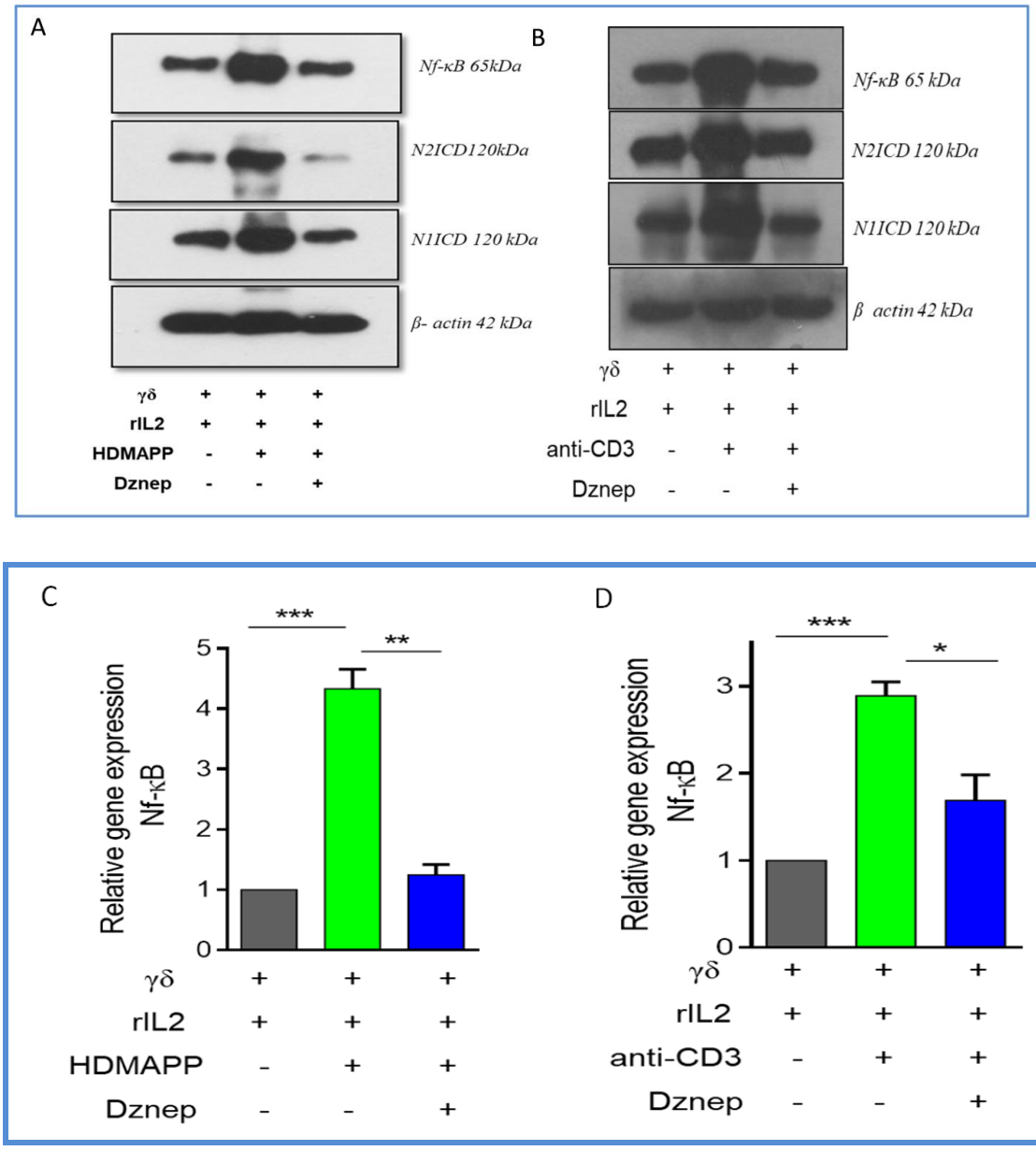

Figure 6.3: Notch signaling in activated γδ T cells abrogated upon methyltransferase Ezh2 inhibition. Protein expression of Nf-κB, N1ICD, and N2ICD in γδ T cells activated for 72h with **(A)** HDMAPP or **(B)** anti-CD3 mAb in the presence or absence of DZnep was detected by western blotting. β-Actin was used as a loading control. Expression of Nf-κB in γδ T cells activated with rIL-2 and HDMAPP **(C)** or anti-CD3 mAb **(D)** were treated with DZnep for 72h. Relative gene expression of Nf-κB was quantitated by real-time PCR where **p < 0.005, ***p < 0.0005, compared with unstimulated γδ T cells.

To further investigate that methyltransferase Ezh2 regulates the TCR mediated Notch signaling in γδ T cells we checked the expression of different Notch signaling downstream targets genes Hey2, Hes1, and NRARP. Activation of γδ T cells with HDMAPP or anti-CD3 mAb and resulted in increased level of these downstream Notch target genes in γδ T cells **(Figure 6.4 A-B).**However, upon treatment of DZnep, decreased expression of these downstream Notch signaling targets was observed in γδ T cells**. (Figure 6.4 A-B).** Thus the data suggest that methyltransferase Ezh2 regulates the TCR mediated Notch signaling in γδ T cells.

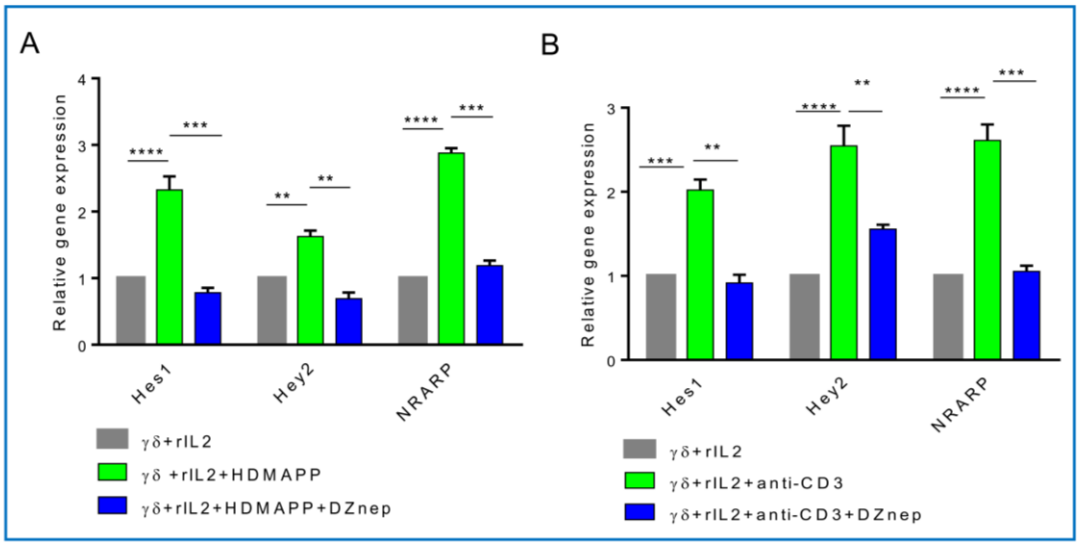

Figure 6.4: Methyltransferase Ezh2 inhibition downregulates Notch target genes in activated γδ T cells. Relative gene expression of Notch target genes (Hes1, Hey2, and NRARP) in γδ T cells activated for 72h with **(A)** HDMAPP or **(B)** anti-CD3 mAb and rIL-2 in the presence or absence of DZnep was quantitated by real-time PCR. The results indicated are the cumulative mean of relative gene expression normalized to 18S rRNA where **p < 0.005, ***p < 0.0005, compared with unstimulated γδ T cells.

6.2.3 Methyltransferase Ezh2 inhibits Notch repressors in antigen-activated γδ T cells through epigenetic mechanism

Methyltransferase Ezh2 is one of the Polycomb group family members that function in transcriptional repression. We hypothesized that methyltransferase Ezh2 may be targeting and repressing Notch repressors and consequently activates Notch signaling in antigen-activated γδ T cells. To investigate this hypothesis, we analyzed the expression of NUMB and FBXW7, two important Notch suppressors in γδ T cells. γδ T cells were activated with phospho antigen HDMAPP or plate-bound anti-CD3 mAb and rIL2 in presence and absence of pharmacological inhibitor DZnep for 72 hours. Activated γδ T cells showed less expression of these Notch suppressors NUMB and FBXW7. However, inhibition of methyltransferase Ezh2 by DZnep in activated γδ T cells leads to an increase in the expression Notch repressors NUMB and FBXW7 **(Figure 6.5 A-B).** The data indicate that methyltransferase Ezh2 may promote activation of Notch signaling in γδ T cells through inhibition of Notch signaling suppressors.

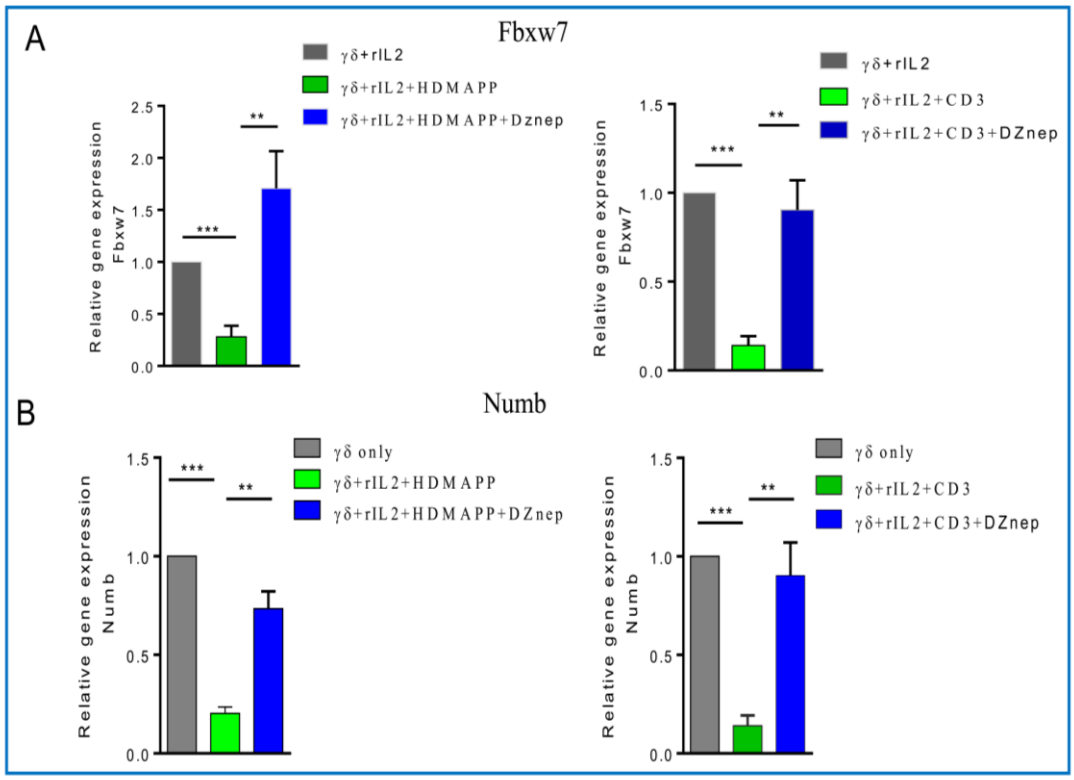

Figure 6.5: Methyltransferase Ezh2 mediated activation of Notch signaling in γδ T cells occurs via inhibition of Notch signaling repressors. γδ T cells activated with rIL-2 and HDMAPP or anti CD3 mAb in the presence or absence of DZnep for 72h. Relative gene expression of Notch signaling repressors **(A)** Fbwx7 and **(B)** Numb was analyzed by real-time PCR. The results indicated are the cumulative mean of relative gene expression normalized to 18S rRNA where **p < 0.005 compared with untreated γδ T cells.

To further elucidate the mechanism behind the suppression of NUMB and FBXW7 in activated γδ T cells we investigated the epigenetic modification H3K27me3 on the proximal promoter region of these genes by chromatin immunoprecipitation (ChIP) assay. ChIP assays revealed high occupancies of transcriptionally repressive histone modification H3K27me3 in the proximal promoter areas of NUMB and FBXW7, in γδ T cells activated with phospho antigen HDMAPP or anti-CD3 mAb and rIL2. However inhibition of methyltransferase Ezh2 in antigen-activated γδ T cells led to decrease in the level of H3K27me3 in the proximal promoter areas of NUMB

(Figure 6.6 A-E) and FBXW7 **(Figure 6.7 A-E)** compared to γδ T cells activated with HDMAPP or anti-CD3 mAb. Normal rabbit IgG was used as a negative control. The data suggest that methyltransferase Ezh2 targets Notch repressors and promote Notch activation and consequently regulates the effector functions of γδ T cells through the activation of Notch signaling.

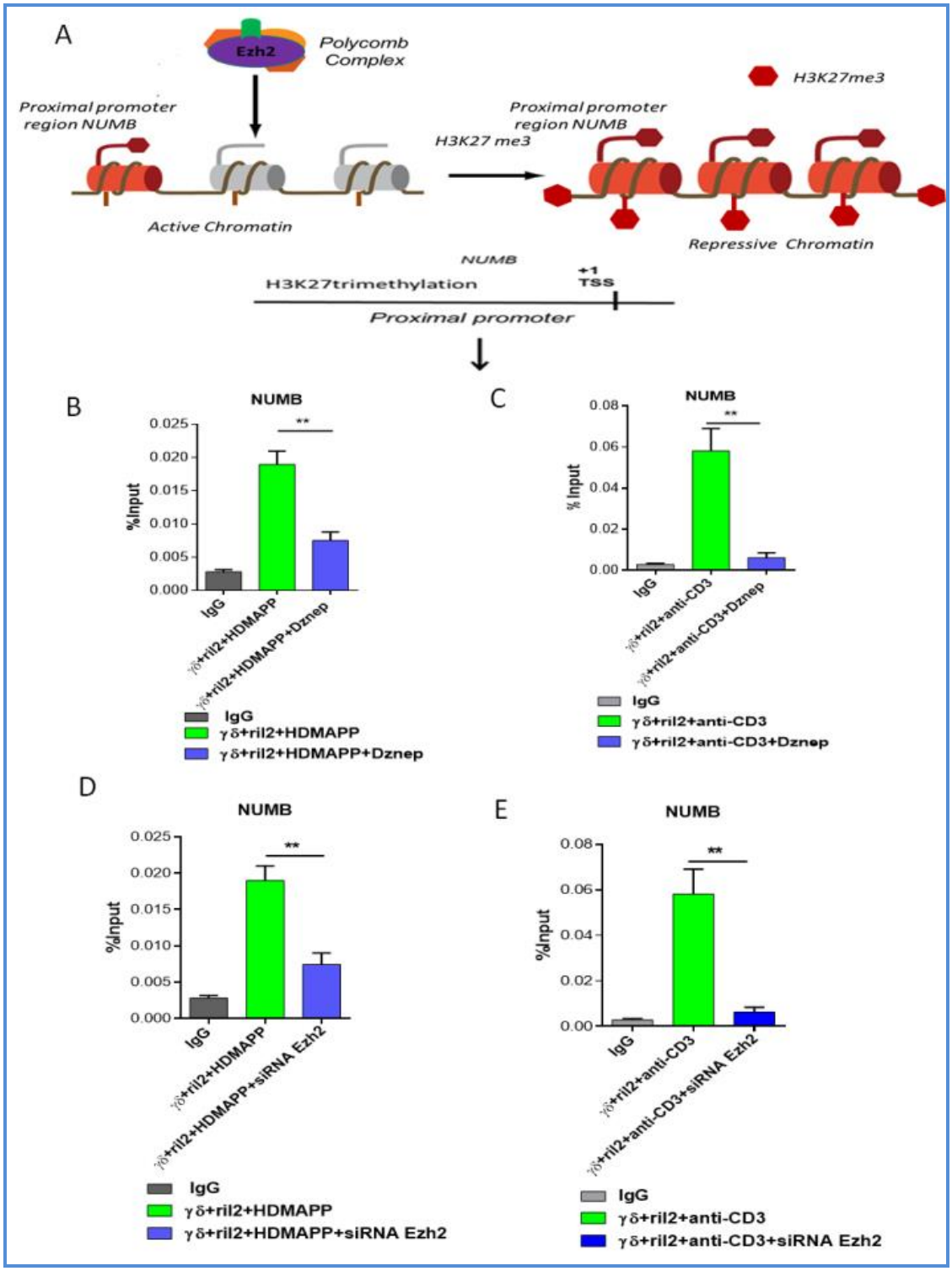

Figure 6.6: Methyltransferase Ezh2 regulates the methylation status of Notch repressor Numb gene on promoter region. (A) Diagrammatic representation of methyltransferase Ezh2 mediated regulation of Numb expression. The image above the graph schematically represents the Numb promoter region including the transcription start site (TSS). Chromatin immunoprecipitation was used to analyze the trimethylation of H3K27 on the proximal promoter region of Numb in γδ T cells activated with **(B)** HDMAPP or **(C)** anti- CD3 mAb in the presence or absence of

DZnep for 72h. Similarly, of H3K27 trimethylation on the proximal promoter region of Numb in **(D)** HDMAPP or **(E)** Anti-CD3 mAb activated γδ T cells transfected with methyltransferase Ezh2 siRNA was analyzed by ChIP assay. ChIP assay was performed by using H3K27 trimethylation specific antibody raised in rabbit. Normal rabbit IgG was used as control. Enrichment of H3K27 trimethylation on Numb promoter region was quantified by qPCR and expressed as a percentage of input DNA. The results indicated are mean ± where **: $p < 0.005$.

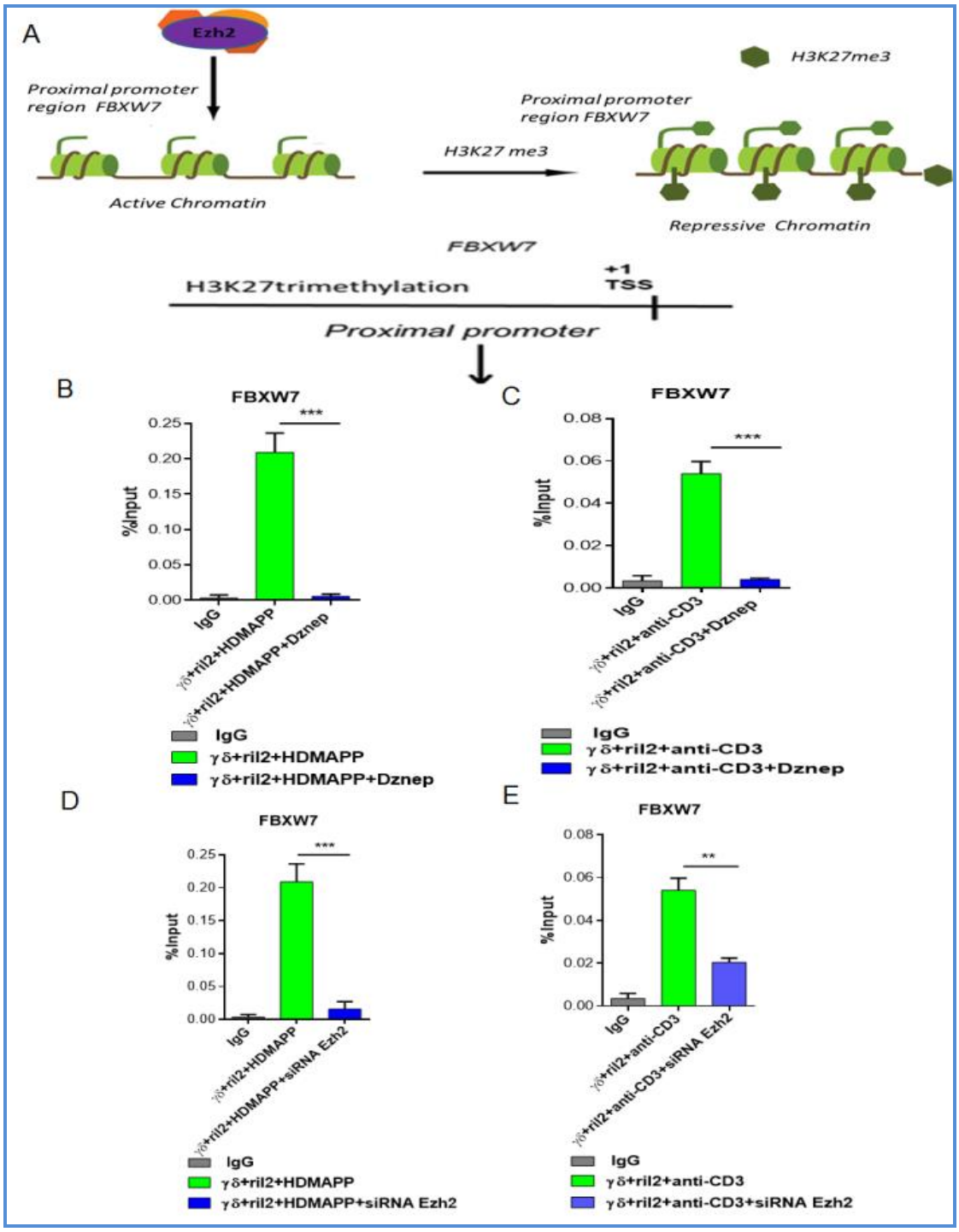

Figure 6.7 Methyltransferase Ezh2 regulates methylation status of Notch repressor Fbwx7 gene on promoter region. (A) Diagrammatic representation of methyltransferase Ezh2 mediated regulation of Fbwx7 expression. The image above the graph schematically represents the Numb promoter region including the transcription start site (TSS). Chromatin immunoprecipitation was used to analyze the of H3K27 trimethylation on the proximal promoter region of Fbwx7 in γδ T cells activated with rIL-2 and **(B)** HDMAPP or **(C)** anti CD3 mAb in the presence or absence of DZnep for 72h. Similarly, trimethylation of H3K27 on the proximal promoter region of Numb in **(D)** HDMAPP or **(E)** anti-CD3 mAb activated γδ T cells transfected with methyltransferase Ezh2 siRNA was analyzed by ChIP. ChIP assay was performed by using H3K27 trimethylation specific antibody raised in rabbit. Normal rabbit IgG was used as control. Enrichment of Fbwx7 promoter region was quantified by qPCR and expressed as a percentage of input DNA. The results indicated are mean ± where **: $p < 0.005$, ***: $p < 0.0005$.

6.2.4:- Methyltransferase Ezh2 in γδ T cells modulate the TCR driven proliferation and cytokine expression

γδ T cells show robust proliferation when stimulated with antigen and also show increased expression of two key cytokines IFN-γ and TNF-α. Data from the previous (**chapter 5)** have shown that Notch regulates the antigen-specific γδ T cell proliferation and IFN-γ and TNF-α expression. This incited us to investigate the effect of methyltransferase Ezh2 inhibition on proliferation of HDMAPP and anti-CD3 mAb stimulated γδ T cells. A marked increase in the proliferation of γδ T cells was observed when stimulated with HDMAPP or anti-CD3 mAb in the presence of rIL-2 as compared to γδ T cells stimulated with rIL-2 alone. However, in the presence of DZnep HDMAPP or anti-CD3 mAb stimulated γδ T cells showed a decrease in the proliferative potential. The data suggested that methyltransferase Ezh2 is an important factor for the TCR driven proliferation of γδ T cells **(Figure 6.8 A-B)**

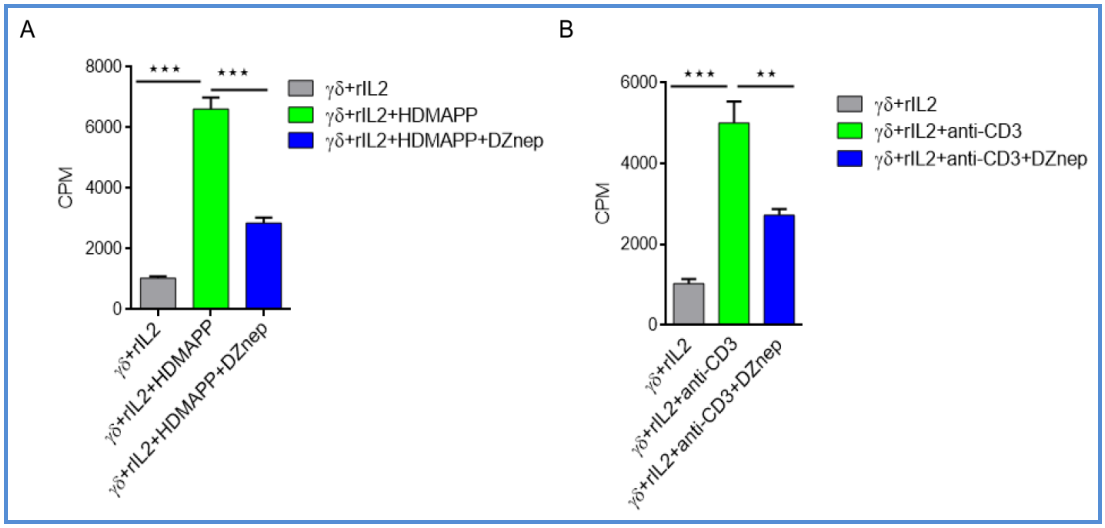

Figure 6.8: Inhibition of methyltransferase Ezh2 impedes antigen-specific proliferation of γδ T cells. The proliferative response of γδ T cells activated with HDMAPP or anti-CD3 mAb was assessed by ^{3}H-thymidine incorporation assay. Sorted γδ T cells were stimulated with phosphoantigen **(A)** HDMAPP or with plate-bound anti-CD3 mAb **(B)** with or without treatment of methyltransferase Ezh2 inhibitor DZnep for 72 h. The graphs illustrate the cumulative mean cpm and are representative of three independent experiments where **p < 0.005, ***p < 0.0005

Upon antigenic activation, γδ T cells secrete copious amounts of cytokines IFN-γ and TNF-α. As discussed in chapter 5 Notch signaling is necessary for the regulation of IFN-γ and TNF-α. To investigate the role of methyltransferase Ezh2 in regulating the expression of IFN-γ and TNF-α, γδ T cells were activated with HDMAPP or anti-CD3 mAb in the presence or absence of DZnep for 72 hours. After 72 hours supernatants were collected, expression of IFN-γ and TNF-α at the protein level was measured by sandwich ELISA. γδ T cells upon stimulation with HDMAPP or anti-CD3 mAb in the presence of rIL2 showed an increased level of cytokines IFN-γ and TNF-α in the supernatants as compared to unstimulated γδ T cells. However pharmacological inhibition of methyltransferase Ezh2 by DZnep in HDMAPP and anti-CD3 mAb stimulated γδ T cells led to a decrease in the level of secreted IFN-γ and TNF-α in cell-free supernatants. We also confirmed the effect of methyltransferase Ezh2 inhibitor DZnep on mRNA expression of IFN-γ and TNF-α by real-time PCR. We

observed a decrease in the expression of IFN-γ and TNF-α **(Figure 6.9 A-B) (Figure 6.10 A-B)**. Our data unraveled the role of methyltransferase Ezh2 in regulating IFN-γ and TNF-α production in activated γδ T cells thus regulating in effector functions of γδ T cells.

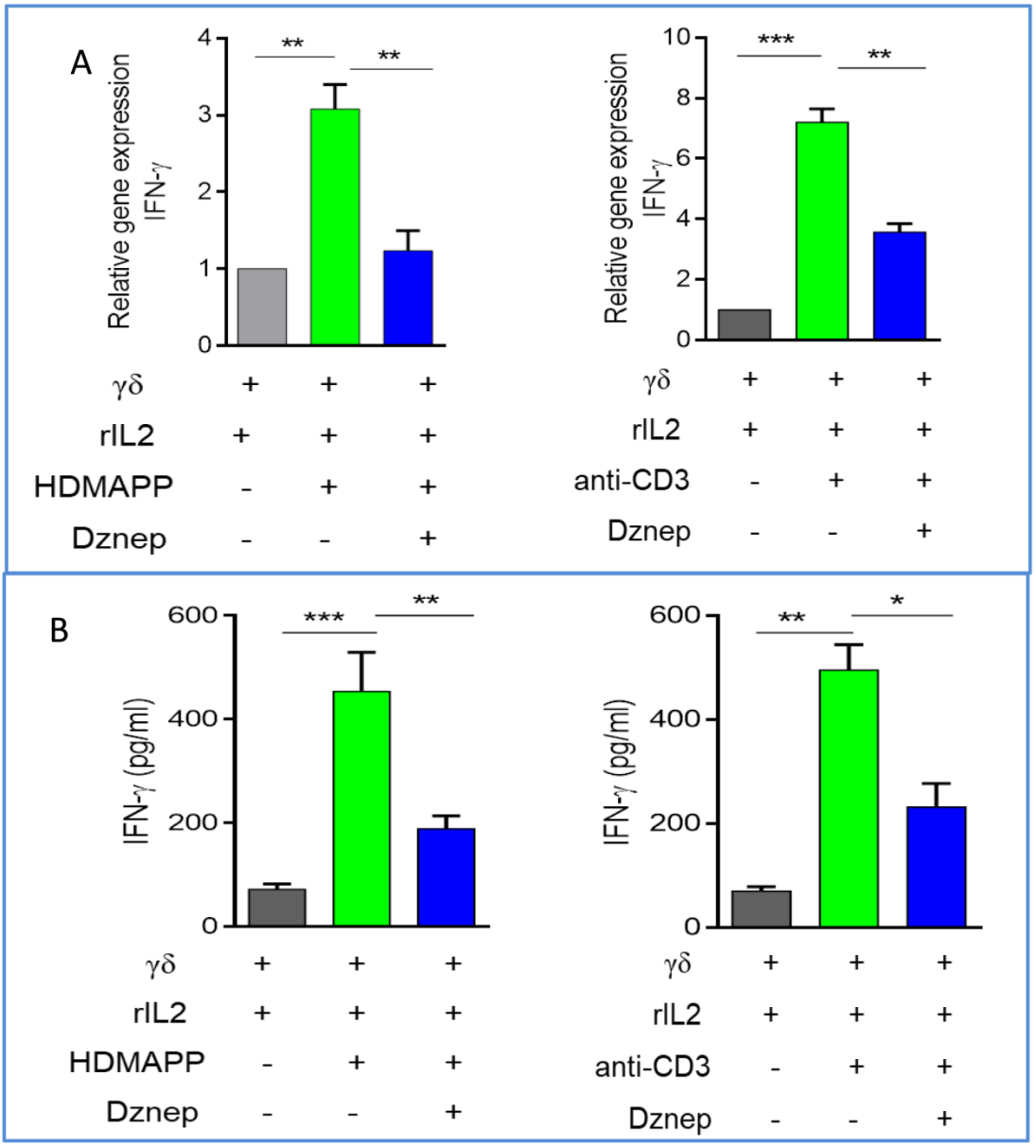

Figure 6.9: Methyltransferase Ezh2 inhibitor downregulates IFN-γ expression in activated γδ T cells. (A) Relative gene expression of IFN-γ in γδ T cells activated for 72h with HDMAPP or anti-CD3 mAb and rIL-2 in the presence or absence of DZnep was quantitated by real-time PCR. The results indicated are the cumulative mean of relative gene expression normalized to 18S rRNA where **p < 0.005, ***p < 0.0005, compared with unstimulated γδ T cells. **(B)** Expression of IFN-γ in cell-free

supernatants collected from HDMAPP or anti-CD3 mAb and rIL-2 stimulated γδ T cells in the presence or absence DZnep for 72h was estimated by ELISA. All the results indicated are mean ± SEM of three independent experiments, where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$

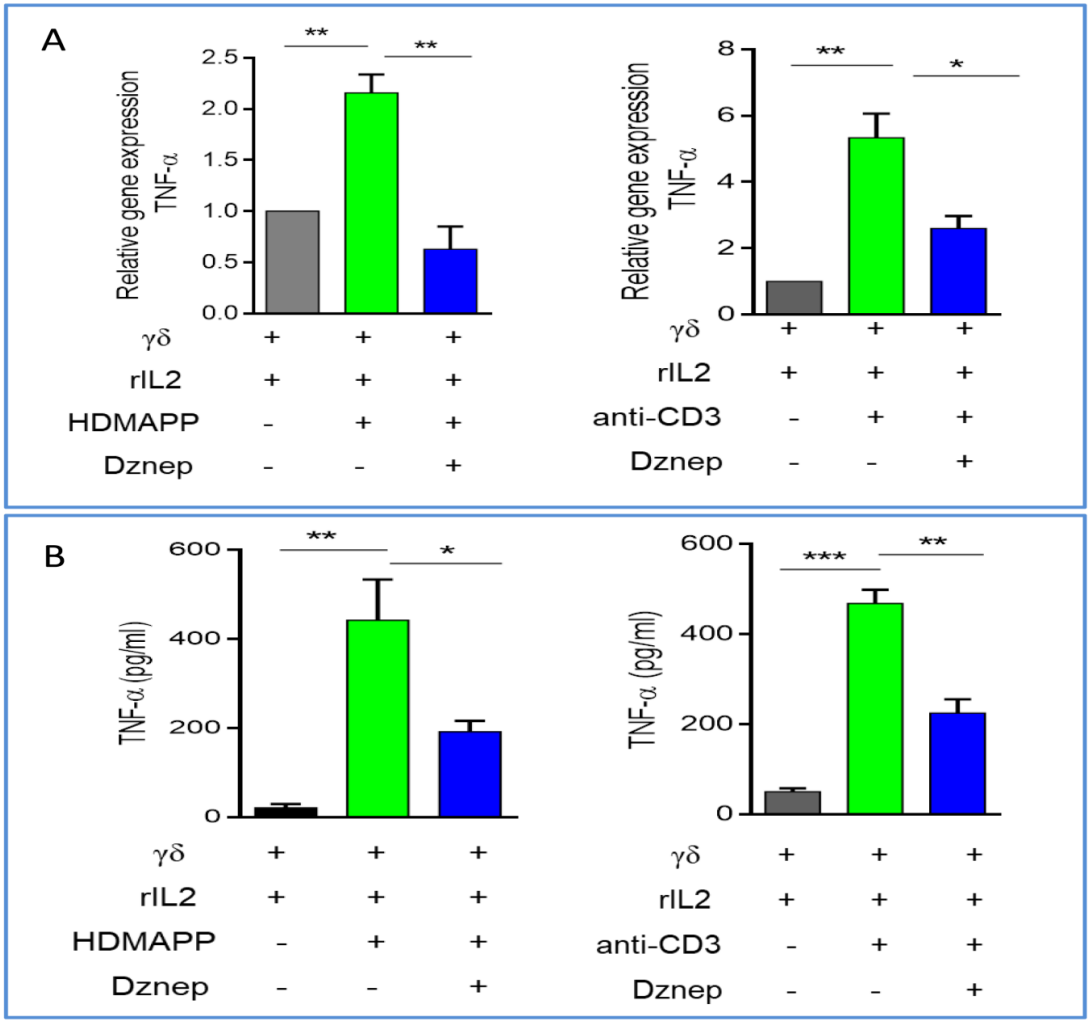

Figure 6.10: TNF-α expression in activated γδ T cells decreased upon methyltransferase Ezh2 inhibition (A) Relative gene expression of TNF-α in γδ T cells activated for 72h with HDMAPP or anti-CD3 mAb and rIL-2 in the presence or absence of DZnep was quantitated by real-time PCR. The results indicated are the cumulative mean of relative gene expression normalized to 18S rRNA where **$p < 0.005$, ***$p < 0.0005$, compared with unstimulated γδ T cells. **(B)** Level of secreted TNF-α in the supernatants collected from HDMAPP or anti-CD3 mAb and rIL-2 stimulated γδ T cells in the presence or absence DZnep for 72h was estimated by ELISA. All the results indicated are mean ± SEM of three independent experiments, where *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$

6.2.5 Methyltransferase Ezh2 modulates the TCR driven expression of principal transcriptional factors Eomes and T-bet in γδ T cells

Downstream of TCR signaling the effector functions of T cells are regulated by the transcription factor Eomes and T-bet. Inhibition of methyltransferase Ezh2 in antigen-activated γδ T cells inhibits the proliferative potential and effector cytokines (IFN-γ and TNF-α) secretion.These events of γδ T cells are controlled by the transcription factors Eomes and T-bet. It has been shown that methyltransferase Ezh2 promotes Th1 response through the transcriptional and posttranscriptional regulation of T-bet. To assess whether methyltransferase Ezh2 inhibition has any impact on the expression of Eomes and T-bet in activated γδ T cells. γδ T cells were activated with the phospho antigen HDMAPP or plate-bound anti-CD3 mAb and rIL2 in presence and absence of methyltransferase Ezh2 inhibitor DZnep for 72 hours. γδ T cells show increased expression of Eomes and T-bet when activated with HDMAPP or anti-CD3 mAb and rIL2 as compared to unstimulated γδ T cells. However, inhibition of methyltransferase Ezh2 in TCR activated γδ T cells led to decrease in the expression of Eomes and T-bet transcription factors both at mRNA **(Figure 6.11 A-B)** and protein level **(Figure 6.12 A-B)**.

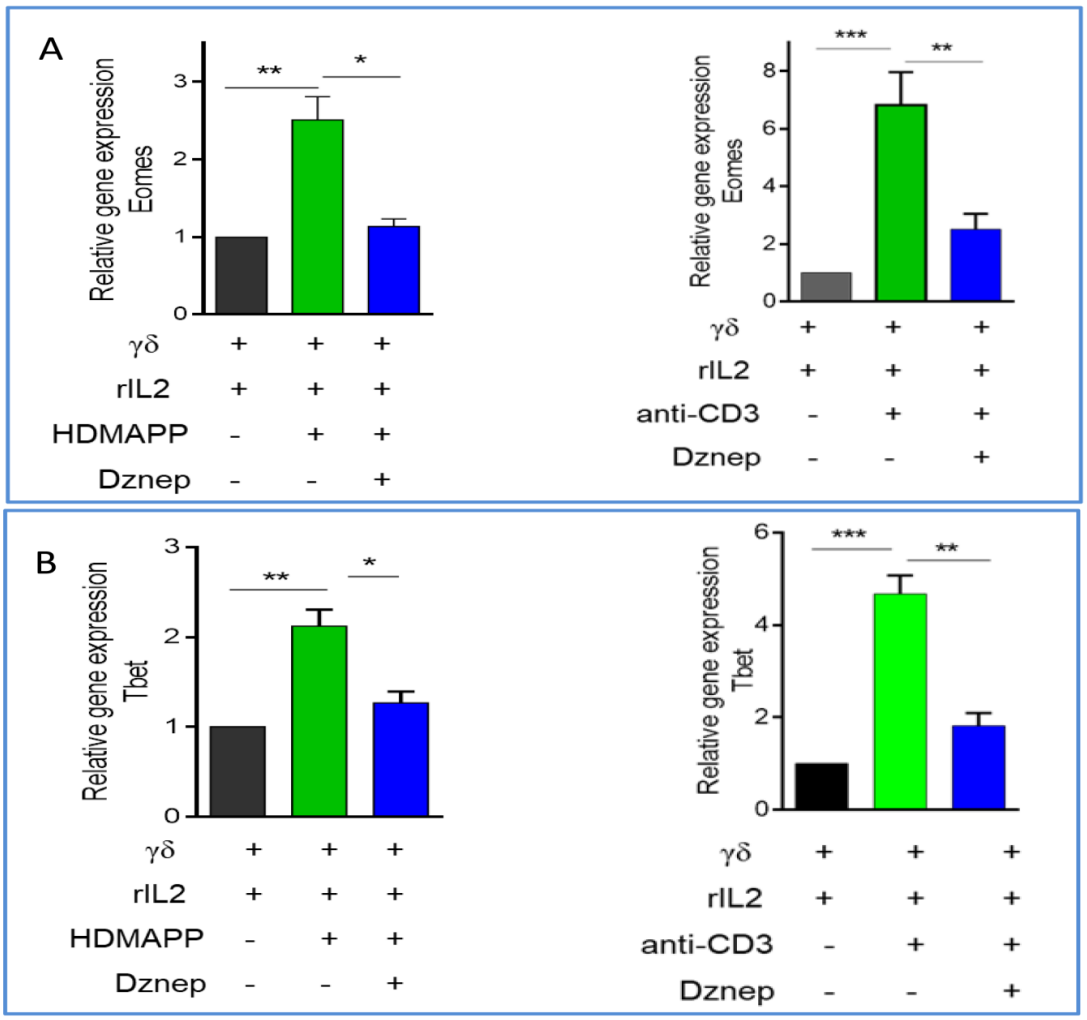

Figure 6.11: Methyltransferase Ezh2 inhibition downregulates the expression of transcription factors in activated γδ T cells. Relative gene expression of transcription factors **(A)** Eomes and **(B)** T-bet in γδ T cells activated for 72h with HDMAPP or anti-CD3 mAb and rIL-2 in the presence or absence of DZnep was quantitated by real-time PCR. The results indicated are cumulative mean of relative gene expression normalized to 18S rRNA where *p < 0.05,**p < 0.005, ***p < 0.0005, compared with unstimulated γδ T cells.

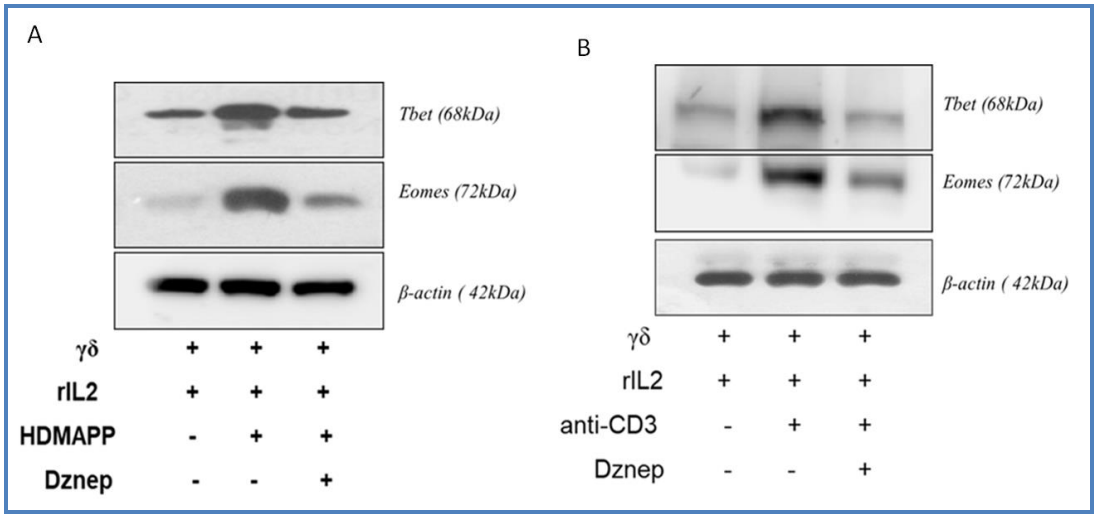

Figure 6.12: Methyltransferase Ezh2 modulates the expression of transcription factors T-bet and Eomes in activated γδ T cells. The protein level expression of transcription factors T-bet and Eomes was detected by western blotting. Cell lysates of γδ T cells, stimulated with **(A)** HDMAPP or **(B)** anti-CD3 mAb after DZnep treatment for 72 hours were probed with, Eomes and T-bet antibody; β-actin was used as a loading control. Decreased expression of T-bet and Eomes was observed upon pharmacological inhibition of methyltransferase Ezh2. The blots shown are representative of three experiments.

6.2.6: Methyltransferase Ezh2 regulates effector molecules Perforin and Granzyme B in γδ T cells

The TCR mediated expression of methyltransferase Ezh2 in γδ T cells coincided with expression of cytokines (IFN-γ and TNF-α) and transcription factors (Eomes and T-bet). We hypothesized that methyltransferase Ezh2 may also regulate the expression of effector molecules Perforin and Granzyme B in γδ T cells. To investigate this hypothesis γδ T cells were Immuno-magnetically sorted from the isolated peripheral blood lymphocytes. Sorted γδ T cells were activated with phospho antigen HDMAPP or plate-bound anti-CD3 mAb and rIL2 in presence and absence of methyltransferase Ezh2 inhibitor DZnep. Perforin and Granzyme B are the cytotoxic mediators of γδ T cells necessary for effector responses. In antigen-activated γδ T cells, the gene expression of Perforin and Granzyme B was increased which we observed to be

diminished upon inhibition of methyltransferase Ezh2 **(Figure 6.13 A-D)**. Similarly, the protein expression of Perforin **Figure 6.14 A-B)**. and Granzyme B **(Figure 6.15 A-B)** was found to be abrogated in antigen-activated γδ T after methyltransferase Ezh2 inhibition as analyzed by flow cytometry. Thus, the results indicate that methyltransferase Ezh2 regulates the effector responses of γδ T cells by controlling the effector molecule expression.

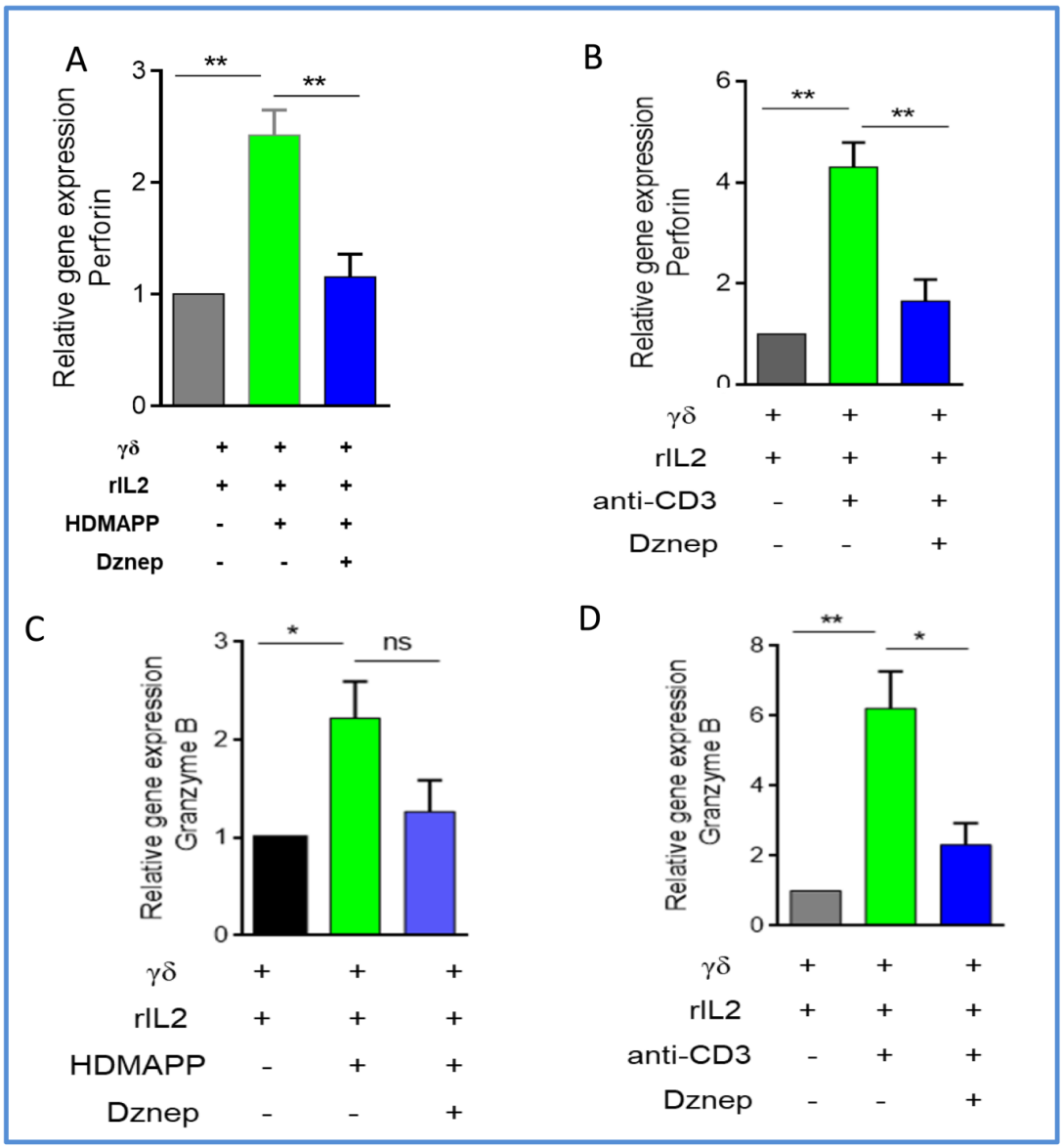

Figure 6.13: Methyltransferase Ezh2 inhibitor abrogates the expression of effector molecules in activated γδ T cells The gene expression of Perforin by γδ T cells stimulated by **(A)** HDMAPP or **(B)** anti-CD3 mAb stimulated with or without DZnep was quantified by quantitative real-time PCR. Similarly, relative gene

expression of Granzyme B by γδ T cells stimulated by **(C)** HDMAPP or **(D)** anti-CD3 mAb stimulated with or without DZnep was measured by quantitative real-time PCR. The results shown are cumulative means of relative gene expression, normalized to 18S rRNA were *: $p < 0.05$ **: $p < 0.005$, ns: not significant and representative of three independent experiments.

To confirm these results, we knocked down the expression of methyltransferase Ezh2 in antigen-activated γδ T cells with methyltransferase Ezh2 specific siRNA. The synthetic siRNA sequences targeting the methyltransferase Ezh2 were transfected into sorted γδ T cells. γδ T cells were transfected with 100 nM of methyltransferase Ezh2 siRNA and control SiGLO siRNA duplexes or left untransfected. Transfection efficiency was checked by flow cytometry. The efficiency of transfection was 70%. After 40 hours post-transfection cells were stimulated with HDMAPP or anti-CD3 mAb in the presence of rIL-2. After 72 hours, cells were harvested and expression of effector molecules Perforin and Granzyme were checked by flow cytometry. Flow cytometry analysis for Perforin and Granzyme B showed that siRNA transfection led to the marked decrease in the expression of Perforin **(Figure 6.14 C-D)** and Granzyme B **(Figure 6.15 C-D)** effector molecules as compared with fluorescent oligonucleotide SiGLO. Thus data confirms that methyltransferase Ezh2 mediates the TCR mediated effector functions through Perforin and Granzyme B in γδ T cells.

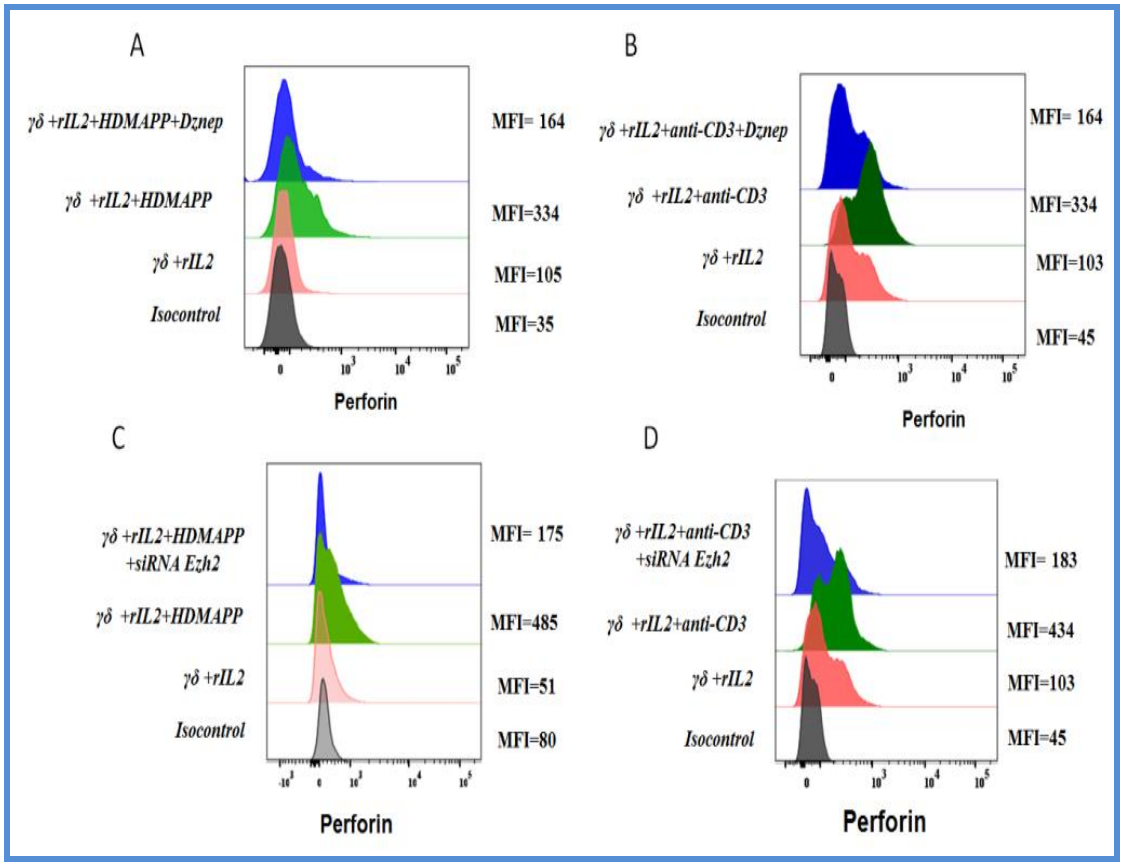

Figure 6.14: Perforin expression in activated γδ T cells regulated by methyltransferase Ezh2. Protein expression of Perforin was studied by flow cytometry respectively. γδ T cells activated by **(A)** HDMAPP or **(B)** anti-CD3 mAb with or without DZnep were stained after 72 hours with the corresponding fluorophore tagged antibody for Perforin and was analyzed by flow cytometry. The values besides the histograms indicate the median fluorescence intensity of Perforin. The histograms are representative of three independent experiments. Alternatively, γδ T cells stimulated with HDMAPP or anti-CD3 mAb were transfected with 100 nM of control siRNA siGLO or methyltransferase Ezh2 siRNA duplexes or left untransfected. siRNA transfected γδ T cells, stimulated with **(C)** HDMAPP or **(D)** anti-CD3 mAb after knockdown of methyltransferase Ezh2 were also stained after 72 hours with fluorophore tagged antibody for Perforin and was analyzed by flow cytometry. The values besides the histograms indicate the median fluorescence intensity of Perforin. The histograms are representative of three independent experiments. Decreased expression of Perforin was observed upon pharmacological inhibition as well as siRNA mediated knockdown of methyltransferase Ezh2

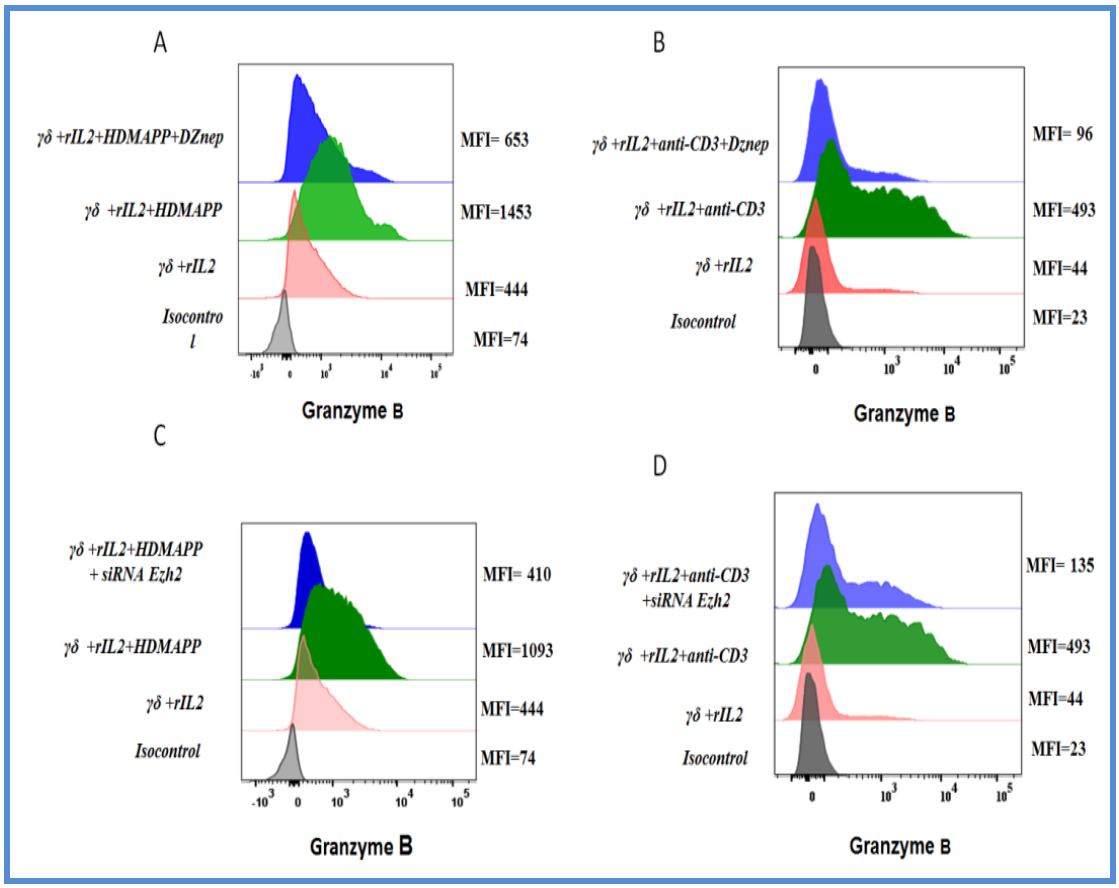

Figure 6.15: Methyltransferase Ezh2 inhibition regulates Granzyme B expression in activated γδ T cells. Protein expression of Granzyme B was studied by flow cytometry respectively. γδ T cells activated by **(A)** HDMAPP or **(B)** anti-CD3 mAb with or without DZnep were stained after 72 hours with the corresponding fluorophore tagged antibody for Granzyme B and was analyzed by flow cytometry. The values besides the histograms indicate the median fluorescence intensity of Perforin. The histograms are representative of three independent experiments. Alternatively, γδ T cells stimulated with HDMAPP or anti-CD3 mAb were transfected with 100 nM of control siRNA siGLO or methyltransferase Ezh2 siRNA duplexes or left untransfected. siRNA transfected γδ T cells, stimulated with **(C)** HDMAPP or **(D)** anti-CD3 mAb after knockdown of methyltransferase Ezh2 were also stained after 72 hours with fluorophore tagged antibody for Granzyme B and was analyzed by flow cytometry. The values besides the histograms indicate median fluorescence intensity of Granzyme B. Histograms are representative of three independent experiments. Decreased expression of Granzyme B was observed upon pharmacological inhibition as well as siRNA mediated knockdown of methyltransferase Ezh2

6.2.7: Inhibition of methyltransferase Ezh2 abrogates the anti-tumor potential of γδ T cells against Zoledronate treated tumor cell lines

Antigen-activated γδ T cells aggressively lyse the Zoledronate treated tumor targets as discussed in chapter 5. This cytotoxic potential of γδ T cells against tumor cells depends on the TCR mediated signaling cascade through recognition of phosphoantigen IPP secreted by Zoledronate treated tumor targets. Inhibition of methyltransferase Ezh2 in TCR activated γδ T cells led to a marked decrease in the expression of effector molecules Perforin and Granzyme B. Perforin create the pores in target cell through which Granzyme is delivered inside the target cell. We wondered whether inhibition of methyltransferase Ezh2 in antigen-activated γδ T cells have any impact on their cytotoxic potential against a panel of Zoledronate treated tumor targets. To investigate this question, we employed a panel of Zoledronate treated tumor cell lines (oral cancer cell line AW13516, colon cancer cell line COLO-205, and B lymphoblastic cell line Raji) and investigated the cytotoxic potential of antigen-activated γδ T cells in presence and absence of methyltransferase Ezh2 inhibitor DZnep. At different effector-target (E:T) ratios ranging from 5:1 to 40:1, HDMAPP-activated γδ T cells in the presence of IL-2 efficiently lysed Zoledronate-treated tumor cells lines (AW13516, COLO- 205, and Raji). Maximum cytotoxicity of γδ T cells was observed at E/T ratio of 40:1 **(Chapter 5 Figure 5.15 A-C)**. This ratio of E: T was used in further experiments, to assess the effect of methyltransferase Ezh2 inhibition on the cytolytic ability of γδ T cells. γδ T cells stimulated with HDMAPP and rIL2 in presence and absence of methyltransferase Ezh2 inhibitor or with GSI-X for 72 h were used as effector against Zoledronate-treated tumor cell lines (AW13516, COLO205, and Raji) as targets at E/T ratio of 40:1. γδ T cells treated with methyltransferase Ezh2 inhibitor DZnep showed a significant decrease in their

cytotoxic potential against Zoledronate treated tumor targets (AW13516, COLO 205, and Raji). The percent decrease in specific lysis upon methyltransferase Ezh2 inhibition was observed to be 67%, 65% and 69% against the three tumor cell lines AW13516, COLO 205, and Raji respectively as compared to cytotoxicity mediated by HDMAPP stimulated γδ T cells (Taken as 100 %)**(Figure 6.16 A).** This further confirms that methyltransferase Ezh2 regulates the anti-tumor effector functions of γδ T cells.

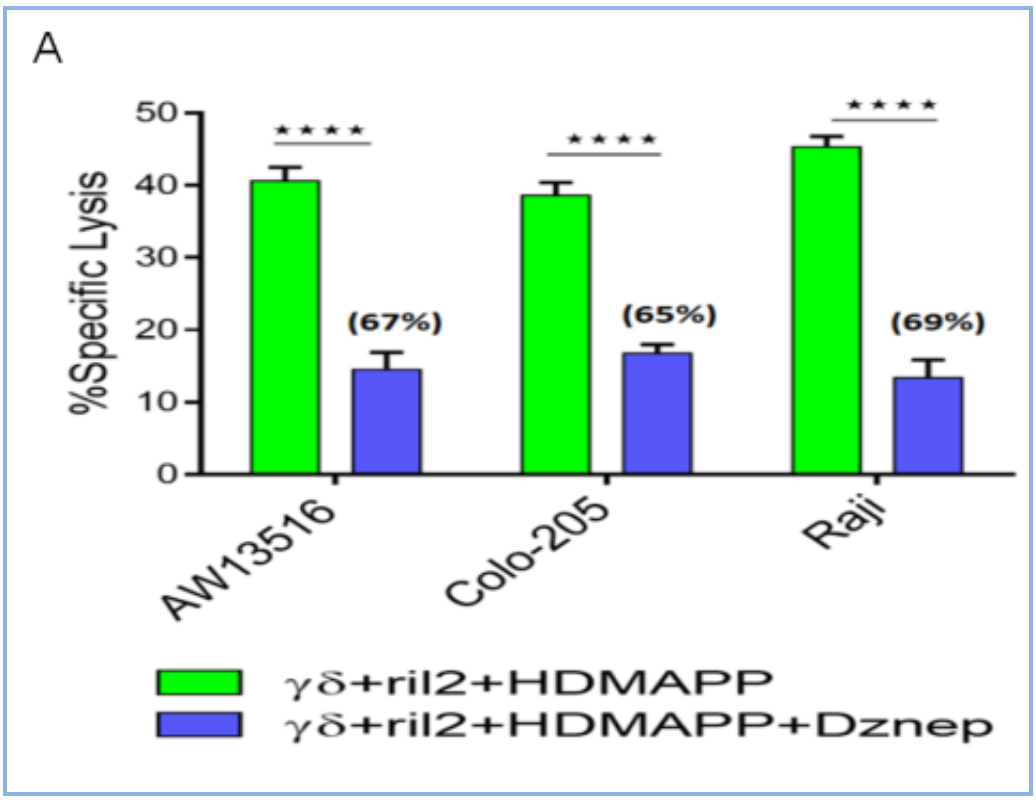

Figure 6.16: Methyltransferase Ezh2 inhibition abrogates the cytotoxic potential of γδ T cells. (A) Cytotoxic potential of γδ T cells activated with HDMAPP and rIL2 in presence and absence of methyltransferase inhibitor DZnep was assessed against three Zoledronate treated tumor targets AW13516, COLO-205, and Raji. Zoledronate tumor targets were co-cultured with γδ T cells for 4 hrs and cytotoxicity was assessed by LDH cytotoxicity assay. The values indicated above the graphs represent the percent decrease in the specific lysis compared to HDMAPP stimulated γδ T cells. The experiment was done along with the data shown in **Figure 5.15 Chapter 5**. The results indicated are percent specific lysis at the effector to target ratio of 40:1 where ****: $p < 0.00005$ when compared with γδ T cells activated with HDMAPP in presence of rIL-2 (n=3).

To further validate the methyltransferase Ezh2 regulate the TCR mediated anti-tumor potential of γδ T cells against a panel of Zoledronate treated tumor targets (AW13516, COLO 205, and Raji) we used siRNA based approach to knockdown the expression of methyltransferase Ezh2 in antigen-activated γδ T cells. The synthetic siRNA sequences targeting the methyltransferase Ezh2 were transfected into sorted γδ T cells. γδ T cells were transfected with 100 nM of methyltransferase Ezh2 and control siRNA SiGLO duplexes or left untransfected. Transfection efficiency was checked by flow cytometry. γδ T cells were 70% transfected. After 40 hours post-transfection, cells were stimulated with HDMAPP in the presence of rIL-2 for 72 hours. γδ T cells showed a decrease in the expression of methyltransferase Ezh2 expression thus confirms the knockdown of methyltransferase Ezh2 expression by siRNAs **(Figure 6.17 A).** These γδ T cells were used as effectors against above-mentioned tumor targets at 40:1 effector ratio. Knockdown of methyltransferase Ezh2 in antigen-activated γδ T cells abrogated the cytotoxic potential of γδ T cells against Zoledronate treated panel of tumor targets (AW13516, COLO 205, and Raji) **(Figure 6.17 B)**. We observed that percent decrease in specific lysis upon siRNA mediated knockdown of methyltransferase Ezh2 was 75%, 64% and 69% for the three tumor cell lines AW13516, COLO 205, and Raji respectively as compared to HDMAPP stimulated γδ T cells. The data confirms that methyltransferase Ezh2 modulates the TCR mediated effector functions of γδ T cells.

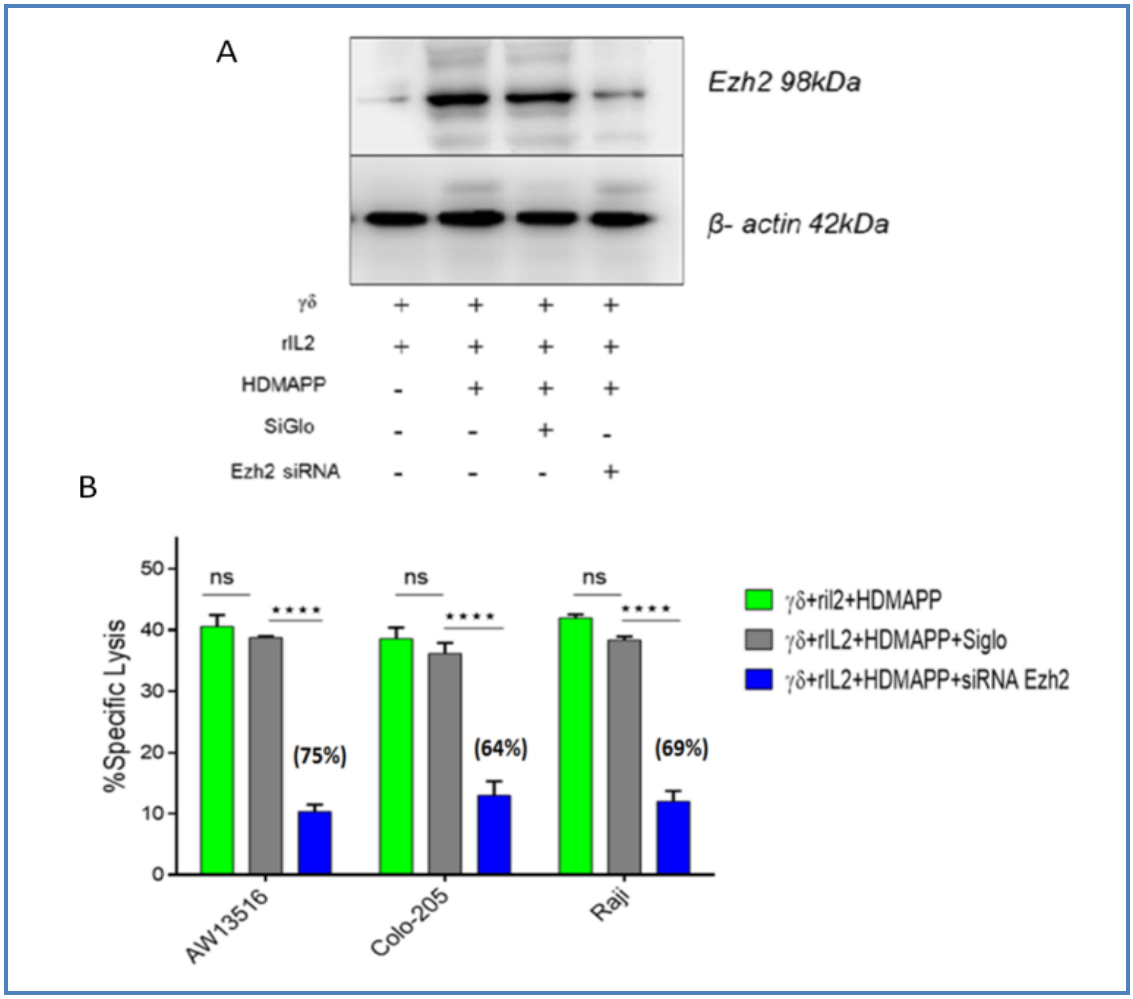

Figure 6.17: *si*RNA mediated knockdown of methyltransferase Ezh2 reduces γδ T cell cytotoxicity. γδ T cells were transfected with 100 nM of control siRNA siGLO or siRNA duplexes or left untransfected. **(A)** After 40 hrs post transfection cells of methyltransferase Ezh2 siRNA, γδ T cells were stimulated with HDMAPP for 72 hours and expression of methyltransferase Ezh2 was detected by western blotting. β-actin was used as a loading control. **(B** Cytotoxic potential of γδ T cells activated with HDMAPP in the presence of rIL-2 transfected with control siRNA siGLO or methyltransferase Ezh2 siRNA duplexes were assessed against three Zoledronate treated tumor targets AW13516, COLO-205, and Raji by LDH cytotoxicity assay. The values indicated above the graphs represent the percent decrease in the specific lysis compared to HDMAPP stimulated γδ T cells. The results indicated are percent specific lysis at the effector to target ratio of 40:1 where ****: $p < 0.00005$ and ns: not significant when compared with untransfected γδ T cells activated with HDMAPP in the presence of rIL-2 (n=3).

6.2.8: Histone modifications regulate the expression of effector molecules Perforin and Granzyme B

γδ T cells are able to rapidly exert effector functions through the immediate expression of effector molecules Perforin and Granzyme B upon encounter with antigen. Rapid expressions of effector molecules Perforin Granzyme B are crucial for anti-tumor immunity. Coordinate and heritable patterns of gene expression are often epigenetically regulated, through histone modifications of chromatin without altering the primary DNA sequence. This led us to investigate the level of transcriptionally active histone modification histone 3 lysine 9 acetylation (H3K9 acetylation) and transcriptionally repressive histone 3 lysine 9 trimethylation (H3K9 trimethylation) in γδ T cells. Sorted γδ T cells were activated with HDMAPP or plate-bound anti-CD3 mAb and rIL2 in the presence of Notch signaling inhibitor GSI-X for 72 hours. After 72 hours total histones were extracted from the γδ T cells unstimulated, stimulated with HDAMPP or anti-CD3 mAb in the presence and absence of GSI-X. Extracted histones were probed with antibodies against H3K9 acetylation, H3K9 trimethylation, and total histone H3. γδ T cells upon activation with HDMAPP or anti-CD3 mAb showed a rapid increase in the level of H3K9 acetylation compared to unstimulated γδ T cells. However upon inhibition of Notch signaling in antigen-activated γδ T cells using GSI-X led to decrease in the level of H3K9 acetylation **(Figure 6.18 A-C)** It was also observed that stimulated γδ T cells showed low levels of H3K9 trimethylation as compared to unstimulated γδ T cells and inhibition of Notch signaling in stimulated γδ T cells by GSI-X led to increasing in the level of H3K9 trimethylation **(Figure 6.19 A-C)**.

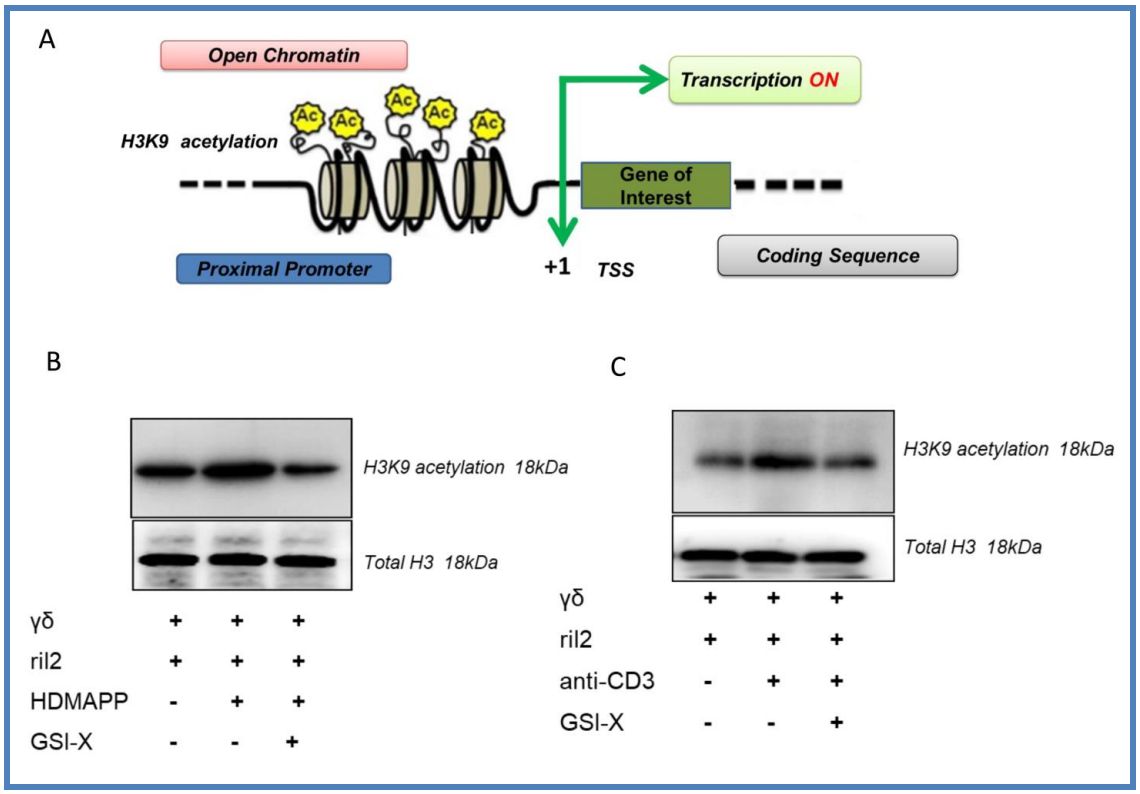

Figure 6.18: Inhibition of Notch signaling decreases H3K9 acetylation in activated γδ T cells. (A) Diagrammatic representation of transcriptional regulation of genes mediated by histone acetylation of H3 at lysine 9. Protein expression of acetylated H3K9 was detected by western blotting in γδ T cells activated with **(B)** HDMAPP and **(C)** anti-CD3 mAb in the presence of rIL-2 with or without treatment with GSI-X. Total H3 was used as a loading control. The blots are representative of three independent experiments.

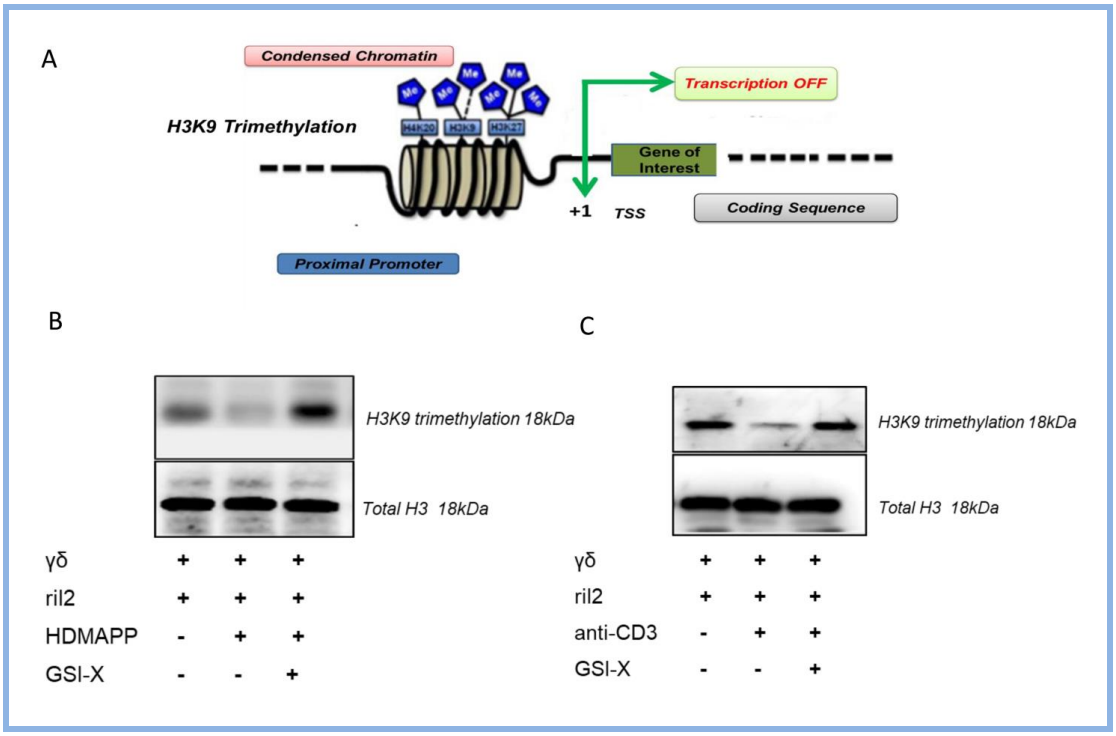

Figure 6.19: Inhibition of Notch signaling enhances H3K9 trimethylation in activated γδ T cells. (A) Diagrammatic representation of transcriptional regulation of genes mediated by histone methylation of H3 at lysine 9. Protein expression of trimethylated H3K9 was detected by western blotting in γδ T cells activated with **(B)** HDMAPP and **(C)** anti-CD3 mAb in the presence of rIL-2 with or without treatment with GSI-X. Total H3 was used as a loading control. The blots are representative of three independent experiments.

We further investigated whether there is a change in the occupancy of histone modifications H3K9 acetylation and H3K9 trimethylation on the proximal promoter regions of effector molecules perforin and Granzyme B by gene by chromatin immunoprecipitation assay. Sorted γδ T cells were activated with HDMAPP or plate-bound anti-CD3 mAb and rIL2 for 72 hours in the presence and absence of Notch signaling inhibitor GSI-X. Chromatin was isolated from these γδ T cells and was used for chromatin immunoprecipitation assay using antibodies specific for H3K9 acetylation and H3K9 trimethylation. H3K9 acetylation is transcriptionally active modification whereas H3K9 trimethylation is a transcriptionally inactive modification. γδ T cells upon stimulation with phosphoantigen HDMAPP or anti-CD3

mAb showed high occupancy of transcriptionally active modification H3K9 acetylation on proximal promoters of Perforin and Granzyme B as compared to unstimulated γδ T cells. On the other hand inhibition of Notch signaling by GSI-X in antigen-activated γδ T cells leads to decrease in the level H3K9 acetylation on proximal promoters of Perforin and Granzyme B **(Figure 6.20 A-C)** but leads to increase in the occupancy of H3K9 trimethylation transcriptionally inactive modifications on the proximal promoter regions **(Figure 6.21 A-C)**. The data is in line with our previous observation that upon activation of γδ T cells showed an increase in expression of effector molecules and inhibition of TCR mediated Notch signaling in γδ T cells decreased the expression of these effector molecules. Thus the data explains that TCR mediated Notch signaling controls the effector molecules and thereby the effector functions of γδ T cells through epigenetic mechanisms.

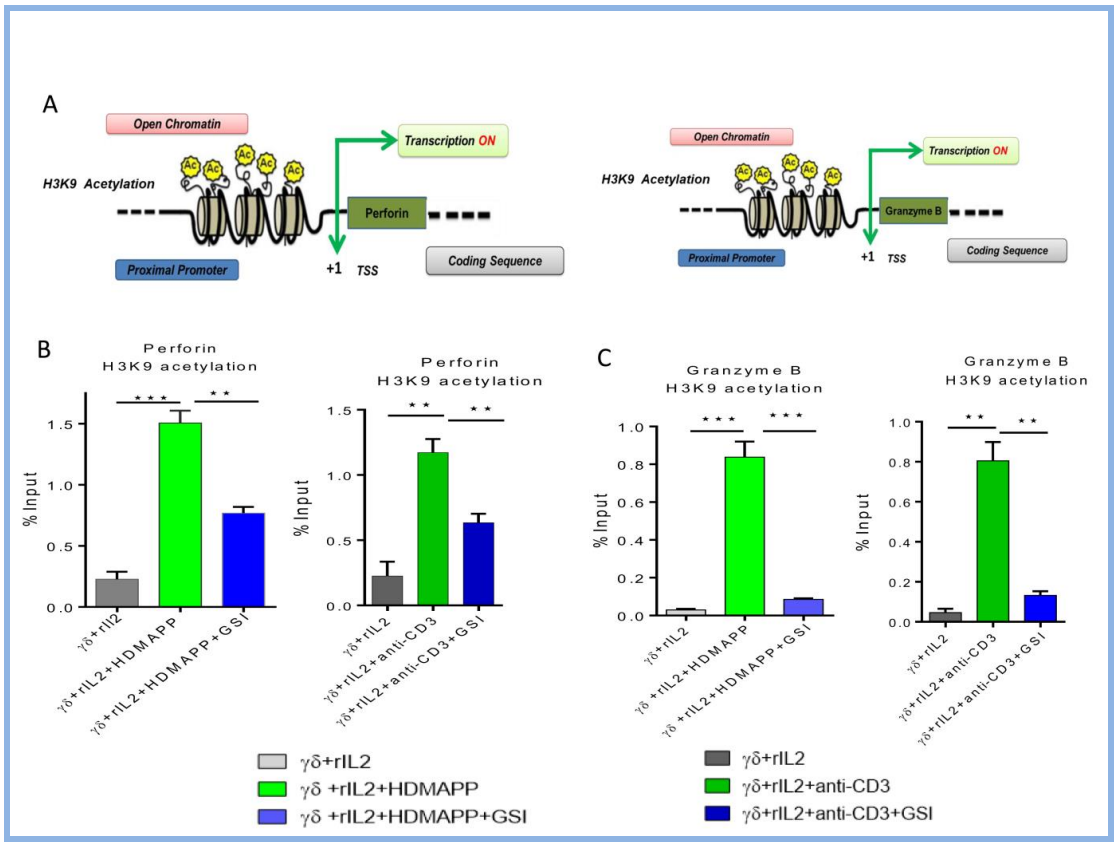

Figure 6.20: H3K9 acetylation level decreases on Perforin and Granzyme B promoters upon Notch inhibition in γδ T cells. (A) Diagrammatic representation of transcriptional regulation of Perforin and Granzyme B expression mediated by acetylation of histone 3 at lysine 9. **(B)** Chromatin immunoprecipitation was used to analyze the acetylation of H3K9 on the proximal promoter region of Perforin in γδ T cells activated with rIL-2 and HDMAPP or anti CD3 mAb in the presence or absence of GSI for 72h. **(B)** Similarly, acetylation status of H3K9 on the proximal promoter region of Granzyme B in HDMAPP or anti-CD3 mAb activated γδ T cells the presence of rIL-2 with or without GSI treatment for 72h Enrichment of Perforin and Granzyme B promoter regions was quantified by qPCR and expressed as a percentage of input DNA. The results indicated are mean $\pm$ where **: $p < 0.005$, ***: $p < 0.0005$a and are representative of three independent experiments.

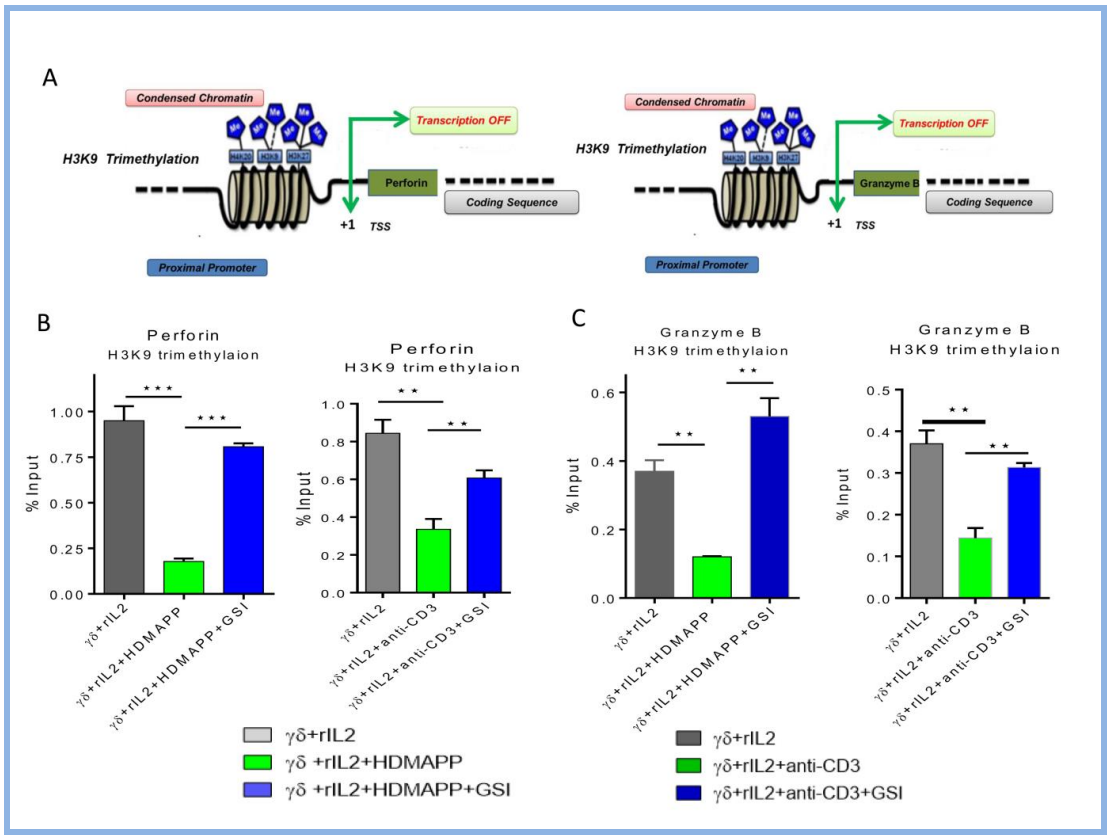

Figure 6.21: Inhibitors of Notch signaling increase repressive histone modification on Perforin and Granzyme B promoters in activated γδ T cells. (A) Diagrammatic representation of transcriptional regulation of Perforin and Granzyme B expression mediated by methylation of histone 3 at lysine 9. **(B)** Chromatin immunoprecipitation was used to analyze the trimethylation of H3K9 on the proximal promoter region of Perforin in γδ T cells activated with rIL-2 and HDMAPP or anti CD3 mAb in the presence or absence of GSI-X for 72h. **(B)** Similarly, the methylation status of H3K9 on the proximal promoter region of Granzyme B in HDMAPP or anti-CD3 mAb activated γδ T cells the presence of rIL-2 with or without GSI treatment for 72h. Enrichment of Perforin and Granzyme B promoter regions was quantified by qPCR and expressed as a percentage of input DNA. The results indicated are mean ± where **: $p < 0.005$ ***: $p < 0.0005$ and are representative of three independent experiments.

Chapter 7
Discussion

Discussion:

Cancer Immunotherapy is gaining considerable attention as it is considered as an attractive strategy for the treatment of cancer among the existing treatment modalities that is surgery, radiotherapy, and chemotherapy. It aims to capture the specificity and memory of the Immune system. The main objective of cancer immunotherapy is to enhance the anti-tumor responses so that tumor cells can be easily eliminated [399, 400]. The classical approaches for the treatment of tumors such as chemotherapy, radiotherapy and surgical removal of tumor tissue either lack specificity or tumors still make a way to metastasize to other sites. On the other hand, immunotherapy can potentially attack both primary tumors and metastasized tumor cells [401-404]. Adoptive immunotherapy is accomplished by expanding immune effector cells *in vitro* and transferring the activated immune cells into the hosts, that target tumor cells or stimulate immune response to eliminate tumor cells[405]. Adaptive immunotherapy depends on understanding the diversity of the adaptive Immune repertoire and utilizing well-defined specificities for the therapeutic interventions making it a personalized cancer treatment.

γδ T cells, the second lineage of T cells that express a unique somatically recommended γδ TCR are unconventional T cells that overcome the limitations of adaptive immunotherapy. Due to attractive roles in immune-surveillance and immune defense against different diseases like cancer. γδ T cells and have become attractive players for cancer immunotherapy [164, 406]. γδ T cells mediate anti-tumor therapy mainly by secreting pro-apoptotic molecules and inflammatory cytokines, through a TCR-dependent pathway [407]. Due to these features γδ T cells are making their way into clinical trials. γδ T cell-based immunotherapies are well tolerated and efficient [44]. They have been extensively targeted against diverse tumors such as melanoma,

renal cell carcinoma, as well as B cell malignancies and have shown promising results in clinical settings[167, 408]. While these therapies have encountered modest clinical success, they have to overcome certain challenges such as limited availability of γδ T cells and rapid exhaustion upon repeated in vitro activation. Hence, combinational approaches have been envisaged with chemotherapeutics, monoclonal antibodies, small molecule inhibitors, etc. Newer treatment modality may include combining γδ T cell immunotherapy with antitumor drugs and other immune-modulating antibodies.

Epigenetic deregulation is one of the hallmarks of cancer. Deregulation of epigenetic modifiers such as histone deacetylases is the main cause of tumor formation. Epigenetic therapy has emerged as a hot issue in cancer research. Hence, epigenetic modifiers such as HDAC inhibitors are being comprehensively explored for their anticancer potential. HDAC inhibitors are under intensive investigation for cancer therapy. Different structurally disparate HDAC inhibitors are under different stages of clinical trials. Besides anticancer properties, HDAC inhibitors have also shown promising results in controlling the other pathological conditions such as neurological disorders and viral infections and are well tolerated [409, 410]. Currently, VPA along with other short-chain fatty acids HDAC inhibitors are being clinically evaluated as anticancer drugs (47). HDAC inhibitors employ a wide range of antitumor mechanisms such as induction of apoptosis, senescence, differentiation, or inhibition of cell cycle [411, 412]. Vorinostat (SAHA), is among the first HDAC inhibitor to be approved by the United States Food and Drug Administration (FDA) for the treatment of relapsed and refractory cutaneous T-cell lymphoma [278]. HDAC inhibitors have been approved for the treatment of hematological malignancies, but the mechanistic. However, clear proof-of-concept data for the clinical efficacy of HDAC inhibitors in solid tumors remains to be established [413]. Although the HDAC inhibitors have

shown the anti-tumor effects the efficacy of HDAC inhibitors is low when used as monotherapy. Studies have demonstrated that HDAC inhibitors exhibit higher therapeutic efficiency when combined with other antineoplastic agents [67]. Hence, there is a growing interest in exploring other combined therapeutic strategies with HDAC inhibitors.

HDAC inhibitors are the potent inducers of growth arrest and differentiation in tumor cells. Besides tumor cells emerging evidence suggests that HDAC play a crucial role in T cell differentiation and effector functions [414]. In severe inflammatory pathological conditions targeting the HDACs have shown to suppress the immune responses of T cells and thus maintain the tolerance. Thus due to this property targeting the HDACs are useful in organ transplantation [414]. Specifically, HDAC inhibitors have shown to induce the regulatory T cell (Tregs) generation or stabilization of Tregs in the inflammatory microenvironment. Regulatory cells are important T cell subtype that maintains the immune suppression. Due to this HDAC inhibitors have shown promising responses in experimental colitis [415]. The effects of HDAC inhibitors on the function of the immune system seem to be diverse and context-dependent.

HDAC inhibitors increase the immunogenicity of tumors by increasing the expression of tumor antigens recognized by the immune cells. The antitumor responses of cytotoxic T lymphocytes like γδ T cells are mediated through recognition of stress molecules (ULBP, HSPs) or danger signals like MICA/B expressed on tumor cells by a class of activating receptors known as NKG2D[416-418]. Studies have demonstrated that HDAC inhibitors upregulate the NKG2D ligands on tumor cells, thereby sensitizing tumor cells to cytotoxicity mediated by γδ T cells in bladder cancer as well as NK cells in other malignancies such as osteosarcoma, pancreatic

cancer, and multiple myeloma[74, 292, 419, 420]. However, the causal effect of HDAC inhibitors on the immune scenario is not well investigated and is contradictory. Several studies have shown that HDAC inhibitors affect each immune subset distinctly either leading to activation as in the case of CD4 T cells and CD8 T cells or by abrogating the effector functions of cells such as NK cell [382, 421, 422]. Furthermore, for a particular immune cell type, the nature of immune regulation differs based on the type of HDAC inhibitor. A recent study demonstrated that NKG2D expression in NK cells is inhibited by VPA[306]. Most of the studies have focused on investigating the impact of HDAC inhibitors on tumor cell lines and immune cells other than γδ T cells. Report by Suzuki et al. demonstrated that the antitumor effect of γδ T cells on bladder cancer was enhanced by treatment with VPA [74]. The study focuses only on the impact of HDAC inhibitor, VPA on bladder cancer cell line. VPA leads to an increase in the expression of MICA and MICB, which are recognized by the NKG2D receptor on γδ T cells. An earlier study by Kabelitz et al. reported that HDAC inhibitor VPA induces differential modulation of cell surface markers on γδ T cells compared to αβ T cells [75]. Although the study shows the direct effect of VPA on γδ T cells, the functional responses of γδ T cells were not investigated in detail.

The present thesis, we have used three different HDAC inhibitors to delineate their effect on the functional responses of a pure and sorted population of γδ T cells. We used clinically relevant concentrations of VPA, TSA, and SAHA in our study, which have also been used in in vitro studies by other investigators [423, 424]. We showed that three different HDAC inhibitors used suppressed the antitumor effector functions of γδ T cells. We observed that γδ T cells activated with the phosphoantigen, HDMAPP in the presence of HDAC inhibitors showed decreased proliferative

potential. One of the mechanisms by which HDAC inhibitors exhibit their anticancer properties is through the induction of cell differentiation and cell cycle arrest at G1 phase [411, 412]. Besides affecting histone proteins, these inhibitors also have several non-histone protein substrates like p53, p21, Rb, and E2F1 in tumors [425, 426]. On the other hand, it was demonstrated that down-modulation of p53 in T cells enhances their antigen-specific proliferative response and also augments antitumor cytotoxic functions [427, 428]. Studies from our lab have shown that CD3-activated T cells upon activation show robust proliferative capacity and decreased expression of p53 and its downstream target p21 [429]. Thus, the decrease in the antigen-specific proliferative response of γδ T cells in presence of HDAC inhibitors incited us to look for the effect of HDAC inhibitors on cell cycle progression and expression of cell cycle regulators p53 and its downstream target p21. The decrease in the proliferation of γδ T cells in the presence of HDAC inhibitors was associated with the increase in the expression of p53 and its downstream target p21. γδ T cells show increased expression of activation markers CD69,CD71,CD25 and phospho-tyrosine when activated with phosphoantigens [378, 430, 431]. We observed that HDAC inhibitors inhibit the expression of CD69, CD25, CD71 activation markers, and phospho-tyrosine. CD25 is the high-affinity IL-2 receptor subunit and IL-2 signaling is necessary for the proliferation of T cells. In our study, HDAC inhibitors not only decreased the expression level of CD25 but also other two subunits of IL-2 receptor CD132 and CD122. It would be logical to conclude that HDAC inhibitors abrogate the IL-2 signaling and thus inhibit the proliferation of γδ T cells. We have used three different HDAC inhibitors VPA, TSA, and SAHA at different concentrations and they showed varied effects on the expression of all the γδ T cell markers we studied. The

likely explanation for the differences observed in their effects could be their structural diversity and also the biological activities they exert may be cell-type dependent [64]. Activated γδ T cells express T-bet and eomesodermin (Eomes) transcription factors. The T-box transcription factors T-bet and Eomes are important for the acquisition of effector functions in cytotoxic T cells including γδ T cells [389, 432]. Eomes and T-bet are highly homologous transcription factors and have cooperative and redundant functions in regulating the expression of different genes involved in the effector functions of CD8 T cells and activated natural killer cells. T-bet and Eomes regulate the expression of Perforin, Granzyme-B, and IFN-γ by binding to promoter regions of these effector genes [11, 388]. Knowing that HDAC inhibitors decrease the activation and proliferation of γδ T cells, we further hypothesized that HDAC inhibitors may modulate the cytotoxic effector functions of γδ T cells by affecting the expression of transcription factors Eomes and T-bet. We observed that treatment of γδ T cells with HDAC inhibitors lead to a decrease in the expression of Eomes and T-bet. To further establish the impact of HDAC inhibitors on the antitumor cytotoxic function of γδ T cells, we used a panel of tumor cell lines (AW13516, COLO-205, and Raji) treated with Zoledronate as tumor targets in the cytotoxicity assay. Previous work from our laboratory and others has demonstrated that tumor cells treated with Zoledronate are aggressively killed by γδ T cells [7, 433]. Our data demonstrated that treatment of HDAC inhibitors retards the ability of γδ T cells to kill Zoledronate-treated tumor targets. Further, we proved that this inhibition of cytotoxic potential of γδ T cells was due to a decrease in the expression of effector molecules Perforin and Granzyme-B in these cells.

For T cell activation, three different signals signal 1 (antigen recognition), signal 2 (co-stimulation) and signal 3 (cytokine priming) are necessary[434]. Co-stimulation

can be either through activating receptors or through inhibitory receptors (Immune checkpoints) [435-437]. The activation of T cells initiated through the T cell receptor is regulated by a balance between co-stimulatory and inhibitory signals. The imbalance between these signals leads to different pathological conditions like tumor [438]. The use of biological agents based on blocking/neutralizing antibodies which interfere with the negative signaling in T cells have improved tumor treatment and revolutionized the field of immunotherapy [435]. For examples Rituximab (B cell-depleting antibody used for leukemia, rheumatoid arthritis, lupus), Infliximab (TNF-alpha-neutralising antibody used in rheumatic diseases) or Ipilimumab (blocking/depleting anti-CTLA4 antibody, used in cancer) [439-442]. Majority of the tumors use these immune checkpoints such as PD-1 or its ligand PD-L1 to escape from the immune surveillance. Immune checkpoint inhibitors have revolutionized the field of tumor immunotherapy[443]. Besides surgery, radiation, and chemotherapy, immune checkpoint inhibitors have surfaced as an important immunotherapeutic approach for cancer treatment. Due to their promising antitumor effects in experimental animal models, preclinical studies and successful clinical trials, immune checkpoint inhibitors have been now approved by the U.S Food and Drug Administration (FDA) for treatment of different malignancies. PD-1/PD-L1 blocking strategy has led to tumor regression in patients with melanoma, renal cell carcinoma, nonsmall cell lung cancer, and bladder cancer [178, 179, 184, 444, 445].

Recent reports have shown that tumors associated with PD-1 expressing NK cells show poor survival. PD-1/PD-L1 signaling axis along with NKG2D signaling axis determines the effector response of NK cells. Blockade of PD1/PD-L1 signaling cascade in NK cells along with other antitumor drugs has shown promising responses in cancer patients [177, 446]. This study supports our observation that HDAC

inhibitors modulate the effector functions of human γδ T cells against tumors via PD1/PD-L1 signaling axis. We observed that γδ T cells show increased expression of immune checkpoints PD1 and PD-L1 upon HDAC inhibitor treatment.

A report by Garcia-Diaz et al. has shown that induction of PD-L1 and PD-L2 on tumor cells is regulated via IFN- γ [447]. In the present study, we have observed that HDAC inhibitors decreased the expression of IFN- γ, and TNF-α in antigen-activated γδ T cells. It has been demonstrated that T-bet transcription factor binds to PD-1 promoter and mediates the suppression of PD-1 expression [448]. In the present study, we have shown that upon HDAC inhibitor treatment of γδ T cells, T-bet protein and mRNA is decreased significantly indicating that less T-bet may be available to bind PD-1 promoter to suppress PD-1 expression. This explains, the IFN- γ independent mechanism of PD-1 expression on γδ T cells. Activated γδ T cells are known to express PD-1. Iwaski *et al*. found that γδ T cells express PD-1 rapidly from day 3 of induction and PD-1^{+} γδ T cells exhibit attenuated effector functions and decreased cytotoxicity against PD-L1 expressing tumors. However, they observed that Zoledronate treatment to tumor cells, which induces IPP to release along with PD-L1 blockade, rescued the γδ T cell cytotoxicity [138]. While our study also confirms that blocking of PD-1 in γδ T cells increases the antitumor cytotoxic potential, our study reports on the effect of HDAC inhibitors on the freshly isolated γδ T cells activated with antigen for 72 h, whereas Iwaski group used γδ T cells which were already in an activated state for their experimental purposes. Another interesting study by Castella et al. explores the multifunctional role of Zoledronate in augmenting γδ T cells responses against multiple myeloma. In this study, Zoledronate-treated autologous DCs were found to efficiently activate γδ T cells and enhance their cytotoxic functions against myeloma cells. Additionally, Zoledronate was also shown to

promote antitumor immunity via suppression of regulatory T cell function, downregulation of PD-L1 expression on DCs, and increased proliferation of tumor antigen-specific CD8 T cells. Although their study has effectively demonstrated the role of Zoledronate in enhancing antitumor responses γδ T cells, it is specific only to multiple myeloma and uses Zoledronate expanded γδ T cells from patient PBMNCs [449]. Converse to our observation, they found that DC-activated γδ T cells did not express PD-1, which might be due to the immune modulation by Zoledronate, which needs further exploration. In our study, we observed that blockade of PD1/PD-L1 signaling partially restores the antitumor cytotoxic function of γδ T cells in the presence of HDAC inhibitors, which reflected in increased expression of effector molecules Granzyme B and Lamp-1. Wei et al. Have demonstrated that PD-1 ligation dramatically shifts the dose-response curve, making $CD8^+$ T cells much less sensitive to TCR generated signals [450]. Although this was shown in $CD8^+$ αβ T cells, it may also apply to γδ T cells. Thus, PD-1 ligation affects TCR signaling and thereby reduces the cytotoxic function of γδ T cells. The role of other activating receptors such as NKG2D interacting with MICA/B and inhibitory receptors KIR2DL2/3 (CD158b) cannot be ignored and it explains the incomplete restoration of cytotoxic effector function γδ T cells upon PD-1 blocking. Our results implicate that HDAC inhibitors along with the immune checkpoint modulating antibodies could be developed as a combination immunotherapy with γδ T cells to treat different malignancies.

In our attempt to further investigate the various cues that influence γδ T cell effector functions we decided to look more closely at TCR signaling events in γδ T cells and other co-stimulatory molecules that may influence this cross talk. The initiation of T cell activation depends upon the plethora of signaling events. The initial T cell antigen

receptor (TCR) signaling is a key step that is essential for T cell activation. Besides the TCR signaling T cell activation also depends on the different co-stimulatory signaling cascades [451]. Initiation of signaling cascades through TCR and co-stimulatory receptors results in the activation and the orchestration of different immune responses. Interaction of antigen with TCR and co-stimulation results in the initiation of different signaling cascades that lead to the expression of different cytokines, chemokines, effector molecules and different activating receptors like NKG2D, NOTCH and TLR, etc [429, 452, 453]. The data presented provide compelling evidence that Notch signaling in γδ T cells is a critical factor in determining the effector anti-tumor immune responses. The study identifies Notch and IL2 as additional signals that regulate the TCR mediated effector function of γδ T cells

During the last several years, published experimental literature has shown that Notch signaling plays an important role during the development stages of T lymphocytes [454, 455]. The notch may also influence other stages of T cell development, including the CD4 vs CD8 and the TCR alpha beta vs gamma delta lineage choices [456-458]. Accumulating data have provided evidence that Notch signaling plays an important role in the differentiation of mature $CD4^+$ T cells [204, 225, 459]. It is well known that TCR mediated signal in CD8 T cells leads to the expression of Notch receptors and ligands [230]. Earlier data from our lab has shown that Notch signaling regulates cytotoxic effector functions of γδ T cells [378]. Further interaction between Notch receptor on CD8 T cells and its ligands on antigen presenting cells is necessary for the effector responses of CD8 T cells [460]. Our data reported in the thesis reveal multiple, previously unrecognized roles for Notch signaling in human γδ T cell effector functions. We observed that Notch receptor and ligand expression is rapidly

induced following γδ T cell activation by phosphoantigen HDMAPP or by plate-bound anti-CD3 mAb. Further, antigen activation in γδ T cells leads to an increase in the expression of downstream Notch target genes as compared to unstimulated γδ T cells. Pharmacologic inhibition of Notch signaling in γδ T cells showed decreased expression of both Notch receptors and ligands which was also reflected in the expression of downstream Notch target gene HES1, Nf-κB, Deltex, and NRARP. Our data support a model in which TCR engagement causes Notch activation and subsequent up-regulation of Notch protein. Thus, our study establishes that TCR mediated signaling in γδ T cells up-regulates the Notch receptor on γδ T cells and there is dynamic crosstalk between Notch signaling and TCR mediated signaling cascades in human γδ T cells.

TCR and co-stimulatory signals are necessary for mediating the T cell effector responses. In the presence of a weak primary signal (TCR signal), sustained co-stimulatory signals are required for T cells to acquire effector functions [461]. In classical αβ T cells, CD28 leads to an increase in the expression of IL-2 which through autocrine mechanism further increases the immune response. The regulation of co-stimulatory signaling is dependent on other signaling cascades like Notch [462]. Our study showed that antigen priming of γδ T cells in the presence of co-stimulatory IL-2 causes increased proliferation and leads to an increase in the expression of Notch receptors. Inhibition of Notch signaling in γδ T cells decreases the proliferative potential of γδ T cells and causes cell cycle arrest. This decrease in the proliferation was p53 and p21 dependent. Thus our data indicate that combined signals generated from Notch receptors and TCR regulate the γδ T cell proliferative response. The decrease in proliferative potential of γδ T cells upon inhibiting Notch signaling by treatment with GSI-X was a due abrogation of the IL-2 signaling pathway. Further

inhibition of Notch signaling also inhibited both proximal and distal IL-2 signaling molecules. Thus our data confirms Notch signaling can influence the strength of TCR signaling through the modulation of co-stimulatory IL2 signaling and thus influences the threshold of activation.

Activation of γδ T cells with phospho antigens causes up-regulation of different activation markers [395, 463]. The observation that Notch regulates the antigen-driven proliferation of γδ T cells prompted us to look at the expression of different activation markers on these cells. Inhibition of Notch signaling in antigen-activated γδ T cells abrogates the expression of early activation (CD69), late activation (CD25) and phospho-tyrosine levels. CD25 (IL-2R) is the high-affinity IL-2 receptor subunit. The decrease in the expression of these markers suggests that Notch signaling effects the distal signaling events in γδ T cells. Upon the antigen stimulus, T cells show an increase in the level of calcium. Calcium acts as a secondary messenger which activates different kinases [464]. We observed that inhibition of Notch signaling in γδ T cells decreased the level of calcium. The data further confirms that Notch modulates the TCR driven signaling in γδ T cells.

Many direct target genes are transcription factors that regulate the second tier of Notch-responsive genes, which in lymphocytes include glucose transporter (Glut1) and iron transporter (CD71). The highly proliferating T cells depend upon the continuous supply of nutrients. One mechanism for this regular supply of nutrients is through the upregulation of different nutrient transporters such as Glucose transporter (Glut1) and iron transporter (CD71). The expression of these transporters is TCR signaling dependent [465, 466]. Our data shows that highly proliferating γδ T cells in response to antigenic stimulation show an increase in the level of iron transporter, CD71. Inhibition of Notch signaling in γδ T cells leads to a decrease in the expression

of CD71. These results indicate that Notch signaling indirectly controls the proliferation of γδ T cells by regulating the expression of nutrients transporters.

Upon activation, T cells undergo activation-induced metabolic changes to meet the demands of proliferation and effector functions. The phosphatidylinositol- 3-OH kinase (PI3K) and mammalian target of rapamycin (mTOR) pathways have emerged as key regulators of T cell antigen priming. Activation of the PI3K pathway, downstream of TCR and IL2, increases nutrient uptake and stimulates the synthesis of proteins, lipids, and nucleotides, which are prerequisites for IL-2 secretion and cell division [462, 467]. We have shown that Notch signaling inhibits both the proximal and distal IL-2 signaling which leads to a decrease in the proliferative potential of γδ T cells.

Sustained T cell activation requires different transcription factors like NFATc1, AP-1, and Nf-κB which lead to the acquisition of effector functions [468]. These transcription factors are in turn regulated through cross-talk with other signaling cascades. A study demonstrated that Wnt signaling downstream mediator GSK- 3β, regulated NFATc1 in T cells, where Phosphoinactivation of GSK- 3β is necessary for nuclear retention of NFATc1 which is required for maintaining effector state of T cells [469]. These transcription factors mediate effector functions in T cells by directly regulating the IL-2 locus. Whereas a separate study showed that differential Notch ligand interaction is essential for the regulation of IL-2 signaling and maintains sustained IL-2 signal via inactivation of GSK- 3β through activation of the PI3K pathway. Thus, TCR and CD28 signaling directly mediate phosphorylation and inactivation of GSK-3, as active GSK-3β inhibits proliferation of T cells [470, 471]. In the present study, we observed that Notch inhibition in activated γδ T cells lead to increased levels of active GSK-3β thereby diminished effector responses reflected in

reduced expression of Granzyme B. However, the effector functions were restored upon pharmacological inhibition of GSK-3β using CHIR. Thus, these results indicate that Notch signaling potentiates effector functions in γδ T cells via regulation of GSK-3β.

Activated γδ T cells are known to secrete copious amounts of IFN-γ and TNF-α [39]. γδ T cells provide an early source of IFN-γ in tumor immunosurveillance and against viral challenge[472, 473]. It has been shown that Notch1 plays a critical role in the expression of IFN-γ in $CD4^+$ and $CD8^+$ T cells [473]. The data presented in the present study demonstrates a role for Notch signaling in IFN-γ and TNF-α production in antigen-activated γδ T cells. It has been observed in $CD8^+$ T cells that Notch regulates the binding of different transcription factors to the promoter region of these cytokines and regulates their expression [230]. We observed that inhibition of Notch signaling in γδ T cells leads to abrogation TCR mediated expression effector cytokines IFN-γ and TNF-α. Thus the results indicate that Notch signaling is indispensable for effector responses of human γδ T cells.

A functional γδ T cell response is an essential component of the immune response against many pathogens and infected cells. Upon engagement, with antigen γδ T cells rapidly expand and produce effector molecules Perforin and Granzyme B. γδ T cells and cytotoxic T cells mediate the effector functions through granule exocytosis pathway that utilizes the pore-forming molecule, Perforin and the Granzyme B a serine protease that induces apoptosis [173]

Notch signaling has been implicated in the regulation of expression of Perforin and Granzyme B, in NK or $CD8^+$ T [230, 474, 475]. It has been demonstrated in CD8 T cytotoxic cells that Notch directly binds to effector molecules Perforin and Granzyme B [476]. However, the regulation of expression of Perforin and Granzyme B in γδ T

cells has not been well-studied. Here we show that Notch signaling in γδ T cells specifically regulates the TCR mediated expression of effector molecules Perforin and Granzyme B. The data presented shows that, in γδ T cells, stimulation with HDMAPP or anti-CD3 mAb, increase the levels of Perforin and Granzyme B expression and blockade of Notch signaling by GSI-X results in reduced expression of these molecules. Moreover, we observed that specific silencing of either Notch1 or Notch2 by siRNA led to the reduced expression of Perforin and Granzyme B in antigen-activated γδ T cells. The previous study from our lab has shown that inhibition of Notch signaling in γδ T cells decreases the cytotoxic potential of γδ T cells against Zoledronate treated tumor targets [7]. The present data further confirms that the decrease in the cytotoxic potential of γδ T cells against Zoledronate treated tumor cells after inhibiting Notch signaling is due to a decrease in the expression of effector molecules Perforin and Granzyme B.

The effector functions of γδ T cells at transcription level are regulated by two main transcription factors Eomes and T-bet. Eomes and T-bet belong to the family of T-box transcription factors and shares 74% homology with each other [388]. The differentiation of naïve $CD8^+$ T cells into cytotoxic effector T cells are regulated by the Eomes and T-bet transcription factors. Eomes and T-bet were shown to induce expression of IFN-γ, Perforin and Granzyme B in CD8 T cells [230, 388]. We observed that activation of γδ T cells with antigen HDMAPP increases the expression of Eomes and T-bet. Inhibition of Notch signaling in antigen-activated γδ T cells by GSI-X decreases the expression of these effector transcription factors which was further confirmed by specific silencing of Notch1 or Notch2 by siRNA. It has been demonstrated that Notch regulates the expression of Eomes and T-bet through directly binding on the promoters of these effector transcription factors [477].

Here we reveal a novel role for Notch signaling in regulating the effector functions of antigen-activated γδ T cells through the modulation co-stimulatory IL-2 signaling and TCR signaling pathway. This direct regulation of Perforin, Granzyme B, Eomes, T-bet and IFN-γ in γδ T cells by Notch may contribute to the effective clearance of tumor cells and other infected pathogens by γδ T cells. Identifying this critical function of Notch signaling in human γδ T cells expands our insight into different pathological diseases like a tumor and may also be useful in the designing of novel therapies for these diseases.

The transition from resting to the activated state upon antigenic stimulus is an essential step for γδ T cells to acquire anti-tumor effector function. To leave the resting state γδ T cells requires TCR signal provided by phospho antigen (HDMAPP) or any other agonist antibodies such as anti-CD3 mAb and co-stimulatory signal (IL2). Upon antigenic stimulus, γδ T cells show expression of different cytokines, effector molecules and transcription factors which are essential to attain the effector state[478, 479]. In order to acquire the effector state resting T cells reqthe uires dramatic changes of chromatin around the key genes involved in anti-tumor effector functions without changing the coding DNA sequence[480, 481]. Downstream events of different signaling pathways are regulated by epigenetic modifications of the histones. The tails of histone proteins undergo different complex and coordinated posttranslational modifications like histone acetylation, methylation, phosphorylation, and ubiquitination [482, 483]. Histone modifications are reversible in nature and are catalyzed by different epigenetic modifiers such as histone acetyltransferases and histone methyltransferases [484]. In the present thesis, we decided to interrogate the role of methyltransferase Enhancer of Zeste Homolog 2 (Ezh2) an epigenetic modifier in regulating the effector functions of γδ T cells.

To identify the molecular mechanism how effector responses are regulated in γδ T cells our findings reveal unpredicted but essential roles of epigenetic modifier Ezh2 in regulating the effector functions. Ezh2 catalyzes H3K27me3 and acts primarily as a gene silencer [313].Previously it has been demonstrated that expression of Ezh2 correlates with proliferation, differentiation, and survival of antigen activated T cells [363, 485]. It has been demonstrated that naïve T cells expressed low levels of Ezh2 but on receiving the TCR signal naïve T cells rapidly upregulated Ezh2[486, 487]. Our findings elucidate that activation of γδ T cells with phospho antigen HDMAPP or anti-CD3 mAb in the presence of rIL2 showed increased expression of Ezh2 as compared to unstimulated γδ T cells. It appears that the expression of Ezh2 in γδ T cells is mediated through TCR signaling. 3-Deazaneplanocin A (DZNep) decreases the expression of Ezh2 in antigen-activated γδ T cells. Inhibition of Ezh2 inhibits the antigen HDMAPP and anti-CD3 mAb mediated proliferation of γδ T cells. These observations demonstrate that TCR mediated expression of Ezh2 in necessary for antigen-driven proliferation of γδ T cells.

The polycomb proteins Enhancer of Zeste Homolog 1 (Ezh1) and Ezh2 form two closely related PRC2 complexes (polycomb protein complex) and are required for maintenance of cellular identity at several stages of development[488]. It has been demonstrated that Ezh2 regulates the Notch signaling and thus determines the cell, fate[362, 364]. Ezh2 has been also associated with the progression of cancer through the induction of cell cycle and inhibition cell differentiation. It has been demonstrated the Ezh2 alters the immunogenicity of the tumor microenvironment. A study by Pang et al has shown that inhibition of Ezh2 and DNA methyltransferase 1 (DNMT1) in a mouse model of ovarian cancer results in increased expression Th1-type chemokines CXCL9 and CXCL10 in cancer cells, leading to the trafficking of CD8 effector T

cells to the tumor, thus decreasing the tumor volume. Conversely, increased expression of Ezh2 leads to decreased CD8 effector T cells in the tumor thus increased the tumor volume [367]. In contrast to this study, Zhao et al demonstrated that down-regulation or inhibition of Ezh2 in tumor-specific T cells increases the tumor burden and the metastatic potential in mouse models of ovarian cancer and melanoma, respectively. The study also demonstrated that high percentage $Ezh2^{+}$ $CD8^{+}$ T cells in the ovarian cancer tissues are a stronger predictor of overall and progression-free survival compared to the low percentage of $Ezh2^{+}$ $CD8^{+}$ T cells. These data support the hypothesis that EZH2 has a key role for the function of tumor-specific effector T cells, while cancer can evade tumor surveillance by targeting T-cell specific expression of Ezh2 in the tumor microenvironment [371]. Both Notch signaling and Ezh2 have been associated with the differentiation of different subtypes of CD4 T cells [227, 228]. Human αβ T cells on TCR mediated activation, show synchronous expression of Ezh2 and the intracellular domain of Notch (NICD) [371, 489]. We observed that γδ T cells upon activation with HDMAPP and anti-CD3MAb showed increased expression of Notch1, Notch2, and Ezh2. Further, we observed that functionally active Notch intracellular domain (NICD) and Ezh2 were synchronously expressed in γδ T cells upon TCR mediated activation. Inhibition of Ezh2 in antigen-activated γδ T cells by DZnep decreased the expression Notch 1 and Notch2 receptors. It was interesting to note that inhibition of Ezh2 leads to abrogation of Notch signaling in antigen-activated γδ T cells as seen by a decrease in the level of functionally active Notch intracellular domains (NICD). Abrogation of Notch signaling in antigen-activated γδ T cells by inhibition of EZh2 also suppressed the expression of Hes1, Hey2, and NRARP, which are direct targets of Notch signaling.

These observations indicate that Ezh2 promotes the TCR mediated activation of Notch signaling in antigen-activated γδ T cells.

Activation of Notch signaling in γδ T cells leads to release of Notch intracellular domain that along with different epigenetic modifiers like p300 modulates the gene expression[231]. It has been demonstrated that stability and availability Notch intracellular domain determines the functional outcome of Notch signaling. The stability and availability of the Notch intracellular domain are regulated by two important mediators Numb and Fbxw7 [371]. Numb and Fbxw7 are negative regulators of Notch signaling as they decrease the availability of the Notch intracellular domain by interacting with it or by proteasomal degradation [488, 490]. Our data shows γδ T cells upon activation with HDMAPP or anti-CD3 mAb show less expression of Notch repressors Numb and Fbxw7.

Inhibition of Ezh2 by DZnep leads to an increase in the expression of Numb and Fbxw7 in antigen-activated γδ T cells. This increase in the level of Numb and Fbxw7 is responsible for the abrogation of Notch signaling in antigen-activated γδ T cells treated with Ezh2 inhibitor DZnep. Although EZH2 is not known as a transcription factor, it has been shown that Ezh2 is involved in defining the survival and polyfunctional cytokine profile of effector T cells [491]. The mechanism through which Ezh2 controls the Notch signaling in T cells is by the direct binding to the promoter areas of genes encoding Notch repressors, NUMB and FBXW7, and repressing their transcription via H3K27me3. This subsequently causes Notch activation, which results in gene activation and effector cytokine expression [371]. Our observation shows that γδ T cells upon activation with HDMAPP or anti-CD3 mAb show increased the level of H3K27me3 which decreases on treatment with DZnep. Further by gene-specific chromatin Immunoprecipitation assay, we observed

high occupancy of repressive histone modification H3K27me3 on the promoter regions of Notch repressors, NUMB and FBXW7 in antigen-activated γδ T cells as compared to unstimulated γδ T cells. Inhibition of Ezh2 in antigen-activated γδ T cells decreases this gene-specific repressive histone modification H3K27me3 on promoters of Notch repressors, NUMB and FBXW7. Thus the decrease in the level of H3K27me3 on Notch repressors, NUMB and FBXW7 abrogate the TCR mediated Notch signaling in γδ T cells. Our observation for the first time demonstrated that TCR mediated expression of Ezh2 plays an important role in the anti-tumor effector functions of γδ T cells.

Ezh2 is involved in the $CD4^+$ T cell differentiation it has been demonstrated that Ezh2 play an important role in pathogenic Th1 cell responses during aplastic anemia [362]. Transcription factor T-bet is indispensable for the differentiation of $CD4^+$ T cells in Th1 subtype, which is the main source of IFN-γ production. The inability of Ezh2-deficient T cells to differentiate into Th1 cells resulted from reduced expression of T-bet transcription factor [492]. Interestingly, although Ezh2 is known to act primarily as a gene silencer, it promotes the expression of T-bet gene in Th1 cells by the direct binding to T-bet gene promoter and thus regulating the expression of T-bet. Besides controlling the expression of T-bet, Ezh2 also regulated the stability of T-bet by preventing proteasome-mediated degradation of T-bet protein [362]. γδ T cells secrete copious amounts of Th1 cytokines IFN-γ and TNF-α upon stimulation which is crucial for mediating the effector functions of γδ T cells [378, 463, 493]. The transcription factors critical for the expression of these effector cytokines in γδ T cells are T-bet and it's homologous protein Eomesodermin (Eomes). Our findings elucidate a new and critical role of Ezh2 in regulating the expression of cytokines IFN-γ and TNF-α in antigen-activated γδ T cells. Inhibition of Ezh2 in HDMAPP or anti-

CD3MAb activated γδ T cells decreased the expression of IFN-γ and TNF-α. Further our data showed that antigen-activated γδ T cells show increased expression of transcription factors T-bet and Eomes. Inhibition of Ezh2 in antigen-activated γδ T cells inhibits the expression of Eomes and T-bet. Moreover, we showed that specific silencing of Ezh2 by siRNA also led to a decrease in the expression of effector cytokines IFN-γ and TNF-α; transcription factors Eomes and T-bet in antigen-activated γδ T cells. Thus our observation reveals that Ezh2 modulates the effector functions of antigen-activated γδ T cells.

Human γδ T-cell subsets exhibit a cytotoxic potential against different tumors or infected cells [7, 171, 494]. This cytotoxic potential is induced through the expression of different cell surface receptors such as γδ TCR (T-cell receptor) and NKG2D (natural killer group 2D)] and is preponderantly mediated by the release of soluble mediators Perforin and Granzymes [123, 495, 496]. As discussed earlier Notch signaling controls the effector functions of γδ T-cell through these effector molecules (Chapter 5). It was also observed that in CD8 cytotoxic T cells Notch signaling regulates the expression of effector molecules Perforin and Granzyme B [229, 230, 460]. Our observation showed that Ezh2 abrogates the Notch signaling and thus leads to decrease in the expression of effector molecules in antigen-activated γδ T cells which may have a direct bearing on the cytotoxic function of γδ T cells. We, therefore, validated the cytotoxic effector functions of γδ T cells against a panel of tumor cell lines. We observed that inhibition of Ezh2 by Dznep in antigen-activated γδ T cells inhibits their ability to lyse Zoledronate treated tumor targets. Earlier data from our laboratory have demonstrated that tumor cells treated with Zoledronate are aggressively killed by γδ T cells [7]. Zoledronate inhibits farnesyl pyrophosphate synthase (FPPS) in the mevalonate pathway. Inhibition of FPPS leads to accumulation

of IPP which results in activation of γδ T cells [111]. Thus our findings reveal the Ezh2 epigenetically suppresses the Notch signaling repressors and thus is responsible for increased cytotoxic potential of γδ T cells against tumor targets.

Active gene transcription depends on the availability of specific transcriptional factors and of the accessibility of chromatin structure for the transcriptional factors and the RNA polymerase machinery at the promoter regions[29, 484]. Our findings of association of H3K27me3 to be associated with the modulation of Notch signaling in antigen-activated γδ T cells through the Ezh2 presented here also raises new questions. First, whether acetylation and methylation of other histone residues such as H3K9 acetylation and H3K9 trimethylation modulate the expression of effector molecules Perforin and Granzyme B. Second, whether the inhibition of Notch signaling in antigen-activated γδ T cells has any impact on these modifications. Accumulating evidence suggests that acetylation of histone H3 lysine 9 (H3K9 acetylation) increases on the promoter regions of IFN-γ on activation of $CD8^+$ T ccells[480, 497]. It is also known that antigen activation in CD8 T cells leads to an increase in the level of H3K9 acetylation on the effector function regulators, Perforin and Granzyme B [37]. Whether such epigenetic changes have any role in the regulation of the effector function of γδ T cells is not known. Our data showed that antigen stimulation leads to an increase in the total H3K9 acetylation as compared to unstimulated γδ T cells and inhibition of Notch signaling decreases the H3K9 acetylation in antigen-activated γδ T cells. Further using promoter-specific chromatin immunoprecipitation assay we showed that high occupancy of H3K9 acetylation on the promoters of genes of effector molecules Perforin and Granzyme. We have already shown that Perforin and Granzyme show higher expression in antigen-activated γδ T cells and inhibition of Notch signaling decreases their expression. We

also observed that antigen activation of γδ T cells leads to a decrease in the level of H3K9 trimethylation which increases on inhibition of Notch signaling. Thus, activation-induced changes in gene expression of effector molecules Perforin and Granzyme B are mediated by acetylation and methylation of histones residues on the promoter regions of Perforin and Granzyme B in γδ T cells.

Chapter 8
Summary and Conclusion

Summary and Conclusion:

Due to their potent cytotoxic potential, γδ T cells have garnered much interest as attractive candidates for the treatment of cancer either by adoptive transfer therapy or in-vivo activation. Several studies have demonstrated promising results of γδ T cells cytotoxicity against tumors in pre-clinical models and clinical studies have been launched for renal carcinoma, melanoma, lung adenocarcinoma, myeloma, and leukemia. However, to be an efficient candidate for immunotherapy, it is necessary to understand how the effector functions of γδ T cells are regulated so that they can be utilized to their full potential.

Activating receptors of γδ T cells stimulate a signaling cascade downstream upon interaction with corresponding ligand or antigen. This signaling cascade culminates in transcriptional activation of effector genes that enable γδ T cells to mediate the cytotoxic effector functions. However, along with TCR signaling, many other signaling cascades function concurrently and contribute to the generation of effector responses of γδ T cells. Apart from the transcriptional regulation, the epigenetic modification also affects the effector fate of γδ T cells. Thus the aim of the study was to understand the epigenetic and transcriptional regulation of effector functions of γδ T cells.

In the present study, we evaluated the effect of HDAC inhibitors on functional responses of γδ T cells. We found that upon treatment with HDAC inhibitors (VPA, TSA, and SAHA) γδ T cells showed decreased antigen-specific proliferative responses and cell cycle arrest in G0/G1 phase. The decreased proliferation was not due to the toxicity of HDAC inhibitors as γδ T cells were viable and showed no apoptosis even after 72h of HDAC inhibitor treatment. The cell cycle arrest was found to be p53 dependent as an expression of p53 and p21 was increased upon HDAC

inhibitor treatment. The decreased proliferative ability further led to dysfunctional effector responses of γδ T cells. We evaluated the effect of HDAC inhibitors on γδ T cell effector functions by different approaches. We found that HDAC inhibitor affected every aspect of γδ T cell functional responses. Pharmacological inhibition of HDAC, in HDMAPP, activated γδ T cell led, to decreased expression of activation markers, IL-2 signaling components as well as activating receptors (NKG2D, CD16) and stunted the activating signals to be recognized by downstream mediators in order to generate a functional response. This hindered activation of γδ T cells upon HDAC inhibition which was reflected in decreased production of cytokines IFN-γ and TNF-α at both gene and protein level. The effector functions of γδ T cells are transcriptionally regulated by a homologous set of transcription factors T-bet and Eomes along with Nf-κB. We found that HDAC inhibitors down-regulated the expression of transcription factors in γδ T cells. Perforin- Granzyme pathway is one of the direct cytotoxic arsenal of γδ T cells to kill tumor cells. HDAC inhibitors abrogated Perforin and Granzyme B expression in γδ T cells, thus proving for the first time that HDAC inhibitors exert control over effector functions of γδ T cells. Downregulation of Perforin Granzyme B expression could be due to the lack of transcription factors that regulate the expression of these effector molecules. The diminished expression of effector molecules in γδ T cells further reduced their cytolytic ability against tumor targets. This study also elucidated that HDAC inhibitors specifically regulate the Perforin Granzyme transcription through hypoacetylation of their promoter regions. The skewed effector functions of γδ T cells due to HDAC inhibition can also be attributed to increased expression of inhibitory receptors (PD-1, PD-L1, and KIR2DL2/3). However, blockade of PD-1 signaling led to the restoration of effector functions in γδ T cells. Thus targeting PD-1 signaling,

could be of therapeutic relevance in developing efficacious γδ T cell-based immunotherapies in combination with HDAC inhibitors

Although, HDAC inhibitor-based therapies have revolutionized the field of tumor chemotherapy, in most cases, these therapies lack specificity. On one hand, HDAC inhibitors have been shown increase the immunogenicity of the tumors but on the other side, HDAC inhibitors also modulate the effector responses of different Immune cells. We have observed in our study that HDAC inhibitors decrease the anti-tumor effector responses of γδ T cells. Further, the structural variations of HDAC inhibitors also add to the complexity of HDAC inhibitor-based therapies. Our study provides proof of the mechanism of how different HDAC inhibitors work in pathological conditions such as cancer. Such inadvertent consequences of HDAC inhibitors on the γδ T cells explains why these epigenetic drugs have not delivered their promise in clinical settings.

The study also focused on elucidating the role of Notch signaling in mediating effector functions of γδ T cells. Notch signaling is evolutionarily conserved signaling pathway which regulates several physiological responses necessary for development and differentiation. Notch signaling also contributes to various aspects of T cell immunity from thymic development, lineage specification to the acquisition of effector functions. We know that Notch signaling is an integral outcome of TCR mediated activation but what role it plays in functional aspects of γδ T cells needs further investigation. In this study, we found that activated γδ T cells rapidly expressed Notch receptors (Notch 1 and 2) and ligands Dll-1 and Jag1 and also showed upregulation of Notch target genes Hes-1, Hey-2, DELTEX, and NRARP. Pharmacological ablation of Notch in antigen-activated γδ T cells however decreased the expression of Notch receptors, ligands and by default the targets genes as well.

Thus proving that Notch signaling partakes in dynamic crosstalk with TCR signaling in γδ T cells. We then evaluated the ways in which this crosstalk influences the γδ T cell function. Inhibition of Notch in activated γδ T cells affected the proliferation and signaling downstream of TCR. We found that γδ T cells in the absence of Notch signaling showed reduced antigen-specific proliferation and led to disrupted cell cycle progression. Activated γδ T cells, when treated with Notch inhibitor (GSI-X), showed upregulated cell cycle checkpoint proteins p53 and p21, thus leading to cell cycle arrest in G0/G1 phase. Expression of activation markers (CD69, CD25, CD71, and phospho-Tyrosine) in γδ T cells was decreased after Notch inhibition. Even in the presence of an activating stimulus in γδ T cells, the proximal (CD122 and CD132) and distal (CD25) IL-2 signaling components as well the downstream effectors were abrogated upon Notch inhibition. We also observed that inactive Notch signal hindered the stored calcium flux in activated γδ T cells which is an essential secondary signal for TCR drove signaling. Thus, this data demonstrates that Notch signal is integral in regulating TCR mediating activation of γδ T cells. We further demonstrated that the anti-tumor response of γδ T cells was decreased after Notch inhibition. In pharmacologically ablated or siRNA mediated knockdown of Notch in γδ T cells, there was decreased secretion of cytokines (IFN-γ and TNF-α) and lowered expression of effector molecules (Perforin and Granzyme B) in the presence of active TCR signal. These effector responses of γδ T cells are paramount in anti-tumor immunity. We observed Notch inhibition led to the lowered cytotoxic potential of γδ T cells against tumor targets. Finally, we elucidated that the decreased effector functions were a result of the abrogated expression of the master regulatory transcription factors T-bet, Eomes and Nf-κB in Notch inhibited γδ T cells. We found that Notch signaling regulates the gene expression of these transcription factors in γδ

T cells. GSK-3β governs the TCR driven activation through negative regulation of major downstream effectors such as NFAT. TCR signaling cascade upon antigen binding mediates phospho inactivation of GSK-3β, to sustain the TCR signal. In γδ T cells we established that Notch acts as a distant regulator of GSK-3β since upon Notch inhibition, γδ T cells showed increased levels of active GSK-3β. We further demonstrated that Notch regulates effector functions in activated γδ T cells through this indirect mechanism of modulating GSK-3β activity. Thus, Notch signaling is integral for TCR mediated activation in γδ T cells and exerts its control through diverse mechanisms, which can be further explored to develop potential therapeutic targets in pathological conditions like cancer and other immune diseases.

Apart from the transcriptional regulation of γδ T cell function, we also studied how epigenetic modifications work to control γδ T cells. Ezh2 is a catalytic subunit of multifunctional protein complex PRC2 that is involved in epigenetic silencing of transcription through chromatin modification. In this evolutionarily conserved complex, Ezh2 function as a methyltransferase and is involved in methylation of histones 3 at specific lysine residues. The key histone modifications, associated transcription repression H3K27me3 are mediated by Ezh2 activity. Extensive studies have shown that lineage-specifying transcription factors and signature cytokines of each subset are epigenetically regulated during T cell differentiation. In this study, we evaluated the role of Ezh2 in the epigenetic regulation of γδ T cell effector functions. In antigen-activated γδ T cells, Ezh2 expression was found to increase with concomitant upregulation of H3K27me3 levels. In γδ T cells, Ezh2 inhibition led to decreased expression of Notch receptors in activated γδ T cells at gene and protein levels and thereby repressed Notch target gene expression as well. To elucidate the role of Ezh2 mediated regulation of Notch we used Chromatin immunoprecipitation assays and found that Notch repressors Fbwx7 and Numb are inhibited by Ezh2. We

observed that Ezh2 increases the H3K27me3 repressive marks on the promoter regions of Notch repressors (Fbwx7 and Numb), thereby positively regulating Notch activation in γδ T cells. Since we observed that Notch signaling is so crucial for γδ T cells effector functions, we went ahead to understand what effect Ezh2 inhibition would have on γδ T cells. We found that Ezh2 is also necessary to mediate effector responses of γδ T cells. Chemical ablation of Ezh2 led to a decreased proliferation in antigen-activated γδ T cells. Decreased proliferation further translates into debilitated functional responses, which we were reflected in decreased cytokine production (IFN-γ and TNF-α), downregulated transcription factor (T-bet and Eomes) expression and culminated in reduced expression of effector molecules (Perforin and Granzyme B). Our study showed that Ezh2 is required to potentiate the anti-tumor functions of γδ T cells, as Ezh2 inhibition decreased γδ T cell cytotoxicity against tumor targets. We also elucidated how Notch signaling regulates the effector functions of γδ T cells through epigenetic regulation of Perforin and Granzyme B. Activated γδ T cells, showed high occupancy of transcriptionally active histone modification H3K9 acetylation on the promoters of effector genes Perforin and Granzyme B. Inhibition of Notch signaling led to loss in H3K9 acetylation on promoters of the effector molecules (Perforin and Granzyme B). However, Notch inhibition led to gain in transcription repressive marks H3K9me3 on the Perforin and Granzyme B promoter regions. Thus, our study demonstrated that both transcriptional and epigenetic mechanisms modulate the effector responses of antigen-activated human γδ T cells. The nexus between the epigenetic and transcriptional control of γδ T cells can be therapeutically exploited to improvea the efficacy of γδ T cell-based immunotherapies for cancer. A detailed summary of the thesis work is graphically explained in **Figure 8.1.**

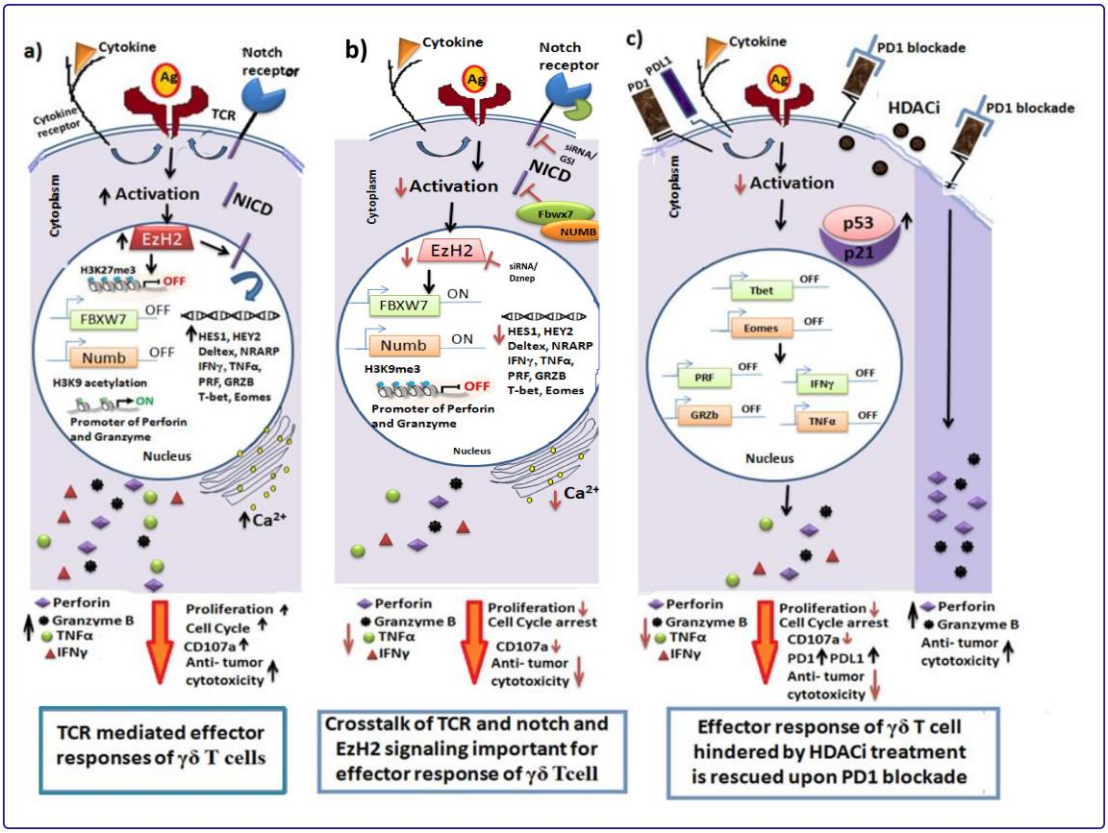

Figure 8.1: Signaling cascade and effector functions of γδ T cells. TCR mediated effector responses of γδ T cells **(A)** TCR dependent recognition of antigen (HDMAPP) by γδ T cells leads to activation of Notch signaling with the release of NICD. Antigen activation also led to the expression of Ezh2 which functions in the nucleus. Ezh2 suppresses the transcription of Notch repressors FBWX7 and NUMB epigenetically. Upon activation of Notch signaling gamma-secretase cleaves the Notch intracellular domain(NICD), which translocates to the nucleus and binds to Notch-responsive elements (Hes, Hey, NRARP, Deltex). Effector molecules Perforin, Granzyme B, IFNγ and TNFα are released bringing about anti-tumor immune response **(B)** Crosstalk of TCR, Notch signaling and Ezh2 is important for the effector response of γδ T cells- Abrogation of Notch signaling either by using siRNA or pharmacological inhibitor GSI, NICD is not translocated into the nucleus. On the other hand, use of Dznep or siRNA against Ezh2, enhances the Notch repressors FBWX7 and NUMB which bind to NICD and marks it for proteasomal degradation. Abrogation of Notch signaling or Ezh2 abrupts the secretion of effector molecules and reduces the antitumor effector function of γδ T cells **(C)** Effector response of γδ T cells hindered by HDAC inhibitor (HDACi) treatment is rescued upon PD1 blockade. Antigen-activated γδ T cells upon treatment with HDACi lose the anti-tumor effector response owing to the reduction of effector molecules Perforin and Granzyme B. PD1 blockade using anti-PD-1 antibody on γδ T cells rescued the expression of Perforin, Granzyme B and the antitumor effector response inhibited by the HDAC inhibitor treatment.

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Checkpoint Blockade Rescues the Repressive Effect of Histone Deacetylases Inhibitors on γδ T Cell Function

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Histone deacetylases (HDAC) are one of the key epigenetic modifiers that control chromatin accessibility and gene expression. Their role in tumorigenesis is well established and HDAC inhibitors have emerged as an effective treatment modality. HDAC inhibitors have been investigated for their specific antitumor activities and also clinically evaluated in treatment of various malignancies. In the present study, we have investigated the effect of HDAC inhibitors on the effector functions of human γδ T cells. HDAC inhibitors inhibit the antigen-specific proliferative response of γδ T cells and cell cycle progression. In antigen-activated γδ T cells, the expression of transcription factors (Eomes and Tbet) and effector molecules (perforin and granzyme B) were decreased upon treatment with HDAC inhibitors. Treatment with HDAC inhibitors attenuated the antitumor cytotoxic potential of γδ T cells, which correlated with the enhanced expression of immune checkpoints programmed death-1 (PD-1) and programmed death ligand-1 in γδ T cells. Interestingly, PD-1 blockade improves the antitumor effector functions of HDAC inhibitor-treated γδ T cells, which is reflected in the increased expression of Granzyme B and Lamp-1. This study provides a rationale for designing HDAC inhibitor and immune check point blockade as a combinatorial treatment modality for cancer.

Keywords: gamma delta (γδ) T cells, phosphoantigen, histone deacetylases inhibitors, effector functions, programmed death-1, programmed death ligand-1

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INTRODUCTION

Gamma delta T cells, the enigmatic brethren of alpha beta (αβ) T cells were discovered coincidently during cloning the αβ T-cell receptor (TCR) locus (1). This small subset of T cells, γδ T cells constitute about 5–10% of the circulating T cell population, which express the variant form of TCR heterodimer (2). γδ T cells manifest the features of both innate and adaptive immunity (3). TheVγ9Vδ2 T cell subset of γδ T cells predominates in peripheral blood, and these cells play an important role in the defense against microbial pathogens, stressed cells, and tumor cells of various origin (4, 5). γδ T cells differ from αβ T cells by their TCR gene usage, tissue tropism, and MHC-independent antigen recognition (6, 7). γδ T-cells display broad functional plasticity, like regulatory potential, antigen-presenting capacity, B-cell helper activity, and have the potential for diverse cytokine production (8). γδ T cells recognize non-peptide phosphoantigens such as isopentenyl pyrophosphate (IPP) or 4-hydroxy-3-methyl-but-2-eneyl pyrophosphate (HMBPP), which are produced through the mevalonate pathway

in mammalian cells or non-mevalonate/Rohmer pathway in non-mammalian cells, respectively (9). γδ T cells are also activated indirectly by aminobisphosphonates such as Zoledronate. Aminobisphosphonates inhibit the key enzyme of mevalonate pathway, farnesyl pyrophosphate synthase and lead to accumulation of IPP. Tumor cells treated with aminobisphophonates show increase in the intracellular level of IPP and, therefore, are easily targeted by γδ Tcells (10, 11).

Activated γδ T cells are known to produce large amounts of the pro-inflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) as well as the chemokines MIP-1 (macrophage inflammatory protein) and RANTES (regulated on activation, normal T cell expressed, and secreted) (12). In addition, cytolytic mediators such as granzyme B and perforin are produced to induce specific lysis of cells with elevated phosphoantigen levels (13). Transcription factors like Eomes and Tbet are known to be expressed upon activation by γδ T cells and are essential for antitumor effector functions (14).

Nucleosome is the basic structure of eukaryotic chromatin, composed of histones and DNA. Each nucleosome comprises 146 bp of DNA wrapped around an octamer of core histones (two H2A–H2B dimers and a H3–H4 tetramer) (15). Histone proteins are rich in basic amino acids lysine and arginine. It is through interaction with these histone proteins that massive DNA is packed inside the nucleus. The tails of histone proteins undergo different complex and coordinated posttranslational modifications like histone acetylation, methylation, phosphorylation, and ubiquitination. According to histone code hypothesis, these modifications are read by specific factors, which ultimately lead to downstream events (16). Histone modifications are reversible in nature and influence many fundamental biological processes. Histone acetylation are directed by histone modifying enzymes, histone acetyl transferases (HAT), and histone deacetylases (HDAC), which participate in potential cross-talk between different modifications (15). Normal physiological functions require a balance between HAT and HDAC. Abrupt alterations that skew this balance can give rise to different pathophysiological conditions like cancer (17, 18).

Histone deacetylases inhibitors, including Trichostatin-A (TSA) and sodium valproate (VPA), can alter the acetylation of histones in chromatin and enhance gene transcription. In the recent decades HDAC inhibitors have received attention as antineoplastic treatment. Extensive evidence suggests that HDAC inhibitors play a role in antitumor immunity (19). HDAC inhibitors lead to growth arrest, induction of apoptosis, and differentiation in tumors. Pan HDAC inhibitors like VPA, TSA, and suberoylanilidehydroxamic acid (SAHA) target Class I (HDAC 1, 2, 3, and 8), Class II (HDAC 4, 5, 7, 9, 6, and 10) HDACs. Hence, their anticancer activities are pleotropic in nature, mediated by altering the expression of various genes that are regulated by class I and II HDACs. Additionally, they also target several non-histone proteins such as transcription factors (p53, E2f1) and cytoplasmic proteins (tubulin, hsp, β-catenin). Hyperacetylation of these histone and non-histone proteins brought about by HDAC inhibiton culminate in induction of cell-death pathways in cancer cells. Several studies have established effective tumor reduction *in vitro* as well as *in vivo* upon HDAC inhibitor treatment (20).

Moreover, HDAC inhibitors inhibit angiogenesis and increase the tumor cell antigenicity (21, 22). HDAC inhibitors mediate elevated expression of antigens on tumor cells so that they can be easily targeted by immune cells (23, 24). Due to these promising antitumor functions, HDAC inhibitors are now assessed in clinical trials and some of them have been approved for treatment (25, 26). Recent reports have demonstrated that HDAC inhibitors enhance response to immune checkpoint blockade in triple negative breast cancer, lung adenocarcinoma, melanoma, and multiple myeloma (27–30).

Although the impact of HDAC inhibitors on tumor cells is well studied, their effect on immune cells has recently surfaced. HDAC inhibitors have been shown to have a dual effect on immune cells, either enhancing their activation in cases of CD4 T cell and Tregs whereas dampening the effector functions of NK cells and CD8 T cells. HDAC inhibitors are also known to inhibit the cytotoxic potential of NK cells. HDAC inhibitors are also reported to downregulate the co-stimulatory molecules and cytokine signals in antigen-presenting cells (31). Previous studies have shown that HDAC inhibitor treated tumor cells are easily targeted by γδ T cells (32), but the impact of HDAC inhibitors on the functional responses of human γδ T cells are not well understood.

For successful immunotherapy, T cell responses are essential. Besides the TCR signal, co-stimulatory signal also determines the functional response of T cells. Co-stimulatory signal may be of positive or negative. Negative co stimulatory signals may be from different receptors like programmed death-1 (PD-1) and PD ligand-1 (PD-L1) interaction. PD-1 and PD-L1 are the members of immunoglobin family like that of CD28. Interaction of PD-1 and PD-L1 leads to functional impairment in T cells (33). It is well-known fact that tumors use this mechanism to escape the immune attack. Blocking antibodies for these immune check points can enhance antitumor responses, and these immune-modulating antibodies have achieved clinical success with FDA approved treatments for several malignancies (34). It has been shown that γδ T cells express PD-1 and PD-L1 and blocking of this signaling lead to increase in the antitumor potential of γδ T cells (35).

The present study focuses on investigating the direct impact of HDAC inhibitors on human γδ T cells. We have studied the effect of three different HDAC inhibitors, TSA, SAHA, and VPA on γδ T cells. We observed that HDAC inhibitors suppress the antigen-specific proliferative responses of γδ T cells and their antitumor effector functions by increasing the expression of immune checkpoints (PD-1 and PD-L1). The study further demonstrates that blocking of immune checkpoints on γδ T cells is capable of augmenting their antitumor cytotoxic potential. The present study will open new avenues in the field of cancer immunotherapy using HDAC inhibitors.

MATERIALS AND METHODS

γδ T Cell Separation

Heparinized peripheral blood was collected from healthy individuals. Peripheral blood mononuclear cells (PBMCs) were isolated by differential density gradient centrifugation using Ficoll Hypaque (Sigma-Aldrich, St. Louis, MO, USA). The study was

approved by the Institutional Ethics Committee (TMC-IECIII Project no. 166) and written informed consent was obtained from the donors prior to collection of blood samples. The experimental conditions and procedures for handling blood samples were performed as per the biosafety guidelines of the Institute Biosafety Committee. In short, blood samples were handled in biosafety cabinets and personnel handling blood samples were vaccinated against Hepatitis B. γδ T cells were purified from PBMCs using immunomagnetic MicroBeads (Miltenyi Biotech, Bergish Gladbach, Germany) by positive selection, as per manufacturer's instructions. The purity of separated γδ T cells was >95% as confirmed by flow cytometry (FACS Aria, BD Biosciences, USA). Isolated γδ T cells were cultured in RPMI 1640 supplemented with 10% heat inactivated AB serum, 2 mM glutamine, and antibiotics.

Cell Viability Assay

The viability of γδ T cells upon treatment with HDAC inhibitors was evaluated with MTT assay and Annexin V and 7-AAD staining. Briefly, 0.1×10^6 γδ T cells, seeded in 96-well flat bottom plates (Nunc), were treated with the following HDAC inhibitors for the given concentration range: VPA (4–0.25 mM; Sigma-Aldrich), TSA (200–25 nM; Sigma-Aldrich), and SAHA (4–0.25 μM; Sigma-Aldrich) along with HDMAPP (1 nM; Echelon) and rIL-2 (50 IU/ml; Peprotech) for 72 h. γδ T cells treated only with HDMAPP (1 nM) and rIL-2 (50 IU/ml) were used as control. DMSO was used as vehicle control. Following 72 h of treatment, MTT (5 mg/ml) was added and incubated for 4 h at 37°C. After incubation, the spent medium was discarded, the formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm by microplate reader (TECAN, Switzerland). Untreated γδ T cells were used as reference for calculating the viability. Concentrations of HDAC inhibitors, which had no impact on viability of γδ T cells were further validated by Annexin V and 7-AAD staining. The concentration of HDAC inhibitors showing viability more or equal to 90% in γδ T cells were used for all the further experimental procedures.

Quantitative Real-Time PCR (qPCR)

The purified γδ T cells, activated with HDMAPP (1 nM) and rIL-2 (50 IU/ml) were treated in the presence or absence of HDAC inhibitors at the given concentrations VPA (2, 1, 0.5 mM), TSA (100, 50, 25 nM), and SAHA (1, 0.5, 0.25 μM) for 72 h. DMSO was used as vehicle control. Total cellular RNA was isolated by using Trizol reagent (Invitrogen Life Technologies, NY, USA) in accordance with the company's instructions and cDNA was synthesized by High-Capacity cDNA Reverse transcription kit (Applied Biosystems) according to manufacturer's instructions. The gene expression of T-bet, Eomes, perforin, granzyme B, IFN-γ, and TNF-α was evaluated by Quantstudio 15k Flex system (Applied Biosystems) using Power SYBR Green reagents (Applied Biosystems) as per manufacture's procedure. All samples were analyzed with the following sequence specific primers: Perforin forward and reverse primer 5′-GACACACAAAGGTTCCTGCG-3′and5′-GACTTTGGCCCTGGTTACAT-3′, respectively, Granzyme B forward and reverse primer 5′-CAACCAATCCTGCTTCTGCT-3′ and 5′-GTCGTCTCGTATCAGGAAGC-3′, respectively, Eomes forward and reverse primer 5′-ATTCCACCGCCACCAAAC TG-3′ and 5′-GCACCACCTCTACGAACAC-3′, respectively, Tbet forward and reverse primer 5′-GTGACCCAGATGATTGTG CT-3′ and 5′-ATGCGTGTTGGAAGCGTTGC-3′, respectively, IFN-γ forward and reverse primer5′-GCATCGTTTTGGGTT CTCTTG-3′ and 5′-AGTTCCATTATCCGCTACATCTG-3′, respectively, TNF-α forward and reverse primer 5′-ACTTTG GAGTGATCGGCC-3′ and 5′-GCTTGAGGGTTTGCTACA AC-3′, respectively, and 18S rRNA forward and reverse primer 5′-AACGGCTACCACATCCAA-3′ and 5′-TTCCAATTACACG GCCTC-3′, respectively. The gene expression was determined by threshold cycle (C_T) method by applying $2^{-\Delta\Delta Ct}$. All the values were normalized to the expression of 18S rRNA as endogenous control.

Western Blot Analysis

1×10^6 freshly isolated γδ T cells were cultured with HDMAPP (1 nM), rIL-2 (50 IU/ml), and with or without HDAC inhibitors at the given concentrations VPA (2, 1, 0.5 mM), TSA (100, 50, 25 nM), and SAHA (1, 0.5, 0.25 μM) at 37°C. After 72 h of treatment, cells were harvested and whole cell lysates were prepared with SDS lysis buffer (1 M Tris–HCl pH 6.8, 10%w/v SDS, glycerol, β-mercaptoethanol, 1M DTT, and bromophenol blue). 10% Polyacrylamide gels were used to resolve the protein samples and transferred to Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The primary antibodies to T-bet (1:1,000) (Cell Signalling Technology), p21 (1:1,000) (Abcam), Eomes (1:1,000) (Abcam), p53 (1:500) (Santa Cruz), NF-κB (1:1,000) (Abcam), total H3 (Abcam) (1:1,000), total H4 (Abcam) (1:1,000), acetyl H3 (Abcam) (1:1,000), acetyl H4 (Abcam) (1:1,000), and β-actin (1:4,000) (Sigma-Aldrich) as loading control were added at different dilution. Immunostaining was performed using appropriate secondary antibody at a dilution of 1:10,000 and developed with ECL plus Western blot detection system (Amersham Pharmacia).

Immunostaining and Cell Cycle Analysis

The magnetically sorted γδ T cells were kept overnight in RPMI supplemented with 10% FBS and were stained for various cell surface markers such as Vδ2 TCR, CD14, CD19, and CD56. Briefly, the cells were harvested from culture, washed with ice cold PBS, and fixed with 1% paraformaldehyde at 4°C for 15 min. The cells were washed with FACS buffer and then labeled with fluorophore tagged antibodies Vδ2-PE, CD3-PECy7, CD14-PerCP, CD19-FITC, and CD56 PerCP Cy5.5 (BD Biosciences, USA) for 30 min at 4°C. Further, the cells were washed and acquired on FACS Aria (BD Biosciences, San Jose, CA, USA). γδT cells treated with or without HDAC inhibitors for 72 h as described earlier were stained with live–dead (LD) fixable dead cell stain kit (Thermo Fischer) as per manufacturer's protocol. After staining with LD dye, the cells were fixed with paraformaldehyde and permeabilized with 1% saponin. Cells were washed and stained with γδ TCR-PE, CD25-PerCPCy5.5, CD69-APC (BD Biosciences, USA), Perforin-BV421, Granzyme B-PECF594, PD-1-PECF594, and PD-L1-PerCP Cy5.5 (BioLegend, San Diego, CA, USA), NKG2D-APC, CD16-BV421, KIRD2L2/3-PE (Miltenyi Biotech,

Bergish Gladbach, Germany). For determination of degranulation marker, Lamp-1 (CD107a) and effector molecule Granzyme B release, purified γδ T cells were activated with rIL2 (50 IU/ml) and HDMAPP (1 nM) in the presence and absence of TSA (100 nM), SAHA (1 μM), and VPA (2 mM) for 72 h at 37°C. Additionally, for PD-1 blockade, anti-PD1 antibody (3 μg/ml; BioLegend, San Diego, CA, USA) was added along with HDAC inhibitors. These effectors were then cocultured with zoledronate treated tumor targets (AW13516 Oral cancer cell line, COLO-205 Colon cancer cell line and Raji B lymphoblastic cell line) for 4 h in polypropylene tubes (BD Biosciences, USA) at effector target ratio of 4:1 in presence of monensin (5 mg/ml; Sigma-Aldrich) as described previously (36). Anti CD107a APC Ab (BioLegend, San Diego, CA, USA) was added at the start of coculture assay. After 4 h, cells were washed, fixed, and stained with anti-human TCR γδ PE and Granzyme B-PECF-594 (BD Biosciences, USA). Cells were acquired on FACS Aria (BD Biosciences, USA) and analysis was done by using FlowJo software (Tree Star, Ashland, OR, USA). The expression of various cell surface markers and intracellular proteins were analyzed on the γδ TCR^+ cells gated populations.

For cell cycle analysis, 1×10^6 γδ T cells treated with HDAC inhibitors VPA (0.5, 1, 2 mM), TSA (25, 50, 100 nM), and SAHA (0.25, 0.5, 1 μM) for 72 h or kept untreated, were harvested, and fixed by adding chilled 70% ethanol. Next day, cells were washed with PBS and stained with DNA intercalating dye propidium iodide (PI) along with RNAse A at a concentration of 40 and 10 μg/ml, respectively. Cells were incubated at room temperature for 30 min. The samples were acquired on FACS Calibur (BD Biosciences, USA) and analyzed using ModFit software.

Proliferation Assay

Proliferation of γδ T cells was analyzed using ^{3}H-Thymidine (3HTdR) incorporation assay. A total of 5×10^4 γδ T cells were treated in the presence or absence of HDAC inhibitors VPA (0.5, 1, 2 mM), TSA (25, 50, 100 nM), and SAHA (0.25, 0.5, 1 μM) along with HDMAPP (1 nM) and rIL2 (50 IU/ml) for 72 h in 96-well tissue culture plates. The cultures were pulsed with 1 μCi [^{3}H] thymidine (Board of Radiation and Isotope Technology, Mumbai) 18 h prior to termination of the assay. Following the incubation, cells were transferred upon glass-wool filters using cell harvester (Perkin Elmer, UK). The radioactivity incorporated into the DNA was measured in a liquid beta scintillation counter (Packard, Meriden, CT, USA). Data were expressed as counts per minute (cpm).

Cytokine ELISA

For cytokine ELISA, supernatants were collected from γδ T cells treated in the presence of different concentrations of HDAC inhibitors VPA (0.5, 1, 2 mM), TSA (25, 50, 100 nM), and SAHA (0.25, 0.5, 1 μM) along with HDMAPP (1 nM) and rIL2 (50 IU/ml) for 24 h. The concentration of secreted cytokines IFNγ and TNFα was measured by human IFN-γ and TNF-α ELISA kit, respectively (BD Biosciences, USA) as per manufacture's procedure.

Cytotoxicity Assay

Cytotoxic potential of γδ T cells against panel of tumor cell lines, oral tumor cell line (AW13516), colon tumor cell line (COLO-205), and B lymphoblastic cell line (Raji) was performed using lactate dehydrogenase (LDH) release assay as described previously (37). Tumor cell lines were treated for 18 h with zoledronate (100 μM; Sigma-Aldrich). γδ T cells were treated with HDMAPP (1 nM) and rIL-2 (50 IU/ml) in presence and absence of HDAC inhibitors, VPA (2 mM), TSA (100 nM), and SAHA (1 μM) for 72 h at 37°C were used as effectors. Additionally, for PD-1 blockade, anti-PD1 antibody (3 μg/ml) was added to HDAC inhibitor treated γδ T cells for 72 h at 37°C and were also used as effectors. Briefly, tumor cell lines were cocultured with effectors at 40:1 effector target (E/T) ratio for 4 h at 37°C in 96-well plates (Nunc, Denmark). After 4 h of coculture, an aliquot of 50 μl of media was used in LDH cytotoxic assay using the LDH cytotoxic assay kit (Thermo Fisher Scientific, USA) according to manufactures protocol. γδ T cell cytotoxicity was defined as % specific lysis = Experimental value − Effector cells spontaneous control − Target cells spontaneous control/Target Cell Maximum Control − Target cells spontaneous control.

Chromatin Immunoprecipitation (ChIP) qPCR Assays

Chromatin Immunoprecipitation assays were performed using MAGnify TM Chromatin Immunoprecipitation System (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Specific anti-acetyl histone H3 (Abcam) and anti-acetyl histone H4 (Abcam) were used to determine the promoter acetylation of perforin and granzyme-B. Normal rabbit IgG was used as negative control. DNA was extracted and analyzed by quantitative real time PCR (qPCR) with specific primers for perforin (region I forward:5′-GATGAGGGCTGAGGACAG-3′; region I reverse:5′-TCTTCACCGAGGCTCCTG-3′; region II forward:5′-CTGCTGGCCTGTTCATCAAC-3′; region II reverse: 5′-CTGTCCTCAGCCCTCATC-3′) and granzyme B (region I forward: 5′-GGGTGGGCAGCATTTACAG-3′; region I reverse: 5′-TTCTCAGGAAGGCTGCCC-3′; region II forward: 5′-CACT TCATAGGCTTGGGTTCC-3′; region II reverse: 5′-CCTCTGG TTTTGTGGTGTCTC-3′). 1% of starting chromatin was used as input. Relative data quantification was performed using $2^{-\Delta\Delta Ct}$ method, using formula: % Input = 2 (Ct Input − Ct ChIP) × Input dilution factor × 100 and expressed in the form of % input as described earlier (38).

Statistical Analysis

Data analysis was done by Student's *t*-test using GraphPad Prism software (GraphPad Software Inc., CA, USA). The comparative CT method was applied in the quantitative real time RT-PCR according to $2^{-(\Delta\Delta Ct)}$ method. Results were indicated as means ± SE and considered significant at $p < 0.05$.

RESULTS

Effect of HDAC Inhibitors on Viability of γδ T Cells

We first studied effects of HDAC inhibitors VPA (0.25–4 mM), TSA (25—200 nM), and SAHA (0.25–4 μM) on the viability of γδ T cells. Magnetically sorted γδ T cells from peripheral blood

of healthy individuals were activated with HDMAPP (1 nM) and rIL2 (50 IU/ml) in presence and absence of above mentioned HDAC inhibitor concentrations for 72 h. HDMAPP is a synthetic analog of IPP and potent activator of γδ T cells. Immunomagnetically sorted γδ T cells were positive for γδ TCR (90%), CD56 (53%), and negative for αβ TCR, CD14, and CD 19 (Figure S2A in Supplementary Material). Viability of γδ T cells was assessed by MTT assay. It was observed that higher concentrations of HDAC inhibitors were toxic to γδ T cells. γδ T cells showed least viability at VPA (3–4 mM); TSA (150–200 nM), and SAHA (3–4 μM). At lower concentrations, these HDAC inhibitors were not toxic and γδ T cell were viable (>90%) (Figure S1 in Supplementary Material). For further validation of viability, γδ T cells activated with HDMAPP and rIL2 in the presence or absence of HDAC inhibitorsVPA (0.5–2 mM), TSA (25–100 nM), and SAHA (0.25–1 μM) were stained with Annexin V and 7-AAD. We observed that at these concentrations HDAC inhibitors did not induce any significant apoptosis. Since HDAC inhibitor concentrations, VPA (0.5–2 mM), TSA (25–100 nM), and SAHA (0.25–1 μM) showed least effect on the viability of γδ T cells (Figures S2B,C in Supplementary Material), these were selected in further experiments.

HDAC Inhibitors Inhibit the Antigen-Driven Proliferation and Cell Cycle Progression of γδ T Cells

γδ T cell show robust proliferation when stimulated with phosphoantigen (HDMAPP) in presence of rIL2. In order to investigate the effect of HDAC inhibitors on proliferation of γδ T cells, γδ T cells were stimulated with phosphoantigen HDMAPP and rIL2 in the presence or absence of different concentration of HDAC inhibitors (VPA; 0.5–2 mM, TSA; 25–100 nM, and SAHA; 0.25–1 μM) and proliferation was monitored using ^{3}H thymidine incorporation assay. γδ T cells showed robust proliferative responses to phosphoantigen HDMAPP in presence of rIL-2, compared to unstimulated γδ T cells. However, in the presence of various concentrations of VPA, TSA, and SAHA, the proliferative responses of γδ T cells were significantly reduced in a concentration-dependent manner (Figure S3A in Supplementary Material), with maximum decrease in proliferation of γδ T cells observed at higher concentration of HDAC inhibitors, VPA 2 mM, TSA 100 nM, and SAHA 1 μM, respectively. Further, we also evaluated the role of HDAC inhibitors on cell cycle progression of γδ T cells. Freshly isolated γδ T cells were stimulated with HDMAPP and rIL2 in presence or absence of different concentrations of HDAC inhibitors. Upon stimulation with HDMAPP and rIL2, significant number of γδ T cells were in S phase and G2/M phase. However, upon treatment of HDAC inhibitors, γδ T cells were arrested in G0/G1 phase (Figures S3B,C in Supplementary Material). This inhibition of cell cycle progression in γδ T cells upon HDAC inhibitor treatment was reflected in the increased expression of p53 and its downstream target p21, suggesting that HDAC inhibitors impede the G0/G1-S phase transition in γδ T cells in p53-dependent manner (**Figures 1A–C**) and (**Figures 1D–I**).

HDAC Inhibitors Regulate Cytokine Production and Activation in γδ T Cells

γδ T cells upon activation secrete copious amount of cytokines such as IFN-γ and TNF-α (36). We examined the effect of HDAC inhibitors on expression of these cytokines in γδ T cells. Marked increase in the expression of cytokines IFN-γ and TNF-α was observed upon stimulation of γδ T cells with HDMAPP and rIL2 compared to unstimulated γδ T cells. Expression of IFN-γ (**Figures 2A,B**) and TNF-α (**Figures 2C,D**) was decreased significantly when treated with HDAC inhibitors TSA, SAHA, and VPA. This inhibition was observed both at mRNA and protein levels. It was observed that inhibition of cytokine expression was concentration dependent for HDAC inhibitors. We also evaluated the effect of HDAC inhibitors on the expression of early activation marker CD69 and late activation marker CD25 on γδ T cells. Treatment of γδ T cells with HDAC inhibitors led to decrease in the expression of early activation (**Figures 3A,B**) and late activation marker on γδ T cells (**Figures 3C,D**). The expression of these activation markers on γδ T cells were significantly reduced in a concentration-dependent manner, with maximum decrease at VPA 2 mM, TSA 100 nM, and SAHA 1 μM, respectively. Percentage of γδ T cells positive for these markers was also less in HDAC inhibitor treated γδ T cells as compared to untreated γδ T cells. To investigate the role of HDAC inhibitors on the expression of other activating receptors like NKG2D, CD16, and inhibitory receptors like KIR2DL2/3 (CD158b), γδ T cells were treated with HDAC inhibitors VPA 2 mM, TSA 100 nM, and SAHA 1 μM for 72 h. We found that HDAC inhibitor-treated γδ T cells show decreased expression of NKG2D (Figure S4A in Supplementary Material) as compared to untreated γδ T cells. On the contrary, γδ T cells treated with HDAC inhibitors VPA 2 mM, TSA 100 nM, and SAHA 1 μM show increase in the expression of inhibitory receptor KIR2DL2/3 (CD158b) (Figure S4B in Supplementary Material). However, we did not observe any change in CD16 expression (Figure S4C in Supplementary Material). Collectively, the data advocate the role of HDAC inhibitors in abating the expression of activation markers (CD69, CD25, NKG2D) and cytokine (IFN-γ, TNF-α) production in γδ T cells.

HDAC Inhibitors Suppress the Expression of Transcription Factors Eomes and Tbet in γδ T Cells

Eomes and Tbet are two main T-box transcription factors expressed in T cells. They are the main transcription factors, which regulate the effector functions of CD8 T cells through the expression of effector genes perforin and granzyme B (39, 40). Besides CD8 T cells, γδ T cells also express Eomes and Tbet (41). Upon activation with phosphoantigen (HDMAPP) and rIL2, γδ T cells show increased expression of these two transcription factors. We hypothesized that HDAC inhibitors may have an impact on the expression of these two transcription factors in γδ T cells. Therefore, the role of HDAC inhibitors was analyzed in regulating expression of Eomes and Tbet in γδ T cells activated with phosphoantigen (HDMAPP) and rIL2. γδ T cells treated with HDAC inhibitors showed decrease in the expression of Eomes and Tbet at both mRNA (**Figures 4A,B**) and protein level (**Figure 4C**).

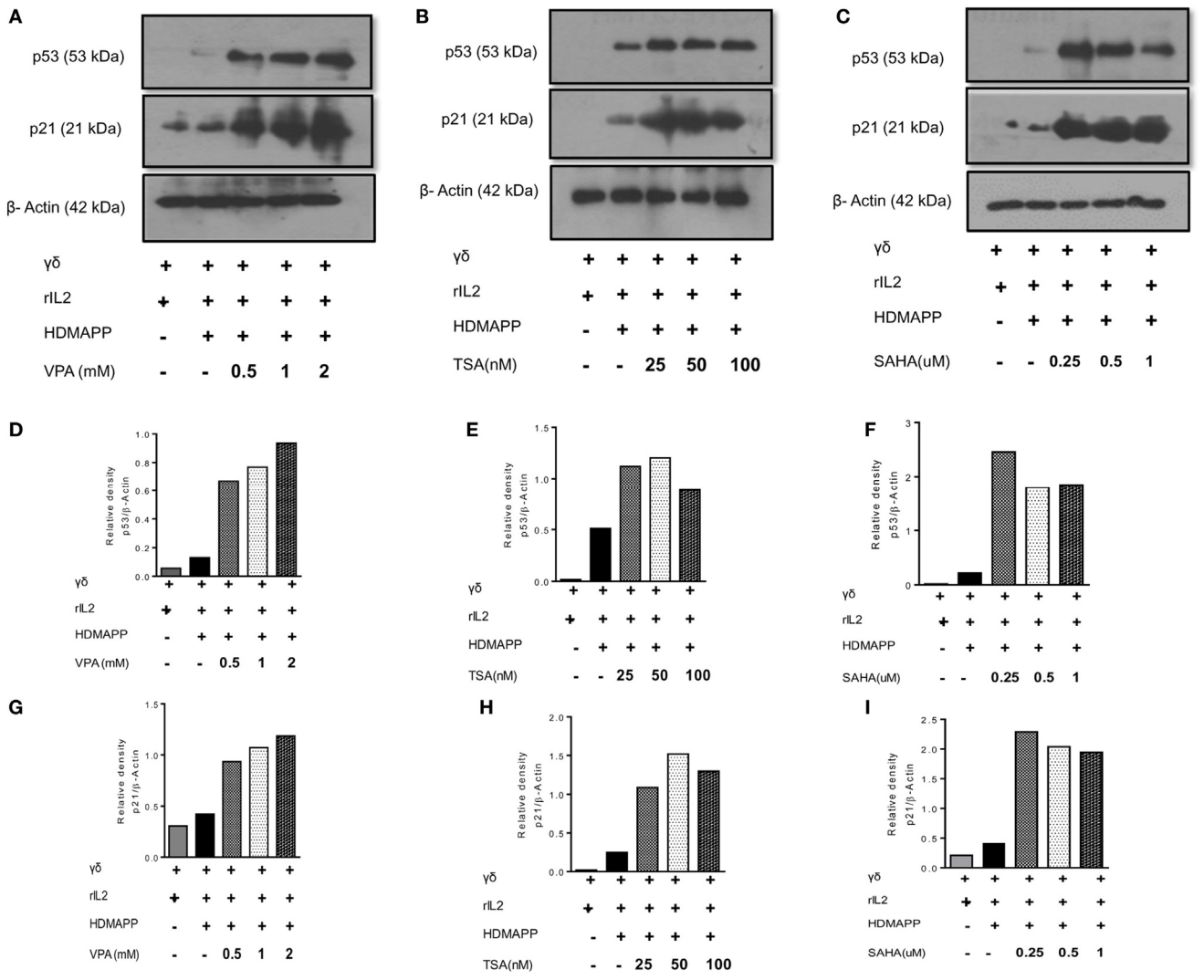

FIGURE 1 | Histone deacetylases (HDAC) inhibitors increase the expression of cell cycle checkpoint proteins p53 and p21. Protein expression of p53 and p21 by γδ T cells upon treatment with **(A)** sodium valproate (VPA), **(B)** Trichostatin-A (TSA), and **(C)** suberoylanilidehydroxamic acid (SAHA) as detected by western blotting. Cell lysates of γδ T cells, stimulated with HDMAPP after treatment with HDAC inhibitors at different concentrations for 72 h were prepared and p53, p21 proteins were detected. β-actin was used as loading control. Densitometry quantification of p53 **(D–F)** and p21 **(G–I)** expression in γδ T cells upon treatment with VPA, TSA, and SAHA, relative to β-actin.

In addition to Eomes and Tbet transcriptional factors, γδ T cells treated with HDAC inhibitors also show decreased expression of NF-κB (Figures S4D–F in Supplementary Material) as compared to untreated γδ T cells. Inhibition of Eomes, Tbet, and NF-κB by HDAC inhibitors clearly demonstrates that HDAC inhibitors regulate the effector functions of γδ T cells.

HDAC Inhibitors Inhibit the Antitumor Cytotoxic Potential of γδ T Cells

To evaluate the role of HDAC inhibitors in modulation of antitumor potential of γδ T cells, we analyzed the expression of effector molecules Perforin and Granzyme B in γδ T cells at mRNA and protein level. Perforin and Granzyme B are the effector molecules, which are responsible for the antitumor functions of CD8 and γδ T cells (42, 43). Freshly isolated γδ T cells activated with phosphoantigen HDMAPP and rIL2 show increased expression of these two effector genes; however, γδ T cells activated in presence of HDAC inhibitors showed decrease in the expression of perforin (**Figures 5A–C**) and granzyme B. (**Figures 5D–F**). Maximum effect on the expression of perforin and granzyme B was observed with VPA 2 mM, TSA 100 nM, and SAHA 1 μM. These concentrations of HDAC inhibitors were used in further cytotoxicity experiments. We next evaluated whether decrease in expression of effector molecules perforin and granzyme B are regulated by histone modifications in γδ T cells. To investigate this, we checked the total histone H3 and H4 acetylation in γδ T cells treated with HDAC inhibitors VPA 2 mM, TSA 100 nM, and SAHA 1 μM. We observed that the total level of H3 acetylation and H4 acetylation increases in γδ T cells after treatment of

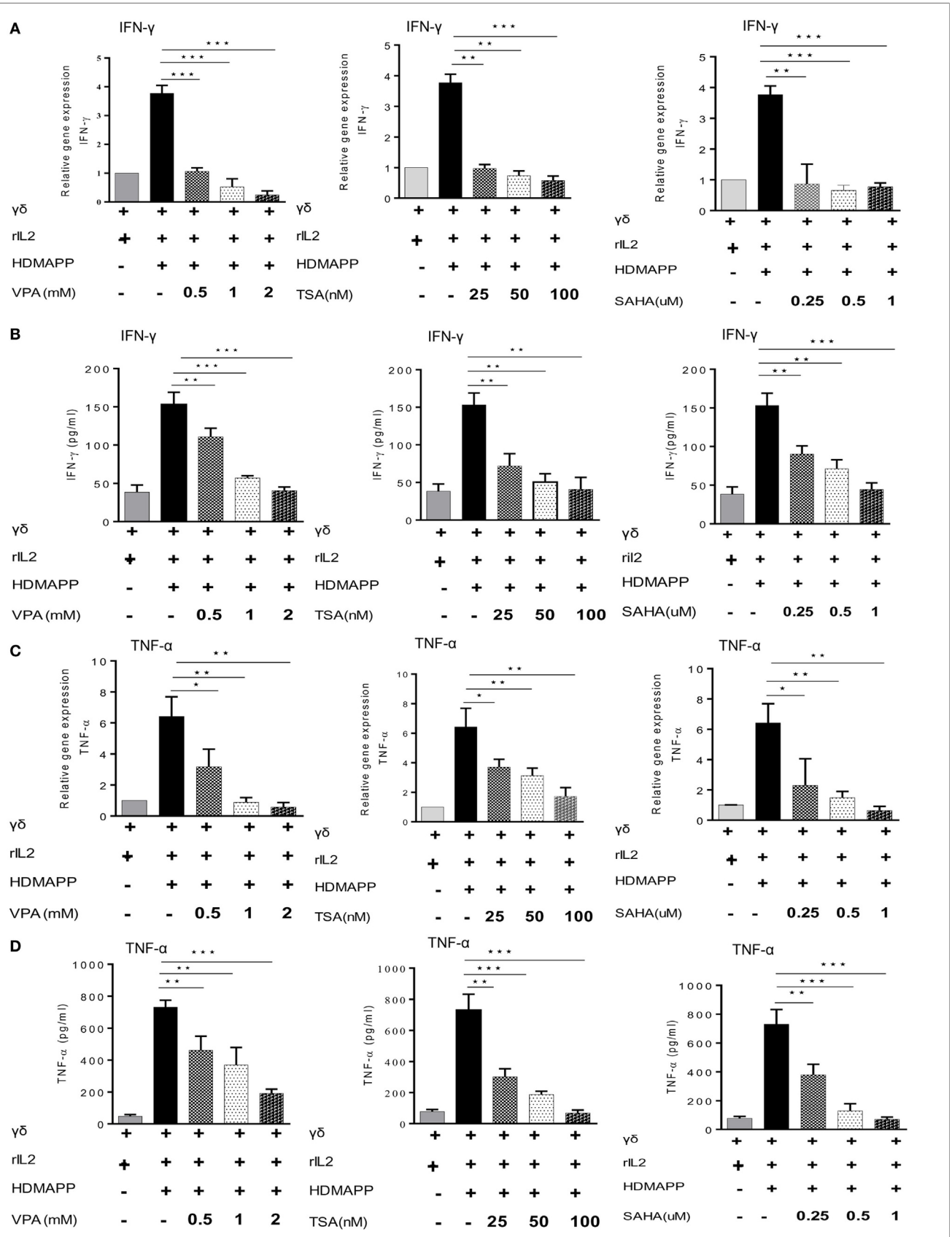

FIGURE 2 | Continued

FIGURE 2 | Histone deacetylases (HDAC) inhibitors regulate cytokine production. Expressions of IFN-γ and TNF-α were detected by quantitative real-time PCR and sandwich ELISA. **(A,B)** IFN-γ expression by γδ T cells stimulated with HDMAPP, treated with or without HDAC inhibitors sodium valproate (VPA), Trichostatin-A (TSA), and suberoylanilidehydroxamic acid (SAHA) at different concentrations at mRNA and protein levels, respectively. **(C,D)** Expression of TNF-α in the supernatants collected from HDMAPP stimulated γδ T cells in the presence or absence of HDAC inhibitors VPA, TSA, and SAHA at different concentrations at mRNA and protein levels, respectively. The expression of different m-RNA transcripts was normalized to 18S r-RNA. All the results indicated are mean ± SEM of three independent experiments, where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

HDAC inhibitors as compared to untreated γδ T cells (Figure S5A in Supplementary Material). However, HDAC inhibitor-treated γδ T cells show less histone H3 acetylation and H4 acetylation on promoters of perforin and granzyme B compared to untreated γδ T cells determined by ChIP qPCR assay. Histone acetylation is positively correlated with transcriptional activity. Thus, our data show that epigenetic changes on promoters of effector molecules perforin and granzyme B control the expression of these molecules in HDAC inhibitor treated γδ T cells (Figures S5B,C in Supplementary Material). The cytotoxic potential of HDAC inhibitor treated γδ T cells was evaluated against panel of zoledronate-treated tumor cells lines (AW13516, COLO-205, and Raji). At different E/T ratios starting from 5:1 to 40:1, HDMAPP-activated γδ T cells in the presence of IL-2 efficiently lysed zoledronate-treated tumor cells lines (AW13516, COLO-205, and Raji). Maximum cytotoxicity of γδ T cells was observed at E/T ratio of 40:1 (**Figures 6A–C**). This ratio of E: T was used in further experiments, to assess the effect of HDAC inhibitors TSA, VPA, and SAHA on cytolytic ability of γδ T cells. γδ T cells stimulated with HDMAPP and rIL2 in presence of HDAC inhibitors VPA (2 mM), TSA (100 nM), and SAHA (1 μM) for 72 h were used as effector against zoledronate-treated tumor cell lines (AW13516, COLO205 and Raji) as targets at E/T ratio of 40:1. Zoledronate, an aminobisphonate drug, inhibits the enzyme farnesyl pyrophosphate synthase in the mevalonate pathway leading to accumulation of IPP, which stimulates γδ T cell activation *via* TCR signaling. γδ T cells treated with different HDAC inhibitors showed significant decrease in their cytotoxic potential against zoledronate treated tumor targets (AW13516, COLO 205, and Raji) (**Figures 6D–F**). It was observed that all the three HDAC inhibitors (VPA, TSA, and SAHA) significantly inhibited the ability of γδ T cells to kill zoledronate treated tumor cell lines.

HDAC Inhibitors Abrogate the Effector Functions of γδ T Cells by Upregulating the Immune Checkpoint Proteins PD-1 and PD-L1

Programmed death-1 receptor and its ligand PD-L1 are commonly expressed on immune cells. PD-1 and PD-L1 belong to the family of immune checkpoint proteins that act as co-inhibitory signaling inducers. Upon activation, T cells show enhanced expression of immune check point PD-1. Interaction between PD-1 and PD-L1 halt the T cell activation, thus maintaining the immune homeostasis. Tumor cells exploit this pathway to evade immune response. The effect of HDAC inhibitors on the expression of PD-1 and PD-L1 on γδ T cells was studied. γδ T cells were treated with different concentrations of HDAC inhibitors and expression of PD-1 and PD-L1 was analyzed by flow cytometry. Upon activation with antigen HDMAPP and rIL-2, expression of PD-1 and PD-L1 increases on γδ T cells. However, the expression of PD-1 and PD-L1 on γδ T cells substantially increased upon treatment with HDAC inhibitors. Maximum increase in the expression of PD-1 (**Figures 7A,B**) and PD-L1 (**Figures 7C,D**) on HDMAPP and rIL-2 activated γδ T cells was observed after treatment with VPA (2 mM), TSA (100 nm), and SAHA (1 μM). To assess the role of PD1/PD-L1 signaling in HDAC inhibitor treated γδ T cells, γδ T cells were activated with HDMAPP and rIL2, treated or untreated with HDAC inhibitors for 72 h. PD-1 blocking antibody was added at the start of culture. After 72 h, these γδ T cells were cultured with zoledronate-treated tumor cell lines AW13516, COLO-205, and Raji for 4 h at E/T ratio of 4:1. Blockade of PD-1 in HDAC inhibitor treated HDMAPP activated γδ T cells rescued the expression of effector molecules Lamp-1 (CD107a) (**Figure 8A**) and granzyme B (**Figure 8B**) as compared to only HDAC inhibitor treated γδ T cells. To further evaluate the role of HDAC inhibitors on the PD1/PD-L1 signaling axis in γδ T cells, we did the similar experiment by coculturing the effectors and above mentioned tumor targets to analyze the cytotoxic potential by LDH release assay at a ratio of 40:1 for 4 h. Blocking of PD-1 in HDMAPP-activated γδ T cells treated with HDAC inhibitorsVPA (2 mM), TSA (100 nM), and SAHA (1 μM) improves the cytolytic potential of γδ T cells as compared to γδ T cells treated with HDAC inhibitor only (**Figure 8C**). Thus, the results shows that blockade of PD-1 and PD-L1 signaling in HDAC inhibitor treated γδ T cells rescue their effector functions.

DISCUSSION

γδ T cell immunotherapy has become the emerging lead in the landscape of cancer immunotherapies due to their distinctive immune features and potent antitumor effector functions. They have been extensively targeted against diverse tumors such as melanoma, renal cell carcinoma, as well as B cell malignancies and have shown promising results in clinical settings (44). While these therapies have encountered modest clinical success, they have to overcome certain challenges such as limited availability of γδ T cells and rapid exhaustion upon repeated *in vitro* activation. Hence, combinational approaches have been envisaged with chemotherapeutics, monoclonal antibodies, small molecule inhibitors, etc. Newer treatment modality may include combining γδ T cell immunotherapy with antitumor drugs and other immune-modulating antibodies.

Epigenetic dysregulation is one of the hallmarks of cancer. Hence, epigenetic modifiers such as HDAC inhibitors are being comprehensively explored for their anticancer potential. Besides anticancer properties, HDAC inhibitors have also shown promising results in controlling the other pathological conditions such as neurological disorders and viral infections and are well tolerated

(45, 46). Currently, VPA along with other short-chain fatty acids HDAC inhibitors are being clinically evaluated as anticancer drugs (47). HDAC inhibitors employ wide range of antitumor mechanisms such as induction of apoptosis, senescence, differentiation, or inhibition of cell cycle (48, 49). Vorinostat (SAHA), is among the first HDAC inhibitor to be approved by United

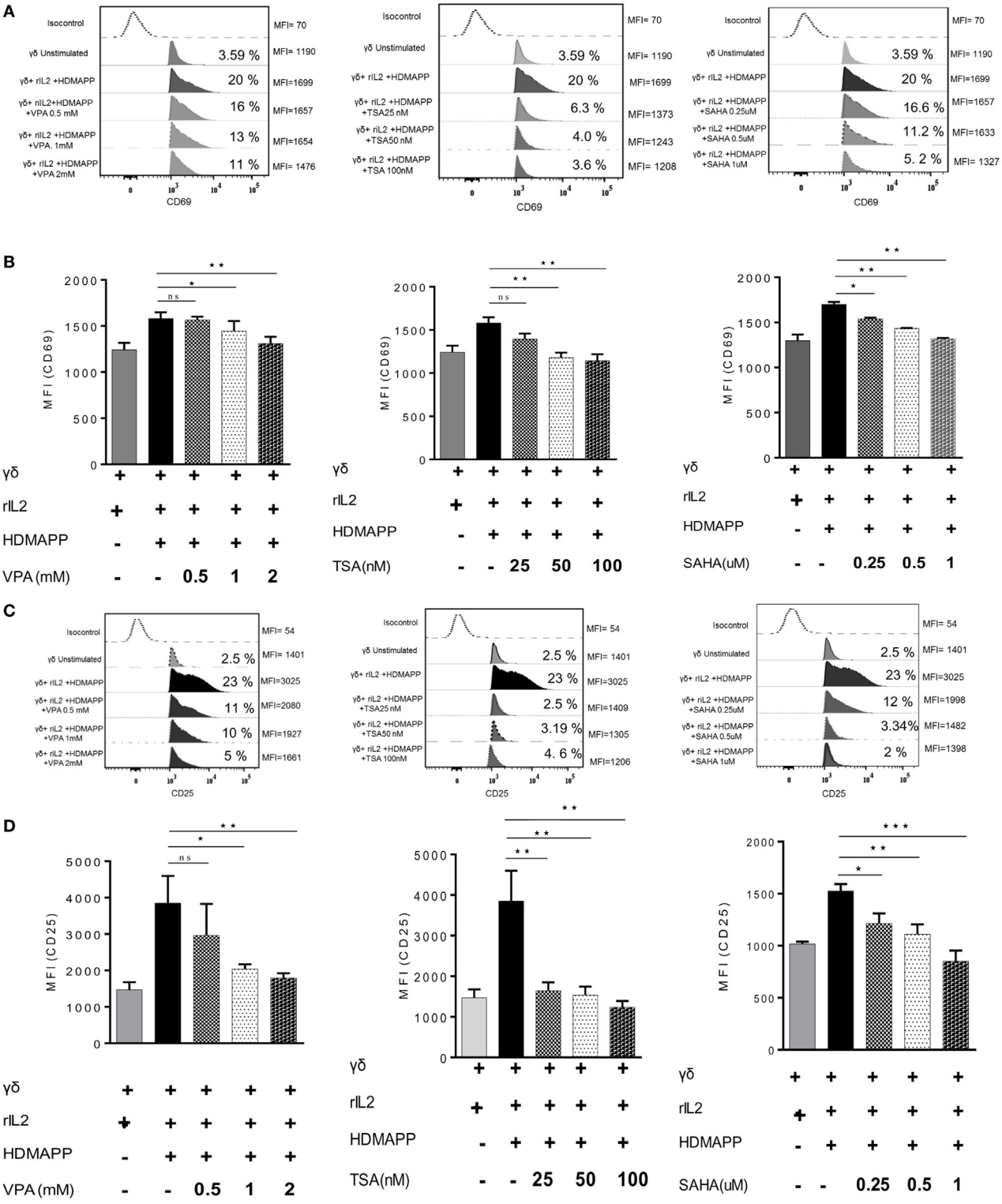

FIGURE 3 | Continued

FIGURE 3 | Histone deacetylases (HDAC) inhibitors affect the activation markers on γδ T cells. **(A)** The expression of early activation marker (CD69) on unstimulated γδ T cells and HDMAPP and rIL-2 stimulated γδ T cells with or without HDAC inhibitor treatment was analyzed by multi-color flow cytometry. Values on right side indicate the median fluorescence intensity (MFI) of CD69, while the values inside the histogram represent the percent CD69-positive γδ T cells. The histograms shown are representative of three independent experiments. **(B)** The cumulative MFI of CD69 expression on γδ T cells is represented as bar graphs. Data shown are representative of three independent experiments where $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$. **(C)** The effect of HDAC inhibitors on expression of late activation marker CD25 was assessed by flow cytometry. Unstimulated γδ T cells and HDMAPP stimulated γδ T cells with or without HDAC inhibitors at different concentrations, after 72 h were stained with the flurophore-tagged antibody and acquired on FACS Aria. Values on right side indicate the MFI of CD25, while the values inside the histogram represent the percent CD25-positive γδ T cells. The histograms depicted are representative of three independent experiments. **(D)** The results shown are cumulative MFI of CD25 expression on γδ T cells. HDAC inhibition decreases expression of CD25. Data shown are representative of three independent experiments where $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

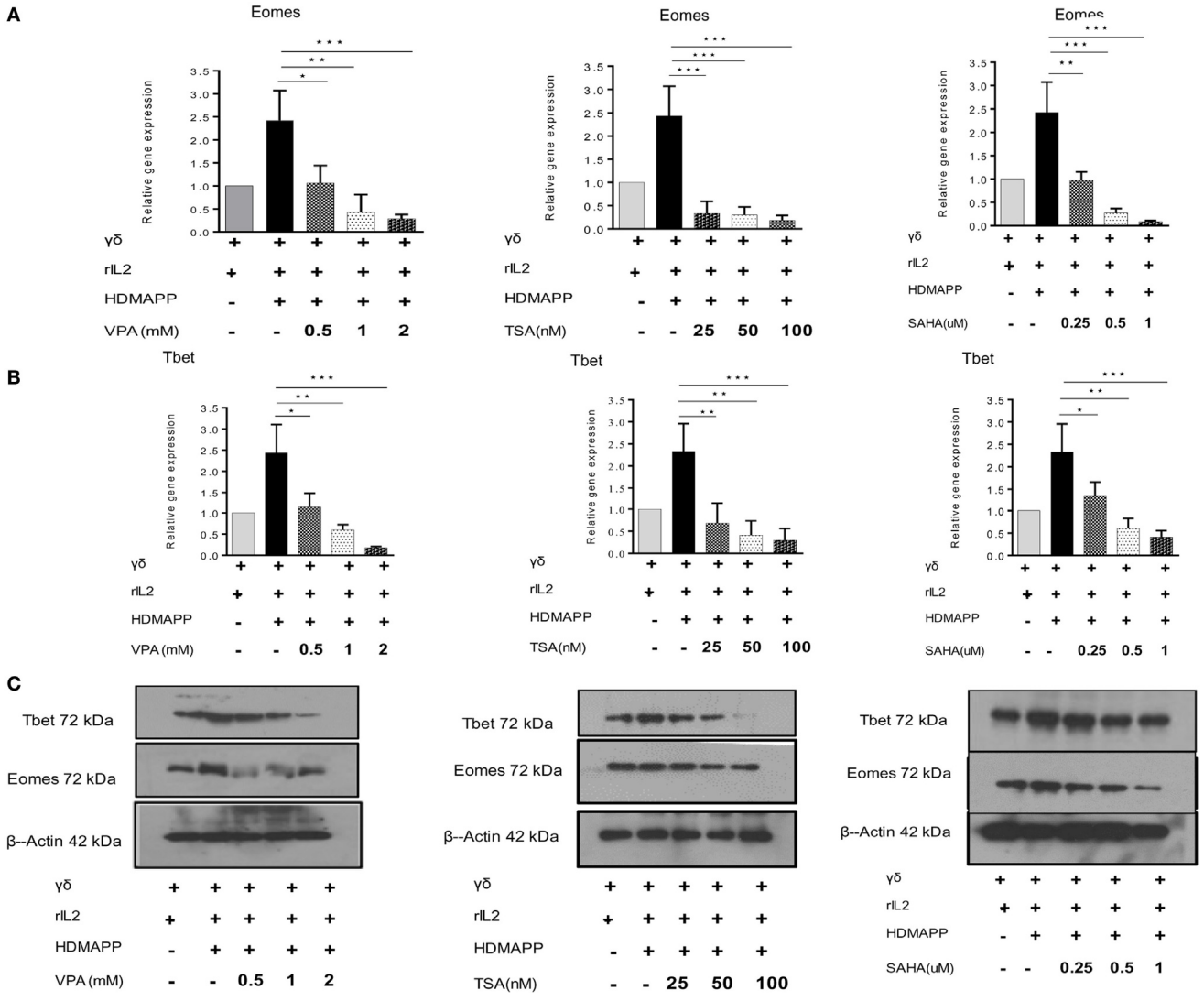

FIGURE 4 | Histone deacetylases (HDAC) inhibition abrogates expression of transcription factors regulating effector functions of γδ T cells. The m-RNA expression of Eomes **(A)** and T bet **(B)** in γδ T cells activated with HDMAPP, in the presence or absence of HDAC inhibitors sodium valproate, Trichostatin-A, and suberoylanilidehydroxamic acid at different concentrations was quantified by quantitative real-time PCR. The results indicated are cumulative mean of relative gene expression normalized to 18S r-RNA where $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$, compared with γδ T cells activated with HDMAPP. The data shown are representative of three independent experiments. **(C)** The protein level expression of T bet and Eomes was detected by western blotting. HDAC inhibitor treatment decreases the expression of T-bet and Eomes. β-actin was maintained as loading control. The blots shown are representative of three experiments.

States Food and Drug Administration (FDA) for the treatment of relapsed and refractory cutaneous T-cell lymphoma (50). Although HDAC inhibitors are approved for hematological malignancies, but clear proof-of-concept data for the clinical efficacy of HDAC inhibitors in solid tumors remains to be established (51). Recent studies have demonstrated that HDAC inhibitors exhibit higher

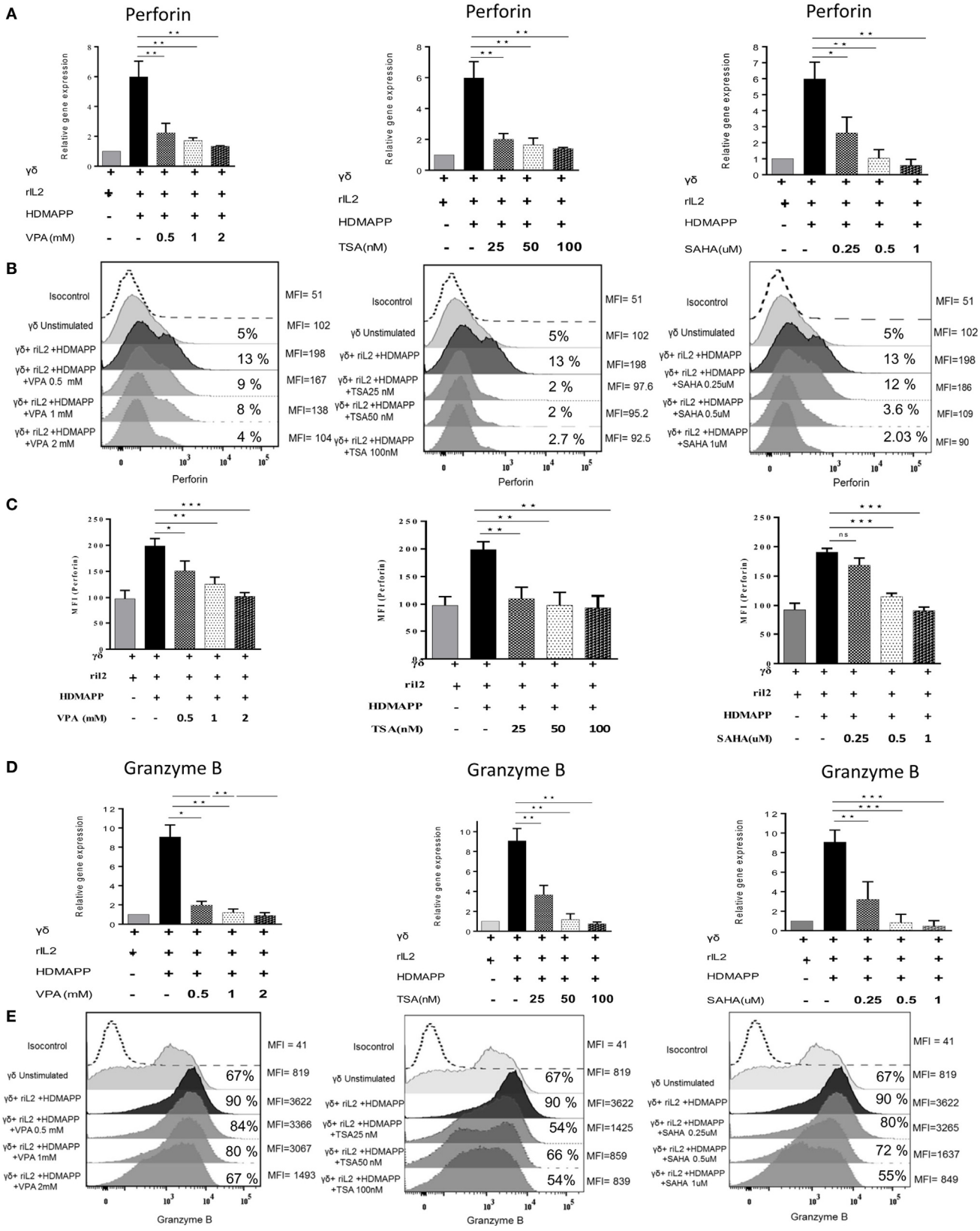

FIGURE 5 | Continued

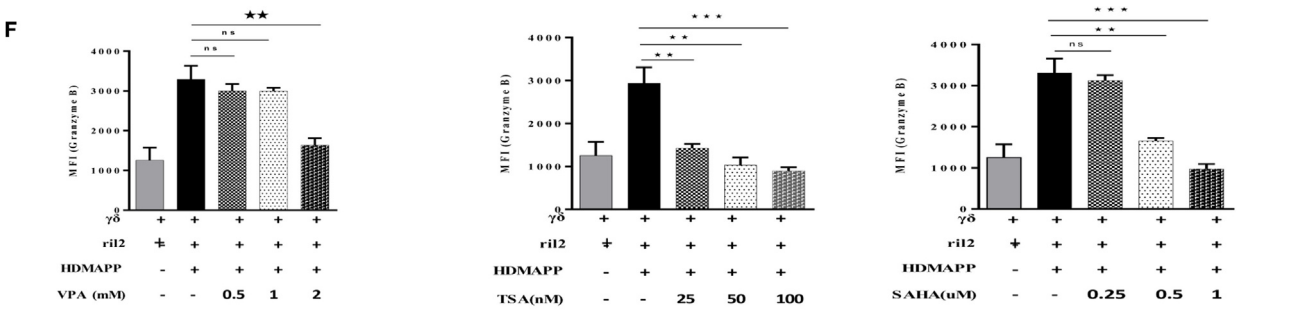

FIGURE 5 | Histone deacetylases (HDAC) inhibitor treatment abrogates the antitumor potential of γδ T cells. Expression of perforin and granzyme was studied at mRNA and protein levels using quantitative real-time PCR and flow cytometry, respectively. The gene expression of **(A)** perforin and **(D)** granzyme B by HDMAPP-stimulated γδ T cells with or without HDAC inhibitor treatment was quantified by quantitative real-time PCR. The results shown are cumulative means of relative gene expression, normalized to 18S r-RNA, and representative of three independent experiments. **(B)** γδ T cells activated by HDMAPP with or without sodium valproate, Trichostatin-A, and suberoylanilidehydroxamic acid at different concentrations were stained after 72 h with corresponding flurophore tagged antibody and expression of perforin was analyzed by flow cytometry. The values on right side of histograms indicate median fluorescence intensity (MFI) of perforin, while the values inside the histogram represent the percent positive γδ T for perforin. **(C)** Bar graphs represent cumulative MFI values where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, compared with γδ T cells activated with HDMAPP. **(E)** Expression of granzyme B by unstimulated γδ T cells and HDMAPP activated γδ T cells with or without HDAC inhibitor treatment was assessed by flow cytometric analysis. The values on right side of histograms indicate MFI of granzyme B, while the values inside the histogram represent the percent positive γδ T for granzyme B. The histograms are representative of three independent experiments. **(F)** The graphs represent MFI values of granzyme B where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

therapeutic efficiency when combined with other antineoplastic agents (52). Hence, there is growing interest in exploring other combined therapeutic strategies with HDAC inhibitors.

Emerging evidence suggest that HDAC play a crucial role in T cell differentiation and effector functions. A number of studies have demonstrated that HDAC inhibitors suppress the immune response of T cells in severe inflammatory conditions and induce tolerance in organ transplantation (53). Specifically, HDAC inhibitors have shown to induce the regulatory T cell (Tregs) generation or stabilization of Tregs in inflammatory microenvironment due to which they have shown promising responses in experimental colitis (54). HDAC inhibitors increase the immunogenicity of tumors by increasing the expression of tumor antigens recognized by the immune cells. The antitumor responses of cytotoxic T lymphocytes like γδ T cells are mediated through recognition of stress molecules (ULBP, HSPs) or danger signals like MICA/B expressed on tumor cells by class of activating receptors known as NKG2D (55–57). Studies have demonstrated that HDAC inhibitors upregulate the NKG2D ligands on tumor cells, thereby sensitizing tumor cells to cytotoxicity mediated by γδ T cells in bladder cancer as well as NK cells in other malignancies such as osteosarcoma, pancreatic cancer, and multiple myeloma (32, 58–60). However, the causal effect of HDAC inhibitors on immune scenario is not well investigated and is contradictory. Several studies have shown that HDAC inhibitors affect each immune subset distinctly either leading to activation as in the case of CD4 T cells and CD8 T cells or by abrogating the effector functions of cells such as NK cell (61–63). Furthermore, for a particular immune cell type, the nature of immune regulation differs based on the type of HDAC inhibitor (64, 65). A recent study demonstrated that NKG2D expression in NK cells is inhibited by VPA (66).

Most of the studies have focused on investigating the impact of HDAC inhibitors on tumor cell lines and immune cells other than γδ T cells. Report by Suzuki et al. demonstrated that the antitumor effect of γδ T cells on bladder cancer was enhanced by treatment with VPA (32). The study focuses only on the impact of HDAC inhibitor, VPA on bladder cancer cell line. VPA leads to increase in the expression of MICA and MICB, which are recognized by NKG2D receptor on γδ T cells. The study does not explain the direct effect of HDAC inhibitors on γδ T cells. Earlier study by Kabelitz et al. reported that HDAC inhibitor VPA induces differential modulation of cell surface markers on γδ T cells compared to αβ T cells (67). Although the study shows the direct effect of VPA on γδ T cells, the functional responses of γδ T cells were not investigated in detail. In the present study, we have used three different HDAC inhibitors to delineate their effect on the functional responses of pure and sorted population of γδ T cells. We used clinically relevant concentrations of VPA, TSA, and SAHA in our study, which have been used in *in vitro* studies by other investigators (68, 69). We showed that three different HDAC inhibitors used suppressed the antitumor effector functions of γδ T cells.

We observed that γδ T cells activated with the phosphoantigen, HDMAPP in the presence of HDAC inhibitors showed decreased proliferative potential. One of the mechanism by which HDAC inhibitors exhibit their anticancer properties is through induction of cell differentiation and cell cycle arrest at G1 phase (48, 49). Besides affecting histone proteins, these inhibitors also have several non-histone protein substrates like p53, p21, Rb, and E2F1 in tumors (70, 71). On the other hand, it was demonstrated that downmodulation of p53 in T cells enhances their antigen-specific proliferative response and also augments antitumor cytotoxic functions (72, 73). Studies from our lab have shown that CD3-activated T cells upon activation show robust proliferative capacity and decreased expression of p53 and its downstream target p21 (74). Thus, the decrease in the antigen-specific proliferative response of γδ T cells in presence of HDAC inhibitors incited us to look for effect of HDAC inhibitors on cell cycle

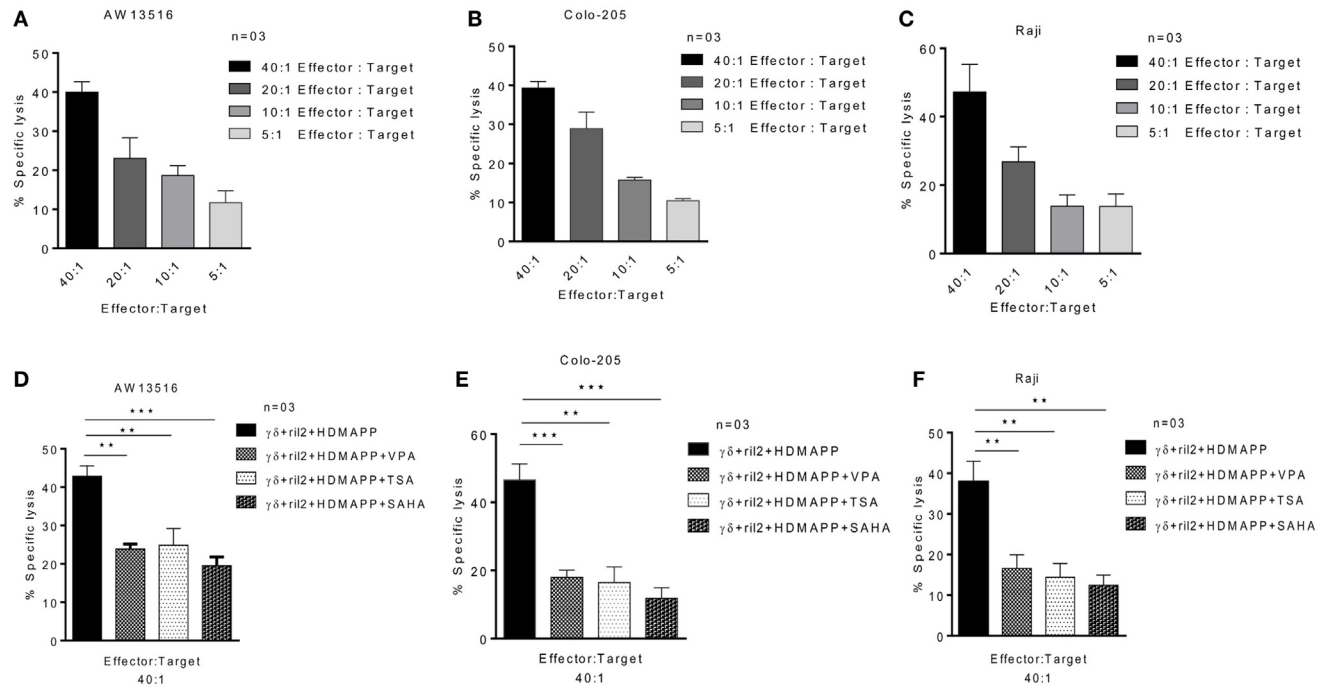

FIGURE 6 | Histone deacetylases inhibitors decrease the cytotoxic effector functions of γδ T cells. Cytotoxic effector function of γδ T cells was assessed against three tumor cell lines **(A)** AW13516, **(B)** COLO205, and **(C)** Raji. Zoledronate-treated tumor targets were cocultured with γδ T cells for 4 h and cytotoxicity was assessed by lactate dehydrogenase (LDH) assay. The results indicated are mean percent specific lysis at different effector:target ratio. Data are representative of three individual experiments. Cytotoxic potential of γδ T cells activated with HDMAPP in the presence of rIL-2 with or without treatment with sodium valproate (2 mM), suberoylanilidehydroxamic acid (1 μM), and Trichostatin-A (100 nM) was assessed against three zoledronate-treated tumor targets AW13516 **(D)**, COLO-205 **(E)**, and Raji **(F)** by LDH cytotoxicity assay. The results indicated are percent specific lysis at the effector to target ratio of 40:1 where $^{**}p < 0.005$ and $^{***}p < 0.0005$ when compared with γδ T cells activated with HDMAPP in the presence of rIL-2 ($n = 3$).

progression and expression of cell cycle regulators p53 and its downstream target p21. Decrease in the proliferation of γδ T cells in presence of HDAC inhibitors was associated with the increase in the expression of p53 and its downstream target p21. γδ T cells show increased expression of activation markers CD69 and CD25 when activated with phosphoantigens (36, 75). We observed that HDAC inhibitors inhibit the expression of CD69 and CD25 activation markers. CD25 is the high-affinity IL-2 receptor subunit and IL-2 signaling is necessary for the proliferation of T cells. It would be logical to conclude that HDAC inhibitors abrogate the IL-2 signaling and thus inhibit the proliferation of γδ T cells. We have used three different HDAC inhibitors VPA, TSA, and SAHA at different concentrations and they showed varied effects on expression of all the γδ T cell markers we studied. The likely explanation for the differences observed in their effects could be their structural diversity and also the biological activities they exert may be cell-type dependent.

Activated γδ T cells express Tbet and eomesodermin (Eomes) transcription factors. The T-box transcription factors T-bet and Eomes are important for acquisition of effector functions in cytotoxic T cells including γδ T cells (41, 76). Eomes and T-bet are highly homologous transcription factors and have cooperative and redundant functions in regulating the expression of different genes involved in the effector functions of CD8 T cells and activated natural killer cells. T-bet and Eomes regulate the expression of perforin, Granzyme-B, and IFN-γ by binding to promoter regions of these effector genes (14, 39). Knowing that HDAC inhibitors decrease the activation and proliferation of γδ T cells, we further hypothesized that HDAC inhibitors may modulate the effector functions of γδ T cells by affecting the expression of transcription factors Eomes and T-bet. We observed that treatment of γδ T cells with HDAC inhibitors lead to decrease in the expression of Eomes and T-bet. To further establish impact of HDAC inhibitors on the antitumor cytotoxic function of γδ T cell, we used panel of tumor cell lines (AW13516, COLO-205, and Raji) treated with zoledronate as target cell line in cytotoxicity assay. Previous work from our laboratory and others has demonstrated that tumor cells treated with zoledronate are aggressively killed by γδ T cells (10, 77). Our data demonstrate that treatment of HDAC inhibitors retard the ability of γδ T cells to kill zoledronate-treated tumor targets. Further, we proved that this inhibition of cytotoxic potential of γδ T cells was due to decrease in the expression of perforin and granzyme-B in these cells.

The activation of T cells initiated through T cell receptor is regulated by balance between co-stimulatory and inhibitory signals (immune checkpoints). Imbalance between these signals lead to different pathological conditions like tumor. Majority of the tumors use these immune checkpoints such as PD-1 or its ligand PD-L1 to escape from the immune surveillance. Immune check point inhibitors have revolutionized the field

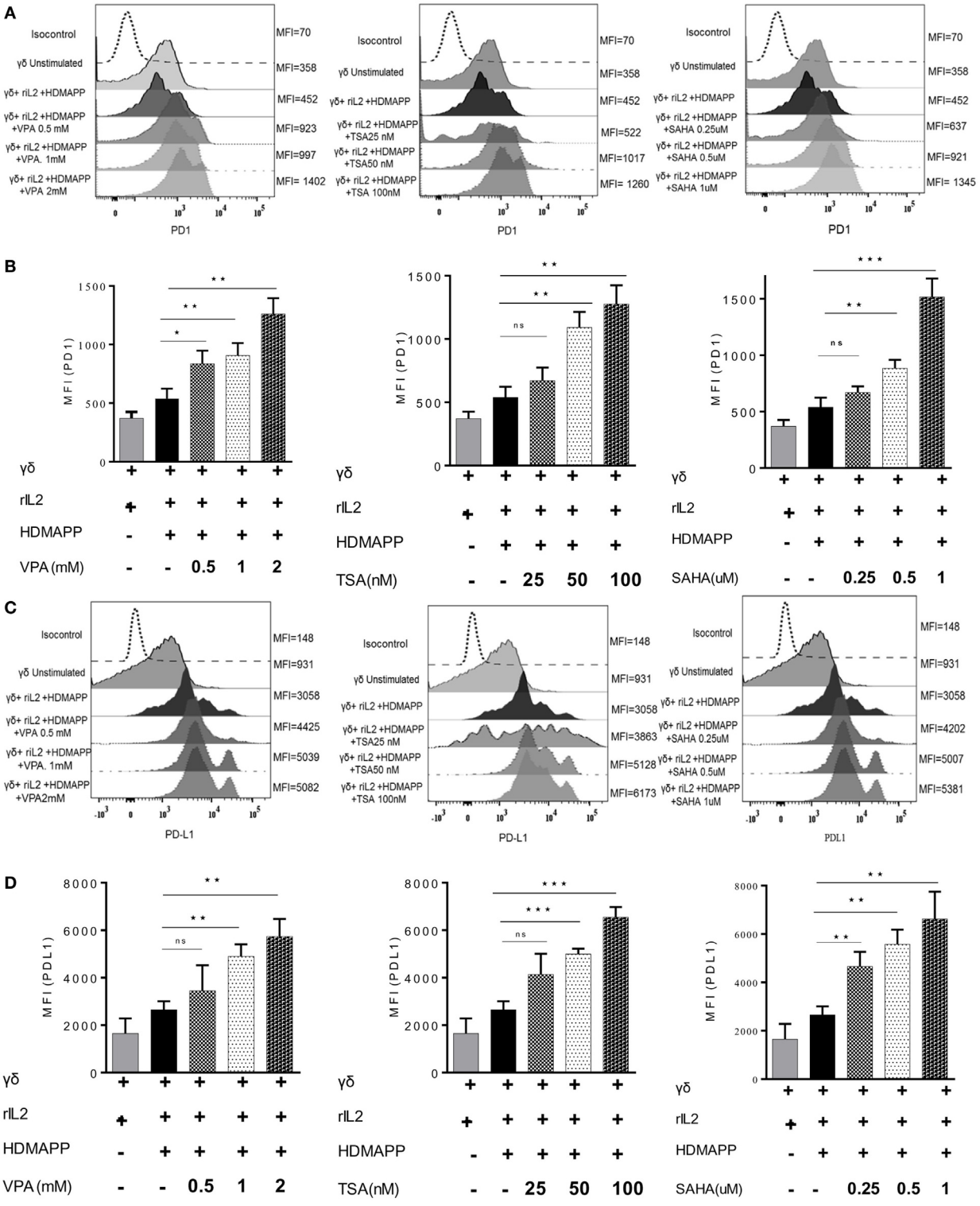

FIGURE 7 | Histone deacetylases (HDAC) inhibitors upregulate the expression of immune checkpoints on γδ T cells. **(A)** The expression of programmed death-1 by HDAC inhibitor-treated γδ T cells at their respective concentration. Histograms are representative of three individual experiments. The values on right side of histograms indicate median fluorescence intensity (MFI) of PD1. **(B)** MFI of PD1 expression as bar graphs where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ and ns, not significant when compared with γδ T cells activated with HDMAPP in the presence of rIL-2. **(C)** The expression of programmed death ligand-1 (PD-L1) by γδ T cells treated with HDAC inhibitors sodium valproate, Trichostatin-A, and suberoylanilidehydroxamic acid at their respective concentration was analyzed by immunostaining. Histograms shown are representative of three individual experiments. The values on right side of histograms indicate MFI of PD-L1. The results indicated in **(D)** are MFI of PD-L1 expression where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ and ns, not significant when compared with γδ T cells activated with HDMAPP.

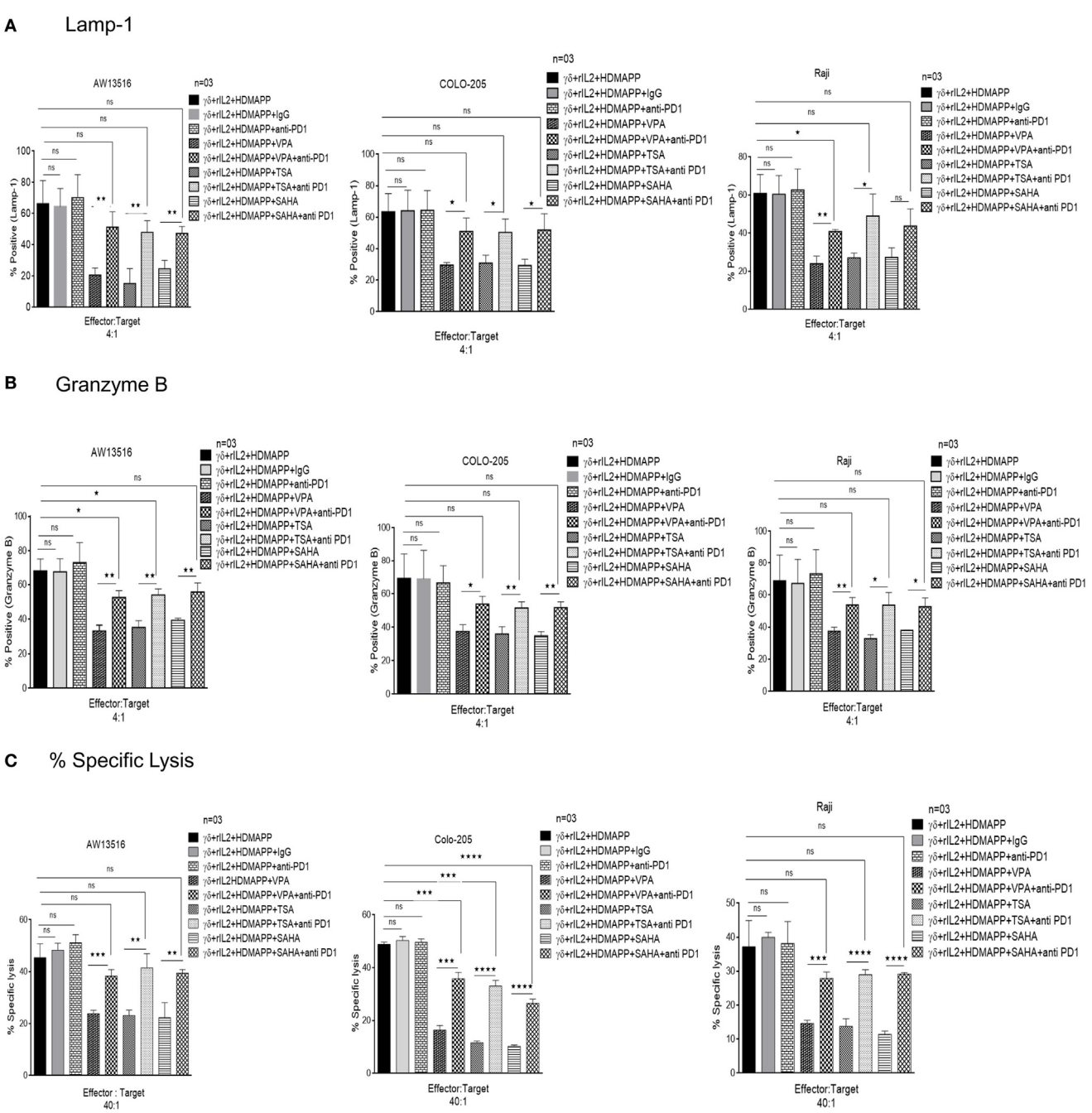

FIGURE 8 | Histone deacetylases (HDAC) inhibitors abrogate effector functions of γδ T cells *via* programmed death-1 (PD-1) upregulation. Expression of of **(A)** degranulation marker CD107a and **(B)** granzyme B by γδ T cells was assessed by flow cytometric analysis. HDMAPP activated γδ T cells upon treatment with sodium valproate (VPA) (2 mM), Trichostatin-A (TSA) (100 nM), and suberoylanilidehydroxamic acid (SAHA) (1 μM) in the presence or absence of PD1 blocking antibody were cocultured with three zoledronate treated tumor targets (AW 13516, Raji, COLO205) cells for 4 h at effector to target ratio of 4:1. The data represent consolidated median fluorescence intensity values of granzyme B and CD 107a expressing cells, indicative of three independent experiments (**$p < 0.005$, *$p < 0.05$, and ns, not significant). **(C)** The cytotoxic ability of γδ T cells treated with HDAC inhibitors TSA, SAHA, and VPA in the presence or absence of PD-1 blocking antibody was assessed against three zoledronate treated tumor targets (AW 13516, Raji, COLO205) by lactate dehydrogenase cytotoxicity assay. HDAC inhibitor treated γδ T cells show increased cytotoxic potential in the presence of PD-1 blocking antibody. The results indicate percent cytotoxicity where **$p < 0.005$, ***$p < 0.0005$, and ns, not significant, when compared with HDMAPP-activated γδ T cells treated with the respective HDAC inhibitor. Data represent three independent experiments.

of tumor immunotherapy (34). Besides surgery, radiation, and chemotherapy, immune check point inhibitors have surfaced as an important immunotherapeutic approach for cancer treatment. Due to their promising antitumor effects in experimental animal models, preclinical studies and successful clinical trials, immune check point inhibitors have been now approved by the U.S Food and Drug Administration (FDA) for treatment of different malignancies. PD-1/PD-L1 blocking strategy has led to tumor regression in patients with melanoma, renal cell carcinoma, non-small cell lung cancer, and bladder cancer (78–82).

Recent reports have shown that tumors associated with PD-1 expressing NK cells show poor survival (83). PD-1/PD-L1 signaling axis along with NKG2D signaling axis determine effector response of NK cells. Blockade of PD1/PD-L1 signaling cascade in NK cells along with other antitumor drugs have shown promising responses in cancer patients (84). This study supports our observation that HDAC inhibitors modulate the effector functions of human γδ T cells against tumors *via* PD1/PD-L1 signaling axis. We observed that γδ T cells show increased expression of immune check points PD1 and PD-L1 upon HDAC inhibitor treatment.

A report by Garcia-Diaz et al. have shown that induction of PD-L1 and PD-L2 on tumor cells is regulated *via* IFN-γ (85). In the present study, we have demonstrated that HDAC inhibitors decrease the expression of IFN-γ and TNF-α in antigen-activated γδ T cells. It has been demonstrated that Tbet transcription factor binds to PD-1 promoter and mediates the suppression of PD-1 expression (86). In the present study, we have shown that upon HDAC inhibitor treatment of γδ T cells, Tbet protein and mRNA is decreased significantly indicating that less Tbet may be available to bind PD-1 promoter to suppress PD-1 expression. This mechanism may explain the IFN-γ independent mechanism of PD-1 expression on γδ T cells.

Activated γδ T cells are known to express PD-1, which was investigated by Iwaski et al., on expanded γδ T cells population. They found that γδ T cells express PD-1 rapidly from day 3 of induction and PD-1$^+$ γδ T cells exhibit attenuated effector functions and decreased cytotoxicity against PD-L1 expressing tumors. However, they observed that zoledronate treatment to tumor cells, which induces IPP release along with PD-L1 blockade, rescued the γδ T cell cytotoxicity (35). While our study also confirms that blocking of PD-1 in γδ T cells increases the antitumor cytotoxic potential, our study reports on the effect of HDAC inhibitors on the freshly isolated γδ T cells activated with antigen for 72 h, whereas Iwaski group used γδ T cells already in activation state for their experimental purposes. Another interesting study by Castella et al. explores the multifunctional role of zoledronate in augmenting γδ T cells responses against multiple myeloma. In this study, zoledronate-treated autologous DCs were found to efficiently activate γδ T cells and enhance their cytotoxic functions against myeloma cells. Additionally, zoledronate was also shown to promote antitumor immunity *via* suppression of regulatory T cell function, downregulation of PD-L1 expression on DCs, and increased proliferation of tumor antigen-specific CD8 T cells. Although, their study has effectively demonstrated role of zoledronate in enhancing antitumor responses γδ T cells, it is specific only to multiple myeloma and uses zoledronate expanded γδ T cells from patient PBMNCs (87). Converse to our observation, they found that DC-activated γδ T cells did not express PD-1, this might be due to the immune modulation by zoledronate, which needs further exploration.

We observed that blockade of PD1/PD-L1 signaling partially restores the antitumor cytotoxic function of γδ T cells in the presence of HDAC inhibitors, which reflected in increased expression of effector molecules granzyme B and Lamp-1. Wei et al. have demonstrated that PD-1 ligation dramatically shifts the dose–response curve, making CD8+ Tcells much less sensitive to TCR generated signals (88). Although, this was shown in CD8+ αβ T cells, it may also apply to γδ T cells. Thus, PD-1 ligation affects TCR signaling and thereby reduces the cytotoxic function of γδ T cells. The role of other activating receptors such as NKG2D interacting with MICA/B and inhibitory receptors KIR2DL2/3 (CD158b) cannot be ignored and it explains the incomplete restoration of cytotoxic effector function γδ T cells upon PD-1 blocking.

Our results implicate that HDAC inhibitors along with the immune checkpoint modulating antibodies could be developed as combination immunotherapy to treat different malignancies. Thus, in future, this strategy may be applied for overcoming the limitations of HDAC inhibitor-based cancer therapies. The underlying mechanistic link of PD-1/PD-L1 may be targeted in developing more efficacious combination γδ T cell-based therapies in the future.

ETHICS STATEMENT

The study was approved by the Institutional Ethics Committee of ACTREC-TMC. All subjects gave written informed consent in accordance with the Declaration of Institutional Ethics Committee, ACTREC-TMC.

AUTHOR CONTRIBUTIONS

SC supervised, contributed conceptionally, and helped to write the manuscript. SB conducted experiments and wrote the manuscript. DV helped in conducting experiments. All authors contributed in final approval of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01615/full#supplementary-material.

FIGURE S1 | Histone deacetylases (HDAC) inhibitors and viability of γδ T cells. HDAC inhibitors affect viability of γδ T cells only beyond specific concentrations. γδ T cells stimulated with HDMAPP in the presence of rIL-2 were treated with HDAC inhibitors sodium valproate (4, 3, 2, 1, and 0.5 mM), Trichostatin-A (250,

150, 100, 50, and 25 nM), and suberoylanilidehydroxamic acid (4, 3, 2, 1, 0.5 μM) for 72 h. The viability of γδ T cells was assessed by MTT assay. The results indicated are mean ± SE percent viability of γδ T cells and are representative of three experiments.

FIGURE S2 | The effect of histone deacetylases (HDAC) inhibitors on γδ T cells viability. **(A)** Purity of sorted γδ T cells. γδ T cells were positively sorted from peripheral blood mononuclear cells and were positive for γδ T-cell receptor (TCR) (90.8%), CD56 (53.2%), and negative for αβTCR, CD14, CD19. **(B)** Effect of HDAC inhibitor treatment on viability of γδ T cells. γδ T cells were activated with HDMAPP and rIL-2. HDAC inhibitors sodium valproate (2, 1, and 0.5 mM), Trichostatin-A (100, 50, and 25 nM), and suberoylanilidehydroxamic acid (1, 0.5, and 0.25 μM) were added to the culture and apoptosis was measured after 72 h with Annexin V and 7-AAD staining. Data shown are representative of three independent experiments. **(C)** The graphs show consolidated γδ T cell viability post HDAC inhibitor treatment ($n = 3$). Data represent mean ± SE.

FIGURE S3 | Histone deacetylases (HDAC) inhibitors impede proliferation of γδ T cells in a dose-dependent manner and leads to cell cycle arrest in G0–G1 phase. **(A)** The proliferative response of γδ T cells was assessed by thymidine incorporation assay. Sorted γδ T cells were stimulated with phosphoantigen HDMAPP, with or without treatment with HDAC inhibitors sodium valproate, Trichostatin-A, and suberoylanilidehydroxamic acid at different concentrations for 72 h. The graphs illustrate the cumulative mean cpm and are representative of three independent experiments where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$. **(B)** Cell cycle analysis of γδ T cells upon HDAC inhibitor treatment. Freshly isolated γδ T cells were activated with HDMAPP with or without HDAC inhibitors for 72 h, and cell cycle progression was analyzed with propidium iodide (PI) staining using FACS calibur. The histograms are representative of three independent experiments. **(C)** The graphs indicate cumulative mean percentage of PI-positive cells in each phase of cell cycle. The graphs are representative of three independent experiments where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

FIGURE S4 | Histone deacetylases (HDAC) inhibitors differentially affects the expression of activating and inhibitory receptors in γδ T cells. The expression of **(A)** NKG2D, **(B)** KIR2D2L2/3, and **(C)** CD16 by γδ T cells in the presence and absence of HDAC inhibitors after 72 h was analyzed by flow cytometry. The histograms are representative of three individual experiments. The values on right side of histograms indicate median fluorescence intensity. HDAC inhibitors reduce NFκB expression in γδ T cells. Protein expression of NFκB by γδ T cells upon treatment with **(C)** sodium valproate, **(D)** Trichostatin-A, and **(E)** suberoylanilidehydroxamic acid as detected by western blotting. Cell lysates of γδ T cells, stimulated with HDMAPP after treatment with HDAC inhibitors at different concentrations for 72 h were probed with NFκB antibody, β-actin was used as loading control. The blots shown are representative of three experiments.

FIGURE S5 | Histone deacetylases (HDAC) inhibitors decrease the acetylation on promoters of perforin and granzyme B in γδ T cells. **(A)** γδ T cells treated with HDAC inhibitors sodium valproate (VPA), Trichostatin-A (TSA), and suberoylanilidehydroxamic acid (SAHA) show increased acetylation of H3 and H4. Expression of acetyl Histone 3 and acetyl histone 4 in γδ T cells was detected by western blotting and total H3 and total H4 were used as loading control. The blots shown are representative of three experiments. Chromatin immunoprecipitation qPCR was used to analyze the histone H3 acetylation **(B)** and histone H4 acetylation **(C)** on perforin region I (−103 to +27 bp), perforin region II (−309 to −130 bp); granzyme B region I (−130 to +11 bp), and granzyme B region II (−263 to −77 bp) from the transcription start site (TSS) of perforin and granzyme B in γδ T cells treated with or without HDAC inhibitors VPA (2 mM), TSA (100 nM), and SAHA (1 μM) for 72 h. Enrichment of promoter regions of Perforin and Granzyme B was quantified by qPCR and expressed as percentage of input DNA. The images above the graphs are schematic representation of Perforin and granzyme B promoter regions including the TSS. All results indicated are mean + SEM where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

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Full Length Article

Inhibition of Notch signalling has ability to alter the proximal and distal TCR signalling events in human CD3$^+$ αβ T-cells

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ABSTRACT

The Notch signalling pathway is an important regulator of T cell function and is known to regulate the effector functions of T cells driven by T cell receptor (TCR). However, the mechanism integrating these pathways in human CD3$^+$ αβ T cells is not well understood. The present study was carried out to investigate how Notch and TCR driven signalling are synchronized in human αβ T cells. Differential expression of Notch receptors, ligands, and target genes is observed on human αβ T cells which are upregulated on stimulation with α-CD3/CD28 mAb. Inhibition of Notch signalling by GSI-X inhibited the activation of T cells and affected proximal T cell signalling by regulating CD3-ζ chain expression. Inhibition of Notch signalling decreased the protein expression of CD3-ζ chain and induced expression of E3 ubiquitin ligase (GRAIL) in human αβ T cells. Apart from affecting proximal TCR signalling, Notch signalling also regulated the distal TCR signalling events. In the absence of Notch signalling, α-CD3/CD28 mAb induced activation and IFN-γ production by αβ T cells was down-modulated. The absence of Notch signalling in human αβ T cells inhibited proliferative responses despite strong signalling through TCR and IL-2 receptor. This study shows how Notch signalling cooperates with TCR signalling by regulating CD3-ζ chain expression to support proliferation and activation of human αβ T cells.

1. Introduction

Notch signalling pathway plays an important role in the regulation of T cell functions (Kuijk et al., 2013; Gogoi et al., 2014). Notch signalling is initiated by the ligand engagement of the Notch receptor. There are four Notch receptors (Notch-1–4) and five Notch ligands, three Delta-like (Dll1, Dll3 and Dll4) and two Jagged (Jag 1 and 2) (Maillard et al., 2003). The engagement of Notch receptor by its ligand cleaves the extracellular and transmembrane domain of Notch through activation of ADAM proteases and gamma-secretase, respectively (Bray, 2006). The second cleavage by gamma-secretase generates Notch intracellular domain (NICD) which is transported to the nucleus and behaves as a transcriptional activator (Schroeter et al., 1998). The Notch signalling pathway controls the development and activation of a variety of immune cells (Yuan et al., 2010). Notch has been implicated in the T versus B lymphocyte differentiation from a common lymphocyte precursor as well as in γδ versus αβ lineage decision (Pui et al., 1999; Tanigaki et al., 2004). The role of Notch in regulating cytotoxic effector functions of γδ T and CD8$^+$ T cells is already reported (Gogoi et al., 2014; Cho et al., 2009).

For T cells to become activated, myriad of signals integrate together, for example, ligation of the TCR accompanied by co-stimulation. The activation of T cells generates intracellular Notch while inhibition of Notch with γ-secretase inhibitors (GSI) decreases T cell activation and proliferation (Gogoi et al., 2014; Palaga et al., 2003; Adler et al., 2003). Despite this, the cross talk between the Notch and TCR signalling in T cells is not well understood. Pharmacological inhibition of Notch activation was reported to decrease the distal TCR signalling events such as the expression of activation markers CD25, CD69, IL-2 and IFN-γ without affecting proximal signalling events such as phosphorylation of Zap70 (Dongre et al., 2014). On the other hand inhibiting Notch signalling has the potential to induce T cell anergy by increasing the

Abbreviations: TCR, T cell receptor; Dll, delta-like ligand; GSI-X, γ-secretase inhibitor; HES1, hairy enhancer of split1; IFN-γi, nterferon-γ; NICD, Notch intracellular domain; Jag, jagged; NRARP, Notch-regulated ankyrin repeat protein

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threshold for T cell stimulation (Dongre et al., 2014). Although anergic T cells show defective cytokine production in response to TCR/CD28 ligation, they produce meaningful levels of cytokines in response to the pharmacological agents- phorbol 12-myristate 13-acetate (PMA) plus ionomycin (Zheng et al., 2008). This suggests that the phenomenon of anergy induced by Notch inhibition in T cells might induce a proximal signal transduction defect. Therefore, a detailed evaluation is required to find out the precise point at which Notch has the capacity to modulate the TCR signalling cascade in human αβ T lymphocytes.

In the present study, the importance of Notch signalling in regulating the proximal and distal TCR-driven signalling molecules was unravelled. We describe the expression of Notch pathway components in human $CD3^+$ αβ T cells. Notch appears to regulate the TCR CD3-ζ chain expression which is known to regulate the proliferative responses and cytokine production in human αβ T cells in response to α-CD3/CD28 mAb stimulation. Our data showed that Notch signalling interacts with TCR-driven signalling and plays an important role in proliferation, cell cycle progression and IFN-γ production by activated human αβ T cells.

2. Material and methods

2.1. Cells

Heparinized peripheral blood samples were collected from healthy individuals. Peripheral blood mononuclear cells (PBMCs) were isolated by differential density gradient centrifugation using Ficoll Hypaque (Sigma-Aldrich, St. Louis, MO). γδ T cells were purified from PBMCs using MicroBeads (Miltenyi Biotcec, Bergish Gladbach, Germany) by positive selection. The negative fraction was used to purify the $CD3^+$ αβ T cells (comprising mostly of CD4 and CD8T cells). 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) (Supplementary Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.molimm.2017.10.013). The study was approved by the Institutional Ethics Committee and written informed consent was obtained from the donors prior to collection of blood samples. The Jurkat T cells were cultured in RPMI supplemented with10% FCS.

2.2. Real time PCR (RT-PCR)

RNA was extracted from immunomagnetically purified $CD3^+$ αβ T cells using Trizol reagent (Invitrogen Life Technologies, N.Y) 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 (Applied Biosystems, Foster City, CA). Samples were analyzed using TaqMan primer sets purchased from Applied Biosystems (NOTCH-1 Hs01062011_m1, NOTCH-2 Hs01050719_m1, NOTCH-3 Hs01128541_m1, NOTCH-4 Hs00270200_m1, DLL1 Hs00194509_m1, DLL3 Hs00213561_m1, DLL4 Hs00184092_m1, JAG1 Hs01070036_m1, JAG2 Hs00171432_m1, HES1 Hs00172878_m1, NF-κB Hs00765730_m1, DTX1 Hs00269995_m1, NRARP Hs01104102_m1, ACTB [β-actin] Hs99999903_m1). All values were normalized to the expression of the housekeeping gene β-actin.

2.3. Semi-quantitative PCR

RNA was extracted from αβ T cells using Trizol reagent (Invitrogen Life Technologies, N.Y) in accordance with the company's instructions. RNA obtained from cells was reverse transcribed in the presence of 5 mM $MgCl_2$, 1X PCR Buffer II, 1 mM dNTPs, 25 u MuLV Reverse Transcriptase, 1 unit RNA guard Ribonuclease inhibitor (Amersham Pharmacia Biotec, Uppsala, Sweden). CD3-ζ chain, ELF-1 and β-actin mRNA levels were measured by semi-quantitative PCR.

2.4. Flow cytometry

The human αβ T cells were gated on the basis of their forward and side scatter characteristics and the fluorescence intensity was measured. Purified unstimulated αβ T cells or cells stimulated with α-CD3/CD28 mAb in presence or absence of GSI-X were stained and analyzed by flow cytometry. Briefly, at different time points cells were harvested and rinsed with cold phosphate buffer saline (PBS) and cold-fixed in 1% paraformaldehyde in PBS for 10 min at 4 °C. The cells were washed and permeabilized for 15 mins with 0.1% saponin in PBS and were stained with CD3-ζ chain, CD25, CD69, CD71 and phosphor-tyrosine (pY) antibodies (BD Bioscience) for 45 min at 4 °C. In the case of Notch-1 intracellular domain (N1ICD) and Notch-2 intracellular domain (N2ICD) expression, cells were first stained with sheep anti-human N1ICD antibody and sheep anti-human N2ICD (R & D Systems, Minneapolis, MN) 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 (Sigma-Aldrich). The data was acquired on the FACSAria flow cytometer (BD Biosciences) with 50,000 events. Cells were analyzed using FlowJo software (Tree Star, Ashland, OR) and expressed as median fluorescence intensity (MFI).

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

2.5. Estimation of intracellular calcium flux

The αβ T cells (1×10^6 cells/ml PBS) were loaded with 5 μM Fluo-3-AM (Sigma–Aldrich, St. Louis, MO, USA) for 30 min at 37 °C. Cells were washed with calcium estimation buffer (137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 5 mM Glucose, 0.5 mM $MgCl_2$, 10 mM HEPES, 1 mM $CaCl_2$, 1 g/L BSA) and acquired on the flow cytometer (FACSAria, BD Biosciences) for 30 s to determine the baseline fluorescence intensity in GSI-X or untreated cells. Soluble α-CD3/CD28 mAb (5 μg/ml) was used as stimulants and Fluo-3 fluorescence intensity was measured immediately for up to 10 min in a continuous manner. Changes in Fluo-3 intensity were analyzed by FlowJo software (Tree Star, Ashland, OR).

2.6. Western blotting

1×10^6 αβ T cells were incubated with α-CD3/CD28 mAb (BD Biosciences) and rIL-2 (PeproTech) at a concentration of 5 μg/ml and 30 U/ml respectively 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 10 μM, or left untreated, before stimulation. 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 dithiothreitol, and 0.1% bromphenol blue), vortexed to reduce sample viscosity, denatured by boiling, and then cooled on ice. Samples were resolved on 10% SDS-PAGE gels, transferred to Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The primary antibodies to N1ICD (1:1000) (R & D Systems, Minneapolis, MN), CD3-ζchain (1:500), p56Lck (1:400), ZAP70 (1:400), GRAIL (1:500), p53 (1:500), c-myc (1:500), (Santa Cruz Biotechnology) NF-κB (1:1000) (Cell signalling Technology), p21

(1:1000) (Abcam) and β-actin (1:4000) (Sigma-Aldrich) as loading control were added at different dilution. Immunostaining was performed using appropriate secondary antibody at a dilution of 1:10000 and developed with ECL plus Western blot detection system (Amersham Pharmacia).

2.7. Proliferation assay

The proliferation of αβ T cells was assayed by ^{3}H-Thymidine (^{3}H TdR) uptake assay. A total of 5×10^{4} αβ T cells were incubated in round-bottom 96-well tissue culture plates with α-CD3 and CD28 mAb (5 μg/ml, BD Biosciences) and rIL-2 (30 U/ml, PeproTech) for 72 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 10 μM, 5 μM, 2.5 μM, and 1.25 μM or left untreated, before stimulation. 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 into the DNA was measured in a liquid beta scintillation counter (Packard, Meriden, CT).The data is expressed as counts per minute (cpm).

2.8. Cytometric bead array (CBA)

The concentrations of IL-2, IFN-γ, IL-4 and IL-10 in cell-free supernatants of αβ T cells were measured using the BD human Th1/Th2 Cytometric Bead Array (BD Biosciences). Human αβ^{+} T cells were treated with α-CD3/CD28 mAb and rIL-2 in the presence or absence of GSI-X (10 μM, 5 μM, 2.5 μM and, 1.25 μM) as described above. After 24 h, cell-free supernatants were collected and the concentration of cytokines was measured following the manufacturer's instructions. Flow cytometry was performed on an FACS Aria (BD Biosciences). The CBA data were analyzed using FCAP Array software version 1.0 (BD Biosciences).

2.9. Statistical analysis

Results are expressed as the mean ± standard error of mean (SEM). Statistical analysis was performed using Prism software (Prism Software, Lake Forest, CA) and the p-value was calculated using student *t*-test. Two-sided *p*-values < 0.05 were considered statistically significant

3. Results

3.1. Activation status of human CD3^{+} αβ T cells regulates expression of Notch signalling pathway

To test the hypothesis that TCR stimulation and Notch signalling regulate each other to control T cell activity and function, the expression analysis of Notch receptors (Notch-1–4) and their ligands (Dll1, Dll3, Dll4, Jag1, and Jag2) in unstimulated and stimulated αβ T cells (MACS sorted, purity Supplementary Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.molimm.2017.10.013) was carried out. The mRNA expression of Notch-1 and Notch-2 genes was observed in αβ T cells (Fig. 1A) while the expression of Notch-3 and Notch-4 was almost undetectable. The α-CD3/CD28 mAb stimulation of αβ T cells led to the marked increase in the expression of Notch-1 and Notch-2 without affecting the Notch-3 and Notch-4 receptors (Fig. 1A). Relatively low expression of Notch ligands Dll1 Dll4, Jag1 and Jag2 mRNA was observed in unstimulated αβ T cells which were enhanced upon TCR stimulation suggesting that TCR-driven signalling has the capability to regulate Notch signalling pathway in T cells (Fig. 1B). Since, there was a dramatic increase in the expression of Notch receptors and ligands on activation, we examined whether inhibition of Notch signalling would have any effect on T cell activation and thereby control the expression of its receptors and ligands. The pharmacological inhibition of Notch signalling using gamma-secretase inhibitor (GSI-X) in α-CD3/CD28 mAb stimulated cells prevented the upregulation of Notch receptors as well as ligands (Fig. 1A and B). This suggests that there is a crosstalk between the TCR and Notch signalling pathways.

To better understand Notch signalling and T cell activity, we focused on two hallmarks of Notch signalling: its dependence on the action of presenilin-dependent γ-secretase activity and the induction of expression of Notch target genes. Using real-time PCR, we demonstrated that unstimulated αβ T cells express the Notch target genes (NF-κB, NRARP, Hes1 and Deltex) at varying degree (Fig. 1C). However, α-CD3/CD28 mAb activation of αβ T cells led to the marked increase in the expression of NF-κB, NRARP, Hes1 and Deltex in αβ T cells which was abrogated on pharmacological inhibition of Notch signalling using gamma-secretase inhibitor (GSI-X) (Fig. 1C). Thus, TCR stimulation results in upregulation of Notch ligands and receptors on T cells which abates the need of adding the external ligands for activation of Notch signalling in these cells.

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

3.2. Notch is required for proximal and distal TCR signalling events in the human CD3^{+} αβT cells

Notch has been demonstrated to influence T cell activation but how T cell signalling intermediates are influenced by Notch signalling remains obscure. The TCR CD3-ζ chain is an important regulator of T cell function as it contains immunoreceptor tyrosine-based activation motif (ITAM) (Pollizzi and Powell, 2014). The activation of αβ T cells with α-CD3/CD28 mAb led to the reduction in the expression of CD3-ζ chain which was further abrogated on GSI-X inhibition (Fig. 2A and B). The inhibition of Notch signalling by GSI-X was not able to alter the expression of CD3-ζ chain or its transcriptional factor ELF1 at mRNA level suggesting that inhibition of Notch signalling may be inducing post-translational downregulation of CD3-ζ chain (Fig. 2C). This was further verified in Jurkat T cells where we found that inhibition of Notch signalling induced downregulation of CD3-ζ chain expression (Fig. 2D) without affecting the downstream molecules like ZAP70 and Lck (Fig. 2E). In human αβ T cells, inhibition of Notch signalling induced GRAIL expression (Fig. 2F). T cell activation is tightly regulated by intracellular signals especially the anergy-related gene (GRAIL) to avoid autoimmunity and maintain tolerance against self-tissues by regulating TCR-CD3 degradation (Nurieva et al., 2010). This indicates that GRAIL might be an essential regulator of T cell tolerance by regulating CD3-ζ chain expression in T cells. It was interesting to note that other signalling molecules like ZAP70 and LCK, downstream of TCR however remained unaltered (Fig. 2G).

Calcium signalling plays a central role in the induction of anergy in T cells, which become functionally inactivated and incapable of proliferating and expressing cytokines following antigen re-encounter (Baine et al., 2009). Stimulation of αβ T using α-CD3/CD28 mAb led to the rapid increase in the intracellular concentration of calcium which reached highest peak at 350 s after stimulation and remains constant for a while but then declines after 500 s of stimulation (Fig. 3A). However, suboptimal stimulation of T cells in the presence of GSI-X led to the gradual increase in intracellular calcium flux which was

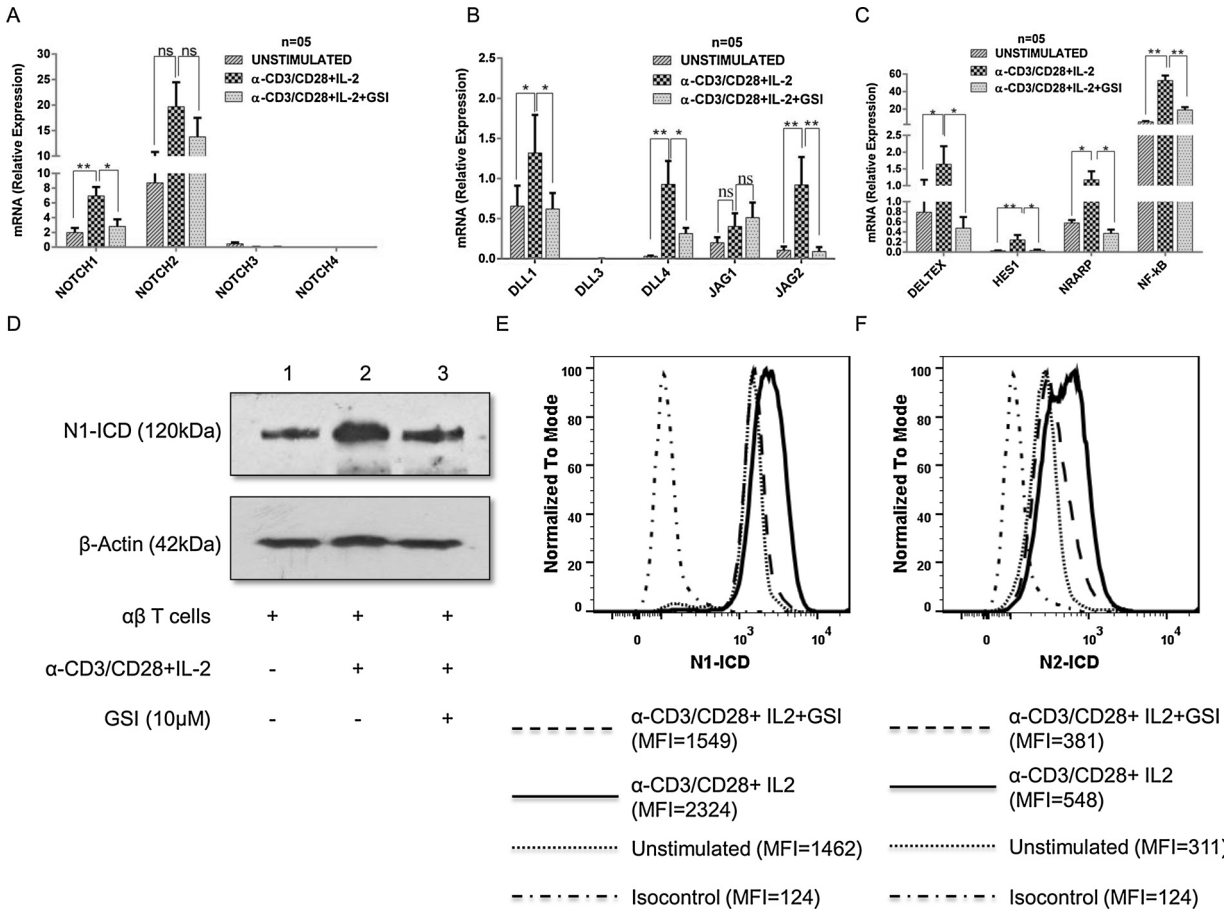

Fig. 1. Expression of Notch receptor, ligands and target genes on human αβ T cells. Human αβ T cells were MACS-purified from the peripheral blood. (A) Real-time quantitative PCR showed mRNA expression of Notch-1, 2, 3 and 4 on αβ T cells. The data represented is the mean of five independent experiments and data was normalized to expression of β-actin. B) Real-time quantitative PCR showed mRNA expression of Dll1, Dll4 JAG1 and JAG2 in d αβ T cells. The data represented is the mean of five independent experiments and data was normalized to expression of β-actin. (C) Human αβ T cells were analyzed for target gene expression (DELTEX, HES1, NRARP and NF-κB). The data represented is the mean of five independent experiments and data was normalized to expression of β-actin (* represents $p < 0.05$, ** represents $p < 0.005$ and was determined by paired student's *t*-Test). (D) Detection of 120 kDa N1ICD in unstimulated (Lane 1), or stimulated with α-CD3/CD28 mAb and rIL-2 (24 h, Lane 2) or in the presence of GSI-X (Lane 3) in αβ T cells using antibodies that recognize the cleaved active form of Notch-1 (N1ICD). (E) Flow cytometric analysis shows the expression of Notch-1 (N1ICD) in unstimulated αβ T cells or in αβ T cells stimulated with α-CD3/CD28 mAb in presence or absence of GSI-X. The data is represented as median fluorescence intensity (MFI). (F) Flow cytometric analysis shows the expression of Notch-2 (N2ICD) in unstimulated αβ T cells or in αβ T cells stimulated with α-CD3/CD28 mAb in presence or absence of GSI-X. The data is represented as median fluorescence intensity (MFI).

maintained at higher levels over the period of time. The levels of calcium in GSI-X treated cells were maintained at higher levels compared to α-CD3/CD28 mAb stimulated cells (Fig. 3A and B). This phenomenon is a characteristic feature of long-lasting anergic T cells suggesting that T cells are intrinsically modified to become tolerant.

Downregulation of CD3-ζ chain has the ability to affect the distal signalling events associated with T cell activation. GSI treatment significantly decreased distal TCR signalling events such as the expression of activation markers CD25, CD69, CD71, and phospho-tyrosine (Fig. 4A–D). The effect of GSI-X was best observed at 48 h after stimulation which is in accordance with data published earlier (Dongre et al., 2014) and can be associated with the lag period of 24–30 h before the activated T cells begin to divide and execute effector functions (Jelley-Gibbs et al., 2008). Collectively, this data suggests that inhibition of Notch signalling has the ability to affect both the proximal and distal TCR signalling events.

3.3. *Notch signalling is involved in proliferation and cell cycle progression of $CD3^+$ αβ T cells*

The defect in the TCR CD3-ζ chain has been correlated with the impaired lymphocyte proliferation and cytokine production in cancer patients (Kulkarni et al., 2009; Dar et al., 2016). In order to investigate the effect of GSI-X induced downregulation of CD3-ζ chain on proliferation of αβ T cells in vitro; T cells were stimulated with α-CD3/CD28 mAb in the presence or absence of different concentration of GSI-X and proliferation was monitored using ^{3}H-TdR incorporation assay. The αβ T cells showed robust proliferative in response to α-CD3/CD28 mAb stimulation (mean 58203 ± 770 cpm) compared to unstimulated αβ T cells (mean 341 ± 11cpm). However, in the presence of various concentrations of GSI-X [10 μM (445.7 ± 147.3 cpm), 5 μM (13526 ± 7630 cpm), 2.5 μM (37409 ± 5116 cpm) and 1.25 μM (44598 ± 772.5 cpm)] the proliferative response of αβ T cells was significantly reduced in a concentration-dependent manner (Fig. 5A), with maximum decrease in proliferation of αβ T cells observed at 10 μM concentration of GSI-X.

We evaluated the effect of Notch signalling pathway in cell cycle progression of αβ T cells. Purified peripheral blood αβ T cells were left untreated or treated with α-CD3/CD28 mAb and IL-2 in the presence and absence of GSI-X. In the absence of strong TCR stimulus, T cells fail to proliferate (Fig. 5B, Upper panel). However in the presence of α-CD3/CD28 mAb and IL-2 stimulation for 72 h a significant increase in the number of cells in S phase (31.4%) and G2/M phase (6.5%) was observed (Fig. 5B, middle panel). The addition of GSI-X (10 μM) prevented the α-CD3/CD28 mAb and IL-2 driven proliferation of αβ T cells and arrested them at G0/G1 phase (Fig. 5B, lower panel). The variation in the percentage of cells in three phases of cell cycle was observed with maximum cells in G_0-G_1 phase for unstimulated and GSI treated αβ T

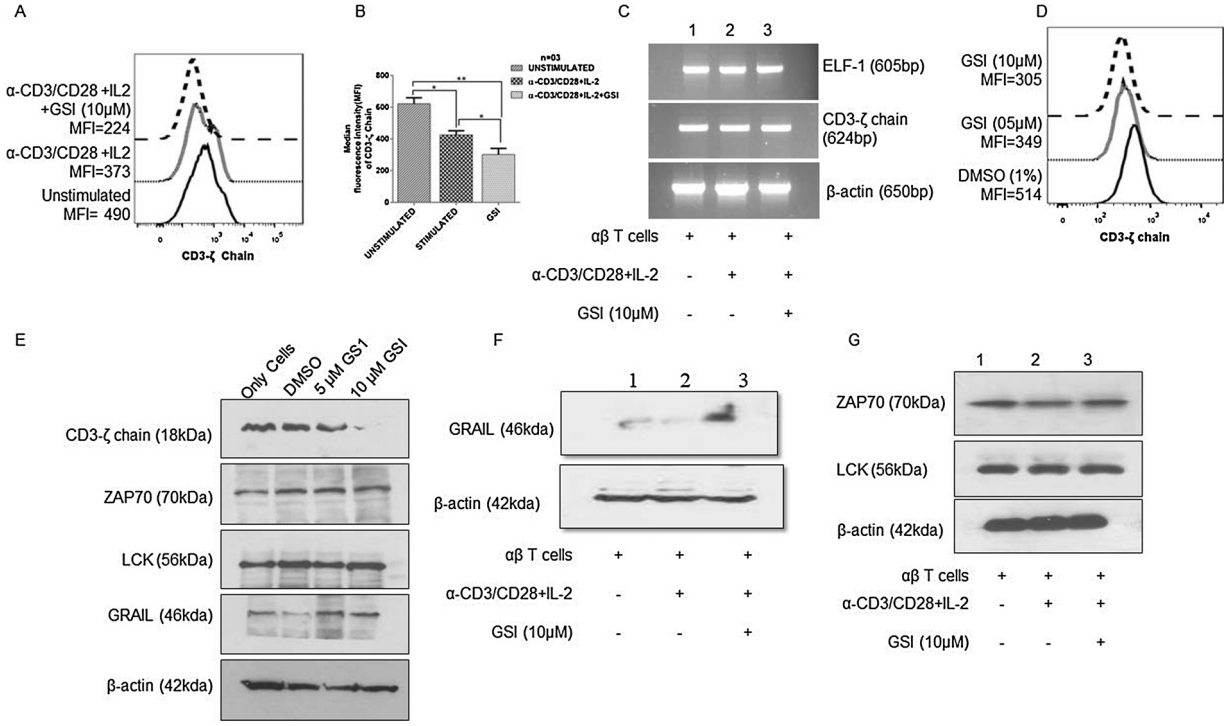

Fig. 2. Notch regulates proximal TCR signalling events in the human CD3$^+$ αβT cells. (A) The half offset histogram shows the median fluorescence intensity of unstimulated αβ T cells (MFI = 490) which on stimulation with α-CD3/CD28 mAb decreases (MFI = 373) which decreased further on inhibition of Notch signalling (GSI-X, 10 μM) (MFI = 224). (B) The graph indicates the expression (Normalized median fluorescence intensity) of CD3-ζ chain in unstimulated, stimulated and GSI-X treated αβ T cells. The graph shown is representative of three independent experiments (* represents $p < 0.05$ and was determined by paired student's *t*-Test). (C). the isolated RNA was converted into cDNA and subjected to PCR to detect CD3-ζ chain, ELF1, and β-actin transcripts. (D) In Jurkat T cells inhibition of Notch signalling induced downregulation of CD3-ζ chain expression. The half offset histogram shows the median fluorescence intensity of Control (1% DMSO) treated Jurkat T cells (MFI = 514) which decrease in a dose-dependent manner after GSI-X treatment. The Jurkat T cells were treated with 5 and 10 μM of GSI-X which reduced the median fluorescence intensity of CD3-ζ chain to 349 (MFI) and 305 (MFI), respectively. (E) Jurkat T cells were treated with different concentration of GSI-X (5 and 10 μM) for 24 h. Total protein extract was used to detect the levels of CD3-ζ chain, ZAP70, LCK, GRAIL and β-Actin by western blotting. β-actin was used as loading control. (F-G) Western blotting experiment shows expression of GRAIL (F), ZAP70 and Lck (G) in presence and absence of GSI-X in human αβ T cells. β-actin was used as loading control.

cells compared α-CD3/CD28 mAb stimulated cells (Fig. 5C). This was also reflected in the increased expression of p53 and its downstream target p21 in αβ T cells after GSI-X treatment suggesting that inhibition of Notch signalling prevents G0/G1- S phase transition in a p53 dependent manner (Fig. 5D).

3.4. Notch signalling regulates activation and cytokine production in activated CD3$^+$ αβ T cells

Given that T cells with low TCR-CD3 ζ chain exhibit a clear proliferative defect, we focused on pathways known to be involved in the proliferation of multiple cell types. The c-Myc proto-oncogene confers proliferative and survival signals in numerous neoplastic tissues. Expression of c-Myc is upregulated following TCR-CD3 ligation and inhibition of Notch signalling prevented c-myc induction in human αβ T cells (Fig. 6A). Like c-Myc, TCR-driven stimulation induced NF-κB in αβ T cells which was inhibited in the presence of GSI-X (Fig. 6A). NF-κB transcription factor is linked to post-differentiation IFN-γ production and regulator of effector functions (Corn et al., 2003). The consequence of GSI-X treatment on the concentration of IFN-γ and IL-10 cytokine

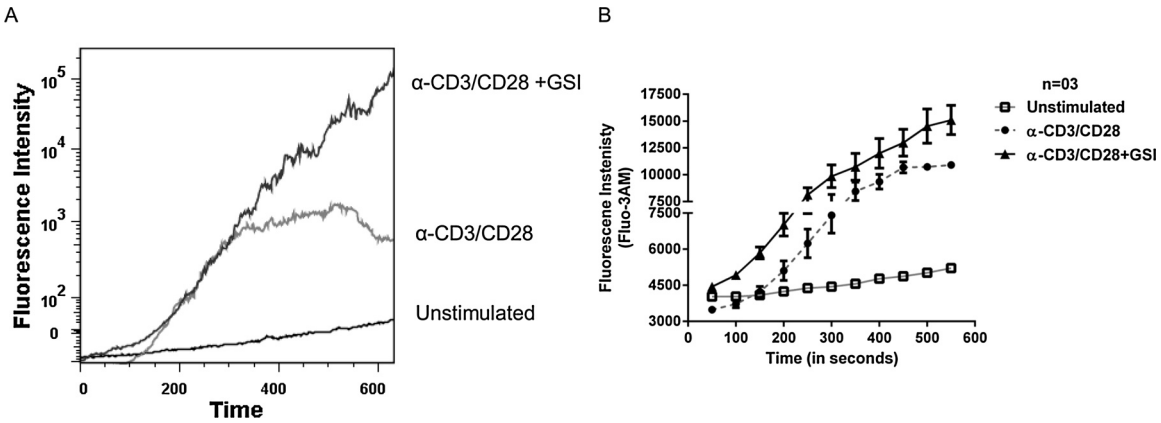

Fig. 3. Inhibition of Notch signalling results in altered calcium flux in T cells. (A) The αβ T cells were loaded with the 5 μM of FLuo-3AM in presence or absence of GSI-X at 37 °C for 30 min. Cells were washed with calcium buffer and acquired on FACSAria for 10 mins. Initially, a 30 s baseline was collected then α-CD3/CD28 was added either alone or in the presence of GSI-X. The graph is a representative figure showing kinetic flux of the intracellular calcium in the T cells stimulated with α-CD3/CD28 mAb in the presence or absence of GSI-X. (B) Each point on the graph represents the average fluorescence intensity over a range of 50 s each. The data represented is the mean of three independent experiments.

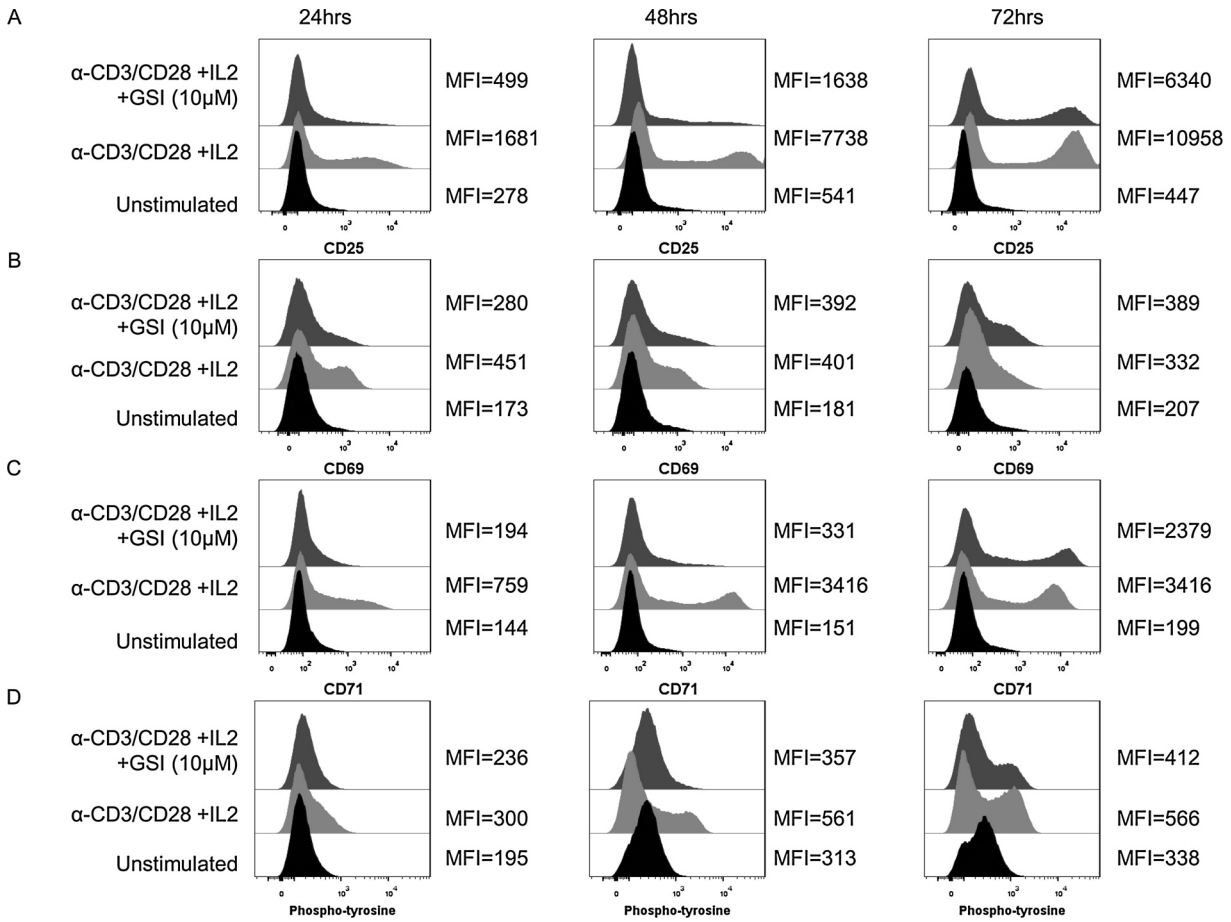

Fig. 4. Notch is required for distal TCR signalling events in the human CD3$^+$ αβT cells. Sorted αβ T cells from healthy individuals were stimulated with plate-bound α-CD3 and CD28 mAb for the indicated times either in presence or absence of GSI-X. Cells were harvested and analyzed by flow cytometry after gating on CD3$^+$ T cells. Median fluorescent intensity (MFI) values are shown in half offset histogram for (A) CD25; (B) CD69; (C) CD71; and (D) phosph-Tyrosine (pY). Data is representative of three independent experiments.

production was analyzed. The marked increase in production of cytokines IFN-γ compared to IL-10 was observed after stimulation with α-CD3/CD28 mAb over unstimulated αβ T cells (Fig. 6B). It was found that inhibition of Notch signalling by GSI-X leads to a marked reduction in IFN-γ release which was proportional to the concentration of GSI-X.

There was no significant increase observed in IL-10 production after α-CD3/CD28 mAb stimulation compared to unstimulated αβ T cells. However, treatment with GSI-X was able to inhibit the IL-10 production in a concentration-dependent manner (Fig. 6C). Thus, a role of Notch in regulating IFN-γ production in activated αβ T cells is evident which suggests that Notch plays a role in effector functions of T cells.

4. Discussion

Notch signalling pathway is essential for the functioning of murine CD4 and CD8 T cells (Cho et al., 2009; Palaga et al., 2003). However, the role of Notch in regulating TCR-driven effector functions of human T cells is not well understood. In the present study, we report that peripheral human αβ T cells express Notch receptors and ligands. Their expression increases after α-CD3/CD28 mAb co-ligation suggesting that TCR-driven signalling has the ability to alter the expression of components associated with the Notch signalling. On the other hand, inhibition of Notch signalling in the presence of TCR stimulation prevented TCR-driven induction of Notch receptors and ligands signifying that there is a dynamic interplay between Notch signalling and TCR-driven signalling in human αβ T cells. Notch ligand binding is necessary to initiate Notch-mediated induction of target genes (Minter and Osborne, 2012). Notch target genes were upregulated similar to Notch receptors and ligands confirming that Notch signalling is regulated by TCR signalling. The Notch activation may be *cis or trans* ligand-dependent which are regulated by TCR signalling on human αβ T cells.

Notch is known to regulate signal strength in thymocytes since constitutive expression of N1ICD in DP thymocytes prevented their maturation into single positive CD4$^+$ and CD8$^+$ T cells by interfering with TCR signal strength (Izon et al., 2001). The higher expression of Notch-1 and Notch-2 compared to Notch-3 and Notch-4 in human αβ T cells could be the outcome of differentiation (Van de Walle et al., 2013). Notch-3 which is a stronger Notch activator and only supports γδ T cell development was absent, whereas Notch-1, a weaker activator supporting both TCR-αβ and −γδ development, was present in αβ T cells (Van de Walle et al., 2013). It can be argued that the lower levels of Notch-3 on human αβ T cells might be the consequence of T cell differentiation to regulate Notch signal strength. The expression of Dll1, Dll4, Jag1 and Jag2 was observed in the human αβ T cells. Dll4 ligand supports both TCR-αβ and −γδ development while as Jag1 induces mainly αβ-lineage differentiation (Lehar et al., 2005). Jag1 is known to regulate the T cell signal strength by inducing an inhibitory signal in T cells. Jag1 might then be regulated by different pathway which has ability to dampen the TCR driven signalling and could be the reason why TCR driven signal failed to alter Jag 1 significantly. Jag1 is also known to regulate the GRAIL expression in T cells which has the ability to decrease the T cell signal strength (Kostianovsky et al., 2007). Jag2 expression is induced after high TCR signal strength which supports the fact that Jag2 mediated Notch activation primarily results in γδ T cell development requiring high TCR cell strength.

In peripheral CD4$^+$ T cells, it has been shown that Notch can influence the strength of TCR signalling and thereby influence the threshold of signalling via the TCR (Dongre et al., 2014). So, inhibiting

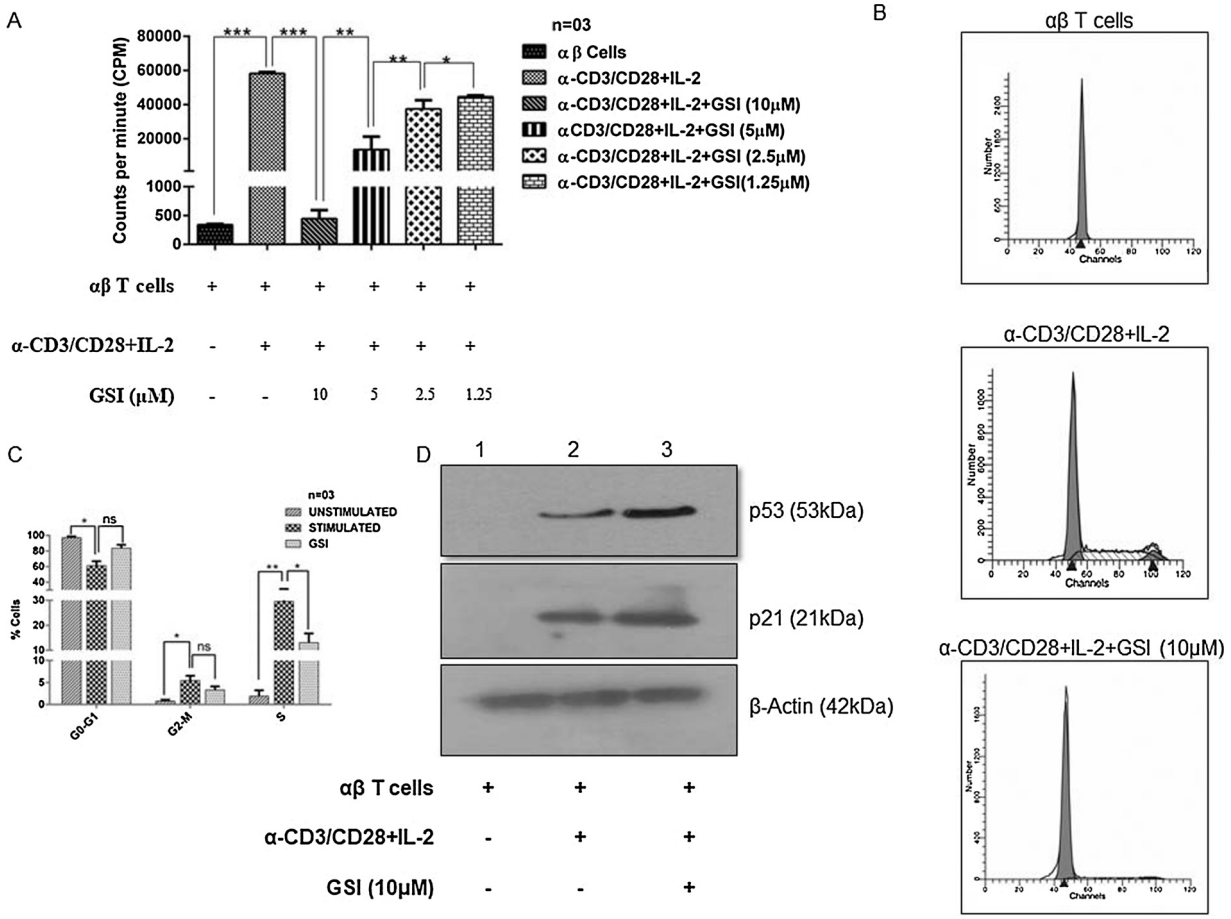

Fig. 5. Inhibition of α-CD3/CD28 mAb induced proliferation and cell cycle arrest of αβ T cells by GSI-X. (A) αβ T cells (5×10^4) were incubated in round-bottom 96-well tissue culture plates with α-CD3 and CD28 mAb (5 μg/ml, BD Biosciences) and rIL2 (30 U/ml, PeproTech) for 72 h. After 72 h, radioactivity incorporated in the DNA was measured in a liquid scintillation counter and represented as counts per minute (CPM). The data shown is the mean ± SEM of three independent experiments. (B) Cell-cycle analysis was performed on unstimulated αβ T cells (upper panel) and **α** -CD3/CD28 mAb plus rIL-2 stimulated αβ T cells without GSI-X (middle panel) or with GSI-X (lower panel). After 72 h cells were stained and analyzed by flow cytometry. FACS plots are representative of three independent experiments. (C) The graph shows the consolidated cell cycle analysis data of three independent experiments. The cell cycle phases G0-G1, G2-M and S phase are shown for unstimulated αβ T cells, αCD3/CD28 + IL2 stimulated αβ T cells and αCD3/CD28 + IL-2, stimulated αβ T cells in the presence of GSI (D) In a similar experiment, after 72 h, total protein lysate of αβ T cells, αCD3/CD28 + IL2 stimulated αβ T cells and αCD3/CD28 + IL-2 stimulation αβ T cells in the presence of GSI were used to detect the levels of p53, p21 and β-Actin by western blotting. β-actin was used as loading control (*** represents $p < 0.0005$, ** represents $p < 0.005$, * represents $p < 0.05$, 'ns' represents non-significant data with $p > 0.05$ and was determined by paired student's *t*-Test).

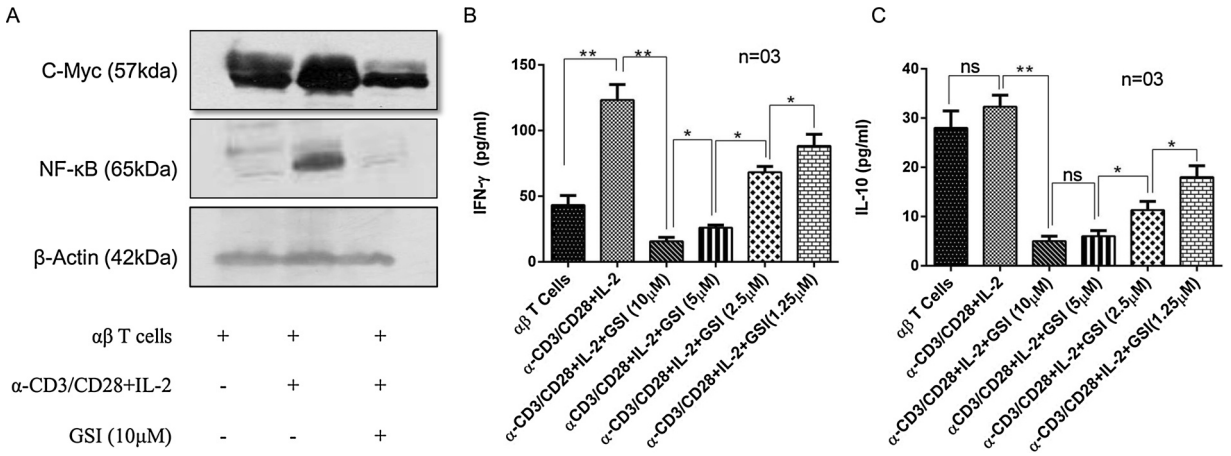

Fig. 6. Notch signalling regulates effector functions of T cells. (A) Human αβ T cells were stimulated with α-CD3/CD28 mAb in the presence of absence of GSI-X (10 μM) for 24 h. The protein extract was used to detect the levels of c-myc, NF-κB, and β-Actin by western blotting. The protein extract (50 μg) was loaded onto the SDS-PAGE and then transferred to the nitrocellulose membrane. β-actin was used as loading control. (B and C) αβ T cells were cultured in the absence or presence of different concentration GSI-X (1.25–10 μM) for 24 h in 96 well plates. Supernatants were collected and levels of IFN-γ and IL-10 cytokines were measured by cytometric bead array. In a concentration-dependent manner, GSI-X treatment decreases both IFN-γ and IL-10 cytokine production by αβ T cells. The data shown is mean ± SEM of three independent experiments (** represents $p < 0.005$; * represents $p < 0.05$, "ns" represents "non-significant" and was determined by paired student's *t*-Test).

Notch signalling will induce T cell anergy by increasing the threshold for T cell stimulation. This might result in the up-regulation of the E3 ubiquitin ligases like c-Cbl, Cbl-b, and GRAIL which are associated with anergy in lymphocytes (Mueller, 2004). Similarly, it was observed that on inhibition of Notch signalling the expression of The E3 ubiquitin ligase GRAIL was induced in human αβ T cells as well as Jurkat T cells. GRAIL also regulates T cell tolerance and regulatory T cell function by mediating T cell receptor-CD3 degradation (Nurieva et al., 2010). Consequently, it was observed that inhibition of Notch signalling induced GRAIL and thereby downregulation of CD3-ζ chain without affecting other T cell signalling molecules associated with proximal signalling. TCR stimulation also led to the reduction in TCR CD3-ζ chain which is known to play an important role in extinguishing the signalling process and reducing T cell responsiveness to antigenic stimulation (Valitutti et al., 1997). This is because effector T cells are susceptible to apoptosis triggered by prolonged TCR stimulation and TCR/CD3ζ down-regulation may serve to protect differentiating effector T cells from apoptosis. It is also reported that in GRAIL$^{-/-}$ naive T cells there are no significant differences in total and phosphorylated levels of ZAP70, phospholipase Cγ1, and MAP kinases p38 and JNK but elevated baseline levels of MAP kinase ERK1/2 (Kriegel et al., 2009). T cells marked with decreased CD3ζ protein expression have up-regulation of the related ITAM-containing signalling subunit FcRγ typically associated with the high-affinity IgE FcR (Krishnan et al., 2001; Blank et al., 1989). In effector cells, the FcRγ subunit forms a new TCR/CD3ε/FcRγ and this complex might have the ability to activate ZAP70. However, how E3 ubiquitin ligases differentially regulate the different proteins associated with T cell signalling needs validation.

GRAIL is induced in anergic T cells, limiting the transcription of cytokines (Anandasabapathy et al., 2003). Following anergic stimuli, calcium flux increases which is known to induce dimerization of NFAT1 leading to the expression of two anergy-inducing genes, *GRAIL* and *Caspase-3* (Baine et al., 2009). This T cell anergy could be the outcome of decreased Notch signalling which increases the threshold for TCR stimulation. This mechanism can also be corroborated with the cancer patients where decreased CD3-ζ expression is observed and could be associated with T cell anergy (Kulkarni et al., 2009; Dar et al., 2016). Manipulating Notch signalling has also been shown to rescue the antigen-specific CD8$^+$ T cells and to overcome the tumour-induced T-cell suppression (Sierra et al., 2014). This could be used as a strategy to enhance the T cell-based immunotherapy in cancer

Activation of Notch signalling has been shown to be involved in the induction of c-Myc and NF-κB in immune cells which in turn are involved in the promotion of cellular proliferation (Cho et al., 2009; Palaga et al., 2003; Guy et al., 2013). Our data showed that the expression of mRNA for non-canonical Notch target gene (NF-κB) was higher in human αβ T cells. Previously, we have shown that stimulation of γδ T cells with phosphoantigen was necessary for induction of cell-cycle regulator, c-Myc as well as upregulation of non-canonical Notch target gene, NF-κB (Gogoi et al., 2014). Our study demonstrate that inhibiting the Notch signal in activated T cells leads to their decreased proliferative response to α-CD3/CD28 mAb stimulation. We have shown that blocking of Notch signalling pathway by GSI-X leads to decreased proliferation of αβ T cells to α-CD3/CD28 mAb stimulation in a concentration-dependent manner. Notch signalling is known to support G1-S phase progression of the cell cycle by regulating cyclin D3 promoter activity in CD4$^+$ T cells in spleens of C57BL/6 mice (Joshi et al., 2009). We observed that α-CD3/CD28 mAb stimulated αβ T cells progress to S-phase of the cell cycle, and their progression was blocked in the presence of GSI-X. Our data indicates that Notch has a role in fine-tuning αβ T cell proliferation in response to α-CD3/CD28 mAb. Notch inhibition diminishes production of IL-2 and the expression of the high-affinity IL-2 receptor (CD25) (Adler et al., 2003). We have shown that inhibition of Notch signalling interferes with antigen non-specific signals of IL-2 by decreasing CD25 expression which limits proliferation of T cells (Gogoi et al., 2014). We believe that Notch signalling may be regulating the cytokine-driven bystander proliferation of memory T cells by interfering with the proximal T cell signalling. This will prevent their unnecessary proliferation and exhaustion in the absence of an antigenic signal in a p53 dependent manner.

It has been reported that Notch-1 can regulate IFN-γ production in activated murine T cells (Palaga et al., 2003). Notch has been reported to also regulate IL-10 production in murine Th1 cells that converts pro-inflammatory Th1 cells into T cells with regulatory activity (Rutz et al., 2008). We observed that GSI treatment of α-CD3/CD28 mAb-activated αβ T cells results in marked reduction of IFN-γ production in activated αβ T cells. Induction of GRAIL in T cells has the capability to inhibit the Th2 effector cytokines (Sahoo et al., 2014) and inhibition of Notch signalling in T cells causes overexpression of GRAIL which could be responsible for the decreased cytokines (IL-10). The expression of Notch in mature T cells and its ability to alter cytokine secretion suggests that Notch signalling has the capability to alter the T cell function and may impact Th1/Th2/Treg differentiation as well.

5. Conclusion

The study has provided insight into mechanisms that integrate TCR and cytokine signals to determine the outcome of T cell response and identified a central role for Notch signalling in this process. Many targets have been unravelled to enhance immunity including methods that can enhance the specificity of immunity by inducing the expansion of T cells and empower T cell based responses. In this context, our study provides a rationale to assume that Notch signalling can be used as a target to harness T cell based immunotherapies.

Conflict of interests

The authors declare no conflict of interest.

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The Jekyll and Hyde story of IL17-producing γδT cells

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In comparison to conventional αβT cells, γδT cells are considered as specialized T cells based on their contributions in regulating immune response. γδT cells sense early environmental signals and initiate local immune-surveillance. The development of functional subtypes of γδT cells takes place in the thymus but they also exhibit plasticity in response to the activating signals and cytokines encountered in the extrathymic region. Thymic development of Tγδ1 requires strong TCR, CD27, and Skint-1 signals. However, differentiation of IL17-producing γδT cells (Tγδ17) is independent of Skint-1 or CD27 but requires notch signaling along with IL6 and TGFβ cytokines in the presence of weak TCR signal. In response to cytokines like IL23, IL6, and IL1β, Tγδ17 outshine Th17 cells for early activation and IL17 secretion. Despite expressing similar repertoire of lineage transcriptional factors, cytokines, and chemokine receptors, Tγδ17 cells differ from Th17 in spatial and temporal fashion. There are compelling reasons to consider significant role of Tγδ17 cells in regulating inflammation and thereby disease outcome. Tγδ17 cells regulate mobilization of innate immune cells and induce keratinocytes to secrete anti-microbial peptides thus exhibiting protective functions in anti-microbial immunity. In contrast, dysregulated Tγδ17 cells inhibit Treg cells, exacerbate autoimmunity, and are also known to support carcinogenesis by enhancing angiogenesis. The mechanism associated with this dual behavior of Tγδ17 is not clear. To exploit, Tγδ17 cells for beneficial use requires comprehensive analysis of their biology. Here, we summarize the current understanding on the characteristics, development, and functions of Tγδ17 cells in various pathological scenarios.

Keywords: γδT cell, IL17, Tγδ17, infection, inflammation, cancer

INTRODUCTION

Decades have passed since the accidental discovery of T cells expressing γ and δ chains (1), yet it is hard to define γδT cells like αβT cells. Ambiguity in understanding the functions of γδT cells is attributed to their unparalleled characteristics as compared to αβT cells. Current understanding of T cell biology has emerged extensively from studies on αβT cells; however, recent findings have underlined the crucial role of γδT cells in shaping the immune response in infections, inflammatory diseases, and cancer. They are involved in early immune response like innate cells, produce proinflammatory cytokines (IFNγ, IL17, and TNFα), and activate adaptive immune cells. The cytokines secreted by γδT cells determine their effector functions. In humans, the major cytokine produced by γδT cells is IFNγ, contributing to its role in antiviral, anti-bacterial, and anti-tumor immunity (2–4). However, upon activation γδT cells can be skewed toward IL17, IL4, or TGFβ producing phenotype governed by the polarizing cytokines present in the surrounding milieu (5). Recent investigations in mice and human have highlighted the role of IL17-producing γδT cells (hereafter referred as Tγδ17) in bacterial infection, inflammatory disease, and cancer (6–8). They are the primary source of IL17 in early disease condition and are pivotal in progression and disease outcome (9, 10). To understand the functional significance of Tγδ17 in pathological conditions, many efforts have made in mouse models but there is scanty literature available on human Tγδ17 cells. In this review, we will discuss the recent findings of Tγδ17 differentiation, mechanisms regulating IL17 production, and their relevance in pathological conditions.

γδT CELLS: UNIQUE BUT VERSATILE

Survival of γδT cells over strong evolutionary selection pressure highlights their exclusive importance and disparate properties from conventional αβT cells. Initially, γδT cells were considered as cells of innate immunity owing to their ability to recognize conserved non-peptide antigens expressed by stressed cells. In addition to this, they recognize pathogen-associated molecular pattern (PAMP) or danger-associated molecular pattern (DAMP) through pattern recognition receptors (PRR) expressed by them (11). Like adaptive immune cells, human γδT cells undergo clonal expansion and exhibit antigen-specific memory (12). Thus, γδT cells link innate and adaptive immunity thereby enhancing the immune response against invading pathogen or danger signal posed by "self" cells. Antigen recognition by murine or human γδT cells does not require antigen presentation by major histocompatibility complex (MHC) class I or class II (13) and the crystal structure of γδTCR has revealed its close homology with immunoglobulins suggesting that antigen recognition by γδT cells is similar to antigen–antibody interaction (14). However, diversity of antigens recognized by γδT cells brands it different from B cells. The antigens exclusively recognized by γδT cells are not peptides of protein antigens rather are small mono- and pyrophosphates of linear C5 isoprenoids called as phosphoantigens (13). These

prenyl pyrophospahtes are metabolites of cholesterol biosynthesis and are recognized through complementarity determining regions (CDRs) of γδT cells (15). In humans, during cholesterol biosynthesis, phosphorylated precursors such as isopentenyl pyrophosphate (IPP) and DMAPP (dimethylallyl pyrophosphate) are synthesized by mevalonate pathway (16). However, microbial pathogens use non-mevalonate pathway to produce these phosphorylated precursors (17). γδT cells respond to these natural or synthetic stimulators with varying degree. Based on this, stimulators are classified either as weak or potent stimulators. HMBPP [(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate], a metabolite of non-mevalonate pathway of bacteria *Mycobacterium tuberculosis* is 10^4 times more potent stimulator of human γδT cells than IPP (18). The exclusive response of γδT cells to these phosphoantigens has a potential therapeutic significance and synthetic pyrophosphates can be used to harness the cytotoxic potential of γδT cells.

Murine and human γδT cells also recognize phycoerythrin (PE) – fluorescent molecule of cyanobacteria and red algae. PE is directly recognized by γδT cells but there is no sequence similarity between PE-specific murine and human γδ TCR (19). Naturally occurring primary alkyl amines activate human Vγ2Vδ2 T cells and enhance immunity against certain microbes and plant-derived antigens (20, 21). Similar to natural killer (NK) cells, human γδT cells also recognize the stress-induced MHC class I-related molecules MICA, MICB, and the UL16-binding proteins that are upregulated on malignant or stressed cells (22, 23). The stress-related molecules are ligands for NKG2D expressed by γδT cells and this engagement also enhances γδT cells' response to non-peptide antigens (24). Human and murine γδT cells recognize lipid antigens presented by CD1 molecules, a classical ligand for NK T cell suggesting the phenomenon similar to MHC-restricted antigen recognition by αβT cells (25–27). The murine γδT cells also recognize non-classical MHC class I molecules like T10 and T22 (β2 microglobulin-associated molecules lacking peptide binding groove) (28, 29). In addition to non-protein and MHC related antigens, murine and human γδT cells also recognize small peptides such as heat shock proteins (HSPs) (30–32). However, they do not require antigen-presenting cells (APCs) and recognition of antigen is MHC unrestricted, resembling B cells (33). Thus, the broad spectrum antigen responsiveness of γδT cells helps them to mount faster immune response.

Like αβT cells, γδT cells develop in the thymus from $CD4^-CD8^-$ (double negative, DN) thymocytes (34); however, they precede αβT cells in T cells ontogeny. γδ TCR rearrangements can be traced in early embryonic stages in mice as well as in humans (35, 36). This highlights their role in neonatal protection as conventional T cells are functionally impaired and APCs are immature in newborns (37). During thymic development, the decision of γδ versus αβ T cell commitment is determined by TCR signal strength or notch signaling (38). In mice, the strong TCR signaling in absence of notch signal induces γδT cells lineage commitment whereas low TCR signal strength in presence of strong notch signaling promotes αβ T cell lineage (39–41). However, notch signaling alone is insufficient to decide γδ/αβ T cell commitment. The intrinsic signals from T cell receptor complex and trans-conditioning by different subsets of thymocytes also determine thymic development of γδT cells (42). In humans, notch has opposite role in αβ versus γδT cell lineage decision, sustained notch signaling is required for the development of γδT cells (43) which is determined by differential notch receptor–ligand interaction importantly Jagged2/Notch3 signaling (44). In human, γδT cells differentiate along two pathways, a notch-independent DN pathway, generating mature DN and $CD8\alpha\alpha^+$ SP (single positive) $TCR\gamma\delta^+$ cells. In the notch-dependent DP (double positive) pathway, immature $CD4^+$ SP, and subsequently DP $TCR\gamma\delta^+$ cells are generated. Human postnatal thymus thus exhibits a scenario of DN, DP, and SP $TCR\gamma\delta^+$ population, which highlights heterogeneity in human γδT cell development (45). The activated extrathymic γδT cells, in humans, express notch receptors, which regulate their effector functions. Inhibiting notch signaling in γδT cells dampened their anti-tumor cytotoxic potential (46). Thus, validates the requirement of notch signaling in both thymic development and functions of human γδT cells. The diversity of human γδ T cell repertoire at birth (majorly contributed by $V\delta1^+$ subset of γδT cells in cord blood) is restricted in adulthood especially to Vγ9Vδ2, a circulating subset of γδT cells. The absolute numbers of Vγ9Vδ2 T cells increase from minor population at birth to more than 75% of γδT cells pool in peripheral blood (35), which constitute around 1–10% of total T cells in humans. The γδT cells exit the thymus as mature T cells and express markers that are associated with antigen-experienced T cells (47).

The other important feature of γδT cells apart from antigen recognition is their tissue tropism. In humans, the first γδT cells to arise from thymus are $V\delta1^+$ (paired with various Vγ chains), which preferentially populate in epithelial tissue and constitute larger proportion of intraepithelial lymphocytes (IELs) (48). They rapidly and innately recognize stressed cells found to be enriched in various tumor tissues (4). The Vγ9Vδ2 is a lymphoid homing subset of γδT cells, which continually expand in response to microbial antigen in circulation and exhibit characteristics of adaptive immune system (49). These cells recognize, expand, and secrete cytokines in response to non-peptide antigens associated with microbes in circulation. In mouse, a substantial proportion of γδT cells reside as the IEL in the skin, intestine, and genitourinary tract. In response to the chemokine signals, $V\gamma5V\delta1^+$ T cells leave the fetal thymus, reside in the epidermis, and form dendritic-like network similar to Langerhans cells. These cells are called as dendritic epidermal T cells (DETCs) and constitute more than 90% of epidermal T cells (50). $V\gamma6^+$ T cells home to tongue and reproductive tract whereas $V\gamma7^+$ T cells home to intestinal tract suggesting that distinct TCR repertoire are present at different anatomical site and respond to antigens unique to their resident tissues (51–53). However, the functions of IELs are determined by the environment at the anatomical site (54) and hence specific γδ T cell subset could be used in tissue repair and generation of effective immune response at different epithelial sites.

γδT cells perform diverse effector functions determined by the TCR expressed, tissue localization, and activation status. Apart from these, MHC-independent recognition of antigens, production of IFNγ, and expression of cytotoxic granules classify γδT cells as potential cytotoxic cells (55). They can kill activated, infected, stressed, and transformed cells using various strategies such as engagement of death-inducing receptors, such as FAS

and TNF-related apoptosis-inducing ligand receptors (TRAILR) and the release of cytotoxic effector molecules such as perforin and granzyme (56, 57). Human γδT cells also recognize HSP (HSP60/70) expressed on tumor cells and enhance its cytolytic activity against the tumors (31, 58). γδT cells support the maturation and activation of other lymphocytes, NK cells, and macrophages with the help of secreted chemokines (CCL3, CCL4, CXCL10) (55). Another chemokine CXC–chemokine ligand 13 (CXCL13) produced by Vγ9Vδ2 cells can regulate B cell organization within lymphoid tissues and help B cells to produce antibodies (59). Human γδT cells can also crosstalk with dendritic cells (DCs) influencing each other functions like the antigen presentation by DCs, activation, and secretion of IL12 and IFNγ by γδT cells, which result in DC maturation (11, 60). These properties of γδT cells aid in generation of the effective immune response in the appropriate condition. Not only this, activated Vγ9Vδ2 cells can take up and process the soluble antigens, opsonize target cells, and can migrate to lymph nodes through CC-chemokine receptor 7 (CCR7) where they upregulate expression of MHCs and co-stimulatory receptors CD80 and CD86 (61, 62). Activated Vγ9Vδ2 cells has also been licensed to act as APC and activate CD4 and CD8 T cells (63). Collectively, these observations highlight the multi-talented role of γδT cells, having both Th- and Tc-like properties along with acting as APC. The special trait of γδT cells is their ability to recognize phosphorylated non-protein antigens and mediate its effector function in spatial and temporal manner making them a robust cell type, which can be manipulated to develop a promising tool for novel immunotherapies against certain types of diseases. However, care should be adapted while designing such immunotherapies because these cells have capacity to secrete various cytokines under different conditions.

Tγδ17: A SUBTYPE OF γδ T CELLS

Unlike αβ T cells, in mice, which leave thymus as naïve cells and are primed in the peripheral compartment, γδT cells undergo subset commitment in the thymus itself. However, in humans, upon activation with different cytokines, Vγ9Vδ2 cells can be polarized toward different effector subtypes like γδ1, γδ2 (64), γδ17 (65, 66), and γδTreg (67, 68). This functional plasticity of γδT cells assists them to tackle the distinct disease conditions and play important role in the early responses to invasive pathogens. The recent findings have stated that γδT cells are major IL17 producers and have shown their involvement in early onset of immune activation (69). Similar to Th17 cells, Tγδ17 cell express RORγt as a lineage determination transcriptional factor (70). Healthy adult human peripheral blood Vγ9Vδ2 T cells distinctively express Th1 signature and 50–80% produce IFNγ but <5% produce IL17 (6). However, Tγδ17cells have been demonstrated to be involved in the pathogenesis of transplantation rejection (71), autoimmune disease (72), allergy (73), and cancer (74) in humans. The biology of Tγδ17 is so naive that it compels us to cross-examine its genesis, functions, and clinical relevance to understand its therapeutic potential.

MOLECULAR EVIDENCES OF Tγδ17 GENESIS

The molecular mechanism of IL17-producing γδT cells remains an enigma. Most of the studies carried out to understand the differentiation mechanisms of Tγδ17cells are based on the murine models. γδT cells preferentially localized to barrier tissues are the initial source of IL17 and are likely to originate from the fetal thymus. These are called as the natural IL17-secreting γδT cells. γδT cells that make IL17 within 24 h fall in this category (75). γδT cells acquire IL17-secreting phenotype in secondary lymphoid tissues after antigen exposure, which is referred to as induced Tγδ17 cells (76, 77).

During development of T cells in thymus, murine γδT cells branch off at the transition of thymocytes from DN3 stage to DN4 stage (34). It is also reported that γδT cells develop from DN2 stage and specifically produce IL17 whereas IFNγ-producing γδT cells can develop from both DN2 and DN3 precursors (78) (**Figure 1**). This suggests that γδT cells do not develop like αβT cells and follow evolutionary ancient path of T cell development. However, the precise DN stage from which γδT cells develop is elusive (79). Fetal thymic γδ T-cell development occurs in successive waves by using the different Vγ and Vδ segments during the embryonic development (34, 80). Successful gene rearrangement of γδ T cells from early thymic precursors ($CD44^{hi}$) lead to the development of naïve γδ T cell characterized by $CD44^{lo}$ $CD27^{+}CD62L^{+}$ phenotype. This phenotype can either leave the thymus to populate in secondary lymphoid organs or it can undergo further intrathymic differentiation that results in the development of multiple γδ T cell subtypes such as dendritic epidermal γδT cell (DETCs), Tγδ17, or NK 1.1^{+} γδ cell (γδNKT cells) (80, 81). Recently, it was described that when thymic lobes of mice at E14 were colonized with DN1a cells from mice at E13 and E18, respectively. It was observed that although both populations (E13 DN1a cells and E18 DN1a cells) generated similar number of γδT cells, only E13 DN1a cells generated $Vγ3^{+}$ DETCs. These observations indicate that precursor lineage of DETCs may be different and needs further investigation (82). DETCs develop at embryonic day 13 (E13) to approximately E17 and readily secrete IFNγ when activated. After the development of DETCs, the next functional developmental wave consists of Tγδ17 cells. Tγδ17 cells are heterogeneous in using TCR chains that mainly include $Vγ6^{+}$ and $Vγ4^{+}$ but also use $Vγ1^{+}$ chain. $Vγ6^{+}$ cells develop by E14 to around birth and finally Vγ1 and Vγ4 cells develop E16 onward (81). The other subtypes of γδ T cells, which develop in thymus, are γδ NKT cells, which are similar to invariant $TCRαβ^{+}$ NKT cells (83, 84).

There are different thymic signaling processes, which determine functional phenotype of γδT cells in thymus before migration to periphery and contribute to the balance between IFNγ committed versus IL17-commited subtypes (85). This biasness toward IL17 or IFNγ depends on the antigen experience in thymus. The γδ T cells that have encountered the cognate antigen interaction in thymus, gain the potential to differentiate into the IFNγ-producing functional phenotype while antigen naïve γδ T cells develop into IL17-producing γδT cells (86). This skewedness also reflects in their distribution outside the thymus. Most of Tγδ17 cells reside in lymph nodes whereas IFNγ-producing γδT cells are mainly found in the spleen and the mechanism for this distribution is not clear (86). Similar distribution is also found in αβT cells and it seems to be logical as the lymph nodes serve as the site of initial exposure to foreign antigens and propagate the wave of inflammation, thus are suited for the earliest source of the IL17 secretion (87).

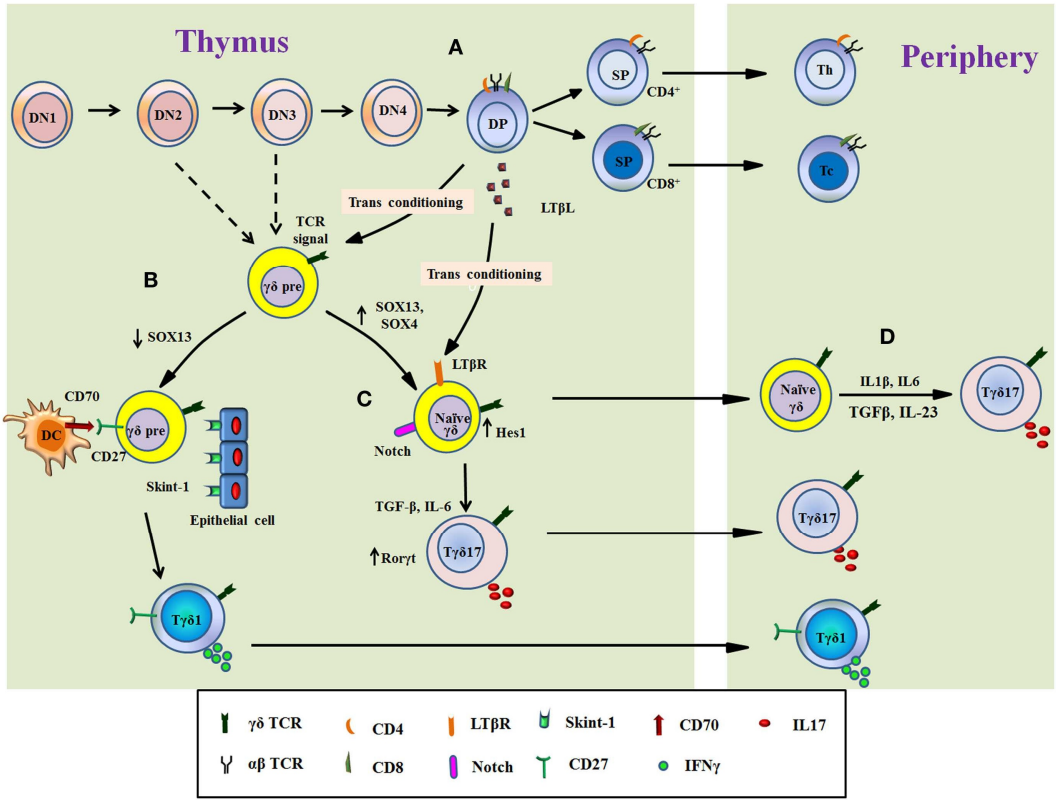

FIGURE 1 | Overview of Tγδ17 cells development. The figure illustrates the differentiation of Tγδ17 cells from T cell progenitors in the murine thymus **(A–C)** and from naïve γδT cells in periphery in human **(D)**. Progenitor T cells differentiate through double negative stage 1 (DN1) to DN stage 4 **(A)**. The decision of αβ or γδ TCR expression takes place at early T cells precursor (from DN2 or DN3 stage) as showed by dashed line. The thymocytes expressing αβ TCR develop into double-positive thymocytes, which support differentiation of functional subtypes of γδT cells called as transconditioning. DP thymocytes secrete LTβL, which support differentiation of Tγδ17. The DP αβ thymocytes then exit the thymus as mature single positive T cells (either $CD4^+$ or $CD8^+$ T cells) **(A)**. The functional programing of γδT cells is determined by TCR signal and/or other related signals. TCR signal, interaction with Skint-1 from epithelial cells, downregulation of SOX13, and signaling through CD27/CD70 divert γδ thymocytes toward IFNγ-producing phenotype (Tγδ1), which migrate to periphery **(B)**. Conversely, signaling through Notch receptor maintain Sox13 levels with increase in Hes1 and RORγt expression induce γδ thymocytes to produce IL17. Progression of γδ thymocytes to Tγδ17 cells is independent of signaling through Skint-1 and/or CD27 but require inputs from IL6 and TGFβ. The natural Tγδ17 cells developed in thymus migrate to tissue or periphery **(C)**. In human, naïve γδT cells, which exit thymus, can also differentiate into Tγδ17 cells in presence of TCR signal and cytokines such as IL6, IL1β, IL23, and TGFβ **(D)**.

Besides the γδ TCR signaling (86), expression of tumor necrosis factor receptor family member, CD27, determines the IL17 versus IFNγ production by γδT cells (88). $CD27^+$ γδT cells differentiate into IFNγ producing cells whereas IL17 production was restricted to $CD27^-$ T cells (89) (**Figure 1**). Thus thymic "imprinting" of the γδT cells as $CD27^+$ or $CD27^-$ regulates effector functions of γδT cells and is preserved in the periphery (89). CD27 is not only associated with IFNγ production but also aids γδT cells to interact with its ligand CD70 expressed on DCs, thymic epithelial cells, and double-positive thymocytes thus acting as a costimulatory receptor (89). Therefore, CD27 conveys an intrathymic message that licenses the $CD27^+$ γδ T cells for the production of IFNγ (47). Another signaling pathway that influences the differentiation of Tγδ17 is the signaling through lymphotoxin-β receptor (LTβR), a member of the tumor necrosis factor receptor family (90). Signaling through LTβR leads to the activation of the alternative nuclear factor (NF)-κB pathway via RelB. Ligands for LTβR regulating this developmental process are produced by $CD4^+CD8^+$ thymocytes (91). The homeostasis of this functional phenotypic differentiation, influenced by other thymic progenitors is known as transconditioning (91), which highlights coordination between different signaling pathways in thymus that occur in physically separate thymic niche (92). LTβR signaling pathway controls Tγδ17 development by regulating transcription factors RORγt and RORα4, required for IL17 expression in γδ thymocytes (93). The role of LTβR signaling, however, remains controversial as LTβR is present downstream to CD27 signaling, which is associated with the IFN-γ production (89).

The maturation of Tγδ17 cells from its precursors requires TCR signaling as mice with reduced ZAP70 show decreased number of Tγδ17 cells (94). However, TCR signaling alone is not sufficient as it also requires other signals (95). An src family kinase, Blk (B lymphoid kinase), is required for Tγδ17 cells development in thymus as Blk-deficient mice was reported to have less number of IL17-producing γδ T cells (96). Similarly, high-mobility group (HMG) box transcription factors, SOX4 and SOX13 are

positive regulators of Tγδ17 development (95, 97). These transcription factors expressed in immature T cells (98) highlight that the development of Tγδ17 is from early precursors (DN2) (78, 95). Other thymic determinant, which is responsible for the functional dichotomy in Tγδ17 and Tγδ1, is Skint-1, a thymic epithelial cell determinant. The interaction between Skint-1^+ cells and γδ thymocytes ($V\gamma5^+V\delta1^+$) induce an Egr3-mediated pathway, leading to differentiation toward IFNγ-producing γδ T cells. Further, it suppresses Sox13 and an RORγt transcription factor-associated Tγδ17 cells lineage differentiation suggesting that the functions of the earliest T cells are substantially preprogramed in the thymus (99). Notch signaling is known to be involved in thymic determination and development of Tγδ17 cells. Hes1, one of the basic helix–loop–helix (bHLH) proteins induced by Notch signaling is critical for the IL17 expression by γδ T cells and its thymic development (100–102). Further, the specific expression of Hes1 in $CD25^+$ and $CD27^-$ γδ T cells and decreased levels of Tγδ17 in Hes1-deficient mice highlights the critical role of Notch–Hes1 pathway in Tγδ17 development in thymus as well as in periphery (101). The thymic development of Tγδ17 is independent of STAT3 but partly dependent on RORγt (101) and most peripheral IL17-producing γδ cells express RORγt and respond rapidly to IL23 (103).

Developmental process of Tγδ17 also requires signaling through different cytokines. TGFβ signaling is necessary for Tγδ17 development (104). It has been shown that in absence of TGFβ1 or Smad3 (a component of the TGFβ signaling), the number of Tγδ17 thymocytes reduced drastically relative to that of wild-type mice (104). As compared to TGFβ, requirement of IL6 for Tγδ17 development is not well understood as there are contrasting reports on its role (72, 105). It is also reported that IL6 does not act directly on uncommitted γδ thymocytes but instead it acts indirectly by regulating the expression of Delta-like ligand 4, a ligand for notch receptor, expressed by thymic epithelial cells that promote the differentiation of Tγδ17 (101, 106). Moreover, IL23 and IL1 produced by DCs are crucial for IL17 production by γδT cells. $IL23^{-/-}$ and $IL23R^{-/-}$ mice showed the significant reduction in Tγδ17 cells after *L. monocytogenes* infection supporting earlier observation (107–110).

Thymic development of human Tγδ17 cells is poorly investigated. Around 80% circulating human Vγ9Vδ2 T cells are IFN-γ producers and express CD27 whereas CD27 negative cells are IL17-producing γδ T cells are <5% (65). Interaction of CD70 with CD27 promotes the expansion of Th1-biased Vγ9Vδ2 T cells in periphery (111). However, such role in their thymic development is unknown. Human Vγ9Vδ2 T cells can be polarized to Tγδ17 cells in periphery upon IPP activation and in the presence of cytokines like TGFβ, IL1β, IL6, and IL23, followed by a week of culture in differentiation medium supplemented with IL2 can induce IL17 in these cells (65, 66). In humans, there are contrasting reports on role of IL6 and IL23 in differentiation of Tγδ17. It has been shown that IL6 is required for differentiation of neonatal Tγδ17, and IL23 is required for the generation of adult IL17-producing γδT cells (65). In another study, it is reported that in the presence of TCR signaling, IL23 promotes the induction of IL17 in neonatal (but not adult) γδT cells (112). However, it appears that IL23 induces γδT cells to coproduce IL17 and IFNγ in adults but support development of Tγδ17 cells in neonates. In addition to the above-mentioned cytokines, IL7 selectively promotes the mouse and human IL17-producing γδT cells. IL7 activates STAT3 preferentially in γδT cells competent to produce IL17 (113). However, the increased IL17 production by γδT cells upon TCR stimulation in presence of IL7 is observed only in case of cord blood cells but not with peripheral lymphocytes. Thus, it is important to note that the antigen naïve γδT cells only can be reprogramed *in vitro* toward Tγδ17 phenotype (66, 113).

The kinetic study of IL17 production by γδT cells has shown that murine γδT cells secrete IL17 within few hours after stimulation (70). This phenomenon can be reasoned by the thymic development of murine Tγδ17 cells and constitutive presence of transcriptional regulators for IL17 production. However, human γδT cells in thymus are functionally immature and can attain their functional differentiation in periphery in presence of cytokines (114). This supports the kinetics of IL17 production by human γδT cells that mRNA expression of IL17 and RORγt peaks by day 3–6 and decrease by day 9 onward, after stimulation. The expression of cytokine receptors (IL1βR, IL6R, TGFβR, and IL23R) on Vγ9Vδ2 T cells peaks on day 3 and decrease by day 6 (66). Thus, coordinated combination of TCR and cytokine stimulation could be necessary for the sustained secretion of IL17 by γδT cells, which highlights the difference in kinetics of IL17 secretion by murine and human Tγδ17 cells. This underscores that human γδ T cells can be "reprogramed" in the periphery into different functional lineages.

Upon antigenic challenge, T cells differentiate to memory phenotype; either central memory (TCM) or effector memory (TEM) (115). Human Tγδ17 cells present in non-lymphoid environment belong to $CD27^-$ $CD45RA^{\pm}$ effector (74) or terminally differentiated (TEMRA) (66) memory phenotype. Similarly, murine Tγδ17 cells also show effector memory phenotype with $CD44^{high}$, $CD45RB^{low}$, and $CD62L^{low}$ (116). Thus, Tγδ17 cells differentiated either in thymus or in periphery, belong to memory phenotype, and licensed to patrol the blood, lymphoid organs, and peripheral tissues.

Tγδ17 IN MICROBIAL INFECTIONS

Tγδ17 cells can rapidly produce IL17 upon Toll-like receptors (TLR) or cytokine stimulation alone even in absence of antigen presentation. The general proinflammatory functions of IL17 [reviewed in Ref. (117, 118)] could be associated with γδT cells as they are major producers of IL17. Studies carried out in various infection models showed that Tγδ17 cells are protective against infection. During mycobacterial infection, IL17 produced by $V\gamma4^+$ and $V\gamma6^+$ cells induce pulmonary granuloma formation by recruitment of granulocytes and monocytes. The IL17 participates in maturation of granuloma by promoting tight cell to cell binding via ICAM1 and LFA1 induction (119). Mycobacteria-infected DCs secrete IL23, which regulate IL17 production by γδT cells emphasizing that the early activation of Tγδ17 cells is important for initiating inflammation and recruiting innate immune cells to the site of infection thereby enhancing bacterial clearance from host (120, 121). Tγδ17 cells also support cell-mediated immunity by inducing Th1cells against pulmonary mycobacterial infection (122).

In *Escherichia coli* infection model also, γδT cells were reported to be the major producers of IL17, which enhanced neutrophil infiltration to the peritoneum. The infiltration of cells diminished after antibody depletion of resident Vδ1$^+$ subtype of γδT cells highlighting its involvement in IL17 secretion in response to IL23 (9). Thus, IL23 and Tγδ17 cells play a dominant role as first line of defense in infection before CD4 T cell activation. In case of *L. monocytogenes* infection, a large number of γδ T cells accumulate in the lymph organs shortly after infection and begin to produce IL17A, signifying the role of Tγδ17 cells in the *Listeria* infection (123). IL17 was also shown to promote proliferation of CD8$^+$ cytotoxic T lymphocytes by enhancing DC cross-presentation *in vitro*. DCs stimulated with IL17 showed upregulation of MHC-I molecule H2Kb and enhanced secretion of cytokines (IL12, IL6, and IL1β). CD8α$^+$ DCs from *Il17a*$^{-/-}$ mice also produced less IL12 and are less potent in activating naive CD8$^+$ T cells (123). This indicate that Tγδ17 cells not only induce innate response but also critical for optimal adaptive cytotoxic response against intracellular bacterial infection. The alliance of IL23 and Tγδ17 is also demonstrated to have a protective role during infections such as *Klebsiella pneumonia* (124), *Citrobacter rodentium* (125, 126), *Salmonella enterica* (127, 128), and *Toxoplasma gondii* (129). The Tγδ17 cells also play a vital role in clearing fungal infections. The rapid production of IL17A was reported in the lungs at a very early stage after intravenous infection with *C. albicans*. Lung resident γδ T cells were the major source of early IL17A production regulated by IL23 and TLR2/MyD88-dependent pathway (130). Presence of Tγδ17 cells were also reported in the lungs of neutropenic mice during *C. neoformans* infection. These Tγδ17 cells played an important role in the chemotaxis of leukocytes and induction of protective immune response (131). Tγδ17 cells thus orchestrate the protective immunity by acting at the early onset in infection models (108).

Relatively few studies have evaluated the role of Tγδ17 cells in human microbial immunity. In patients with tuberculosis (TB), elevated levels of Tγδ17 cells were found in peripheral blood and were major producers of IL17 (6). As a protective role, in response to bacterial antigens, IL17-producing Vγ9Vδ2 T cells induce neutrophil migration through secretion of CXCL8 and promote their phagocytic activity (66). Tγδ17 cells also induce epithelial cells to secrete anti-microbial peptides like β-defensins in response to bacterial antigens (66). This signifies the modulatory effects of Tγδ17 cells on keratinocytes and other immune cells in anti-microbial defense. In children with bacterial meningitis, the population of IL17$^+$ Vγ9Vδ2 T cells significantly increase in peripheral blood and at the site of infection (cerebrospinal fluid). The reversal of this pattern after successful anti-bacterial therapy clearly suggests the anti-microbial role of Tγδ17 cells (66). Collectively, these studies provide new insight into the functions of γδ T cells as the first line of host defense against bacterial and fungal infection in human and may pave a path in designing newer treatment modalities.

TOLL-LIKE RECEPTORS REGULATE IL17 PRODUCTION IN Tγδ17 CELLS

γδT cells express various chemokine receptors, cytokine receptors, and PRRs, which regulate IL17 production. TLRs are the well-studied PRRs expressed by DCs, macrophages, and γδT cells. The unique microbial molecules called as PAMP are recognized by TLRs, which orchestrate the anti-microbial response in γδT cells (11). In malarial infection, MyD88 deficiency results in severe impairment of IL17A producing γδT cells levels, but not IFNγ producing γδT cells highlighting differential control by innate signaling through TLRs in infections (132). Murine Tγδ17 cells specifically express TLR1 and TLR2 but not TLR4. High number of Tγδ17 cells were induced upon *in vivo* stimulation with Pam3CSK4 (ligand for TLR2) but not with LPS (TLR4 ligand) or CpG (TLR9 ligand) (70). Interestingly, it has been shown that TLR4 indirectly controls IL17 generation by γδT cells through IL23 secreted by TLR4 expressing macrophages in response to HMG Box 1 (HMGB1, a damage-associated protein and TLR4 ligand) (133). Moreover, Tγδ17 cells promote experimental intraocular neovascularization (134) as well as early acute allograft rejection (135) in response to HMGB1. Signaling through TLR2 is indispensable for Tγδ17 in anti-microbial functions. Absence of TLR2 or MyD88 in cutaneous *Staphylococcus aureus* infection, or in *Candida albicans* infection, caused an impaired IL17 production and poor microbial clearance in the skin infiltrated with Vγ5$^+$ γδT cells (130, 136). Tγδ17 cells also express DC-associated C-type lectin 1 (dectin 1) and intraperitoneal injection of curdlan (dictin 1 ligand), induced IL17 production by γδT cells (70). In imiquimod (IMQ)-induced psoriasis-like model, dermal γδT cells spontaneously secreted a large amount of IL17 in IMQ-treated skin cells. Thus, it appears that TLR7/8 (receptor of IMQ) may regulate the IL17 production by γδT cells. It is important to note that the modulatory effects of TLRs on γδT cells as showed in *in vivo* murine models are mediated through IL23 and/or IL1β cytokines. The direct stimulation of CD27$^-$ γδT cells by TLR ligands (LPS or PAM) show no effect on IL17 production (132). This suggests that TLR signaling indirectly modulates Tγδ17 function.

RECEPTOR REPERTOIRE EXPRESSED BY Tγδ17 CELLS

The receptor profile of Tγδ17 cells is similar to Th17 cells. In mice, the majority of IL17-producing CD4 cells belong to CCR6$^+$ compartment compared to CCR6$^-$ (137). Sorted CCR6$^+$ γδT cells showed increased mRNA expression of IL17, IL22, IL23R, Rorγt, and aryl hydrocarbon receptor (AhR) compared to CCR6$^-$ γδT cells (70, 138). This suggests that CCR6 can be a phenotypic surface marker of Tγδ17 cells. Besides CCR6, Tγδ17 cells express various chemokine receptors including CCR1, CCR2, CCR4, CCR5, CCR7, CCR9, CXCR1, CXCR3, CXCR4, CXCR5, and CXCR6 (7). The early onset recruitment of Tγδ17 to the site of inflammation is determined by the type of chemokine receptor on them. Tγδ17 cells expressing CCR6 and CCR9 show selective migration toward allergic inflamed tissue in response to CCL25 (ligand for CCR9). α4β7 integrin expression is indispensable for this migration and transendothelial crossing of Tγδ17 cells. (139). Since migration through CCL2/CCR2 axis is determinant for total γδT cells, CCL25/CCR9-mediated migration seems to be specific for Tγδ17 subtype (140, 141).

In humans, Tγδ17 cells express CCR6 but not CXCR3, CXCR5, CCR3, CCR4, or CCR5. However, they express granzyme B, FASL, and TRAIL but not perforin (66). The lack of granzyme B and perforin coexpression may be responsible for absence of cytolytic activity of Tγδ17 cells. On the contrary, it has been shown that the

human colorectal tumor-infiltrating Tγδ17 cells do not express FASL or TRAIL but express CD161 and CCR6 (74). The inconsistency in expression of cytolytic markers and their relevance on Tγδ17 cells needs to be understood in detail. The AhR is indispensable for Tγδ17 cells as it promotes differentiation of naïve Vγ9Vδ2 T cells toward Tγδ17 phenotype (66).

In mouse model, it has been shown that $Ahr^{-/-}$ Tγδ17 cells express IL17 but fail to produce IL22 (70). Moreover, in mouse model of *Bacillus subtilis* induced pneumonitis, deficiency of Ahr resulted into low IL22 production but IL17 levels were maintained (142). Thus, although Ahr promotes IL17, it is indispensable for IL22 production by Tγδ17 cells.

INFLAMMATORY DISORDERS AND MANIA OF Tγδ17

Th17 cells and Tγδ17cells are essential in disease progression and are pathogenic in autoimmune disease. Dysregulated levels and sustained secretion of proinflammatory cytokines by γδ and/or CD4 T cells have devastating effects on autoimmune disease progression. In a collagen-induced arthritis (CIA) model (resembling human rheumatoid arthritis), IL17-producing $Vγ4/Vδ4^+$ T cells selectively increase in joints and lymph nodes. Depletion of γδ T cells by anti Vγ4 antibody, markedly reduced the disease severity score revealing its pathogenic nature (143). Interestingly, both Th17 and Tγδ17 are present in the joints but Th17 cells localize proximal to the bone, which facilitates its interaction with osteoclast. Selective depletion of Th17 cells abrogated the bone resorption suggesting that Th17 but not Tγδ17 cells are responsible for bone destruction. Thus, Tγδ17 cells may be responsible for enhancing joint inflammation and exacerbate CIA (144). In contrast, absence of Tγδ17 was reported in patients with rheumatoid arthritis and in murine model of autoimmune arthritis (SKG model) (145). The SKG mouse model has defects in the differentiation of Tγδ17 cells (94), which might result into low Tγδ17 cells in the inflamed joints. Thus, the role of Tγδ17 cells in autoimmune arthritis need to be evaluated comprehensively.

Tγδ17 also enhanced experimental autoimmune encephalomyelitis (EAE) (mouse model for human multiple sclerosis). Upon immunization of mice with myelin oligodendrocyte glycoprotein (MOG) peptide in complete Freund's adjuvant (CFA), $Vγ4^+CCR6^+IL23^+$ γδT cells accumulate in the central nervous system (CNS), which expand by 20-fold in absolute number during development of clinical signs of the disease (72). In contrast, IFNγ-producing γδT cells are low in CNS and marginally increase during course of EAE (103). The mechanism behind aggravation of EAE could be attributed to restraining the development of $Foxp3^+$ regulatory T cells (Tregs) functions by Tγδ17 cells. Supernatants from IL23-activated γδT cells inhibited the TGFβ driven conversion of naive $Foxp3^-$ αβT cells into Foxp3 expressing T cells and also reversed the suppressive effect of Treg cells (72). Similar function of Tγδ17 was reported in cardiac transplantation in mice. IL17, majorly produced by γδT cells, accelerates acute rejection of transplanted heart but IL17 deficiency enhanced Treg expansion and prolonged allograft survival (71). In ischemic brain injury, Tγδ17 were reported to be present at the infract areas (146). Tγδ17 rather than Th17 was the major source of IL17 whereas IFNγ was majorly produced by Th1 cells. In mice, genetically deficient for IL17 or IL23, the infract areas were reduced suggesting a role of Tγδ17 as a key contributor of neuroinflammation (146). Overall, this suggests that in chronic inflammatory condition, innate cytokines IL23 and IL1β promote infiltration and generation of IL17-producing γδT cells, which aggravate the disease.

Experimental silicosis is a useful model for depicting chronic lung inflammation, tissue damage, and fibrosis. Tγδ17 along with Th17 accumulated in the lung in response to IL23 expressing macrophages by third day after silica treatment but interestingly did not induce lung fibrosis (73). On the contrary, in allergic lung inflammation, Tγδ17 cells are known to be protective (147, 148). Functional blockage of both IL17 and γδT cells impaired the resolution of airway lung inflammation (148). It is claimed that this protective role is mediated by prostaglandins (PGs), which are abundant at the site of inflammation. PGI2 analog iloprost enhanced IL17 production by γδT cells in the thymus, spleen, and lungs, reducing airway inflammation (147). This highlights the role of PGI2 analogs that can be exploited in the development of immune response in immunotherapeutic approaches. Age-related macular degeneration (AMD) is another chronic inflammation associated disease, characterized by choroidal neovascularization (CNV). In an experimental model, Tγδ17 cells along with $Thy\text{-}1^+$ ILCs (innate lymphoid cells) infiltrate the eye after laser treatment and promote neovascularization. This recruitment is in response to IL1β but not IL23 produced by macrophages (134).

Tγδ17 CELLS AS HEROES OR VILLAINS IN CANCER

The unmatched characteristics of human γδT cells to have MHC unrestricted tumor directed cytotoxicity, release of copious amounts of IFNγ, and recognition of cancer cells through variety of mechanisms render them as potential candidate for cancer immunotherapy (4, 149). Upon activation, γδT cells show cytotoxicity against myeloma (150), lymphoma (151), leukemia (152, 153), and other epithelial carcinomas (57, 154, 155) *in vitro*. Several clinical trials have been launched using γδT cells based therapies in cancer patients. The hallmark characteristic of γδT cells to be used for therapy is their ability to infiltrate tumors (156). *In vivo* activation by phosphoantigens or adaptive transfer of preactivated autologous γδT cells have proved successful in cancer treatment (157). However, the role of Tγδ17 cells as anticancer effector cells is not well defined.

In a chemotherapeutic approach, Tγδ17 cells are reported to play decisive role in several transplantable tumor models (EG7 thymoma, MCA205 sarcoma, CT26 colon cancer, and TS/A mammary carcinomas). Tγδ17 ($Vγ4^+/Vγ6^+$) cells were shown to invade the tumor bed early in response after drug treatment. This was followed by infiltration and induction of IFNγ-producing CD8 (Tc1) cells to the tumor bed. This infiltration of Tγδ17 and Tc1 cells was correlated and associated with tumor regression post radio or chemotherapy (158). Thus, IL17-producing $Vγ4^+/Vγ6^+$ cells are critical for the induction of Tc1 response in tumor tissue in response to drug treatment or radiation. Another study in bladder cancer supports the helper function of Tγδ17 cells in cancer treatment. Tγδ17 cells induce neutrophil infiltration to the tumor site and show anti-tumor effect upon *Mycobacterium bovis* BCG treatment (159).

In contrast to anti-tumor role of Tγδ17 cells, they also promote tumor development. With the notion that IL17 is a proangiogenic

cytokine (160), Tγδ17 cells promote angiogenesis in tumor model. In IL17$^{-/-}$ tumor bearing mice, the blood vessel density was markedly decreased compared to wild type. In addition, IL17 induced the expression of Ang-2 (angiopoietin) and VEGF (vascular endothelial growth factor) in tumor cells (8). In ovarian cancer model, it has been reported that CD27^{-} Vγ6^{+} cells produced higher IL17 and induce VEGF and Ang-2 in peritoneal exudates of tumor bearing mice after 6 weeks of post-tumor inoculation (161). Additionally, Tγδ17 cells induce mobilization of protumor small peritoneal macrophages (SPM) to the tumor bed, which express IL17-dependent proangiogenic profile (*Il1b, Il6, vegfa, tgfb, mif, cxcl1, cxcl8*, and *tie2*). SPMs also enhance ovarian cancer growth by stimulating tumor cell proliferation (161). In hepatocellular carcinoma mouse model, it was reported that IL17, majorly produced by Vγ4^{+}γδT cells, induced CXCL5 production by tumor cells, which enhance migration of MDSCs (myeloid-derived suppressor cells) expressing CXCR2 to the tumor site. In addition, IL17 also enhanced suppressive functions of MDSCs by inhibition of T cells proliferation and cytokine (IFNγ and TNFα) production (162). In return, MDSCs induced γδT cells to produce IL17 through IL23 and IL1β secretion forming positive feedback loop for Tγδ17 activation (162). Thus, Tγδ17 cells interact with myeloid cells and counteract tumor immune-surveillance.

In human colorectal cancer, IL8 and GM-CSF secreted by Tγδ17 promote migration of MDSCs while IL17 and GM-CSF enhanced their proliferation. Tγδ17 cells also support survival of MDSCs through IL17, IL8, and TNFα (74). Thus, it is possible to speculate that Tγδ17 cells might be responsible for gradual shift from initial inflammatory to immunosuppressive tumor environment in advanced stage cancer (163). In human colorectal carcinoma, Tγδ17 cells were positively correlated with advancing tumor stages as well as with clinicopathological features including tumor size, tumor invasion, lymphatic and vascular invasion, lymph node metastasis, and serum CEA (Carcinoembryonic antigen) levels suggesting their pathogenic role (74).

Collectively, these findings highlight the apparently opposite roles of Tγδ17 cells in cancer immunity. It seems that during tumor development, inflammatory environment (IL1β and IL23) modulate the cytokine profile of γδT cells from primary IFNγ toward proinflammatory IL17, which support tumor progression.

CONCLUDING REMARKS

Despite the small percentage in total T cell population, γδT cells have emerged as an important modulator of early immune responses. The development of functional subtypes of γδT cells require polarizing cues including molecular and cellular

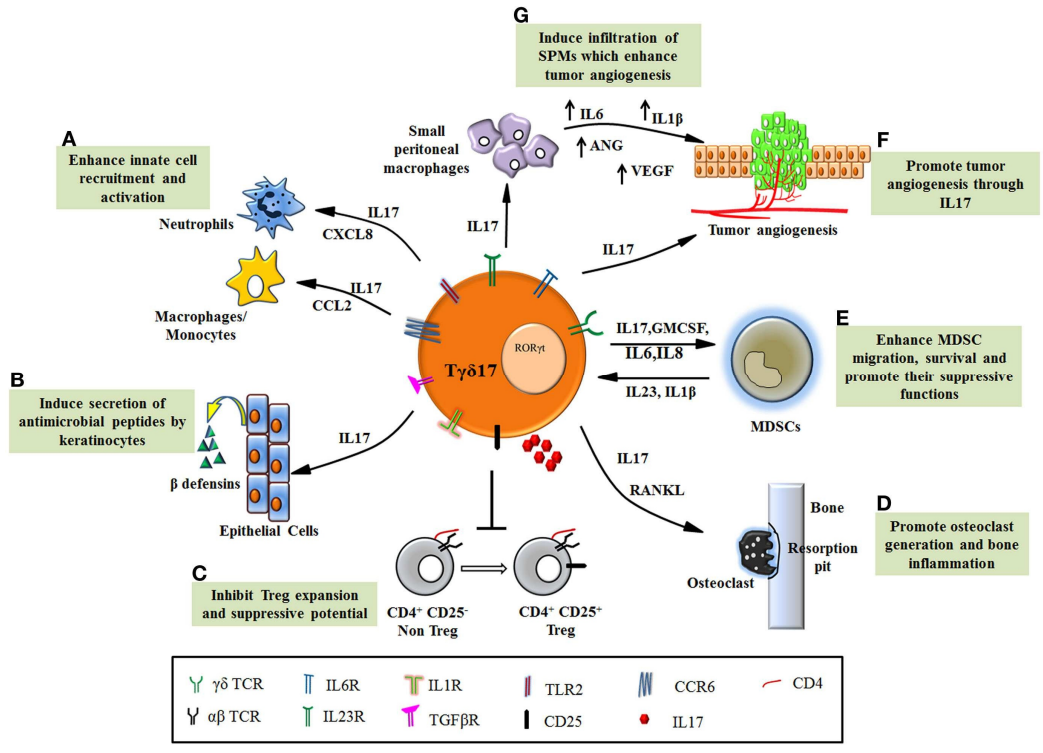

FIGURE 2 | Functions of Tγδ17 cells in pathological conditions. **(A)** Tγδ17 cells promote infiltration of neutrophils and monocytes/ macrophages to the site of inflammation through chemokines. **(B)** IL17 secreted by Tγδ17 cells induces keratinocytes to produce anti-microbial peptides such as β defensins and protect host in infections. **(C)** Dysregulated Tγδ17 cells in autoimmune diseases inhibit Treg expansion and its ability to suppress autoreactive cell, thereby exacerbating the disease. **(D)** The inflammatory condition in arthritis is worsened by IL17, which foster osteoclast formation through induction of RANKL. Tγδ17 cells are involved in bone resorption and enhance joint inflammation. **(E)** Human Tγδ17 cells support MDSC migration, survival, and promote their suppressive functions through IL17, GMCSF, and IL8. MDSCs also form feedback loop and promote Tγδ17 differentiation through IL23 and IL1β. **(F)** Tγδ17 cells secrete IL17 and induce tumorigenesis by their proangiogenic activity. **(G)** Murine Tγδ17 cells recruit small peritoneal macrophages to the tumor bed, which induce angiogenesis.

interaction and combination of multiple cytokines and chemokine receptors that regulate their distribution. This suggests that the functional determination of γδT cell subtypes is dictated by the local environment (thymus or peripheral blood or the inflamed tissue) in which they are present. Tγδ17 is a special γδT cell subset, distinctly present at early immune response in the tissue and can modulate the functions of other immune and epithelial cells but their relevance in disease outcome remains controversial. In response to microbial antigens, Tγδ17 cells promote infiltration of neutrophils and macrophages and induce production of antimicrobial peptides resulting in clearance of microbial load. Such protective behavior of Tγδ17 cells in infections can be exploited to develop newer approaches to tackle the microbial pathology (**Figure 2**).

The opposite side of Tγδ17 functions has revealed its detrimental role in enhancing inflammation in autoimmunity and cancer (**Figure 2**). The mechanism, which regulates such dual personality of Tγδ17 cells is unknown. It appears that the obvious common role executed by these cells is enhancement of inflammation but due to functional heterogeneity and their complex interdependency on other cells (innate and adaptive); the emerging scenario of their biology is far from complete. This provokes us to consider contextual behavior of Tγδ17 cells in disease pathology. Current progress in understanding the significance of Tγδ17 cells in inflammatory diseases has revealed their novel but debilitating functions such as suppression of Tregs in autoimmunity, induction of angiogenesis, and recruitment and activation of MDSCs in various malignancies. Thus, in inflammatory disorders, Tγδ17 cells can be targeted using various immunotherapeutic approaches. However, need of hour is to expand the understandings of Tγδ17 in humans and develop a protocol for their propagation and activation. The future therapies will rely on regulating the key transcription factor RORγt by designing suitable antagonists that will help in fine tuning Tγδ17 differentiation and eventually their function in chronic inflammation and infection.

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