Role of Notch Signaling in T Cell Polarization

Shilpa Keerthivasan

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LOYOLA UNIVERSITY CHICAGO

ROLE OF NOTCH SIGNALING IN T CELL POLARIZATION

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR BIOLOGY

BY

SHILPA KEERTHIVASAN

CHICAGO, ILLINOIS

AUGUST 2011
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supported me during tough times of PhD, rather guided me and helped me troubleshooting experiments. My daughter Pragnya for her immense love. I dedicate my thesis to them.
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<tr>
<td>AHR</td>
<td>Aryl Hydrocarbon Receptor</td>
</tr>
<tr>
<td>ANK</td>
<td>Ankyrin repeats</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cells</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen Induced Arthritis</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSL</td>
<td>CBF-1/RBP-Jk, mammals; Suppressor of Hairless, Drosophila; and Lag-1, C. elegans</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C) motif ligand 12</td>
</tr>
<tr>
<td>DAPT</td>
<td>3,5-Difluorophenylacetyl)-L-AlanylPhenyl Glycinebutyl Ester</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DLL</td>
<td>Delta Like</td>
</tr>
<tr>
<td>DNER</td>
<td>Delta/Notch like epidermal factor related</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental Autoimmune Encephalomyelitis</td>
</tr>
<tr>
<td>GATA</td>
<td>Ability to bind DNA sequence “GATA”</td>
</tr>
</tbody>
</table>
GSF   Granulocyte Stimulating Factor
GSI   Gamma Secretase Inhibitor
HLX   H2.0 like homeobox
IBD   Inflammatory Bowel Disease
IFN   Interferon
IL    Interleukin
ILCHO Z-Ile-Leu-Aldehyde
IRF-4 Interferon Regulatory Factor-4
Itk   IL-2 inducible T cell kinase
kDa   KiloDalton
LCMV  Lymphocytic Choriomeningitis Virus
MAGP  Myofibril associated glycoprotein
MHC   Major Histocompatibility Complex
MMPs  Matrix Metalloproteases
MOG   Myelin Oligodendrocyte Glycoprotein
MS    Multiple Sclerosis
MTS   3-(4,5-dimethylthiazol-2-yl)-5-(3carboxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt
N^{EC} Notch Extracellular
N^{IC} Notch intracellular
NIK   Nuclear Factor kappa B-Inducing kinases
NLS   Nuclear Localization Signal
NOD   Nucleotide binding Oligomerization domain
<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NOV</td>
<td>Nephroblastoma Overexpressed gene</td>
</tr>
<tr>
<td>N&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>Notch transmembrane</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Patterns</td>
</tr>
<tr>
<td>PEST</td>
<td>Proline-Glutamate-Serine-Threonine rich</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid peptide</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen related receptor</td>
</tr>
<tr>
<td>PTPRC</td>
<td>Protein Tyrosine Phosphatase Receptor type C</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RAM</td>
<td>Recombination-signal-protein-J (RBP-J) associated molecule</td>
</tr>
<tr>
<td>ROR</td>
<td>Retinoic acid receptor related orphan receptor</td>
</tr>
<tr>
<td>RUNX</td>
<td>RUNT related transcription factor</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activators of Transcription</td>
</tr>
<tr>
<td>T-bet</td>
<td>T box expressed in T cells</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell Receptor</td>
</tr>
<tr>
<td>Tec</td>
<td>Tyrosine kinase expressed in Hepatocellular carcinoma</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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</tbody>
</table>
ABSTRACT

The differentiation of CD4$^+$ T cells to different effector lineages in response to pathogenic stimuli is the core of the adaptive immune system. One of the effector subsets recently discovered is T-helper 17 (Th17) and it plays a predominant role in autoimmune diseases and inflammatory disorders. Within a short span of time extensive literature has been available on this subset. This subset not only helped us to better understand basic adaptive responses but also unlocked a complete new area of T cell plasticity. Presently we are aware of several Th17 lineage determining factors such as cytokine environment (TGF$\beta$+IL-6) and transcription factor ROR-$\gamma$t. The role of cell surface receptors in Th17 differentiation is not known and has remained an understudied area of investigation.

In my thesis, I aimed to study the role of Notch cell surface receptors in Th17 differentiation. Using in vitro Th17 differentiation assays of human naïve CD4$^+$ T cells, I have shown that Notch signaling, particularly Notch1, plays a crucial role in Th17 polarization. I have demonstrated that expression of Notch1 is increased in human in vitro differentiated Th17 cells. By using pharmacological Notch signaling inhibitors and specific knockdown of Notch1, I have shown that Notch1 is necessary for Th17 differentiation. Overexpression of Notch1 in naïve
CD4⁺ T cells followed by Th17 differentiation resulted in an increase in IL-17 secretion, which further supported my data. Furthermore, IL-17 promoter assays and chromatin immuno-precipitation (ChIP) experiments revealed that IL-17 and ROR-γt are transcriptional targets of Notch1.

I have also observed that in vitro differentiated Th17 cells are plastic, as during the polarization of T cells towards Th17, they also secrete Th1 associated cytokine IFNγ. This suggests that there is flexibility in IL-17 positive cells to become IFNγ positive, and/or Th17 and Th1 share an overlapping differentiation program. This concept of flexibility in Th17 differentiation is very intriguing. The presence of these IL-17⁺/IFNγ⁺ double producer cells both in vitro and in vivo under inflammatory conditions makes it an area of active investigation. In this study I have also shown that Notch signaling not only regulates Th17 subset differentiation, but also controls Th1 component of the Th17 differentiation process. By using pharmacological Notch inhibitors and specific Notch1 knockdown in human in vitro Th17 differentiation assays, I have shown that Notch1 signaling regulates IL-17 and TNFα expression but not IL-5 secretion. Notch1 overexpression led to increase in IL-17 and TNFα but not IL-5 secretion in Th17 differentiated cells. Chromatin immuno-precipitations and human T-bet promoter assays support that T-bet and ROR-γt, but not GATA3 are direct targets of Notch1 under Th17 differentiating conditions.

A role of Notch signaling in Th17 as well as Th17/Th1 co-differentiation is further validated in vivo. Experimental Autoimmune Encephalomyelitis (EAE)
model is used to detect the effects of Notch signaling inhibition on Th17 and Th1 subsets (in collaboration with Dr Barbara Osborne, University of Massachusetts, Amherst). In this mouse model of human multiple sclerosis several publications suggest a role of both IL-17 and IFNγ in EAE pathogenesis. We observed that Notch signaling inhibition by oral Gamma Secretase Inhibitors (GSI) in the EAE model ameliorates the symptoms as well as delays the onset of disease. We also observed a decrease in IL-17 and IFNγ cytokine expression levels among restimulated splenocytes and mononuclear cells of the CNS in GSI fed EAE mice as compared to animals fed with control chow.

My study demonstrates a role of Notch signaling in dictating Th17 differentiation. Further it determines the role of Notch signaling in regulating Th17/Th1 double producer population also. Thus I propose use of Notch inhibitors (GSI) as a potential therapeutic strategy to treat Multiple Sclerosis due to its ability to regulate pathogenic cytokines such as IL-17 and IFNγ.
CHAPTER I

INTRODUCTION

Activation of naïve CD4$^+$ T cells and differentiation

After completing their developmental process in thymus, naïve T cells enter the blood stream, and they circulate continuously from blood stream to lymphoid organ and back to the blood until they encounter antigen. While circulating between lymphoid organs and blood stream these naïve T cells make contact with several antigen presenting cells (APCs) in lymphoid tissue. These contacts allow naïve T cells to undergo positive selection for self MHC: nonself peptide recognition as well as to experience positive survival signals. Naïve T cells (1 in $10^4$-$10^6$ is specific for particular antigen) that recognize their specific antigen by T cell receptor (TCR) cease to migrate, and then become activated to proliferate and differentiate into effector T cells. The most important professional APCs are highly specialized dendritic cells and macrophages. This recognition of antigen with respect class II MHC on APCs by the TCR of naïve T cells is called antigen specific “Signal 1”. Clonal proliferation of naïve T cells also requires second or costimulatory signals called “Signal 2” by the same APC (Kindt et al., 2007).

Naïve T cells express CD62l (L-Selectin) which facilitates their homing to lymphoid organs by interacting with CD34 and GlyCAM1 (Nicholson, 2002).
Another cell surface marker by which naïve T cells are commonly characterized by is CD45RA. The cytoplasmic tail of CD45 antigen has intrinsic phosphatase activity that removes phosphate from tyrosine kinase, specifically Lck, in T cells and helps in the activation of T cells (Zamoyska, 2007). There are various isoforms of CD45; CD45RA is located on naïve T cells and CD45RO is expressed on activated T cells and memory T cells (Tchilian and Beverley, 2002). Naïve T cells are negative for activation markers CD25, CD44, CD69 as well as memory cell marker CD45RO (De Rosa and Roederer, 2001).

<table>
<thead>
<tr>
<th>Naïve T cells</th>
<th>Activated effector T cells</th>
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After activation, naïve CD4⁺ T helper cells begin to divide and give rise to effector cells. These effector cells are CD4⁺ and are commonly divided into T helper 1, T helper 2 and more recently identified T helper 17 based on distinct cytokine profile and immuno-regulatory functions. T helper 1 (Th1) cells secrete cytokines IFNγ and TNFα and are important for eliminating intracellular infections by viruses, bacteria and provide an effective anti-tumor protection. T helper 2 (Th2) cells secrete cytokines IL-4, IL-5, IL-10 and IL-13 and are important in the defense against parasitic infections (Kidd, 2003). Over the years Th1/Th2
paradigm of T helper cells differentiation, which was first introduced by Mosmann and Coffman in 1986, helped us to explain phenomenon of adaptive immunity (Mosmann et al., 1986). Recently, this paradigm has been expanded with the discovery of a third subset known as T helper 17 (Th17) (Harrington et al., 2005; Langrish et al., 2005; Park et al., 2005). These cells secrete IL-17, IL-17F, IL-21 and IL-22. The primary function of this subset is the induction of inflammatory responses against extracellular bacterial and fungal infections.

The Th1, Th2 and Th17 cells differentiation process is mutually antagonistic as well as self-reinforcing, that is: only one of the subsets is dominant in response to a given pathogen at any time. The important question that arises is what determines the differentiation pattern and immunological response against a particular pathogen?

Although other subsets of CD4⁺ T cells are also been described in literature, for example T regulatory cells and other minor T helper subsets (see Table 1) including TGFβ producing Th3 (Chen et al., 1994), IL-10 producing TR1 (Groux et al., 1997), IL-9 producing Th9 (Veldhoen et al., 2008b) and T follicular helper cells (Nurieva et al., 2008), the primary focus of this thesis lies in differentiation of Th17 subset of T helper cells.
# T-Helper cells subsets

<table>
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<tr>
<th></th>
<th>Th1</th>
<th>Th2</th>
<th>Th17</th>
<th>Th9</th>
<th>Tfh</th>
<th>Treg</th>
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<tbody>
<tr>
<td><strong>Signature cytokine</strong></td>
<td>IFNγ, TNFα, TNFβ</td>
<td>IL-4, 5, 10, 13</td>
<td>IL-17, IL-21, IL-22</td>
<td>IL-9</td>
<td>IL-21</td>
<td>TGFβ, IL-10</td>
</tr>
<tr>
<td><strong>Transcription factor</strong></td>
<td>Tbet, STAT4, Hlx</td>
<td>STAT6, GATA3, c-maf</td>
<td>ROR-γt</td>
<td>TGFβ, IL-4</td>
<td>FoxP3</td>
<td></td>
</tr>
<tr>
<td><strong>Polarizing cytokine</strong></td>
<td>IL-12, IFNγ</td>
<td>IL-4</td>
<td>TGFβ/IL-6, TGFβ/IL-6/IL-21/IL-1β</td>
<td>IL-21</td>
<td>TGFβ, IL-10, IL-2</td>
<td></td>
</tr>
<tr>
<td><strong>Host defense</strong></td>
<td>Intracellular bacteria, Viruses.</td>
<td>Parasitic Infections</td>
<td>Similar to Th1, mucosal immunity, Extracellular bacteria, fungi, parasites</td>
<td>Helminthes</td>
<td>Suppressor of immune response</td>
<td></td>
</tr>
<tr>
<td><strong>Pathogenic role</strong></td>
<td>Autoimmune Diseases.</td>
<td>Asthma and allergies</td>
<td>Autoimmune Disease, psoriasis</td>
<td>Self tolerance</td>
<td></td>
<td></td>
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### Table 1: Different T-helper subsets

Different subsets of T-helper cell subsets are shown. This table emphasizes the differential cytokine environment, transcription factor, signature cytokines secretes and role in normal as well as pathogenic response of each subset.
Th1/Th2 differentiation and its regulation.

The concept of T helper 1 (Th1) and T helper 2 (Th2) type of cytokine production was emerged in the late 1980 by Mosmann and Coffman, originating from the observation that mouse CD4\(^+\) T cells clones could be classified into different subtypes of T helper population differing in cytokine secretion and immune-regulatory functions (Mosmann et al., 1986). This observation later revealed to be valid for human T cells as well (Romagnani, 1991).

Naïve T cells undergo preactivation state called Th0 (Th\(_{\text{precursor}}\)) followed by differentiating towards either Th1 or Th2. This process is called polarization of T cells. Polarization of T helper cells into Th1, Th2 or recently discovered Th17 is a highly complex phenomenon which is regulated at multiple levels. It involves activation of cytokine genes specific for particular T helper cell subset and commitment of these polarized cells to effector phase. Simultaneously it also involves antagonizing expression of alternate cytokine genes. There are number of factors involved including the nature and affinity for cognate antigen, type of TCR signaling, cytokine environment, transcription factors involved and epigenetic mechanisms that regulate the T cell differentiation process. These factors are discussed in detail below.

Factors governing Th1/Th2 differentiation
Role of Antigen Presenting Cells (APC): The generation of immune response is antigen controlled by the interaction of antigen with APC. Evidences suggest that APCs including monocytes and macrophages themselves polarize into Th1 promoting DCs (DC1) or Th2 promoting DCs (DC2) depending on nature of processed antigen and then subsequently bias the polarization of T-helper cells (Hertl, 2005). These DCs selectively express co-receptors, cytokines and other polarizing signals that promote differentiation of naïve T helper cells towards Th1 or Th2, for example DC1 (including macrophages) secrete IL-12 to promote Th1 polarization whereas DC2 (including mast cells and CD4+ NK like cells) secrete IL-4 to promote Th2 polarization (Liu, 2001; Rissoan et al., 1999). In response to antigen and associated adjuvants, recognition and binding via pathogen associated molecular patterns (PAMPs), APCs are driven to become DC1 and DC2 subtypes through toll like receptors (TLRs) expressed by APCs. Associated adjuvants such as polyI:C, LPS and CpG which act through TLR 3, 4 and 9 respectively on DCs have a role in Th1 inducing signals (Dabbagh and Lewis, 2003). By contrast binding of PAM3Cys to TLR2 promotes Th2 inducing signals in DCs (Redecke et al., 2004). Preliminary data suggests that differential up-regulation of MAP kinase pathway explains the activation of DC to either the DC1 or the DC2 subtypes with p38 promoting DC1 and pERK promoting DC2 (Agrawal et al., 2003).
T cell receptor (TCR) signaling and role of co-stimulatory molecules:

Polarization of naïve T helper cells towards Th1 or Th2 subsets is regulated antigen regulation via TCR and by subsequent downstream intracellular signaling, for example activation of PKC, Ca\(^{2+}\) and signaling molecules such as NF-κB and NFAT (Noble et al., 2000). In addition of TCR signaling, costimulatory signals also regulate T helper cell differentiation, for example B7 and ICOS (Van Gool et al., 1996).

It has been documented that early TCR signaling events differ between Th1 and Th2 subsets, for instance tyrosine phosphorylation profiles in Th2 cells are reduced as compared to Th1 cells. This may be due to difference in tyrosine phosphatase activity between Th1 and Th2 cells (Hannier et al., 2002). Among the costimulatory signals, the B7 family of protein plays an important role in the T cells differentiation process. It has been reported in in vitro differentiation assays that addition of anti B7.1 increases IL-4 production whereas neutralizing B7.2 enhances production of IFNγ (Kuchroo et al., 1995). Similarly, another costimulatory molecule ICOS is particularly for Th2 responses as ICOS\(^{-/-}\) T cells are specifically deficient in IL-4 production (Coyle et al., 2000; Nurieva, 2005; Watanabe et al., 2005).

Cytokine environment:
The cytokine milieu generated by antigen presenting cells upon interaction with pathogen is the most influential factor regulating T helper cell differentiation. Th1 differentiation is initiated by secretion of IL-12 by macrophages and dendritic cells (Hsieh et al., 1993). A role of IFNγ is also appreciated in this process as in vitro neutralization of IFNγ inhibited Th1 differentiation (Farrar et al., 2002; Lighvani et al., 2001). It has been postulated that IL-12 promotes the release of IFNγ by DCs and natural killer cells, which in turn acts on DCs to produce more IL-12. Moreover, IFNγ released by APCs also acts as an inhibitor of Th2 cell proliferation. In the case of Th2, IL-4 produced by natural killer cells, eosinophils and mast cells regulate Th2 differentiation (Farrar et al., 2002; Lafaille, 1998). These cytokines upregulate and bind to their receptors expressed in naïve CD4⁺ T cells, and then downstream signaling pathways involving various intracellular signaling cascades and transcription factors regulate specific T helper cell differentiation (Refer Fig.1).

Transcription factors network:

T helper differentiation is determined by the cytokine milieu and the resultant transcription factor network which is up-regulated specifically in either Th1 or Th2 subset differentiation. The crosstalk and interplay between these transcription factors culminates specific lineage. Differentiation of naïve CD4⁺ T-helper cells towards the Th1 subset is initiated by Signal Transducers and
Activators of Transcription family of transcription factors (STAT) 1 and 4, which are activated in response of IFNγ and IL-12 respectively (Kaplan et al., 1996; Lighvani et al., 2001). STAT1 inturn regulates the T-bet promoter, a transcription factor important for Th1 differentiation (Afkarian et al., 2002). T-bet directly binds and transactivates the IFNγ promoter. Moreover it also induces the expression of H2.0-like homeobox (HLX) and RUNT related transcription factor 3, (RUNX 3) and binds as a complex with them to the IFNγ promoter (Djuretic et al., 2007; Mullen et al., 2002). T-bet indirectly regulates Th1 differentiation by binding to the GATA-3 promoter, negatively regulating its expression (Usui et al., 2006). It also complexes with RUNX3 and silences the IL-4 gene locus (Djuretic et al., 2007). T-bet binds to the IFNγ promoter even when DNA is methylated (repressive mark) (Chen et al., 2006a; Tong et al., 2005) and directly recruit histone demethylases (Jumonji domain containing protein demethylases) which removes the repressive Histone 3 Lysine 27 (H3K27) trimethylation mark (Miller et al., 2008). This probably provides the mechanism by which overexpression of T-bet can reprogram differentiation of T cells towards Th1 in already committed Th2 subsets. Interestingly, STAT 4, T-bet and HLX form a multimolecular complex and regulate the IFNγ promoter, thereby reinforcing Th1 commitment (Refer Fig.1A).

Similarly Th2 differentiation is governed by a network of transcription factors. Differentiation is initiated by activation of STAT 6, which is activated by IL-4 secreted by mast cells (Ansel et al., 2006). STAT 6 then binds and activates
GATA-3, which in turn activates transcription factor MAF and activates the IL-4 promoter. GATA-3 along with MAF and STAT 6 regulates IL-4, IL-5 and IL-13 promoters (Ansel et al., 2006) (Refer Fig.1B).

Epigenetic mechanisms:

During the differentiation process, T-helper cells undergo intrinsic cellular changes which are passed on to daughter cells. These intrinsic changes are very important for maintenance of self-reinforced differentiation status. These changes are mediated by epigenetic modifications which include chromatin remodeling by DNA methylation and histone modifications. A study by Bird et al has convincingly shown that epigenetic modifications can regulate T helper cells differentiation (Bird et al., 1998). They demonstrated that the use of either demethylating agent azadecoxycytidine or histone hyperacetylating agent trichostatin A can drive cells towards Th2 differentiation in presence of IL-4 even in STAT6 deficient T cells, despite the importance of this transcription factor for Th2 differentiation. Consistent with this data, the chromatin remodeling complex component Brahma Related Gene 1 (BRG1) regulated the IFNγ promoter in mouse Th1 cells and MLL (H3K4 methyltransferase) regulate GATA3, IL-4, IL-5 and IL-13 expression (Yamashita et al., 2006; Zhang and Boothby, 2006).

Identification of Th17 cells, novel subset of T helper cells
Novel discovered Th17 subset secretes IL-17 as the predominant cytokine, and it has immunoregulatory and effector functions different from the Th1 and Th2 subsets. Development of this subset requires a completely different cytokine milieu as compared to Th1 and Th2 subsets, and also requires crosstalk between different transcription factors to initiate differentiation.

Initially Th1 cells were known to play predominant role in autoimmunity, but this concept was challenged when IFNγ and IFNγ receptor deficient mice were not protected from several autoimmune disorders, but rather developed aggravated form of disease (Krakowski and Owens, 1996; Zhang et al., 2003a). Mice lacking the expression of IL-12 p35 subunit, IL-12 receptor or IL-18 (crucial cytokines for Th1 differentiation) were also not protected against autoimmune disorders (Gran et al., 2002; Gutcher et al., 2006). This suggested the existence of a distinct lineage of T helper cells having a role in development of autoimmune disorders.

The Th17 lineage was originally discovered in the year 2000 as a result of computational analysis which led to the identification of the p19 subunit. Interestingly this protein was later found to form a heterodimer with the p40 chain of IL-12, together was coined the novel cytokine IL-23 (Oppmann et al., 2000). This could explain the previous discrepancies as all earlier studies were based on antibody neutralization of the p40 subunit of IL-12 (subunit shared between IL-12 and IL-23) and which could alleviate autoimmune disease. This strongly
suggested that IL-23, rather that IL-12, has a role in autoimmune disorders. To further elucidate the precise mechanism a systematic approach was used involving IL-23p19 and IL-12p35 knockout mice. As predicted it was found that IL-23p19−/− mice were incapable of developing autoimmune disease whereas IL-12p35−/− mice were susceptible (Cua et al., 2003; Murphy et al., 2003). IL-23 is a regulator of IL-17 cytokine production as in IL-23p19−/− mice, the number of IL-17 producing T cells were dramatically reduced (Langrish et al., 2005). On the basis of this finding a novel subset of T-helper cells “Th17” was proposed. After the discovery of the Th17 subset, it has become active area of research. Below is an overview of role of Th17 subset in health and diseases.

Importance of Th17 subset in health and diseases

Th17 cells as well as the cytokines released by them (IL-17A, IL-17F, IL-21, IL-22) play a role in a plethora of diseases, including inducing inflammatory diseases, autoimmune disorders and cancer (Dong, 2008). IL-17, signature cytokine of Th17 subset, is a member of a cytokine family group consisting of IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F since they all share common protein structure. Only IL-17A and IL-17F (around 50% amino acid identity) are expressed in subgroup of activated T cells during inflammation and induce release of chemokines (CXCL-1, CXCL-8) and cytokines (IL-6, IL-1β) from eosinophils which contributes to inflammation. Th17 cells exert their effect of
nonimmune cells by promoting release of matrix metalloproteinases (MMP 1,2,3 and 9) which play a role in tissue remodeling and mucosal degradation. They induce secretion of several chemokines (CXCL-1,2,5,10,11,19,20) and cytokines (G-CSF, IL-6 and TNFα) which attract neutrophils and granulocytes, thus causing inflammation. Another important function of Th17 cells lies in regulating release of antimicrobial compounds, for example mucins (MUC5AC, MUC5B), β-defensins and S100 which facilitate their role in immunity against anti-microbial immunity (as reviewed by (Dong, 2008).

The protective role of this subset is against bacteria (Propionibacterium, Citrobacter rodentium, Klebsiella pneumoniae, Mycobacterium pneumonia), viruses (rotavirus, HIV), parasites (Toxoplasma gondii) and fungi (Candida albicans, Pneumocystis carinii) (Chung et al., 2003; Huang et al., 2004; Infante-Duarte et al., 2000; Khader et al., 2007; Rudner et al., 2007). Besides playing as antimicrobial role in physiological adaptive immune responses, Th17 cells can become pathogenic in autoimmune diseases for example Multiple sclerosis (Matusevicius et al., 1999), Rheumatoid arthritis (Kirkham et al., 2006), Inflammatory Bowel disease (Duerr et al., 2006), and psoriasis (Krueger et al., 2007).

Protective role of Th17 cells

Host defense:
As mentioned before Th17 cells play a major role in antimicrobial immunity. A vast majority of publications suggest that a wide range of microbial disease can be prevented by the Th17 subset supporting a crucial role in adaptive immunity. Initially, the antimicrobial role of Th17 cells became evident in Lyme disease, caused by Borrelia burgdorferi. Authors used lysate from these bacteria and found that it increases Th17 differentiation of T cells in \textit{in vitro} differentiation assays (Infante-Duarte et al., 2000). Subsequent studies further established the role of Th17 cytokines in immunity against microbial organisms. Given below is the role of IL-17 and other Th17 subset associated cytokines in host defense.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Effect</th>
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<tr>
<td>IL-23</td>
<td>IL-23 p19 knockout mice are susceptible to K. pneumoniae infections (Happel et al., 2005).</td>
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<tr>
<td>IL-17</td>
<td>IL-17 knockout mice are susceptible to K. pneumoniae infections. IL-17A play role in neutrophils recruitment as well as clearance of bacterias (Aujla et al., 2008). IL-17 increases bactericidal properties in macrophages against B. pertussis (Higgins et al., 2006). IL-17A and IL-17F increases neutrophils infiltration and clearance of M. pneumonia (Wu et al., 2007). IL-17R knockout mice are susceptible to Candida albicans</td>
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<td>Cytokine</td>
<td>Function and Examples</td>
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<td>IL-17</td>
<td>Infections and injection of IL-17 protects against C. albicans infections in murine models (Huang et al., 2004).</td>
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<tr>
<td>IL-22</td>
<td>Neutralization of IL-22 leads to severe of K. pneumoniae infection (Aujla et al., 2008). IL-22 promotes recovery of human bronchial epithelial cells after injury (Aujla et al., 2008). IL-22 induces secretion of antimicrobial peptides by epithelial cells (Liang et al., 2006).</td>
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<tr>
<td>IL-21</td>
<td>IL-21 levels decreases in HIV infected patients, associating this cytokine with disease progression (Iannello et al., 2008).</td>
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Role of Th17 in autoimmune diseases

Multiple Sclerosis (MS): MS is an autoimmune inflammatory disorder in which myelin sheaths around neuronal axons of central nervous system (CNS) (brain and spinal cord) are damaged, leading to demyelination and associated physical and cognitive disability. Various pro-inflammatory cytokines play a role in MS, for example IL-6, IFNγ, TNFα and IL-1β (Porrini and Reder, 1994; Rovaris et al., 1996; Woodroofe and Cuzner, 1993). In 1999, first association between Th17 cells and MS patients was presented by detecting high level of IL-17 mRNA.
transcripts in blood and CNS of MS patients (Matusevicius et al., 1999). Furthermore patients with severe, opticospinal MS have higher levels of IL-17 in their cerebrospinal fluid than conventional MS patients and control subjects (Ishizu et al., 2005). Magnetic resonance studies have also suggested that there is a positive correlation between the extent of CNS inflammation, intracranial IL-17 levels as well as breach of the blood brain barrier (Kebir et al., 2007). A recent study has shown that Th17 cells may directly interact with neuronal cells and can lead to their damage (Siffrin et al., 2010).

Role of Th17 subset in MS is supported by using EAE, mouse model of MS. In this model, CNS proteins (Myelin basic protein, proteolipid peptide and myelin oligodendrocytes glycoprotein) are injected into mice which elicit auto-reactive immune response, mimicking human MS symptoms. It has been shown that in IL-23p19 and IL-23 receptor deficient mice are protected from EA (Langrish et al., 2005; Zhang et al., 2003b). In addition IL-17 A knockdown attenuated the disease (Hofstetter et al., 2005).

Rheumatoid arthritis (RA): RA systemic inflammatory autoimmune disorder that affects synovial joints. It also causes inflammation in organs like lungs, pericardium, pleura and sclera (Majithia and Geraci, 2007). A positive correlation between increased IL-17 in sera and severity and extent of inflammation in RA patients has been shown (Hwang and Kim, 2005 Chabaud, 1999 #15276;
Kirkham et al., 2006). The role of IL-17 in pathogenesis of RA was substantiated in vitro experiments where IL-17 can promote secretion of cytokines (IL-1β, IL-6 and IL-23) and chemokines (CXCL-1,2,5,8) from chondrocytes, osteoblasts and fibroblasts (reviewed by Lundy et al., 2007).

Role of Th17 cells in RA is further emphasized by its mouse model, Collagen induced arthritis (CIA) in which collagen II is injected along complete Freund's adjuvant. The symptoms of CIA mimic rheumatoid arthritis pathology including synovial hyperplasia, infiltration of T cells and damage of cartilage. It has been shown that IL-23p19 and IL-17 knockout mice were protected from collagen induced arthritis (CIA) (Murphy et al., 2003). Other cytokines produced by Th17 cells also play pathogenic role in CIA as blocking IL-21 attenuated CIA (Jungel et al., 2004; Young et al., 2007).

Inflammatory bowel disease (IBD): IBD is a group of inflammatory disorder of the intestine including ulcerative colitis and Crohn’s disease. Correlation studies suggest association between IL-23R and IBD (Duerr et al., 2006). Promising results have been obtained in phase I and II clinical trials using STA 5326, a oral drug inhibiting both IL-12 and IL-23, in IBD patients (Billich, 2007; Burakoff et al., 2006). IL-23 p19 knockout studies revealed protection of mice from T cell dependent colitis, also supporting a pathogenic role for Th17 cells (Elson et al., 2007; Hue et al., 2006). It is suggested that Th17 cells, the inflammatory
cytokines that induce them (IL-1β, IL-6 and IL-23) and Th17 downstream mediators (chemokines, MMPs) all promote gut inflammation and are potential targets for treatment of IBD.

Inflammatory skin disease and psoriasis: Psoriasis is an inflammatory skin disorder marked by hyperkeratosis, epidermal hyperplasia and infiltration of several immune cells. The presence of Th17 associated cytokines, IL-17A, IL-17F and IL-22 in the serum and lesional skin of psoriasis patients (Arican et al., 2005) as well upregulation of transcript levels of RORC, IL-6, IL-1β and IL-23 in psoriatic skin (Wilson et al., 2007) suggest a role for the Th17 subset in the pathogenesis of this inflammatory disorder. Genetic Polymorphism studies also suggest a correlation between IL-23R variant and susceptibility to psoriasis (Capon et al., 2007). Presently a phase III clinical trial of human IL-12/IL-23 monoclonal antibody (ustekinumab) is ongoing in psoriasis patients (Papp et al., 2008).

Role of Th17 in cancers

Th17 cells and associated cytokines are implicated in several autoimmune and inflammatory diseases. The role of Th17 subset in cancer is very controversial and knowledge is currently limited. Th17 cells are present in tumors of both mouse and human origin (Kryczek et al., 2007). The important question
yet to be answered is whether these Th17 cells are tumor promoting or protective. Intradermal administration of IL-23 in carcinogen induced papillomas led to a decrease in intra-tumoral \( \text{CD8}^+ \) T cells infiltration and enhanced tumor growth (Langowski et al., 2006) suggesting tumor promoting function of Th17 subset. In contrast, intra-tumoral implantation of IL-23 transduced dendritic cells in glioma model resulted in an effective anti-tumor response involving infiltration by CD8\(^+\) T cells, CD4\(^+\) T cells and NK cells (Hu et al., 2006). Direct involvement deficient mice are highly susceptible to melanoma and their metastases to lungs (Martin-Orozco et al., 2009b). Administration of tumor specific Th17 cells led to marked reduction in tumor burden (Martin-Orozco et al., 2009b). In an another report using \textit{in vitro} differentiation of melanoma antigen TRP specific CD4\(^+\) T cells towards the Th17 subset and adoptively transferred to mice carrying established melanoma tumors resulted in an effective anti-tumor response (Muranski et al., 2008). Interestingly Th17 cells mediated anti-tumor protection was dependent on IFN\(\gamma\), as this effect was abrogated by IFN\(\gamma\) depleting antibodies (Muranski et al., 2008). Functional importance of Th17 cells has also been reported in ovarian cancer patients where the presence of IL-17 positive cells correlated with enhanced survival of patients (Horlock et al., 2009). Further, in breast cancer patients a reduction in Th17 cells was shown and treatment with trastuzumab led to an increase in Th17 cells infiltration and increased overall survival of patients (Horlock et al., 2009).
An important question yet to be answered is that if IFNγ secreted by Th17 cells is so important for anti-tumor response, what differentiates the role of Th17 cells from Th1 differentiated cells (Muranski et al., 2008). Another crucial future area of investigation is defining the dynamics of Th17 cells and T regulatory cells within the tumor microenvironment.

Regulation of Th17 differentiation

Cytokine environment: In 2006, three independent laboratories indicated that IL-6 along with transforming growth factor (TGFβ) have a role in initiation of Th17 differentiation (Bettelli et al., 2006; Mangan et al., 2006). Later it was shown that IL-1β also has a role Th17 differentiation (Veldhoen et al., 2006). The indispensable role of TGFβ in Th17 differentiation is quite surprising as earlier reports showed TGFβ is an immunosuppressive cytokine which plays a role of T-regulatory cells differentiation. Studies from Bettelli et al using GFP-FOXP3 transgenic reporter mice has shown clearly that when present alone TGFβ leads to T-regulatory cells differentiation, but when present along with pro-inflammatory cytokines IL-6 and IL-1β, TGFβ leads to Th17 differentiation (Bettelli et al., 2006). Recently it has also been suggested that the concentration of TGFβ can play very important role in balance between T regulatory cells and Th17 cells, with low concentration of TGFβ supporting Th17 differentiation by increasing expression of the IL-23 receptor (Zhou et al., 2008).
Another cytokine which is important for Th17 differentiation is IL-6. It has been demonstrated both *in vivo* and *in vitro* that IL-6 induces IL-21 production in a STAT3 dependent manner, which in combination with TGFβ regulates IL-17 production from naïve T helper cells (Zhou et al., 2007).

IL-1β is another cytokine which is particularly important for Th17 differentiation and expansion, as IL-1 receptor knockout mice display defective differentiation of Th17, but not Th1 and Th2 subsets (Sutton et al., 2006), along with a low incidence of EAE (Sutton et al., 2006).

IL-23, composed of IL-12p40 and p19 subunits, plays an important role in Th17 differentiation as IL-23 knockout mice are resistant to EAE (Experimental autoimmune encephalomyelitis) (Cua et al., 2003), CIA (Collagen induced arthritis) (Murphy et al., 2003) and IBD (Inflammatory Bowel disease) (Yen et al., 2006). But the precise role of IL-23 in Th17 differentiation is not known as IL-23 alone cannot differentiate naïve T helper cells (Mangan et al., 2006). This may be due to the fact that naïve T helper cells do not express the IL-23 receptor, requiring IL-6 and IL-21 for its expression (Nurieva et al., 2007; Yang et al., 2007; Zhou et al., 2007). So the IL-23 is not required for initial differentiation of Th17 cells but rather for maintenance of differentiation.

Besides aforementioned positive regulators, IFNγ and IL-4 are very important negative regulators of Th17 differentiation. Neutralizing IFNγ and IL-4 increases Th17 producing cells upon *in vitro* polarizations (Harrington et al.,
2005) and IFNγ and IFNγ receptor deficient mice are highly susceptible to EAE, this is due to an increase in IL-17 producing cells (Harrington et al., 2005; Iwakura and Ishigame, 2006).

Many studies, including ours, have shown the presence of IFNγ and IL-17 double positive cells. This suggests that IFNγ may be important for Th17 cell pathogenicity or that both subsets share an overlapping differentiation program. The precise molecular mechanism of development of these cells or the role of double positive population is not at all clear.

Transcription factor network: Complex interplay between transcription factors and cytokines is the most important Th17 lineage determining factor. Below is the overview of transcription factors playing role in Th17 differentiation:

STATs: STAT1 and STAT6, transcription factors which define Th1 and Th2 differentiation respectively seem to negatively regulate Th17 differentiation (Harrington et al., 2005). By contrast, STAT3 plays a supportive role in Th17 differentiation. SOCS3 knockout mice (lacking the Suppressor of Cytokine Signaling) T cells have increased IL-17 production, which is due to hyperactive STAT3 signaling (Chen et al., 2006c). STAT3 binds directly to the IL-17 promoter (Chen et al., 2006c) as well as to the ROR (Retinoic acid related Orphan Receptor) family of transcription factor promoters (RORγt and RORα) (Ivanov et al., 2006; Yang et al., 2008b), key Th17 lineage determining transcription factors.
Retinoic acid related orphan receptor (RORs): RORα, β, γ, are each encoded by RORC gene using different promoters. RORα is important for development of the cerebellum and the lymph nodes, for lipid metabolism and for regulating immune responses. Its endogenous ligand is melatonin. The function of RORβ is not known but it is highly expressed in brain and retina. RORγ, particularly splice variant ROR-γt expressed in T cells defines Th17 differentiation (Jetten, 2004). During in vitro Th17 differentiation assays, ROR-γt expression is upregulated. Moreover retroviral transduction of naïve CD4⁺ T cells with ROR-γt vector leads to Th17 differentiation (Ivanov et al., 2006). Interestingly in ROR-γt knockout mice, the Th17 subset population is greatly reduced and protected from EAE (Ivanov et al., 2006). Another ROR family transcription factor, RORα, has been recently implicated in Th17 differentiation. RORα knockdown leads to impaired Th17 differentiation both in vivo and in vitro (Yang et al., 2008b). It has been shown that ROR-γt and RORα can bind to IL-17 gene locus and can cooperatively regulate IL-17 cytokine production (Akimzhanov et al., 2007; Yang et al., 2008b). Therefore interplay between STAT-3 and the ROR family of transcription factor regulate Th17 differentiation. (Refer Fig.2C)

IRF-4 (Interferon regulatory factor 4): ROR-γt interacts with another transcription factor IRF-4. Overexpression of ROR-γt in IRF-4⁻/⁻ T cells failed to fully differentiate into Th17. Also IRF-4⁻/⁻ T helper cells have low IL-17 production and
exhibited higher FoxP3 levels (Brustle et al., 2007). Thus IRF-4 may be a determinant of Th17 and T regulatory cells balance (Yang et al., 2008a).

FoxP3: The reciprocal relationship between T regulatory cells and Th17 population is an active area of investigation. This follows as extensive immune responses have to be controlled by suppressive actions of T regulatory cells. TGFβ alone leads to differentiation towards T regulatory cells subset but together with proinflammatory cytokines (IL-6 and IL-1β) drives Th17 differentiation. A reciprocal relationship between Th17 and T regulatory subsets is regulated by a fine balance between FoxP3 and ROR-γt transcription factors. FoxP3 can directly bind to ROR-γt leading to inhibition of its transcriptional activity (Zhou et al., 2008). FoxP3 can also bind to RORα through LXXLL sequence (Du et al., 2008), explaining the molecular basis of inter-relationship reciprocal relationship. When TGFβ is present in elevated concentrations it up regulates FoxP3 which in turn binds and inhibit both ROR-γt and RORα, thus inhibiting Th17 differentiation.
Cross talk between cytokines and transcription factor
In Th1, Th2 and Th17 differentiation.

Figure 1: Schematic representation of cross-talk between cytokines and transcription factors in differentiation of Th1, Th2 and Th17 subsets.
AHR (Aryl hydrocarbon receptors): AHR are cell membrane receptors which are expressed in both T regulatory cells and Th17 cells. Surprisingly these receptors regulate Th17 and T regulatory cells in a ligand dependent manner. 2,3,7,8 tetrachlorodibenzo p-dioxin (TCDD) regulate generation of T regulatory cells while another ligand of AHR, 6 formylindolo (3,2-b)-carbazole promoted Th17 differentiation (Quintana et al., 2008; Veldhoen et al., 2008a).

Nature and affinity of antigen: T-helper cell differentiation can also be regulated by the interaction of pathogen (antigen) with APCs. Factors such as antigen dose, toll like receptors (TLRs) activated and downstream TCR signal strength can regulate Th17 differentiation. Antigens including fungal wall component zymosan (a TLR2 activator on DCs), C-type lectins (pathogen associated molecular patterns recognition receptors on DCs) and NOD (Nucleotide binding oligomerization domain) like receptors upregulate IL-23 and skew T cells response towards the Th17 subset (Bird, 2010). TLR signaling, particularly via TLR2, plays a role in Th17 differentiation (for example, Pam3CysSerLys4 a TLR2 agonist, skew T cells response specifically towards Th17) (Bird, 2010).

A recent study published by Iezzi G has clearly shown the role of antigen dose, pathogen associated molecular patterns (PAMPs) and TCR stimulation in Th17 differentiation (Iezzi et al., 2009). Using in vitro differentiation of naïve CD4+ T cells specific to LCMV (Lymphocytic Chorimenigitis Virus) glycoprotein gp61-80
in presence of increasing concentrations of peptide, the authors have shown that at the highest dose of peptide antigen used (10µM), IL-17 producing cells were observed even in the absence of polarizing cytokines. Polarization was further increased using TGFβ and IL-6 or IFNγ and IL-4 neutralizing antibodies. In contrast low antigen dose (0.01-1µM) leads to Th2 differentiation and intermediate-high antigen dose to Th1 subset. The authors have also shown that high antigen dose and PAMPs synergistically regulate Th17 differentiation. They exposed splenic DCs to various TLRs agonists and pathogen related receptors (PRRs) and observed that TLR9 (CpG) and Dectin 1 ligands (zymosan and curdlan) upregulated Th17 differentiation which was further increased by high antigen dose (Iezzi et al., 2009).

TCR and co-stimulatory molecules: The next step after antigen recognition in eliciting a specific Th17 immune response is downstream TCR signaling, which may be differentially upregulated in Th1, Th2 and Th17 differentiation. Several signaling events downstream of TCR engagement regulate Th17 differentiation: TCR proximal signaling: One of the important TCR proximal signaling that defines Th17 differentiation is through nonreceptor tyrosine kinases Tec (tyrosine kinase expressed in hepatocellular carcinoma). Knockdown of Tec family kinases, particularly Itk (IL-2 inducible T cell kinase), lead to defective IL-17A
production in *in vitro* differentiation assays. This defect was linked with impairment of Ca\(^{2+}\)/NFAT pathway (Gomez-Rodriguez et al., 2009).

Ca\(^{2+}\)/NFAT: T helper cells differentiation can be regulated by Ca\(^{2+}\) signaling. Th1, Th2 and Th17 cells can be regulated by the duration, amplitude and pattern of Ca\(^{2+}\) signaling. Intracellular Ca\(^{2+}\) profiles of Th17 cells were different from Th1 and Th2 cells. Th17 cells maintain sustained intermediate levels of Ca\(^{2+}\) in between Th1 and Th2 where Th1 display the highest intracellular Ca\(^{2+}\) levels. Interestingly authors have shown that these differences in Ca\(^{2+}\) levels may be associated with variation in phosphoPLC\(\gamma\)-1 where Th1 cells display the highest level of PLC\(\gamma\)-1 (Th1>Th17>Th2) (Weber et al., 2008).

NF-\(\kappa\)B: Noncanonical NF-\(\kappa\)B signaling (processing of NF\(\kappa\)B precursor p100 to p52 and Rel B) plays a role in Th17 differentiation. CD4\(^+\) T cells from NIK\(^{-/-}\) mice (lacking NF-\(\kappa\)B inducing kinase, having distinct role in noncanonical NF\(\kappa\)B signaling) were used to show that these cells fail to differentiate into Th17 cells, although differentiation towards to Th1 and Th2 proceeded normally. NIK\(^{-/-}\) mice also failed to develop EAE. When comparing pSTAT3 (Tyr 705) expression levels between NIK\(^{-/-}\) and NIK\(^{+/+}\) mice, authors found that NIK\(^{-/-}\) mice lack up-regulation of pSTAT3 by TCR and IL-6R signals (Jin et al., 2009).
miRNA: miRNA are short sequences (around 22nt in length) that regulate several biological processes including immune responses. miRNA species maintains secondary structure which is processed by the activity of RNAase III enzymes such as Dicer, Pasha and Drosha. Resulting short RNA duplexes then bind to the 3’UTR of transcripts, inhibiting translation and/or hindering mRNA stability (Shivdasani, 2006). Initial studies of the role of miRNA in T cell differentiation showed that Dicer⁻/⁻ T cells when differentiated to Th2, still showed IFNγ production suggesting miRNA plays a negative role in Th1 differentiation (Muljo et al., 2005). Furthermore miR155 was proposed to have role in T regulatory cell differentiation (Lu et al., 2009) and miR326 plays important role in Th17 differentiation as well as pathogenesis of multiple sclerosis (Du et al., 2009). The miR326 regulates Th17 differentiation and EAE disease onset by inhibiting the translation initiation of Ets protein, which is a negative regulator of Th17 differentiation. The miR326 expression levels correlate with IL-17 levels in MS patients. Furthermore a high level of miR326 transcripts were detected in peripheral blood of MS patients, thus suggesting a prognostic importance of miR326 detection for MS patients.

Epigenetics: Since Th17 is a novel subset, regulation of IL-17A/IL-17F locus by epigenetic mechanism is currently an area of active investigation. The IL-17A/F locus is heavily histone 3 acetylated at eight different conserved noncoding
sequences (CNS) in Th17 differentiated mouse CD4\(^+\) T cells (Akimzhanov et al., 2007). Corroborating this data CNS2 is regulated by both ROR-\(\gamma\)t and RUNX-1 (Zhang et al., 2008) transcription factors emphasizing a contribution of chromatin remodeling to Th cell differentiation.

Plasticity of Th17/Th1 subset

The concept of mutually exclusive and irreversible commitment towards Th1, Th2 and Th17 differentiation is mostly based on \textit{in vitro} differentiation assays. Although these assays contribute to our understanding of basic mechanisms, \textit{in vivo} conditions are much more complex. For example CD4\(^+\) T cells expressing IFN\(\gamma\)^+/IL-17\(^+\) double positive population are commonly observed \textit{in vivo} and \textit{in vitro} in human T cells (Acosta-Rodriguez et al., 2007a; Acosta-Rodriguez et al., 2007b; Wilson et al., 2007). Interestingly these double positive cells co-express markers of both Th1 and Th17 subtypes (for example CCR6/CXCR3 and Tbet/ROR-\(\gamma\)t)(Acosta-Rodriguez et al., 2007b; Annunziato et al., 2007). T bet (transcription factor specific for Th1 differentiation) can be induced by IL-23 in Th17 cells and facilitate upregulation of IL-23R (Chen et al., 2006b), supporting such plasticity.

The presence of this population of mixed lineage cells sharing cytokines and transcription factors represents either flexibility of lineages to transform to another lineage, efficiently regulating immune process or merely representing
undecided population of cells during the differentiation process. This needs to be further evaluated. A number of factors can potentially regulate plasticity including a heterogeneous cytokine environment in vivo, dual epigenetic modification, intermediate pathogen doses and T cell memory (Lee et al., 2009). Further discussion of Th17/Th1 cells is in Chapter 5.

Notch structure and function:

Another area of investigation which is currently understudied is role of cell membrane receptors as a potential participant in differential T helper cell differentiation. Among membrane bound proteins, Notch is an important candidate for having a role in T cell differentiation. Firstly Notch receptors are present on naïve CD4+ T cells (Palaga et al., 2003) and their ligands on antigen presenting cells (Ross and Kadesch, 2004; Yamaguchi et al., 2002). Secondly Notch has been shown to be activated after T cell receptor stimulus and interestingly co-localized with CD4+ at T cells-APC interface suggesting its role in modulating early T cells signaling events (Adler et al., 2003; Benson et al., 2005). Thirdly Notch is a cell fate determinant for T cells, driving a decision between T cells vs B cells (Pui et al., 1999), CD4 vs CD8(Robey et al., 1996) and αβ CD4 vs γδ CD4 T cells (Washburn et al., 1997). Therefore we can assume that Notch play important role in peripheral T cell differentiation also.
Notch structure

The Notch is 300 kilodalton, class I heterodimer transmembrane receptors. In humans, there are four different paralogs of Notch receptors, Notch1-4 and five different types of Notch ligands Delta-like 1, 3 and 4 (DLL-1,3,4) and Jagged1, and 2 (Jag1,2). Notch consists of extracellular domain (N\text{EC}), transmembrane domain (N\text{TM}) and intracellular domain (N\text{IC}). The N\text{EC} domain consists of variable glycosylated EGF-like repeats followed by cysteine rich Lin repeats which prevents ligand independent activation of Notch. The N\text{TM} consists of RAM domain (RBP-J associated molecule) which helps in interaction of Notch with effector transcription factor CSL; ankyrin repeats (ANK), which facilitates protein:protein interaction; transactivating domain (TAD), which is important for transcription and PEST (proline-glutamate-serine-threonine rich) domain which regulated Notch degradation (Radtke et al., 2010).

Canonical Notch signaling:

The Notch receptor is synthesized as a single large pre-protein in endoplasmic reticulum (ER). In Golgi, it gets O-glucosylated (by O-glucosyltrasferase Rumi) followed by O-linked fucosylation (by O-focosyltransferase, POFUT1). In Golgi, full length Notch receptor undergoes first cleavage by furin like proteases (S1 site) to generate non-covalent linked heterodimeric Notch. Before going to cell membrane, the extracellular domain
undergoes N-acetylglucosamination (by family of Fringes, Manic, Radical and Lunatic). The post-translational modifications by Fringes are very important for ligand binding affinity of Notch receptor with either Delta or Jagged. On the cell membrane it undergoes further cleavage by an ADAM (a disintegrin and metalloproteinase) protease at S2 site. The final processing of Notch by γ-secretase complex at S3 site yields activated form of Notch1 (NIC) (Radtke et al., 2010). NIC translocates to the nucleus and activates downstream transcription. γ-secretase is known to cleave vast number of type 1 transmembrane protein substrates (Kopan and Ilagan, 2004). In fact, γ-secretase inhibitors (GSI) are under clinical trial to treat Alzheimer’s disease since it cleaves β amyloid protein (Borchelt et al., 1996). One of the other targets of GSI is Notch (De Strooper et al., 1999). There are number of pharmacological inhibitors of γ-secretase available including DAPT (GSI IX, Ly 374973,(3,5-Difluorophenylacetyl)-L-Alanyl-PhenylGlycinebutyl Ester) (Dovey et al., 2001), Ly411,575 (DAPT derivative) (Searfoss et al., 2003), L685458 and L852647 (Merck) (Shearman et al., 2000). The NIC rapidly translocates to the nucleus where it interacts with the DNA-binding repressor protein known as CSL (CBF-1/RBP-Jk, mammals; Suppressor of Hairless, Drosophila; and Lag-1, C. elegans). In the absence of Notch signaling, CSL is bound to DNA in a complex with several repressor proteins consist of SMRT (silencing mediator of retinoic acid and thyroid hormone receptor), histone deacetylases (HDACs), CIR1 (CBF1-interacting co-repressor)
and SHARP (SMRT/HDAC associated repressor protein) (Hsieh et al., 1999; Oswald et al., 2002; Shi et al., 2001). N^{\text{IC}} translocation to the nucleus and binding to CSL results in disruption of the repressor complex followed by recruitment of several co-activator proteins including SKIP (Ski-interacting protein), Mastermind like (MAML) and Histone acetyl transferases (HAT). This results in the initiation of transcription of genes located downstream of Notch/CSL complex (Miele, 2006; Osborne and Minter, 2007).

Noncanonical Notch signaling:

Interestingly non-canonical Notch signaling is also documented in literature involving noncanonical Notch ligand (Delta/jagged) or CBF-1 independent signaling. Various Notch ligands, besides conventional Delta and Jagged, are reported for example DNER (Delta/Notch like epidermal factor related receptor), Contactin, NOV (nephroblastoma overexpressed gene) and MAGP (myofibril associated glycoprotein) (Cui et al., 2004; Eiraku et al., 2005; Hu et al., 2003; Miyamoto et al., 2006; Sakamoto et al., 2002). The intriguing question is whether they play any role in immune cells? Another discussion is about their mechanism of action. It has been hypothesized that these ligands destabilize the heterodimeric domains of Notch and thus leads to the activation of downstream signaling. CSL independent signaling is originally described in Drosophila melanogaster, through E3 ligase deltex (Hori et al., 2004). But its role
in immune system is questionable as Deltex1 and 2 deficient mice showed no change in immune system development (Lehar and Bevan, 2006). Most studied and published Notch downstream signaling, besides canonical CSL pathway, is through NF-κB (Oakley et al., 2003; Oswald et al., 1998). A close examination of consensus binding sites of NF-κB and CSL reveals that both share overlapping motifs, suggest that may be there is competition between them to regulate gene expression (Lee et al., 2000; Vales and Friedl, 2002).

Paradoxical role of Notch in Th1 and Th2 differentiation.

Role of Notch in Th1 differentiation: Several reports suggest role of Notch signaling in Th1 differentiation. Pathogens such as virus (respiratory syncytial), bacteria (Acinetobacter lwofii and Lactococcus lactis) and TLR4 (CpG) upregulate Notch ligand DLL4 in dendritic cells through the Myd88 dependent pathway promote Th1 differentiation (Debarry et al., 2007; Napolitani et al., 2005; Rudd et al., 2007; Skokos and Nussenzweig, 2007). This is further supported by overexpressing Notch receptors (Notch 1 and Notch3) in CD4+ T and culturing in plate crosslinked DLL-Fc (DLL1 and DLL4) and observed upregulated Th1 differentiation (Maekawa et al., 2003; Minter et al., 2005). Evidences showing contribution of canonical (CSL) and noncanonical (NF-κB) signaling in Th1 differentiation exist. Supporting a CSL dependent mechanism, Notch directly binds and activates Tbx21, the gene that encodes T-bet, in T cell hybridomas
Another mechanism by which Notch promote Th1 differentiation is through interaction with NF-κB. Notch is known to interact with p50 and p65 and regulates the IFNγ promoter (Shin et al., 2006).

Role of Notch in Th2 differentiation: Role for Notch signaling in Th2 differentiation is also reported. In contrast to DLL-Notch signaling regulating Th1 differentiation it was observed that Jagged-Notch signaling regulates Th2 differentiation, suggesting that the Notch ligand involved may specify T cell fate (Amsen et al., 2004). Antigens such as Schistosoma mansoni egg antigen (SEA), Vibrio cholera and prostaglandin E2 upregulate Jagged on dendritic cells and regulate Th2 differentiation (Amsen et al., 2004; Kaisho et al., 2002; Krawczyk et al., 2008; Schnare et al., 2001). Genetic studies involving mouse CD4+ T cells deficient in CSL or dominant negative form of co-activator mastermind (MAML) resulted in abrogated Th2 responses in vitro and in vivo (Amsen et al., 2007; Tanigaki et al., 2004; Tu et al., 2005). To rule out a Notch independent effect of CSL and MAML, Amsen et al used Notch1 and Notch2 double knockout mice and observed a similar defect on Th2 differentiation (Amsen et al., 2007; Barolo et al., 2000; McElhinny et al., 2008). Later GATA3 and IL-4 promoters were found to be direct targets of Notch, providing a molecular mechanism by which Notch can regulate Th2 differentiation (Amsen et al., 2004). The diagrammatic representation of the
paradoxical role of Notch signaling in both Th1 and Th2 differentiation is shown in fig.2.
Role of Notch signaling in differentiation of peripheral T cells

Figure 2: Schematic representation of role of Notch signaling in T cell differentiation: Notch1 is known to bind NF-κB subunits (p50 and p65) and regulate IFNγ promoter. Additionally Notch binds to Tbx promoter and regulate Th1 differentiation. On the other hand dominant negative mastermind or conditional deletion of CSL led to defective Th2 phenotype. Role of Notch in regulation of Th17 differentiation is not known.
Hypothesis and specific aims

Notch is an important regulator of peripheral T cell differentiation. Role of Notch signaling in Th17 differentiation is not known. A recent publication suggested role of Notch ligand DLL4 in promoting expression of ROR-γt and expansion of mouse Th17 CD4+ T cells (Mukherjee et al., 2009). This led to my hypothesis that Notch signaling plays a defining role in Th17 differentiation, and inhibiting Notch signaling can be effective means of preventing Th17 mediated autoimmune diseases.

Role of Notch in Th1 and Th2 differentiation is known, but contradictory reports are present. The important question is whether these paradoxical results are due to different experimental method used, different Notch constructs or gamma secretase inhibitors used. In the current study, I used in vitro differentiation assays to study the role of Notch in T cell differentiation, particularly Th17. In addition, I also make efforts to compare and contrast role for Notch in Th1, Th2 and Th17 differentiation using same experimental system.

Aim 1: To delineate the role of Notch signaling in Th17 polarization.

My first aim is to determine whether Notch signaling regulates human Th17 differentiation. Using in vitro Th17 differentiation as my model system, I have shown that Notch signaling, particularly Notch1, regulates Th17 cytokine production including IL-17A, IL-17F and IL-22. The data presented in CHAPTER
2 demonstrate that in Th17 differentiated cells, there is increase in Notch1 expression and downstream signaling. Blockage of Notch expression (by pharmacologic inhibitors or specific siRNA) results in reduced expression of Th17 associated cytokines. Further overexpression of Notch1 in naïve CD4\(^+\) T cells resulted in increase in IL-17 production.

Aim 2: To determine the molecular mechanism through which Notch regulates Th17 polarization.

I further investigated the regulation of human ROR-\(\gamma\)t and IL-17 promoter by Notch1 to explain its mechanism of action. Specific knockdown of Notch1 and Chromatin immunoprecipitations (ChIP) are performed. Data shown in CHAPTER 3 has shown that ROR-\(\gamma\)t is direct transcriptional target of Notch1. Moreover human IL-17 promoter luciferase assays and ChIP assays also suggest that IL-17 promoter is also regulated by Notch1.

Aim 3: To determine whether Notch signaling inhibitors can prevent Th17 mediated diseases \textit{in vivo}.

EAE (Experimental Autoimmune Encephalomyelitis), mouse model of multiple sclerosis is used for this aim. The data presented in CHAPTER 4 using \(\gamma\)-secretase inhibitors demonstrate that Notch signaling regulates Th17 mediated
pathogenesis in EAE model and GSI reduced the disease symptoms and delayed the onset of disease as well.
Framework of proposed study

Naïve T cells → APC

Th17 CD4+ → RORγt promoter (key transcription factor of Th17 cells)

IL-17 IL-22

Promotes Th17 differentiation and autoimmune disorders

GSI

AIM 1

Notch L (Delta 4)
Mukherjee S et al 2009

AIM 2

CSL/MAML

AIM 3
CHAPTER II

NOTCH FUNCTION AS A REGULATOR OF TH17 DIFFERENTIATION.

Introduction

Th17 cells as well as cytokines released by them (IL-17A, IL-17F, IL-21, IL-22) are very important for the immunity against bacterial and fungal infections. When the immune system is deregulated, for example autoimmune diseases, chronic disorders and cancers, the Th17 subset play a pathogenic role. Th17 cells induce rapid accumulation of neutrophils at site of infection and promote secretion of inflammatory cytokines, chemokines and matrix metalloproteases (MMPs) (Korn et al., 2009).

Various TLR signals, particularly TLR9, upregulate Notch ligand DLL4 dependent IL-17 production in DO11.10 splenocytes (Mukherjee et al., 2009). Furthermore, adding DLL4 protein to mouse CD4+T cells upregulates the transcript levels of IL-17A, IL-17F, IL-22 and IL-23R suggesting a role of DLL4 in Th17 differentiation. Another study has utilized a pulmonary granuloma formation model in mice to emphasize role of DLL4 in Th17 regulation (Ito et al., 2009). They have shown that mycobacterial antigen, through TLR9, upregulate DLL4 in granuloma leading to Th17 response. In addition, TLR9 knockout mice have an impaired Th17 response and displayed reduced DLL4 expression on dendritic
Publications by the Elyaman and Jurynczyk groups have also provided evidence of role for Notch involvement in the Th17 mediated autoimmune disorder Multiple Sclerosis (Elyaman et al., 2007; Jurynczyk et al., 2008b). They have shown that demyelinating lesions in MS as well its mouse model EAE express notch receptors and their ligands and observed active downstream Notch signaling within them.

These observations led us to ask whether Notch signaling plays a role in human Th17 differentiation and which receptors this would involve. Under this aim we studied the involvement of Notch receptors in human Th17 differentiation. To follow human Th17 polarization we in vitro polarize naïve human CD4+ T cells towards Th17 subset.

Results

Differentiation of naïve human CD4+ T cells towards Th17 subset.

In order to study the role of Notch signaling in human Th17 differentiation, I used human in vitro Th17 polarization assay. I purified naïve CD4+ T cells from peripheral blood mononuclear cells by negative selection using MACS separation. The purity of naïve CD4+ T cell was assessed by staining with antibodies against CD4 and CD45RA. I obtained 93-96% pure population of naïve CD4+ T cells (Fig.3A). These cells were then stimulated and cultured with anti-CD3/CD28 beads under Th0 and Th17 polarizing conditions as mentioned in
materials and methods. I observed 3.5 fold increase (p≤0.01) in IL-17 levels starting from 24hrs when Th17 differentiated cells were compared with Th0 differentiated cells (Fig.3B). I also compared human Th17 in vitro polarization using anti-CD3/CD28 beads vs coated plates and observed comparable (6-8 fold) increase (p≤0.01) in IL-17 levels (Fig.4). Th17 differentiation was confirmed by intracellular staining of IL-17. I observed that intracellular IL-17 increased from 1.25% to 11% when compared between Th0 and Th17 differentiated cells at 72hrs (Fig.5). Interestingly, I also observed increase in IL-17+/IFNγ+ double positive cells (0.8 to 4%) in Th17 differentiated subset. When comparing ROR-γt transcript levels in Th17 differentiated cells with Th0 by real time PCR, I observed significant increase (4.5 fold; p≤0.01) in transcript levels starting from 24hrs after differentiation (Fig.6). Levels of other Th17 subset associated cytokines were also measured in in vitro Th17 differentiated cells by ELISA. I observed 2 fold increase in IL-17F levels (p≤0.5) and 3 fold increase in IL-22 levels (p≤0.01) in Th17 differentiated cells as compared to Th0 cells (Fig.7). Thus in vitro differentiated Th17 cells produce IL-17, IL-17F and IL-22 and have elevated ROR-γt transcript levels.
**Figure 3:** *In vitro* Th17 polarization of human naïve CD4⁺ T cells: Naïve CD4⁺ T cells were isolated from PBMC of healthy donors and were stimulated with anti-CD3/CD28 coated magnetic beads in Th0 (no cytokines) and Th17 (with skewing cytokines cocktail as mentioned in materials and method) conditions. A) Naïve T cells purity was checked by staining with CD4PE and CD45RAFITC.B) Supernatants were collected at indicated time points and analyzed for IL-17 by ELISA. Data shown here represents one of at least three independent experiments performed in triplicates. *p≤.05, **p≤.01, ***p≤ .001.
Figure 4: Comparing anti CD3/CD28 coated vs beads in *in vitro* Th17 polarization of human naïve CD4+ T cells: Naïve CD4+ T cells were isolated from PBMC of healthy donors and were stimulated with anti-CD3/CD28 coated magnetic beads or plate coated anti-CD3/CD28 and differentiated towards Th0 and Th17 conditions. Supernatants were collected after 72hrs and analyzed for IL-17 by ELISA. Data shown here represent the mean± SD for two independent experiments performed in triplicates. **p≤.01.
Figure 5: Intracellular IL-17 levels in human *in vitro* Th17 differentiated cells. After 72hrs of *in vitro* Th17 differentiation, cells were stimulated with PMA/Ionomycin for 4hrs and monensin for another 2 hrs. Cells were then fixed and permeabilized as mentioned in materials and methods, followed by staining with anti-humanIL-17PE, anti-human IFNγ-APC and anti-humanIL-4FITC and analyzed by flowcytometry.
Figure 6: ROR-γt transcripts increases in human *in vitro* Th17 differentiated cells as compared to Th0. Total RNA was isolated from invitro differentiated Th17 cells at indicated time points, cDNA was synthesized and ROR-γt transcript levels were measured by quantitative real time PCR. Data represented were normalized against 18S RNA. Shown is mean±SD of three independent experiments performed in triplicates.
Figure 7: Upregulation of IL-17F and IL-22 in human in vitro Th17 as compared to Th0 differentiated cells. Naïve CD4+ T cells were stimulated with anti-CD3/CD28 coated magnetic beads and skewed towards Th0 and Th17 subset. Supernatants were collected at indicated time points and ELISA of IL-17F (A) and IL-22 (B) was performed. Data shown represent mean±SD experiments performed in triplicates. *p≤.05, **p≤.01.
Upregulation of Notch1 in human \textit{in vitro} Th17 differentiation assays.

Expression level of Notch isoforms upon \textit{in vitro} Th17 differentiation was assessed to study the role of Notch signaling in Th17 differentiation. Whole cell lysates were prepared at several time points (24-72hrs) of Th17 differentiated cells and western blot of activated Notch1 was performed in these lysates. Two fold increase in Notch1 expression levels was observed in Th17 differentiated cells even at 24hr time point as compared to Th0 cells (Fig.8). Upregulation (5.3 fold) of Notch1 expression was even observed in naïve T cells stimulated with anti-CD3/CD28 stimulus (Th0) as compared to unstimulated cells (Fig.8). This supports the previously published data (Palaga et al., 2003) that activation of T cells results in upregulation of Notch signaling. Expression of other isoforms of Notch (Notch 2, 3 and 4) was also tested, but no significant increase in their levels was observed in Th17 differentiated cells as compared to Th0 (Fig.9).

Upregulation of Notch1 was also observed in extended culture of Th17 cells (Fig.10). Real time PCR of Notch downstream target Hey1 was also performed in \textit{in vitro} Th17 differentiated cells to check the activation of Notch signaling. Seven fold increase (p≤0.01) in Hey1 transcript levels were observed in Th17 differentiated cells as compared to Th0 cells (Fig.11). Thus expression of Notch1 as well as activation of Notch target gene Hey1 correlates with human \textit{in vitro} Th17 differentiation.
Figure 8: Expression of active Notch1 is increased in *in vitro* Th17 differentiated cells as compared to Th0 and unstimulated naïve T cells. A) Whole cell lysates were prepared from naïve CD4+ T cells un-stimulated (US), or differentiated under Th0 and Th17 conditions and immunoblotted for intercellular active Notch1. β-actin was used to confirm equal loading. B) Densitometry of active Notch1 was performed and normalized to actin. Data shown above is plotted as arbitrary units (AU).
**Figure 9: Expression of Notch1, but not Notch2, 3 or 4 favors in vitro Th17 differentiation.** Whole cell lysates were prepared from naive CD4+ T cells un-stimulated (US), or differentiated under Th0 and Th17 conditions at indicated time points and immunoblotted for Notch1, Notch2, Notch3 and Notch4. β-actin was used to confirm equal loading. Lysates from 293T cells transfected with active Notch1IC, Notch2IC, Notch3IC, and Notch4IC were used as positive controls. E: 293T cells transfected with empty vector.
Figure 10: Expression Notch1 remains elevated at later time points (day 6 and 7) of *in vitro* Th17 differentiation. Whole cell lysates were prepared from naive CD4⁺ T cells differentiated under Th0 and Th17 conditions at indicated time points and immunoblotted for Notch1. β-actin was used to confirm equal loading. Notch₁^{FL}: Notch full length, Notch₁^{TM}: Notch transmembrane, Notch₁^{IC}: intracellular active Notch1, AU: Arbitrary units.
**Figure 11:** Upregulation of Notch signaling in \textit{in vitro} Th17 differentiated cells shown by quantitative real time PCR of Notch downstream target gene Hey1. Complementary DNA was synthesized from \textit{in vitro} differentiated Th17 cells and Hey1 transcript levels were measured by quantitative real time PCR at 24hrs. Data represented here is relative Hey1 transcript levels normalized against 18S and is mean±SD of two independent experiments performed in triplicates.**p≤0.01.
Pharmacologic and genetic knockdown of Notch signaling reduces *in vitro* human Th17 differentiation.

Next I examined whether pharmacologic inhibitors of Notch signaling pathway could block Th17 differentiation. *In vitro* Th17 polarization assays were used to study the effects of gamma secretase inhibitors (GSI) on naïve CD4⁺ human T cells differentiating towards the Th17 phenotype. Naïve CD4⁺ T cells were pretreated with either DMSO as a vehicle control or individual GSIs (Compound E, Z-ILE-LEU-Aldehyde (IL-CHO), Ly 411,575 (LY), DAPT (3,5-Difluorophenylacetyl)-L-Alanyl-PhenylGlycinebutyl Ester) and cultured for 24-72hrs in Th17 inducing conditions. IL-CHO is a competitive peptide aldehyde inhibitor of γ-secretase activity that is thought to modify the active sites, while compound E is a non-peptide, non-transition state, non-competitive inhibitor of γ-secretase. LY 411,575 and DAPT are chemically similar to compound E. A significant reduction in IL-17 secretion was observed after treatment with GSIs at 24hrs (Fig. 12). Similar reduction in IL-17 levels was observed at 48 and 72 hrs after GSI treatment (data not shown). Levels of other Th17 cytokines, including IL-17F and IL-22, after GSI treatments were assessed by ELISA. Statistically significant decrease (approximately 2 fold) in IL-17F and IL-22 cytokines was observed after IL-CHO and compound E treatment (Fig.13). MTS assay was performed to confirm a decrease in IL-17A, IL-17F and IL-22 secretion by GSI was not due to an effect on cell viability and I observed no change (Fig.14). Next
I checked if Notch signaling inhibition affects already differentiated Th17 cells. Interestingly, addition of GSI after 5 days of Th17 differentiation did not change IL-17A, IL-17F and IL-22 cytokine levels suggesting Notch signaling plays role in initial time points of Th17 differentiation process (Fig.15).

Thus, I concluded that blocking Notch signaling pathway by gamma secretase inhibitors decreases Th17 polarization. This suggests a role of Notch signaling in Th17 differentiation, however GSI has several targets besides Notch, for example CD44, β amyloid, E-cadherin, ErbB-4. Therefore I planned to perform specific knockdown of Notch1 and study its effects on Th17 differentiation.

Specific knockdown of Notch 1 by siRNA in human naïve CD4$^+$ T cells followed by Th17 polarization leads to decreased IL-17 secretion. I observed upregulation of Notch signaling, particularly Notch1 and Hey1, in human in vitro Th17 differentiation assays, and GSI inhibited Th17 differentiation. To determine whether Notch1 is a functional target of GSIs during Th17 polarization, expression of Notch1 was reduced by delivery of siRNA to naïve CD4$^+$T cells. Naïve CD4$^+$ T cells were nucleoporated with scrambled and Notch1 specific siRNA. Cells were subsequently polarized to the Th17 lineage and harvested 48 h after transfection. Western blot analysis of Notch1 protein and quantitative RT-PCR confirmed that Notch1 siRNA reduced the expression
of Notch1 protein (Fig.16A) as well as mRNA (Fig.16B). Western blot of Notch 2, 3 and 4 expression was also performed to confirm the specificity the siRNA for Notch1 (Fig.17) and no significant change were observed in levels of other Notches after Notch1 knockdown. Notch1 knockdown (40-50% as compared to scrambled siRNA) significantly inhibited IL-17A production (50%, p≤0.05) under Th17 polarizing conditions (Fig.16C). I also checked levels of other Th17 cytokines, IL-17F and IL-22, after siRNA mediated Notch1 knockdown. I observed that similar to IL-17A, IL-17F cytokine production was also significantly reduced by 50% (p≤0.05) after Notch1 knockdown in human in vitro differentiation assay (Fig.18A). Surprisingly I did not observe any significant change in IL-22 cytokine production (Fig.18B). Taken together GSI and Notch1 siRNA data, it seems that IL-22 expression is regulated by other paralog of Notch, but not Notch1. Next I checked cell viability of Th17 cells in Notch1 siRNA treated cells to assess if decrease in IL-17A and F was due to decrease in cell survival. MTS assays were performed to check cell survival and it revealed no changes in viability between cell treated with scrambled siRNA or Notch1 siRNA (Fig.16D).
Figure 12: Gamma secretase inhibitors (GSIs) significantly reduce IL-17 levels in human in vitro Th17 polarization assays. (A) Semi quantitative RT-PCR of Hey1 and Hes5 showing GSI mediated down regulation of Notch signaling. (B)ELISA of IL-17 in supernatants of Th17 polarized naïve human CD4+ T cells treated with GSIs. Purified human CD4+ T cells were pretreated with indicated concentration of GSIs or DMSO as a vehicle control and then cultured in Th0 and Th17 polarizing conditions. Supernatants were collected at 24 hrs and were analyzed for IL-17. Mean±SD of two experiments performed in triplicates. *p≤0.05
Figure 13: Gamma secretase inhibitors (GSIs) significantly reduce IL-17F and IL-22 levels in human in vitro Th17 polarization assays. ELISA of IL-17F (A) and IL-22 (B) in supernatants of Th17 polarized naïve human CD4+ T cells treated with indicated concentration of GSIs at 24-72 hrs. Purified human CD4+ T cells were pretreated with indicated concentration of GSIs or DMSO as a vehicle control and then cultured in Th0 and Th17 polarizing conditions. Supernatants were collected at 24, 48 and 72 hrs and were analyzed for IL-17F and IL-22. Data represented the mean±SD of two experiments performed in triplicates.*p≤0.05, **p≤0.01.
Figure 14: MTS assay to assess cell viability of *in vitro* differentiated Th17 cells. Cells were assayed for viability after GSI treatment using MTS assays. Data depicted here is absorbance of formazan product (which measures reductase expressed by viable cells) measured at 490nm using ELISA plate reader, representing viability of the cells.
Figure 15: GSI treatment after 5 days of *in vitro* Th17 differentiation does not affect IL-17F and IL-22 cytokine levels. ELISA of IL-17F (A) and IL-22 (B) in supernatants of Th17 differentiated naïve human CD4+ T cells treated with 2μM IL-CHO after 5 days of *in vitro* Th17 differentiation. Supernatants were collected at 24 hrs after IL-CHO treatment and analyzed for IL-17F and IL-22. Data shown represent the is mean±SD of two experiments performed in triplicates.
Figure 16: Notch 1 controls human Th17 polarization. 10^7 purified human naive CD4+ T cells were nucleoporated with Notch1 specific siRNA or scrambled siRNA. After transfection, the cells were cultured under Th17 skewing conditions and whole cell lysates and cDNA were prepared. (A) Immunoblot of Notch1 expression and β-actin (loading control). (B) Quantitative RT-PCR using Notch1 specific primers to assess Notch1 transcript levels. (C) ELISA of IL-17 was performed on the supernatants of naïve CD4+ T cells nucleoporated with control siRNA and Notch1 siRNA followed by in vitro Th17 polarization. (D) Cells were assayed for viability after nucleoporation using MTS assay. Data depicted here represent absorbance at 490nm, representing relative viability using ELISA plate reader. Data shown is mean±SD of three independent experiments done in triplicates.*p≤0.05
Figure 17: Knockdown of Notch 1 by siRNA does not affect other Notch isoforms. Western blot of Notch1IC, Notch2IC, Notch3IC and Notch4IC after nucleoporation of Notch1 siRNA in naïve CD4+ T cells followed by Th17 differentiation. 293T cells transfected with Notch1IC, Notch2IC, Notch3IC and Notch4IC constructs were used as a positive control.
Figure 18: Notch 1 controls IL-17F, but not IL-22 cytokine expression in human in vitro Th17 polarization. Naïve CD4+ T cells were nucleoporated with Notch1 specific siRNA or scrambled siRNA. After transfection, the cells were cultured under Th17 skewing conditions and supernatant was collected. ELISA of IL-17F (A) and IL-22 (B) was performed on these supernatants. Data shown represent mean ±SD of three independent experiments performed in triplicates. *p≤0.05.
Overexpression of Notch 1 by retrovirus resulted in increase in human in vitro Th17 differentiation.

The role of Notch1 in human Th17 differentiation was confirmed by over-expressing activated Notch1 (intracellular domain of Notch1 cloned in the LZRS retroviral construct) in naïve human CD4+ T cells followed by Th17 polarization. Western blot of Notch1 was performed to confirm Notch1 over-expression (Fig. 19A). Naïve CD4+ T cells overexpressing Notch1IC LZRS produced higher levels of IL-17A compared to control cells in Th17 differentiation conditions (Fig. 19B). Interestingly Notch1 overexpression also increased IL-17 secretion in cells activated under Th0 conditions, suggesting that Notch1 is a necessary signal to promote IL-17 secretion even in the absence of differentiating cytokines and the presence of differentiating cytokines during Th17 polarization enhances its effect.

Assessment of Notch Ligands in human Th17 differentiation.

The presence of Notch ligands (DLL-1, DLL-3, DLL-4, Jag1 and Jag2) were assessed by quantitative real time PCR in Th0 and Th17 differentiated cells (Fig.20). My preliminary results suggest that transcripts of Notch ligands are present on naïve CD4+ T cells. Upon in vitro Th17 differentiation, there is upregulation of DLL-1 and DLL-4, and downregulation of Jagged 1 and 2 transcript levels (Fig.20, 21).
Figure 19: IL-17 release by naïve CD4+ T cells in response to Notch 1IC expression in naïve CD4+ T cells under in vitro Th17 differentiation condition. (A) Immunoblot of Notch1IC after transduction of naïve human CD4+ T cells with Notch1IC LZRS or control LZRS followed by Th17 differentiation. (B) ELISA of IL-17 performed after naïve CD4+ T cells transduced with control LZRS and Notch1IC LZRS followed by Th17 differentiation. The data is representative of three independent experiments performed is triplicates. * p≤.05.
Figure 20: Upregulation of Notch ligand DLL-4 and DLL-1 correlates with \textit{in vitro} Th17 differentiation. RNA was prepared from naive CD4+ T cells unstimulated (US), or differentiated under Th0 and Th17 conditions at indicated time points and quantitative reverse transcriptase PCR for Notch ligands DLL1 (A), DLL3 (B) and DLL4 (C) was performed. Data represented here is relative Notch ligand transcript levels normalized against 18S. Data are representative of two independent experiments performed in triplicates.
Figure 21: Downregulation of Jagged-1 correlates with *in vitro* Th17 differentiation. RNA was prepared from naive CD4+ T cells un-stimulated (US), or differentiated under Th0 and Th17 conditions at indicated time points and quantitative real time PCR for Notch ligands Jagged-1(A) and Jagged-2(B) was performed. Data represented here is relative Notch ligand transcript levels normalized against 18S. Data is representative of two independent experiments performed in triplicates.
Discussion

Several studies have highlighted the importance of the Th17 subset in normal immune responses as well as disease pathology, for example in autoimmune disorders and in inflammatory diseases. In this aim I examined the regulation of Th17 subset differentiation. The role of Notch signaling in peripheral T cell differentiation, particularly of the Th17 subset differentiation, has been implied recently, although the clear mechanism has not been clarified (Jurynczyk et al., 2008b; Mukherjee et al., 2009). Here I have shown that Notch signaling regulates in vitro human Th17 differentiation. Upon in vitro differentiation of naïve CD4+ T cells towards the Th17 subset, there is upregulation of activated Notch 1 expression and of downstream Notch target gene Hey1 transcript levels. Using pharmacologic Notch signaling inhibitors as well specific Notch1 siRNA, I have demonstrated that Notch signaling, is involved in human Th17 differentiation.

Upon in vitro differentiation of naïve human CD4+ T cells towards Th17, there is an upregulation of IL-17A, IL-17F and IL-22 production as measured by ELISA. Also, real time PCR of ROR-γt and intracellular staining of IL-17 showed that these cells are successfully differentiated to Th17 subset. Surprisingly I observed insignificant levels of IL-21, another cytokine which is known to be produced by Th17 subset. It may be possible that in humans, IL-21 is not the major cytokine produced by Th17 cells whereas in mouse there are significant levels of IL-21 secreted by Th17 cells (Wei et al., 2007). This differential
production of IL-21 in mouse and humans Th17 cells is very interesting and a potential area of future investigation.

Using pharmacological inhibitor of γ-secretase *in vitro*, I have shown that loss of Notch signaling resulted in down-regulation of IL-17A, IL-17F and IL-22 produced by Th17 cells. Because GSI blocks activation of all four Notch proteins, experiments described here implicate role of Notch signaling in Th17 differentiation, but do not determine which unique Notch receptor/s are crucial for this process. There are several other potential targets of GSI within the immune system. In addition to Notch, CD43 and CD44 are also known to be cleaved by γ-secretase (Kopan and Ilagan, 2004). Therefore the interpretation of data obtained using only GSI can be inconclusive. Therefore I performed specific knockdown of Notch1 in naïve CD4⁺ T cells followed by differentiating them towards the Th17 subset. Notch1 siRNA in naïve CD4⁺ T cells led to 50-60% decreased Notch1 expression levels as compared to scrambled siRNA measured by real time PCR, however no differences were observed in Notch2, 3, and 4. Upon knockdown of Notch1, there is decrease in IL-17A and IL-17F but not IL-22. Taking together both GSI and Notch1 siRNA data, it seems Notch signaling regulates IL-22, but may be Notch1 independent and depend on other Notch paralogs. Indeed in Notch1 deficient mice there is no change in IL-22 production (Alam et al., 2010). Recently role of Notch2 in regulation of IL-22 secretion has
been reported (Alam et al., 2010). It may be possible that different Notch receptors regulate IL-17 versus IL-22 cytokine production.

Another area of active investigation is the role of Notch ligand in Th17 differentiation. It has been suggested that different Notch ligands expressed on APCs drive differing T cell responses (Amsen et al., 2004). Data from several laboratories suggest that antigen presenting cells (APCs) expressing Delta like-4 (DLL-4) drive the differentiation of Th1 cells (Kassner et al., 2010; Liotta et al., 2008; Skokos and Nussenzweig, 2007) while APCs expressing Jagged1 promote differentiation of Th2 cells (Liotta et al., 2008). Lukacs and colleagues (Mukherjee et al., 2009) recently revisited and expanded this observation and determined that DLL4 expression is induced on APCs by pathogen-associated signals and this ligand promotes expression of ROR-γt and expansion of Th17 CD4⁺ T cells. These studies have raised the interesting possibility that distinct ligands may initiate different Notch responses in peripheral T cells. My preliminary results suggest that Notch ligands are present on naïve CD4⁺ T cells and upon Th17 differentiation; there is upregulation of DLL-4 and together with downregulation of Jagged1 transcript levels even after 4hrs as measured by real time PCR. This is an interesting finding as Notch ligands are known to be present only on antigen presenting cells. Role of these ligands in Th17 differentiation has to be further evaluated by knockdown techniques.
In this aim I have shown the role of Notch signaling, particularly by Notch1, in regulating Th17 differentiation. Next I studied the molecular mechanism by which Notch regulates Th17 differentiation and whether blocking the Notch signaling pathway by GSI is capable of inhibiting Th17 mediated diseases.
CHAPTER III

NOTCH FUNCTIONS AS A REGULATOR OF ROR-γt AND IL-17 PROMOTER

Introduction

ROR-γt (Retinoic acid related orphan receptor-γt) is a member of the Retinoic acid receptor related orphan nuclear receptor family. The family consists of RORα, RORβ and RORγ differing in chromosome location, function and regulation (He, 2002). Due to the use of alternate promoter and exon splicing, each member of this family exists in several isoforms differing in tissue distribution and function. The RORγ gene map to 1q21.3 and has two isoforms, γ1 and γ2 (Hirose et al., 1994). RORγ1 is present in liver, adipose and skeletal muscles whereas RORγ2 (ROR-γt) is exclusively present in the cells of immune system (Eberl et al., 2004). Initially ROR-γt is shown to be crucial for LTi (lymphoid tissue inducer) expressing cells and thus explains the absence of lymph nodes and Peyers patches in ROR-γt deficient mice (Eberl et al., 2004). The role of ROR-γt in Th17 differentiation was revealed later, when expression of this transcription factor was observed in the lamina propria T lymphocytes that produce IL-17, whereas and these CD4+IL-17+ T cells were absent in ROR-γt deficient mice (Ivanov et al., 2006).

The ROR-γt transcription factor is found to be induced in human and mice Th17 in vitro differentiation assays (Ivanov et al., 2007). Studies have shown that
cocktails of IL-6+TGF-β (Ivanov et al., 2007) or IL-6+TGFβ+IL-21+IL-1β (Manel et al., 2008) upregulate the transcript levels of ROR-γt in purified CD4⁺ T cells of mouse and human origin respectively prior to expression of IL-17. Furthermore, Th0 cells (precursor T helper) isolated from ROR-γt deficient mice failed to differentiate towards Th17 and this defect can be rescued by overexpression of ROR-γt in these cells (Yang et al., 2008b). ROR-γt transcription factor binding site AGGTCA preceded by 5bp of A/T rich sequence is present within IL-17A, IL-17F and IL-23R promoters, and thus it directs the expression of inflammatory cytokines (Jetten et al., 2001). Subsequently RORα is also been shown to play role in Th17 differentiation (Yang et al., 2008b). Although both ROR-γt and RORα regulate Th17 differentiation but ROR-γt is considered to be major player as ROR-γt deficiency has more pronounced effect on Th17 differentiation than RORα.

There are very few studies performed on regulation of ROR-γt promoter. A recent study suggests that Notch ligand DLL4 regulates expression of ROR-γt and thus Th17 differentiation (Mukherjee et al., 2009). They have demonstrated that various TLRs upregulate Notch ligand DLL4 in antigen presenting cells which binds to CSL on ROR-γt and IL-17 promoter in mouse CD4⁺ T cells. However binding of CSL to these promoters in response to TLR signals does not make sense as CSL is usually bound to Notch regulated promoters and provide repressive signals. Binding of the intracellular domain of Notch to CSL leads to
recruitment of several coactivators including Mastermind and histone acetyltransferases, and thus CSL becomes part of transcriptional activator complex. In the current aim, I studied the molecular mechanism by which Notch regulates human Th17 differentiation process and whether human ROR-γt and IL-17 promoters are direct transcriptional targets of Notch.

Results

Notch1 regulates Th17 polarization by regulating ROR-gammaT promoter.

I utilized in vitro Th17 differentiation assay to study the mechanism by which Notch signaling regulates Th17 differentiation. First key candidate is the orphan nuclear receptor ROR-γt, the key transcription factor that orchestrates the differentiation of the Th17 effector cell lineage. To determine whether Notch1 influences human Th17 polarization by regulating ROR-γt expression, naïve human CD4+ T cells were purified, and nucleoporated with Notch1 specific siRNA and scrambled siRNA as a control, followed by culture under Th17 polarizing conditions. Quantitative RT-PCR of ROR-γt was then performed. Notch1 knockdown resulted in 40% decreased levels of ROR-γt transcripts (Fig. 22A). Results from the collaborative laboratory of Dr. Barbara Osborne also corroborated my data by showing that GSI treatment down regulates ROR-γt transcripts to around 50% in mouse CD4+ T cells as quantified by real time PCR in Th17 differentiated cells (Fig. 22B). Taken together, these data indicated that
Notch1 regulates the expression of ROR-γt. Further, I explored the possibility that Notch1 may directly bind and regulate the human ROR-γt promoter. The human ROR-γt promoter was analyzed for putative CSL binding sites by TF-binding search software (cbrc.jp/research/db/TFSEARCH.html). Two potential CSL sites were identified within the proximal 3kb promoter, upstream of the ROR-γt transcriptional start site: CSL1 (-1156 to -1161bp) and CSL2 (-1800 to -2082bp) (Fig.23A). Chromatin immune-precipitation analysis (ChIP) using an anti-human Notch1 antibody was performed after 24hrs of Th17 in vitro polarization assays to determine whether Notch1 binds directly to the ROR-γt promoter. ChIP experiments indicated that Notch1 binds directly to putative CSL binding sites in the human ROR-γt promoter (Fig.23B, C). In particular, Notch1 bound at the CSL1 site, which could be inhibited by treatment with GSI (Fig 23B, C).

Quantitative real time PCR of ChIP assays suggested that Notch1 is recruited to human ROR-γt CSL1 site even under Th0 condition (2.5 fold), and Notch1 recruitment is further increased to around 9 fold under Th17 driving condition. This suggests that Notch1 binds the ROR-γt promoter and may be one of the mechanisms by which it regulates Th17 differentiation.
Figure 22: Notch 1 regulates ROR-γt promoter activity. (A) Human naïve CD4+ T cells (1x 10⁷) were nucleoporated with Notch 1 specific siRNA or scrambled siRNA followed by in vitro Th17 polarization. Cells were harvested and ROR-γt expression was determined by quantitative RT-PCR. Transcript abundance was normalized to 18s rRNA expression. Data shown represent the mean±SD for three independent experiments. (B) In vitro IL-CHO treatment down-regulates ROR-γt and IL-17 mRNA expression. Total RNA was isolated from mouse CD4+ T cells pretreated with 25μM IL-CHO or DMSO as a vehicle control and cultured in Th17 polarizing conditions and analyzed by quantitative real time PCR.
Figure 23: Notch 1 binds to the human ROR-γt promoter. (A) Schematic representation of putative CSL binding sites in human ROR-γt promoter. (C) Specific primers were used to amplify putative CSL binding sites on the human ROR-γt promoter. ChIP assay was performed to determine recruitment of Notch1 on ROR-γt promoter. Data shown represents fold recruitment of Notch1 on CSL binding sites of the human ROR-γt promoter with respect to control IgG normalized with 1% input DNA. (B) Regular PCR was also performed using specific primers against CSL sites in ROR-γt promoter to confirm transcript size. Data represents mean ± SD of two independent experiments performed in triplicates. **p≤.01.
Notch 1 regulates human IL-17 promoter activity.

Recent study by Lukacs and colleagues suggested that Notch ligand DLL4 can regulate the IL-17 promoter and thus Th17 differentiation (Mukherjee et al., 2009). Additional evidence came from collaborative Osborne Laboratory where treatment with GSI led to reduction in IL-17 transcript levels by around 90%. This binding of Notch, both to ROR-γt and IL-17 promoter, may be a mechanism by which Notch tightly regulates differentiation program such that once fate of naïve CD4⁺ T cells is decided, it is irreversible. Therefore I also checked this possibility that Notch may also regulate the IL-17 promoter in addition to the ROR-γt promoter. Co-transfection of 293T and Jurkat T cells cells with a human IL-17 promoter luciferase construct in combination with an activated Notch1 expression vector construct (N1IC) resulted 2.5-3 fold increase in human IL-17 promoter activity (Fig.24A,B) in both the cell types. This suggests that Notch1 regulates the human IL-17 promoter also. Next I assessed if Notch 1 binds directly to human IL-17 promoter under *in vitro* Th17 polarization conditions. The human IL-17 promoter (3kb) upstream of the transcription start site (TSS) was therefore analyzed for putative CSL binding sites (cbrc.jp/research/db/TFSEARCH.html) (Fig. 25A). I found four putative CSL binding sites within this region: CSL1 (-167 to -172bp), CSL2 (-454 to -459 bp), CSL3 (-1210 to -1215 bp) and CSL4 (-1725 to -1730 bp) (Fig. 25B). ChIP assays were performed using anti-Notch1 antibody after 24 hrs of *in vitro* Th17 differentiation. The ChIP assays revealed that Notch1
binds to putative CSL binding sites in the human IL-17 promoter, particularly at CSL1 and 4 (Fig. 25C), but not at CSL2 and 3. The binding was inhibited by treatment with GSI (Fig.25C). These data suggest binding of both ROR-γt and IL-17 promoters, by Notch1.

Differential upregulation of Notch isoforms under in vitro Th1, Th2 and Th17 differentiation.

Although several studies are performed studying the role of Notch signaling in peripheral T-helper cells differentiation, particularly Th1 and Th2 differentiation, concrete conclusions could not be withdrawn. I used human Th1, Th2 and Th17 in vitro differentiation assays to address this question. Upon human in vitro differentiation of naïve CD4+ T cells towards Th1, Th2 and Th17, differential upregulation of Notch isoforms was observed (Fig.26). Expression of Notch1 was found to be increased in Th17 differentiation and Notch3 expression in Th1 differentiation. This is very interesting as different Notch paralogs can regulate T helper cell differentiation to Th1, Th2 or Th17. In addition even the expression of individual Notch paralog was found to be different in each T helper subset.
Figure 24: The human IL-17 promoter is regulated by Notch1. (A) 293 T cells were co-transfected with intracellular activated Notch expression vector construct (Notch1IC) along with a human IL-17 promoter construct cloned upstream of the firefly luciferase gene along with renilla luciferase plasmid. Luciferase assay was performed and data was normalized to renilla luciferase depicted as relative luciferase units (RLU). Immuno-blotting of Notch1 was performed to confirm overexpression. Results are shown as mean±SD of three independent experiments performed in triplicates.*p≤0.05

(B) Jurkat T cells were electroporated with separate plasmids encoding Notch1IC expression vector and human IL-17 luciferase promoter. After 48hrs, cells were stimulated with PMA/I for 6hrs and luciferase assays were performed. Data shown here is normalized to renilla luciferase and depicted as relative luciferase units (RLU). Experiments are done in triplicates and data shown is mean±SD of two independent experiments.
Figure 25: Notch 1 binds to human IL-17 promoter (A) Schematic representation of putative CSL binding sites in human IL-17 promoter. (C) ChIP assays were performed to determine the recruitment of Notch1 on human IL-17 promoter. Data shown represents fold recruitment of Notch1 to the human IL-17 promoter with respect to isotype control IgG normalized to input DNA. (B) PCR performed using specific primers against different putative CSL binding sites in human IL-17 promoter. Data shown here represents the mean±SD of two independent experiments performed in triplicates.*p≤.05,**≤0.01
Figure 26: Differential upregulation of Notch isoforms under *in vitro* Th1, Th2 and Th17 differentiation. Naïve human CD4+ T cells were *in vitro* differentiated towards Th1, Th2 and Th17 subsets. (A) ELISA of Th1 associated cytokine (TNFα), Th2 associated cytokine (IL-5) and Th17 associated cytokine (IL-17A) was performed. (B) At 72 hrs, whole cell lysates were made and western blotting was performed for active Notch1 and active Notch3. β actin was used as a loading control. Data depicted here is a representative of two independent experiments.
Discussion:

T-helper cells differentiation is a highly regulated process involving several molecular events to determine the fate of naïve CD4+ T cells. In this study, I assessed the role of the Notch signaling pathway in Th17 differentiation. I provided evidence that Notch1 binds and regulates both ROR-γt and IL-17 promoter providing a mechanism by which Notch1 dictates Th17 differentiation. A recent publication has shown that in response to LPS signal, there is secretion of IL-17 which can be blocked by antibody to Delta like ligand-4 (DLL-4) (Mukherjee et al., 2009). Furthermore addition of DLL-4, but not jagged 1, promotes Th17 differentiation. In my in vitro Th17 differentiation, I observed increase in DLL-4 transcripts from 5 folds to 12 folds at 12 and 24 hrs respectively after Th17 polarization (Fig.20). Combining all the results together, it seems that Notch1-DLL-4 signal regulates Th17 differentiation.

Previous studies showed a paradoxical role of Notch in differentiation of both the Th1 and Th2 CD4+ T cells subsets. Next important question to be answered is how Notch signaling dictates mutually exclusive Th1, Th2 and Th17 differentiation program? Expression of Notch1 was found to be increased in Th17 differentiation and Notch3 expression in Th1 differentiation. In addition even the expression of individual Notch paralog is different in each T helper subset. Knockdown of individual Notch receptor as well as expressing Notch receptors in varied levels would further resolve this issue.
Notch binds and regulates the T bet and IFNγ promoters during Th1 differentiation, and GATA3 and IL-4 promoter in Th2 differentiation (Amsen et al., 2007; Minter et al., 2005). In this study I have shown that Notch1 can also bind to ROR-γt and IL-17 promoter in Th17 differentiation. It is possible that Notch promiscuously binds to promoters defining more than one differentiation pathway or there is difference in co-activator complex which is recruited to these promoters resulting in differential T helper cell differentiation pathways. It has been shown that coactivator Mastermind 3 (MAML3) has high Notch 4 binding preference in contrast to MAML1 which favorably binds Notch3 (Wu et al., 2002). Further, it has also been shown that Notch binds as a dimer to c-myc and pre TCR promoter (Liu et al., 2010). Interestingly in both human ROR-γt and T-bet promoter, I observed two adjacent putative Notch binding sites. Another assumption can be that combination of different Notch paralogs result in differential T helper cell differentiation. Further experiments have to be performed to assess these assumptions.

Till now, I have demonstrated the role of Notch as an important determinant of Th17 differentiation under in vitro conditions. Next I studied the biological significance of Notch signaling in Th17 mediated autoimmune disease.
CHAPTER IV

NOTCH SIGNALING INVOLVEMENT IN TH17 MEDIATED DISEASE, EAE (EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS), MOUSE MODEL OF MULTIPLE SCLEROSIS

Introduction

EAE (Experimental autoimmune encephalomyelitis) is an animal model that resembles human inflammatory demyelinating disease, Multiple Sclerosis (MS) (Sospedra and Martin, 2005). Histologically MS is characterized by presence of inflammatory T cells and macrophages in active lesions accompanied by demyelination, loss of oligodendrocytes and axonal damage. This damage to the myelin sheath hampers neuronal transmission and causes debilitating symptoms in MS patients including loss of sensation, muscle weakness, visual impairment and paralysis (Compston and Coles, 2002; Compston and Coles, 2008). The symptoms of MS can be broadly divided into two subtypes: relapsing-remitting or progressive disease. The relapsing-remitting type is characterized by the presence of disease symptoms alternating with asymptomatic periods. Progressive disease is marked by continuous neurological symptoms without remission (Lublin and Reingold, 1996). This disease usually affects young adults, most commonly females, with a frequency of 2-150 per 100,000 (Rosati, 2001). There is no known cure for MS. Current
regimens aims to prevent new attacks and stabilizing patients (Compston and Coles, 2002).

In mice this disease is induced by injecting brain extracts, CNS proteins (Myelin basic protein) or small molecule derivative of CNS proteins (Proteolipid peptide, Myelin oligodendrocyte glycoprotein) emulsified in complete Freund’s adjuvant (CFA) (Mannie et al., 2009; Miller and Karpus, 2007). The presence of adjuvant facilitates the generation of an inflammatory response towards CNS proteins. Along with CFA, mice are also injected with pertussis toxin which helps break the blood brain barrier and allows immune cells to attack the CNS (Miller and Karpus, 2007).

Classically EAE was considered to be a Th1 mediated disease with a predominant role for IFNγ (Kuchroo et al., 2002). Later, this paradigm was challenged and systematic studies utilizing mice knock-out for IL-12p19 and IL-23p35 subunits revealed that indeed IL-23, but not IL-12, is important for EAE development (Cua et al., 2003; Langrish et al., 2005; Langrish et al., 2004). IL-23 is an indispensable cytokine for Th17 development, that is required for maintenance and expansion of this subset (Harrington et al., 2005). Subsequently several studies were performed to study role of Th17 subset in EAE pathogenesis. Two photon microscopy of the brainstem of mice having progressive EAE showed a direct interaction between Th17 cells and neurons, which leads to an intracellular Ca^{2+} imbalance and damage to neuronal cells.
Moreover, with co-culture experiments between glial cells and autoreactive T cells it was shown that T cells secreting IL-17 are responsible for inducing an inflammatory milieu with in CNS. IL-17 also promotes neuronal inflammation by inducing MHC class II and costimulatory molecule expression on glial cells (Domingues et al., 2010). Recent studies using adoptive transfer of myelin oligodendrocyte glycoprotein (MOG) specific Th1 or Th17 monoclonal T cell clones expressing the same TCR into lymphopenic Rag2−/− knockout mice revealed interesting results. Rag2−/− knockout mice that received Th1 cells develop classical EAE with paralysis progressing from tail to head, whereas those receiving Th17 cells develop atypical EAE with an ataxia and an unbalanced gait followed by tail to head paralysis. In a cutting-edge article utilizing IL-17A fate reporter mice (Il17a<sup>Cre</sup>R26R<sup>eYFP</sup>) it was shown that under chronic inflammatory conditions in EAE, Th17 cells switch to an IFNγ producing phenotype, again supporting a role for Th17 cells in EAE pathogenesis (Hirota et al., 2011).

Since my in vitro experiments have demonstrated the role of Notch signaling in regulating Th17 differentiation, therefore I hypothesized that Notch inhibitors can prevent Th17 mediated diseases. Besides Notch signaling regulating human Th17 differentiation, parallel data have been obtained in mouse Th17 differentiation assays in Dr. Osborne laboratory, University of
Massachusetts, Amherst. Summary of results obtained in mouse in vitro Th17 differentiation assays are given below:

| **Gamma secretase inhibitors (GSI) significantly down-regulate Th17 associated cytokine levels in murine Th17 in vitro polarization assays.** |
| **Upregulation of Notch1 in mouse Th17 differentiation assays, which decreases upon treatment with GSI.** |
| **Downregulation of intracellular IL-17 levels upon treatment with GSI in mouse Th17 differentiated cells.** |

Combining both mouse and human data led us to check in vivo if Notch inhibition ameliorates EAE, a Th17 mediated disease.

This work is done in conjunction with Dr Barbara Osborne laboratory, University of Massachusetts, Amherst.

Results

Gamma Secretase inhibitor (GSI) ameliorates the severity of EAE induced inflammation and Th17 differentiation in-vivo.

To investigate the role of Notch signaling in regulating Th17 mediated disease in vivo, we immunized the SJL/J strain of mice with proteolipid peptide (PLP) in complete Freund’s adjuvant (PLP$_{139-151}$-CFA) to induce EAE, a Th17 dependent chronic autoimmune disease. Before inducing EAE, SJL mice were
fed control chow or orally active GSI (LY) chow for 5 weeks. We used doses between 2.5 and 5 mg/kg/day of GSI, which is known to be safe and effective in reducing Notch activity. Higher doses cause the secretory diarrhea due to goblet cell metaplasia of the intestine. In control mice (fed with normal chow), the initial signs of EAE were observed at day eight post immunization. Delayed onset of disease, ten days after immunization, was observed in mice fed with Notch signaling inhibitor GSI (Fig.27A). This suggested that inhibition of Notch signaling by oral GSI delayed the onset of EAE. Next we wanted to check whether Notch inhibitors affect the disease symptoms. The progression and severity of EAE disease was monitored and scored from 0-5 as follows: Score 0-no disease; 1-limp tail, 2-hind limb weakness; 3-hind limb paralysis; 4-hind and fore limb paralysis; 5-morbidity and death (Serada et al., 2008). We observed that in control mice (mice fed with normal chow) the mean clinical score at the peak of disease was 2 (hind limb weakness) while in GSI fed mice the clinical mean score was reduced to 0.2 (Fig.27A). Therefore GSI treatment delayed the disease progression as well as reducing the severity of EAE symptoms.

Next we wanted to assess whether this relief of symptoms in EAE disease (in SJL/J mice by GSI) was due to an effect on Th17 responses. Splenocytes were obtained from control or GSI fed mice, and were stimulated with PLP\textsubscript{139-151} \textit{in vitro}. It was observed that PLP stimulated splenocytes cultured from SJL/L mice fed with GSI showed significantly lower (approximately four fold, \(p<0.05\)) IL-
17 levels as compared to control chow fed mice (Fig.27B). Similarly, supernatants obtained from mononuclear cells isolated from spinal cords showed lower IL-17 levels in the GSI-treated mice than in the control group (Fig.27C). This suggested that GSI treatment hampered the potential of immune cells among splenocytes, and mononuclear cells within the CNS to elicit Th17 response towards the PLP peptide. Since Notch signaling is important for T cells development also, another possibility is that in GSI fed mice there is reduction in total T cell numbers, rather than an effect on Th17 differentiating potential. Therefore we quantified staining spinal cord infiltrating cells for CD4⁺ and CD8⁺ surface markers in GSI fed mice (Fig.28). We observed no significant differences between control chow or GSI fed mice in the number of T cells infiltrating the spinal cord. This showed that the decrease in IL-17 in the group of mice treated with GSI was not due to a difference in infiltrating T cell numbers, but rather due to selective effects of GSI on Th17 cells.
FIGURE 27: GSI treatment reduces EAE-induced inflammation and the development of PLP\textsubscript{139-151}-specific Th17 responses. (A) Clinical scores of SJL/J mice given GSI formulated chow, at 2.5mg/kg alternated with 5mg/kg for 4 weeks. Control mice were given regular chow. n=5 mice in each group. Results represent the mean disease score grouping each group. Splenocytes (B) and cells from the spinal cords (C) of EAE-induced mice were restimulated ex vivo with PLP\textsubscript{139-151} and were then analyzed for IL-17 by ELISA.

By Reem Suleiman, Dr Osborne Laboratory
FIGURE 28: GSI treatment does not affect total number of CD4 and CD8 T cells. After feeding SJL/J mice with GSI formulated chow, total spinal cord cells were stained with CD4 and CD8 antibodies and analyzed by flow cytometry.

By Reem Suleiman, Dr Osborne Laboratory
Discussion:

In this study, we asked whether Notch signaling would influence Th17 mediated immune responses \textit{in vivo}. We used the EAE (Experimental autoimmune encephalomyelitis) mouse model of the human demyelinating disease Multiple Sclerosis. Initially a role for the Th17 subset in autoimmune diseases was discovered within this model, leading to several publications highlighting the role of the Th17 subset and associated cytokines in the axonal damage and demyelination in the EAE model system (Cua et al., 2003; Domingues et al., 2010; Hirota et al., 2011). Thus the Th17 subset is considered to make a major contribution to EAE pathogenesis. We have shown that Notch signaling plays a role in Th17 differentiation using \textit{in vitro} Th17 differentiation assays. Therefore, targeting the Notch pathway in the EAE disease model can be an effective means to regulate Th17 differentiation and EAE associated symptoms. Interestingly, oral Notch signaling inhibitors GSI were able to delay the onset of disease as well as the clinical score of EAE in SJL/J mice. As GSI treatment did not impact on the total number of T cells, this suggested that GSI is effecting EAE disease progression by altering the inherent ability of T cells to differentiate towards Th17 subset.

The effect of GSI on EAE disease progression can also be partially due to role of Notch signaling in Th1 differentiation. The concept that Th17 is the only subset responsible for CNS inflammation is challenged by several reports
showing that both Th1 and Th17 cells are involved in EAE (Kroenke et al., 2008; Stromnes et al., 2008). It has been shown that both ROR-γt and T-bet deficient mice are resistant to EAE (Bettelli et al., 2004; Ivanov et al., 2006). Moreover, analysis of T cells infiltrating the CNS during EAE development showed presence of both Th1 and Th17 cells (Korn et al., 2008; Langrish et al., 2005). Notch is already reported to regulate Th1 differentiation and EAE pathogenesis by regulating IFNγ levels (Minter et al., 2005). Taken together, Notch inhibitors can be an effective therapy against EAE pathogenesis as it regulates both Th1 and Th17 proinflammatory subsets. In support of our data, difluroketone MW167, another GSI was injected to the lateral cerebral ventricle of mice with EAE, and these mice showed enhanced recovery as compared to control mice. In GSI injected mice there was decreased inflammation accompanied by remyelination, emphasizing that targeting Notch signaling can also be an effective therapy for EAE (Jurynczyk et al., 2005). During development Notch negatively regulates oligodendrocytes maturation by inhibiting transcription factors Mash1 and NeuroD (Lee, 1997). It may be possible that Notch is re-expressed in multiple sclerosis/EAE lesions and prevent oligodendrocytes maturation and thus remyelination. This could explain restoration of myelination after Notch signaling inhibition by GSI treatment.

LY450139, yet another GSI, is already in Phase II clinical trials to treat Alzheimer’s disease, since β-amyloid is another GSI target (Fleisher et al., 2008;
Siemers et al., 2005). This drug is found to be well tolerated, with minor side effects including drug rashes, hair color changes and gastrointestinal symptoms (Fleisher et al., 2008). Thus targeting Notch signaling to treat MS patients by GSIs seems to be an attractive therapeutic tool. Other targets, for example antibodies against specific Notch receptors or Notch ligand Delta can also be used to manipulate Notch signaling more specifically in the treatment of multiple sclerosis and autoimmune diseases in general.
CHAPTER V

PUTATIVE ROLE OF NOTCH SIGNALING IN Th1/Th17 DOUBLE PRODUCER CELLS.

Introduction

The Th1-Th2 paradigm introduced by Mosmann and Coffman helped us to understand adaptive immunity and the potential of the immune system to acquire alternate differentiation program, in response to different pathogenic stimuli. This helper CD4⁺ T cell differentiation program was considered to be mutually exclusive and self-reinforcing. The discovery of Th17 has not only further extended our understanding of adaptive immunity, but also led us to appreciate the non-rigid aspects of the T helper cell differentiation program. The identification of TGFβ and IL-6 as important cytokines required for Th17 differentiation, and ROR-γt as a Th17 lineage determinant transcription factor, led to the acceptance of Th17 as a novel and distinct T-helper cell subset (Ivanov et al., 2006; Yang et al., 2008b). Nevertheless substantial evidence has since accumulated pinpointing to the plastic nature of this subset. Th17 differentiated cells readily convert to IL-17⁺IFNγ⁺ double positive cells in vitro (Annunziato et al., 2007; Bending et al., 2009; Lee et al., 2009; Martin-Orozco et al., 2009a; Shi et al., 2008), which may be the reason for the initial supposition that Th17 cells
originated from the Th1 subset, rather than being a separate subset (Bettelli and Kuchroo, 2005). It was also hypothesized that this Th17/Th1 plasticity only occurs in *in vitro* cultures, but not *in vivo*, due to the absence of a complete cytokine environment providing strong Th17 polarizing signals in culture. This was refuted by using an IL-17 reporter system *in vivo* in which IL-17 producing cells cannot only be detected but also traced. Upon induction of EAE by immunization with MOG-CFA (Myelin Oligodendrocytes Glycoprotein in Complete Freund’s Adjuvant) in *Il17a*Cre*Rosa26*eYFP mice, eYFP+ (IL-17A secreting) cells develop from IFNγ*IL-17A* secreting cells to IFNγ+IL-17A+, and finally into predominantly IFNγ secreting cells (Hirota et al., 2011). Thus this report provides evidence of Th17 cells being plastic even under *in vivo* disease conditions. Furthermore when Th17 differentiated MOG specific CD4+ T cells were adoptively transferred to Rag2−/− mice, they spontaneously converted to a Th1 phenotype in an EAE model system (Domingues et al., 2010). Similar studies have been performed in the autoimmune colitis mouse model (Lee et al., 2009) and in an adoptive transfer model of diabetes (Bending et al., 2009) revealing parallel results of conversion of the Th17 subset to a Th17/Th1 dual phenotype and its role in disease pathogenesis. Importantly Th17/Th1 cells are also detected in human diseases, for example in inflammatory bowel disease (Kleinschek et al., 2009) and rheumatoid arthritis (Nistala et al., 2010).
Studies have been performed to understand the mechanism of Th17/Th1 plasticity. The most important driving factor appears to be the cytokine environment. IL-6 and TGFβ up regulate ROR-γt and RORα, which in-turn increase expression of the IL-23 receptor and IL-12 receptorβ-2 in Th17 precursor cells. This may explain the responsiveness of Th17 precursor cells to both IL-23 and IL-12 (Lee et al., 2009). Therefore, Th17 precursor cells transform from a Th17 to a Th1 phenotype depending upon the balance between cytokines with a TGFβ promoting Th17 phenotype, low TGFβ and high IL-23 promoting the dual Th17/Th1 phenotype and high IL-12 promoting a Th1 phenotype. IL-23 is also been shown to upregulate T-bet in Th17 precursor cells. In this regard IL-23p19 deficient Il17CreR26RεYFP reporter mice failed to upregulate T-bet after MOG-CFA immunization suggesting a role for IL-23 in not only Th17 subset development but also in double expression of IL-17 and IFNγ by regulating the T-bet transcription factor (Hirota et al., 2011).

The presence of an IL-17+IFNγ+ double positive population both in vitro and in vivo in mice and humans, and their role in the pathogenesis of several diseases makes Th17 and Th17/Th1 double producers an area of active investigation. Studies to resolve the molecular mechanisms underlying the differentiation of naïve CD4+ T cells towards Th17 and Th17/Th1 plasticity will help to understand adaptive immune responses and their dysregulation in autoimmune diseases. In this study I have shown that Notch binds to both ROR-
γt and IL-17 promoters, consistent with role of Notch signaling in Th17 differentiation. Previously a role for Notch signaling in Th1 differentiation has also been shown (Minter et al., 2005) suggesting that similar signals are involved in the polarization towards either subset. Moreover, Notch ligand Delta plays a role in both Th1 (Skokos and Nussenzweig, 2007) and Th17 (Mukherjee et al., 2009) differentiation, suggesting Notch-Delta interaction play role in co-differentiation of Th17/Th1 subsets. Therefore it is very interesting to identify whether Notch signaling similarly defines Th17/Th1 plasticity.

Results
The presence of IL-17+/IFNγ+ double positive cells in human in vitro Th17 differentiation assay.

In order to study the role of Notch signaling in Th17/Th1 double producer population, I wanted to check if IL-17+/IFNγ+ double positive cells are detectable in human in vitro Th17 differentiation assays. Naïve CD4+ T helper cells (CD4+CD45RA+) were purified from human peripheral blood mononuclear cells and were cultured under Th0 and Th17 polarizing conditions. After 24 hrs, supernatants were collected and analyzed for TNFα as a cytokine marker for Th1 cells by ELISA. In human Th17 in vitro differentiation assays, I observed a two fold increase in TNFα levels as compared to Th0, but no changes in IL-5 levels were detected (Fig.29). This observation was further confirmed by performing
intracellular staining of IFN$_\gamma$ and IL-4 in Th0 and Th17 in vitro polarized subsets (Fig.30). I observed that within human in vitro Th17 differentiated subset, there is an increase in IL-17$^+/\text{IFN}_\gamma^+$ expression by 5.6 fold as compared to Th0 as detected by flowcytometry, with no change in IL-4 expression. Furthermore transcript levels of T-bet were also up-regulated two fold within Th17 differentiated subset as compared to Th0 as detected by real time PCR (Fig.31). Taking together all these results, I concluded that during human in vitro Th17 differentiation, a mosaic IL-17$^+/\text{IFN}_\gamma^+$ population is present. Another interesting observation is the presence of T-bet transcripts within the Th17 differentiated subset. Next I wanted to study role whether Notch signaling also plays a role in Th17/Th1 population differentiation.
Figure 29: GSI affects Th1 (TNFα) but not IL-5 under Th17 polarization. Naïve CD4⁺ T cells were in vitro polarized to Th0 or Th17 and pretreated with different concentrations of GSI or DMSO as a vehicle control. Supernatants were collected after 24 hrs and ELISA was performed for Th1 cytokine TNFα (A) and Th2 cytokine IL-5 (B). Concentrations of GSI used were DAPT (10, 25, 50µM), CompE (500nm, 2µM and 5µM), Ly (10, 25, 50µM) and IL-CHO (2, 5, 10µM). Data is representative of three different experiments done in triplicates. For T test, values are compared with Th17 (DMSO). *p≤0.05, **p≤0.01.
IFNγ+/IL-17+ mosaic population in both Th1 and Th17 differentiated cells. Naïve human CD4+ T cells were in vitro differentiated towards Th1 and Th17 subsets. Intracellular staining with anti-humanIL-17PE, anti-human IFNγ-APC and anti-humanIL-4FITC was performed after 72hrs. Cells were stimulated with PMA/Ionomycin for 4hrs and monensin for further 2 hrs. Cells were then fixed and permeabilized, followed by staining with aforementioned antibodies and analyzed by flowcytometry.
Figure 31: Upregulation of T-bet transcripts in *in vitro* Th17 differentiated cells shown by quantitative real time PCR. cDNA was synthesized from *in vitro* differentiated Th17 cells and T-bet transcript levels were measured by quantitative real time PCR at 24hrs. Data shown here represent relative T-bet transcript levels normalized against 18S transcripts and are mean±SD of two independent experiments done in triplicates. *p≤0.05.*
Pharmacologic and genetic inhibition of Notch signaling in Th17 differentiation reduces Th1 (TNFα) associated cytokines with no change in Th2 (IL-5).

In order to study the role of Notch signaling in differentiation of Th17/Th1 mosaic population, pharmacologic inhibitors of the Notch signaling pathway were used. naïve CD4⁺ T cells were pretreated with either DMSO as a vehicle control, or individual GSI, namely {Compound E (GSI XXI, (S,S)- 2-[2-(3,5-Difluorophenyl)-acetylamino]-N-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)-propionamide), IL-CHO (GSI XII, Z-ILE-LEU-Aldehyde), Ly (Ly-411575) and DAPT (GSI IX, Ly 374973,(3,5-Difluorophenylacetyl)-L-Alanyl-PhenylGlycinebutyl Ester}). I observed that with increasing concentrations of different GSIs, there is significant decrease in levels of TNFα (p≤.05) and IL-17A (p≤.05), IL-17F (p≤.05) and IL-22 (p≤0.5) at 24hrs with no change in cell viability as measured by MTS assays (Fig.29A,12,13). Interestingly I did not observe any change in IL-5 levels even at the highest GSI concentrations by ELISA (Fig.29B). This suggested that Notch signaling is playing a role in the initial steps of differentiation of both Th1 associated cytokine TNFα and Th17 associated cytokines IL-17A, IL-17F and IL-22 in human in vitro differentiated Th17 cells. It also suggests that Notch signaling does not regulate Th2 associated cytokine IL-5 in human in vitro Th17 differentiation assays. However the downside of using GSI is its lack of specificity, exemplified by a number of alternate targets including β amyloid, CD44, ErbB-4 and E-cadherin.
Therefore the effects of GSI of the Th1 and Th17 subsets can be indirectly due
effects on other downstream targets. Next I studied the specific role of Notch
signaling on regulating Th1 and Th17 associated cytokines in Th17 differentiated
cells.

I observed an up-regulation of only Notch1 expression, but not of other
Notches in human in vitro Th17 differentiated cells, which also contained
IFNγ+/IL-17+ double positive cells (Fig.9). Therefore I planned to specifically
knock down Notch1 by siRNA in naïve CD4+ T cells followed by Th17
differentiation. Naïve CD4+ T cells were nucleoporated with scrambled and
Notch1 specific siRNA and were subsequently polarized to the Th17 lineage. I
observed that upon Notch1 downregulation (50% as compared to scrambled
siRNA), there is a significant decrease in IL-17A (p≤0.5), IL-17F (p≤0.5) and
TNFα (p≤0.5) levels as measured by ELISA (Fig.32A,16C,18A). Surprisingly,
Notch1 down-regulation reiterated the same results as GSI treatment, having no
effect on IL-5. Rather, a statistically significant increase in IL-5 levels was
observed upon Notch1 knockdown (Fig.32B). This suggested that Notch1 is
controlling Th1 and Th17 associated cytokines as upon downregulation of
Notch signaling either by chemical inhibitors or Notch1 specific siRNA, it led to a
decrease in Th1 associated cytokines (TNFα) and Th17 associated cytokines (IL-
17A and IL-17F). Unexpectedly Notch inhibition shifted differentiation program
towards the Th2 lineage (as I observed an increase in IL-5). It is very surprising
as even under Th17 differentiating conditions, the absence of Notch1 can reduce the differentiation of naïve CD4$^{+}$ T cells towards the Th17 lineage while promoting IL-5 secretion.

I further addressed the role of Notch1 in regulating human Th17/Th1 mosaic population differentiation by over-expressing activated Notch1 (intracellular domain of Notch1 cloned into the LZRS retroviral construct) in naïve human CD4$^{+}$ T cells followed by Th17 polarization. Notch1 overexpression led to a statistically significant increase in IL-17 levels (p≤0.5) (Fig.19). Although I did not observe a statistically significant difference, I did observe a trend towards increased TNF$\alpha$ levels. (Fig.33A). No changes were observed in IL-5 cytokine levels measured by ELISA (Fig.33B).

Therefore I concluded that Notch signaling, particularly by Notch1, is regulating Th17 and Th1 populations upon in vitro Th17 differentiation, but not Th2. The next important question to be answered is molecular mechanism by which Notch1 is regulating the Th17/Th1 double producers.
Figure 32: Notch1 knockdown results in a decrease of TNFα and an increase in IL-5 levels. Naïve CD4+ Tcells were nucleoporated with scrambled or Notch1 siRNA followed by \textit{in vitro} Th17 differentiation. Supernatants were collected after 48hrs. ELISA was performed for TNFα (A) and IL-5 (B). Data shown is mean±SD of two different experiments performed in triplicates. *p≤0.05.
Figure 33: Notch1 overexpression results an increase in TNFα, but no change in IL-5 expression upon in vitro Th17 differentiation. ELISA of TNFα (A) and IL-5 (B) performed after naïve CD4+T cells were transduced with control LZRS and Notch IC LZRS followed by Th17 differentiation. Data shown is mean±SD of two independent experiments performed in triplicates.
Notch1 binds and regulates T-bet promoter activity, but not to GATA3 upon \textit{in vitro} Th17 differentiation.

To examine the molecular mechanism by which Notch signaling regulates Th17/Th1 differentiation, I studied the human T-bet, ROR-γt and GATA3 promoters and their regulation by Notch1. Firstly I down-regulated Notch1 in human naïve CD4⁺ T cells followed by Th17 differentiation, and quantified transcripts encoding T-bet, ROR-γt and GATA3 by real time PCR (Fig.34, 22). I observed that upon Notch1 knockdown there is a 50% decrease in T-bet transcripts (p≤0.05) and a 40% decrease in ROR-γt transcripts (p≤0.05). Interestingly, there were no changes observed in human GATA3 transcript levels upon Notch1 knockdown. This suggested that Notch1 is regulating T-bet and ROR-γt transcription, but not GATA3 in human \textit{in vitro} Th17 differentiated cells.

Next I studied regulation of human T-bet promoter by Notch1 in 293T cells. 293T cells were co-transfected with a construct encoding human T-bet promoter (2.5kb) inserted upstream of renilla luciferase, and an activated Notch1 expression vector construct. Notch1 induced a 7 fold increase in human T-bet promoter activity (Fig.35). This suggested that Notch1 regulates expression from the human T-bet promoter. Next I assessed regulation of human T-bet, ROR-γt (Chapter 3) and GATA3 promoter by Notch1 \textit{in vivo}. The human T-bet promoter (3Kb upstream of transcription start site) was analyzed for putative CSL binding site using the TFSEARCH software program (cbrc.jp/research/db/
Two putative CSL binding sites were found within this region, CSL1 (-1732 to -1737bp) and CSL2 (-2478 to -2497 bp) (Fig. 36A). Naïve CD4+ T cells were in vitro differentiated towards Th17, and ChIP assays were performed after 24 hrs of differentiation. The ChIP assays showed that Notch1 binds to putative CSL binding site CSL2 within the human T-bet promoter (Fig. 36B), but not to CSL 1. The binding was inhibited by pretreatment with GSI (Fig.36C). Under the same conditions, a ChIP assay was performed on the human GATA3 promoter. The human GATA3 promoter was scanned for putative CSL binding sites using TFSEARCH software and two sites were identified: CSL1 (-138 to -152 bp) and CSL2 (-1741 to -1746bp). When a ChIP assay was performed, Notch1 was not immunoprecipitated with either of these putative CSL binding sites within the human GATA3 promoter (Fig.37). Thus these data support binding of human T bet and ROR-γt promoter activity by Notch 1, consistent with Notch mediated regulation of Th17 and possibly Th17/Th1 double producer population. Moreover, Notch1 do not bind to human GATA3 promoter under in vitro Th17 differentiating conditions.

Thus these results insinuate towards a role for Notch signaling, particularly of Notch1, in Th17/Th1 differentiation by binding ROR-γt and T-bet promoters.
Figure 34: Decrease T-bet but not GATA3 transcript levels following Notch1 knockdown. Naïve CD4+ Tcells were nucleoporated with scrambled or Notch1 siRNA followed by in vitro Th17 differentiation. RNA was isolated and quantitative real time PCR was performed for T bet (A) and GATA3 (B). Data shown is mean±SD of two different experiments performed in triplicates. *p≤0.05.
Figure 35: The human T-bet promoter is regulated by Notch1. 293 T cells were co-transfected with an intracellular activated Notch expression vector construct (Notch1IC) along with a human T-bet promoter construct cloned upstream of the firefly luciferase gene. Luciferase assays were performed and data were normalized to renilla luciferase depicted as relative luciferase units (RLU). Results are shown as mean±SD of three independent experiments performed in triplicates. **p≤0.01
Figure 36: Notch1 binds to human T-bet promoter in \textit{in vitro} Th17 differentiation. (A) Schematic representation of putative CSL binding sites on human T-bet promoter. ChIP assays were performed to determine the recruitment of Notch1 on human T-bet promoter. (C) Data shown represents fold recruitment of Notch1 with respect to isotype control IgG on human T-bet promoter normalized to input DNA. (B) Regular PCR was also performed using specific primers against different putative CSL binding sites in human T-bet promoter to confirm transcript size. Data depicted is representative of two independent experiment performed in triplicates. $^*p \leq 0.05$
Figure 37: Notch1 do not bind to human GATA3 promoter under in vitro Th17 polarization conditions. (A) Schematic representation of putative CSL binding sites on human GATA3 promoter. (C) Data shown represents fold recruitment of Notch1 on human GATA3 promoter with respect to isotype control IgG normalized to input DNA. (B) Regular PCR was also performed (2μl of DNA eluates) using specific primers against different putative CSL binding sites in human T-bet promoter to confirm transcript size. Data is repeated two times in triplicates.
FIGURE 38: GSI treatment reduces EAE-induced inflammation and the development of PLP\textsubscript{139-151}-specific Th1 responses. SJL/J mice given GSI formulated chow and control mice were given regular chow. n=5 mice in each group. Splenocytes of EAE-induced mice were restimulated ex vivo with PLP\textsubscript{139-151} and were then analyzed for IFNγ by ELISA. By Reem Suleiman, Dr Osborne Laboratory
Figure 39: Differential regulation of Fringes during Th1, Th2 and Th17 differentiation. RNA was prepared from naive CD4+ T cells differentiated under Th0, Th1, Th2 and Th17 conditions at indicated time points and quantitative real time PCR for Fringes were performed. Data represented here is relative Fringes transcript levels normalized against 18S.
Discussion:

In this study I have demonstrated that Notch signaling along with TCR activation provides a polarizing signal to naïve CD4$^+$ T cells to adopt a Th17/Th1 phenotype. By using pharmacologic inhibitors of Notch signaling (GSI) as well as specific inhibition of Notch1, I observed that there is a reduction in TNF$\alpha$ and IL-17 cytokine levels, but no change in IL-5 levels in \textit{in vitro} differentiated Th17 cells. Furthermore, down-regulation of Notch1 in naïve CD4$^+$ T cells followed by Th17 differentiation resulted in a decrease in T-bet and ROR-$\gamma$t transcript levels but no change in GATA3 transcripts. ChIP assays performed on \textit{in vitro} differentiated Th17 cells have shown that Notch1 binds to human T-bet and ROR-$\gamma$t promoter but not to the GATA3 promoter despite the presence of consensus binding sites in all promoter sequences assessed, which contributes to our understanding of the molecular mechanism by which Notch1 drives Th17/Th1 differentiation. This observation is also valid \textit{in vivo} as GSI reduces the symptoms of EAE, also down-regulating both IL-17 and IFN$\gamma$ levels (Fig.27.38) (Minter et al., 2005).

After discovery of Th17, several studies suggested a role for Th17 but not Th1 in the pathogenesis of autoimmune diseases including EAE (Cua et al., 2003). It has been argued that these studies were performed using harsh treatments, such as actively immunizing animals with complete Freund’s adjuvant that would affect the overall immune response of the animals. Later
studies were performed by adoptively transferring polarized Th1 or Th17 into lymphopenic mice, showing a pathogenic role for both subsets in the EAE mouse model (O'Connor et al., 2008; Stromnes et al., 2008). Moreover, in a spontaneous model of EAE, there is a presence of both Th1 and Th17 cells in the CNS suggesting role of both subsets in its pathogenesis (Krishnamoorthy et al., 2006; Pollinger et al., 2009). Currently this concept is again modified because of the presence of IL-17^{+}IFN_{\gamma}^{+} double positive populations under in vitro culture conditions as well as in vivo inflammatory conditions, suggesting an intricate relationship between Th17 and Th1 differentiation programs. I have shown that T-bet, a transcription factor which is considered to be Th1 specific, is also upregulated during Th17 differentiation. The presence of T-bet in Th17/Th1 or only Th17 cells has been demonstrated by other investigators also (Yang et al., 2009). This suggests that there is possible an IL-12 independent, but IL-23 dependent pathway for an up-regulation of T bet. This is shown to be true as the absence of IL-23 signaling in Th17 cells prevented T- bet up-regulation (Hirota et al., 2011). In this study I have shown that Notch1 expression is increased when naïve CD4^{+} T cells were stimulated with anti CD3/CD28 signal along with Th17 skewing cytokines. Furthermore I demonstrated that Notch1 binds to the ROR-\gamma t and T-bet promoter under the Th17 differentiating conditions. It seems that IL-23 (present in Th17 skewing cytokine cocktail) upregulates both ROR-\gamma t and T-bet expression in Th17 precursor cells. In this respect, Notch1 (upregulated by the
TCR signal) binds and activates both promoters, resulting a Th17/Th1 mosaic population. The current literature only supports plasticity of Th17 cells and argues against a conversion of Th1 cells to Th17 subtype (Lee et al., 2009). But in my experiments, upon in vitro Th1 differentiation, I could observe IFNγ⁺/IL-17⁺ double positive cells. Further experiments have to be performed to study transformation of Th1 to Th17 subset and vice-versa. It may be possible that IL-12 and IL-23 have convergent signaling pathways due to a shared IL-12 receptor β1 subunit, further contributing to the Th1/Th17 mosaic phenotype.

Th1/Th17 double producing cells are present in several autoimmune diseases in human for example multiple sclerosis (Edwards et al., 2010), rheumatoid arthritis(Nistala et al., 2010) and inflammatory bowel disease (Kleinschek et al., 2009). Since Notch signaling regulates the differentiation of either subset, Notch inhibitors can be a potential therapeutic intervention for these diseases.
CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

With research related to the Th17 subset of CD4+ T cells gaining momentum in the field of immuno-biology and due to its newly discovered role in various autoimmune diseases, HIV infections and cancer, the study of the regulation of this subset is of utmost importance. Notch signaling plays a role in several stages of T cell development. The importance of Notch in T cells can be emphasized by the fact that in absence of Notch signaling, lymphoid progenitors fail to become T cells in thymus and acquire a B cells phenotype by default (Radtke et al., 1999; Tan et al., 2005; Wilson et al., 2001). Moreover, overexpression of Notch signaling in immature thymocytes leads to T cell leukemia (Lin et al., 2006) (Bellavia et al., 2000; Dumortier et al., 2006). In this study, I have demonstrated the role of Notch in dictating peripheral T cells differentiation, particularly towards the Th17 lineage. I have observed that in the absence of Notch signaling, Th17 associated cytokines are significantly reduced even in the presence of a Th17 polarizing environment. This strongly suggests that Notch signaling is one of the important Th17 lineage determining factors. The effects of Notch inhibition on Th17 differentiation are observed only within 24-48hrs, but not at later time points (after 5 days) suggesting that Notch signals are required for the very initial steps.
of Th17 differentiation. It has been shown before that Notch colocalizes with the CD4 coreceptor in TCR activated T cells, suggesting that it acts at the T cell-APC interface to regulate T cell differentiation (Benson et al., 2005). A role for Notch as a regulator of T helper cell differentiation is absolutely fascinating, but remains very controversial. Notch is known to direct activated T cells towards both Th1 (Minter et al., 2005) or Th2 (Amsen et al., 2004) differentiation. This paradoxical role of Notch signaling specified by several laboratories may be due to differences in experimental models, Notch constructs and even gamma secretase inhibitors used. In the current study, I tried to answer this question by using in vitro differentiation assays, polarizing naïve CD4+ T cells towards Th1, Th2 or Th17 subsets. I observed that not only there is differential upregulation of Notch paralogs but also different levels of expression of individual Notch isoforms in different Th subsets. It has been shown previously that Notch3 expression increases when Th cells commit to the Th1 pathway (Maekawa et al., 2003). Consistent with this finding, my results have also shown upregulation of Notch3 in Th1 differentiated cells. On the contrary, Notch1 is upregulated in Th17 differentiated cells. It may be possible that different Notch paralogs have variable downstream target genes. Alternatively strength of Notch1 signaling might correlate with T helper cell differentiation with maximum Notch1 expression favoring Th17 subset differentiation. Another possibility is that a combination of different paralogs and their binding to promoters can result in different outcomes.
In both ROR-γt and IL-17 promoters, I observed adjacent putative CSL binding sites, suggesting cooperative and/or competitive binding of different Notch paralogs to these promoters. Binding of Notch as a dimer (Notch1 and 3) is already shown to regulate the c-myc and pre-TCR promoters (Liu et al., 2010). This is an attractive reasoning, but specific knockdowns of each Notch paralogs and ChIP assays have to be performed to reach a definitive conclusion.

ROR-γt is the most important Th17 lineage determining factor. It is a transcription factor which is necessary and sufficient for Th17 differentiation (Manel et al., 2008). It binds directly to the IL-17 promoter and regulates Th17 differentiation (Jetten et al., 2001). Consistent with previous literature, I have also observed upregulation of ROR-γt expression in Th17 differentiated cells. I found by chromatin immuno-precipitation that Notch1 binds to the ROR-γt promoter in Th17 cells. Interestingly Notch1 also binds to the IL-17 promoter in Th17 differentiated cells, suggesting that Notch1 drives Th17 polarization by binding both the proteins. Further, I observed an increase in T-bet transcript levels in the Th17 differentiated cells. This is very intriguing, as T-bet is a Th1 specific transcription factor (Afkarian et al., 2002). This presence of T-bet transcripts may explain the presence of IFNγ/IL-17 double positive cells in Th17 cells. Since T-bet is a transcription factor downstream of the IL-12R, but my in vitro Th17 differentiation protocol does not involve IL-12, but rather IL-23, the important questions to be asked are what causes IL-12 independent upregulation of T-bet,
and is Notch involved in T-bet promoter regulation. It has been shown that IL-
23R can also activate T-bet as it shares IL-12R-β1 subunit with IL-12R (Hirota et
al., 2011). Surprisingly we observed that Notch1 also binds and regulates the T-
bet promoter in Th17 differentiated cells as shown by ChIP assays and luciferase
assays. Thus we proposed a putative role for Notch in Th17/Th1 cells co-
differentiation. This observation is validated in vivo in our studies as feeding EAE
mice with Notch signaling inhibitor GSI abrogated the disease. Further GSI
treatment downregulates both IFNγ and IL-17 expression in restimulated
splenocytes. Therefore Notch, being a cytokine modulator, is a promising

My study emphasizes the importance of Notch as a modulator of immune
response. It’s ability to bind to the various promoters and recruit histone acetyl
transferases (p300, PCAF (p300/CBP associated factor) and GCN5 (general
control of amino acid synthesis 5)) (Kurooka and Honjo, 2000; Oswald et al.,
2001; Wallberg et al., 2002) may indicate an indispensable role of Notch in
opening up chromatin and transcriptional activation. Future studies on the
transcriptional complexes recruiting Notch to these promoters have to be
performed to explain the pleiotropic effects observed for Notch.

It is clear from several studies that different ligands drive different
outcomes in peripheral T cells, with DLL ligands preferentially driving Th1
(Amsen et al., 2004) and Th17 responses (Mukherjee et al., 2009) and Jagged
ligands (Amsen et al., 2004) driving Th2 responses. Consistent with a recent published report (Mukherjee et al., 2009), my experiments have also shown upregulation of DLL-4 ligand in Th17 differentiation. Notch ligands are expressed by antigen presenting cells, but surprisingly, my experiments have shown them to be present on CD4^+ T cells as well. It has been known that in order to achieve signal directionality, a signal receiving cell often downregulates Notch ligand expression (Bray, 2006). It is thus possible that when CD4^+ T cells are present together with antigen presenting cells, CD4^+ T cells downregulate their ligands. In the absence of antigen presenting cells as represented by my experiments, CD4^+ T cell apparently combine the signal sending and signal receiving functions. Therefore they do not downregulate their ligand expression. Again this explanation warrants further evaluation and experiments have to be performed including specific knockdowns of individual Notch ligands to study their physiological significance. The next question that comes to mind is what regulates differential upregulation of Notch receptors and their interaction with Notch ligands in different T helper subsets? Fringes are N-acetylglucosaminyl-transferase that add N-acetylglucosamine (GlcNAc) sugar to the O-fucose group of the Notch extracellular domain in the Golgi, followed by addition of galactose and sialic acid (Moloney et al., 2000). It has been shown recently that Fringes (Lunatic, Radical and Manic) regulates the level of Notch glycosylation and its subsequent interaction with Delta or Jagged (LaVoie and Selkoe, 2003). In the
presence of Fringe, Notch signals strongly when interacted with Delta, but reduced signaling is observed following interaction with Jagged (Bruckner et al., 2000). My preliminary results using in vitro differentiation (Fig. 39), have suggested differential expression of Fringes in Th1, Th2 and Th17 subsets, suggesting that different pathogenic stimuli and/or cytokine environments can affect expression of Fringes, which can further dictate Notches’ ability to bind and signal through different ligands, and thus different T helper cells differentiation.

Significance: Notch inhibitors, potential therapeutic targets for Multiple Sclerosis. Abnormalities in Notch signaling have been associated with different diseases, for example cancers (T-cell acute lymphoblastic leukemia) (Sharma et al., 2007) and genetic disorders (Alagille syndrome, CADASIL (Cerebral Autosomal Dominant Arteriopathy with Sub-cortical Infarcts and Leukoencephalopathy)) (Joutel et al., 2000; Oda et al., 1997). More recently, Notch overexpression has been observed in patients with rheumatoid arthritis (Jiao et al., 2010), autoimmune thrombocytopenia (Ma et al., 2010) and systemic lupus erythematosus (Sodsai et al., 2008). A primary role for Notch in multiple sclerosis lies in modulating immune responses as Notch affects autoreactive Th1 cell differentiation (Jurynczyk et al., 2005; Minter et al., 2005). My results have shown that Notch plays role in Th17 differentiation as well. Moreover gamma secretase inhibitors significantly reduce both Th1 and Th17 associated cytokines
in EAE model. A secondary contribution for Notch signaling lies in inhibiting oligodendrocyte maturation, preventing myelin repair in multiple sclerosis (Genoud et al., 2002; Stidworthy et al., 2004). Thus inhibiting Notch signaling can be an effective way of treating MS patients, as it will prevent pathogenic cytokine secretion and promote remyelination.

Besides the studies presented here, Notch signaling inhibitors have been used to treat EAE, mouse model of MS. In our studies, pharmacological γ-secretase inhibitors (Ly 411,575) downregulated Th1 and Th17 responses and ameliorated the disease. Injection of specific Notch3 neutralizing antibody also decreased Th1 and Th17 responses in vivo (Jurynczyk et al., 2008b). Moreover, DLL1 neutralizing antibody decreases Th1 responses and EAE symptoms, while Jagged 1 neutralizing antibody was shown to aggravate disease (Elyaman et al., 2007).

Gamma secretase inhibitors are the drugs of choice as they are already in clinical trials for treatment of Alzheimer’s disease and T cell leukemia (Fleisher et al., 2008; Siemers et al., 2005). However potential side effects of this drug should also be taken into consideration, particularly toxicity to GI tract (Wong et al., 2004). Long term use can also lead to problems in lymphoid development and to myeloproliferative diseases (Qyang et al., 2004; Wong et al., 2004) or skin cancers (Nicolas et al., 2003).
Future studies have to be performed aiming to minimize side effects of Notch inhibition. For example, specific Notch inhibitors or GSI has to be synthetically modified to facilitate their homing to particular organs and restrict Notch inhibition to diseased site. In case of Multiple Sclerosis, specific Notch1 siRNA or GSI has to be targeted to blood-brain barrier endothelial cells (either tagging them with homing receptor for specific brain endothelial cells-along with transferrin, such drug is taken up by transferrin receptor. Recently role of nanotechnology in delivering drug in across blood brain barrier is also been investigated (Silva, 2008).
Notch inhibitors, potential novel therapeutic target for Multiple Sclerosis.

![Diagram](image)

**Figure 40: Regulation of Th17 differentiation and Th17 mediated disease by Notch:** Invitro differentiated Th17 cells upregulate Notch1 expression. Notch1 binds to ROR-γt and IL-17 promoter and regulate Th17 differentiation. In addition, Th17 cells also has upregulation of expression of T-bet transcription factor and presence of IFNγ/IL-17 double positive cells. Notch binds to T-bet, but not GATA3 in Th17 cells. Inhibition of Notch signaling by gamma secretase inhibitors ameliorates EAE symptoms consistent with downregulation of IL-17 and IFNγ.
CHAPTER VII
MATERIALS AND METHODS

Cell lines and constructs:

HEK293 T cells and Jurkat T cells were obtained from the American Type Culture Collections and used for luciferase assays (Manassas, VA). The human plasmid Notch\textsuperscript{IC} (1-4) constructs were generated by cloning Notch\textsuperscript{IC} into BamH1 and EcoRI sites of pcDNA3.0 (Invitrogen). Pheonix-Eco cells of retroviral packaging cell lines were transfected for producing retroviral supernatants for Notch1 overexpression experiments. Active Notch1\textsuperscript{IC} was expressed from LZRS retroviral construct.

Media:

293T cells and phoenix packaging cells (Phoenix-Ampho) were cultured in DMEM (Invitrogen). Jurkat T cell were cultured in RPMI (Mediatech, Inc, Manassas, VA) Primary cells were cultured in serum free X-VIVO 20 media (BioWhittaker, Walkersville, MD). Transfections were done in serum free Opti-MEM media (Invitrogen).

Drugs and chemicals:
Gamma secretase inhibitors DAPT (GSI IX, Ly 374973, (3,5-Difluorophenylacetyl)-L-Alanyl-PhenylGlycinebutyl Ester)(Sigma Aldrich, St Louis, MO), Compound E(GSI XXI, (S,S)-2-[2-(3,5-Difluorophenyl)-acetylamino]-N-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)-propionamide)(Alexis Biochemicals, San Diego, CA), Ly 411,575 ((Searfoss et al., 2003) and IL-CHO (GSI XII, Z-ILE-LEU-Aldehyde)(Palaga et al., 2003) were dissolved in DMSO and used in concentration as indicated in figure legends.

Cell purification:

Peripheral Blood Mononuclear cells (PBMC) were prepared from buffy coats obtained from human healthy donors (Lifesource, Glenview, IL) on Ficoll-PAQUE gradient (GE healthcare, Uppsala, Sweden). Naïve CD4+ T cells were purified from PBMC by depletion of Non T helper cells and memory CD4+ T cells using LS columns according to manufacturer’s instructions (Miltenyi Biotech, Sunnyvale, CA). Briefly, human purified CD4+ T cells were incubated with a cocktail of biotinylated CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, anti-TCRγ/δ, anti-HLA-DR, and CD235a (glycophorin A) antibodies. These cells were subsequently magnetically labeled with anti-Biotin microBeads for depletion and passed through magnetic column. Naïve CD4+ T cells (CD4+CD45RA+CD45RO-) were verified to around 93-96% pure before proceeding with polarization experiments.
Cell culture and *in vitro* polarization:

Jurkat T cells were cultivated in 37°C incubator and 5% CO₂ in RPMI 1640 medium (Mediatech, Inc, Manassas, VA) supplemented with 10% FBS (Cellgro, Mediatech, Manassas, VA), 2mM glutamine, 1mM pyruvate, 10mM HEPES and 0.1 mM nonessential amino acids (all supplements are from Lonza, Walkersville, MD). 293 T cells were cultured in DMEM medium (Mediatech, Manassas, VA) supplemented with 10% FBS (Cellgro, Mediatech, Manassas, VA), 2mM glutamine, 1mM pyruvate (Lonza, Walkersville, MD). Human naïve CD4⁺ T cells were *in vitro* polarized towards Th17 subset in X VIVO-20 serum free medium (BioWhittaker, Walkersville, MD) supplemented with 2mM glutamine. For *in vitro* polarization assays naïve CD4⁺ T cells were plated at the density of 2×10⁶ per ml in 24 well plates (1.5ml/well) with beads coated with anti-CD3 and anti-CD28 (Dynabeads, Invitrogen, Norway) and pulsed with 10U/ml recombinant human IL-2 on day 0. All antibodies and recombinant cytokines used in Th subset polarization were purchased from R&D systems, Minneapolis MN. The following antibodies and cytokines were added at the time of plating in 24 well plates: Th1: 1ng/ml IL-12, 10μg/ml anti-IL-4; Th-2: 10 ng/ml IL-4 and 10 ug/ml IFN-γ; Th-17: 10 ng/ml TGF-β1, 10 ng/ml IL-6, 10 ng/ml IL-23, 10 ng/ml IL-1β, 10μg/ml anti-IL-4 (AB-204-NA) and 10μg/ml anti-IFN-gamma (AB-285-NA).

ELISA:
Supernatants were collected and stored at -20 until use. 100 μl supernatant fluids were analyzed in triplicate by standard ELISA (ebioscience) for detection of human IFN-gamma, TNF-alpha, IL-5, IL-17A, IL-17F, IL-21 and IL-22 (representative of each T helper subset). Briefly, these ELISA kits make use of capture antibodies clone NIB42, MAB TRFK 5, 64cap17, SHLR17, H17F10A7, eBio2B2-G20 (2B2-G20), 22URTI respectively and detection antibodies Clone 4S.B3, JES-5A10, 64Dec 17, eBio3A3-N2 (3A3-N2), IL22JOP respectively. The detection antibodies are labeled with biotin and avidin-horse radish peroxidase labeled enzyme was used (eBioscience, San Diego, CA). Tetramethylbenzidine (TMB) substrate (ebioscience) is used for development of the assay. Color development is stopped by addition of stop solution consisting of 0.16M sulphuric acid (ebioscience). Standard solutions are plated to represent a range of 0 to 1000 pg of cytokine to generate a standard curve. Analysis is performed in an ELISA reader ( BioWhittaker, ELX 808) at 450 nm. Cytokine concentration was analyzed based on standard curve of known concentration of standards.

Dual Luciferase assay:

293 T cells were plated in 60mm culture dishes and were co-transfected with 1μg Notch1IC expression vector cloned in pcDNA3 along with 1μg human IL-17 promoter cloned in firefly luciferase pGL3basic (kind gift from Dr Sarah Gaffen) and 0.1μg pRLTK plasmid that contains Renilla luciferase gene to
normalize transfection. Briefly transfections were performed with 12μl FuGene 6 (Roche) and 100μl Opti-MEM media, luciferase assays were performed 48 hours after transfection according to manufacturer’s instructions. Harvested cells were washed with 1xPBS and incubated with 500μl 1xPassive Lysis Buffer for 30 minutes. After centrifugation at 4°C at 14000rpm for 5 minutes, 10μl of the supernatant was assayed. Luciferase was measured using Sirius Luminometer (Berthold Detection Systems). Firefly luciferase values were normalized with renilla luciferase values.

Surface and intracellular staining:

For intracellular cytokine staining, cells were incubated for 6hrs with phorbol 12-myristate 13-acetate (PMA)(50ng/ml;Sigma, St Louis MO), ionomycin (500ng/ml;Sigma, St Louis, MO) and Golgi-Stop (BD biosciences, San Jose, CA). Cell staining was performed by incubation for 30 minutes on ice with fluorescently labeled antibodies; Phycoerythrin (PE) labeled anti-human CD4, Fluorescein Isothiocyanate (FITC) labeled anti-human CD45RA (Becton Dickinson, NJ). The cytofix/cytoperm (BD, Bioscience, CA) was used to fix and permeabilize cells according to manufacturer’s instructions. Briefly cells were fixed with 200μl of 2% paraformaldehyde for 15 minutes at room temperature followed by washing with 1xPBS. Then fixed cells were permeabilized with buffer containing 0.1% saponin, 0.1% Na-azide, 4% Heat inactivated FBS in 7.5 ml
1xPBS). For intracellular staining, antibodies used were Allophycocyanin (APC) labeled anti-human IFNγ, PE labeled anti-human IL-17A, FITC labeled anti-human IL-4 (ebioscience, CA). Flowcytometry were performed using FACSCalibur and analysed using FLOWJO software.

Production of retroviral particles and retroviral infection:

Phoenix-Ampho cells (2x10^5/ml) were plated one day before transfection in 100mm culture dishes. Next day, 4μg of DNA construct (N1IC in LZRS retroviral construct or LZRS empty vector) were incubated for 30 minutes with 12μl of FuGene 6 OptoMEM to a final volume of 200μl. This mixture was then added to Phoenix –Ampho cells. After 72 hrs of transfection, Phoenix-Ampho cells were positively selected by adding 1μg/ml puromycin. After selecting them, cells were cultured in puromycin free DMEM. Retroviral particle were obtained after 48 and 72hrs and were filtered through 0.45μm filter (Nalgene). For retroviral infection, naïve CD4^+ T cells were stimulated for 24 hrs with anti-CD3 and anti-CD28 and then retroviral particles were added to these stimulated cells along with 4μg/ml polybrene (Millipore) and spun for 1hr at 300xg.

Semiquantitative RT-PCR and quantitative Real time PCR:

RNA isolation
Total RNA was extracted using RNeasy mini kit (Qiagen), following manufacturer's instruction. Briefly cells were resuspended in 1 ml of RPE buffer (containing guanidium thiocynate) and passed through QIAshredder (Qiagen) to homogenize the cells. Then 70% ethanol is added to the lysate to promote binding of RNA to RNeasy columns. RNA bound to RNeasy column would be washed by ethanol and eluted with RNAase free water. Extracted RNA was quantified by optical density of 260nm.

Semiquantitative RT-PCR

RNA was treated with DNase(Promega) and cDNA was synthesized with reverse transcription (Superscript III, Invitrogen) of 1μg of RNA with random hexamers (Invitrogen). For semi-quantitative PCR, following primers were used:

Hey1 forward: TGGATCACCTGAAAATGCTG
Hey1 reverse: TTGTTGAGATGCGAAACCAG
Hes5 forward: TCAGCCCCAAAGAGAAAAAC
Hes5 reverse: TAGTCCCTGGTGCAGGCTCTT

Quantitative real time PCR:

Real time PCR was performed using the iSYBR Green Supermix (Bio-Rad, CA) and 7300 real time PCR (Applied biosystem, CA). The expression of each gene was normalized to expression of 18S by $2^{\text{ddCT}}$ method (Livak and
Schmittgen, 2001). Error bars were calculated based on triplicate measurements of each gene. PCR primers used are Notch1 Forward: 5’-GTC AAC GCC GTA GAT GAC C- 3’, Reverse: 5’-TTG TTA GCC CCG TTC TTC AG-3’; T-bet Forward: 5’-ACA GCT ATG AGG CTG AGT TTC GA-3’, Reverse: 5’-GGA CGC GGC GCA GTA C-3’, Reverse: 5’-TGC CTT GAC CGT CGA TGT TA-3’; ROR-γt Forward: 5’-TTT TCC GAG GAT GAG ATT GC-3’, Reverse: 5’-CTT TCC ACA TGC TGG CTA CA-3’; 18SrRNA Forward: 5’-GGC GCC CCC TCG ATG CTC TTA G-3’, Reverse: 5’-GCT CGG GCC TGC TTT GAA CAC TCT-3’. Immunoblotting analysis:

Protein lysis and quantification:

The cell pellet was resuspended in 100μl of RIPA lysis buffer (1%NP-40, 10mM Tris (pH7.6), 150mM NaCl, 0.1%SDS, 0.5% deoxycholate and 2mM EDTA) with protease inhibitor cocktail (Pierce). The cells in RIPA buffer were incubated on ice for 15 minutes. Finally cells are centrifuged at 14000rpm at 4°C for 15 minutes. The cell lysate obtained was quantified for protein concentration by Bradford assay. Bovine serum albumin was used as a standard to measure protein concentration.
Western blot:

For immunoblot analysis, 30-50 μg of protein from whole cell lysate is mixed with 6x Laemmli sample buffer (4% SDS, 200mM DTT, 120mM Tris-HCl (pH 6.8), 50% glycerol and 0.02% bromophenol blue) and ran on 6-12% Sodium Dodecyl Sulfate- Polyacrylamide gel electrophoresis (SDS-PAGE) gels. After proteins got resolved in gel, they were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked with 5% nonfat dry milk in PBST followed by probing with primary antibody overnight. Next the membrane was washed with TBST (0.05% Tween 20 in 1xPBS) three times and incubated with fluorescent secondary antibodies Secondary antibodies (goat anti-rabbit IgG-Alexa Fluor 680 (Invitrogen), donkey anti-rabbit IgG-IRDye 800 (Rockland) and goat anti-mouse IgG-AlexaFluor-680 (Molecular Probes, and donkey anti-mouse IgG IRDye 800 (Rockland) for detection using the LI-COR infrared scanning system (LI-COR Biosciences). Primary antibodies used were: rabbit polyclonal anti human Notch1 (C20) (Santa Cruz, CA), rabbit polyclonal anti-activated Notch1 (Rockland, PA), rabbit polyclonal anti-human Notch3 (M134) (Santa Cruz, CA) and rabbit polyclonal anti Notch4 (H225) (Santa Cruz, CA). β-actin (Sigma Aldrich, MO) was used as a loading control.

RNA-mediated interference:
To knock down of Notch1, naïve CD4\(^+\) T cells were purified and were nucleoporated with siRNA specific for human Notch1 or scrambled siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using Amixa Nucleoporator system according to manufacturer’s instructions. Briefly, 5\(-10 \times 10^6\) CD4\(^+\) cells were resuspended with 100 μl of nucleofector solution and transfected with 100nM siRNA using nucleofector program U-014 (Amixa, Lonza, Switzerland). After transfection cells, were incubated for 6h at 37\(^{0}\)C, and then were stimulated with anti- CD3/CD28 coated magnetic beads and were cultured in Th-17 polarizing conditions for additional 48hrs.

MTS assay:

To assess cell viability MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS) and electron coupling reagent, phenazine methosulfate (PMS) were used. 1x10\(^5\) cells/ml treated with GSI or DMSO was plated in 96 well plates in triplicates. 20μl of Cell Titer 96Aqueous one solution reagent was added in each well of 96 well plates containing 100μl of culture media. Plate was incubated for 4 hrs at 37\(^{0}\)C in humidified 5% CO\(_2\). Absorbance was measured at 490nm using 96 well plate reader POLARstar Omega (BMG Labtech, Germany).

Chromatin immunoprecipitation assay (ChIP):
ChIP assay was performed using $1 \times 10^7$ naïve CD4$^+$ T cells stimulated with anti CD3/CD28 coated magnetic beads (concentration of 1 bead/cell) differentiated to Th0 (no cytokines) and Th17 (presence of Th17 skewing cytokines cocktail as described in Invitro polarization section) pretreated with DMSO and GSI (10μM) for 24 hrs at $37^\circ$C and 5%CO$_2$ and analyzed using ChIP assay kit (Upstate Cell Signaling Solutions). Briefly cells were fixed in 1% formaldehyde for 10 minutes, and then 250μM glycine was added to quench unreacted formaldehyde for 5 minutes. Five hundred microliters of cell lysis buffer was added following by 500μl of nuclear lysis buffer. Chromatin-DNA were sonicated using ultrasonic processor (GEX -130 PB, 50% of maximum power) with 5 sec pulse and 1 min standing on ice. Lysate was diluted in ChIP dilution buffer and 5% lysate was saved as input DNA. For immunoprecipitations 5μg of anti-humanNotch1 or anti human IgG was added in 500μl of cell lysate for 2 hrs at $4^\circ$C followed by 60μl of protein A/G agarose suspension (Calbiochem, CA) and again incubated at $4^\circ$C overnight with rotation. DNA–protein complex were eluted in elution buffer, and crosslinks were reversed with at 65$^\circ$C overnight in the presence of 5M NaCl. Released proteins were digested with 10mg/ml proteinase K for 1 hr at 45$^\circ$C, and DNA was recovered using DNA purification columns (Qiagen, Valencia, CA). The following primers were used for quantitative as well as standard PCR. IL-17 primer sets are 17 CSL1 (expected size 170bp) (forward) 5’-TTGACCCATAGCATAGCAG-3’, (reverse) 5’-
TTCAGGGGTGACACCATT-TT-3'; 17CSL2 (expected size 199bp) (forward) 5'-GAAAATCTCGTGCTCTTGAACC-3', (reverse) 5'
TTCCTCACAGATTCTTGGC-3'; 17CSL3 (expected size 147bp) (forward)- 5'
TTCCACTTTCCACTTCCAC-3' (reverse) 5'- TTCCTCCCTGTCCCTGCTCTA-3';
17CSL4 (expected size 136bp) (forward) 5'- CAATTGGGAAAAGCAAGCAT-3',
(reverse) 5'- CCCTACTGCCCTCTCTAC-3'. RORC primer sets are RCBF1
(expected size 129bp) (forward) 5'- ATCTCCAGCTCAGCTTTGA-3' (reverse) 5'
GATGCCCTGTCTTCTTGGAG-3'; RCBF2 (expected size 223bp) (forward) 5'-
AGAGGGACTCCTTGCCTCTC-3'. Antibodies used were rabbit anti human-Notch1, normal rabbit IgG (both were Santa Cruz biotechnology). Conditions for
real time PCR are 50°C 2 min., 95°C 10 min, 95°C 15 sec, 60°C 1min (40
cycles). Conditions for PCR are 95°C 5 min, 95°C 30s, 55°C 1min, 72°C 30s (35
cycles), 72°C 2min.

In vivo GSI treatment:

For in-vitro polarization assays C57BL/6 mice were purchased from
Jackson laboratory. For the EAE experiments, 8-12 weeks old female SJL mice
were purchased from Charles River Laboratory (Wilmington, MA). All mice were
housed in the animal care facility at the University of Massachusetts, Amherst in
accordance with the Institutional Animal Care and Use Committee (IACUC)
guidelines. The GSI administered in vivo was LY-411,575 (LY) formulated for two
doses 5mg/kg and 2.5 mg/kg. Mice were fed 5mg/kg LY chow for four weeks. They were immunized at this point, and then fed 2.5mg/kg LY chow for two weeks before being returned to the 5mg/kg LY chow until the end of the experiment.

EAE Evaluation:

EAE was induced by immunizing mice in the flank with 50μg PLP \textsuperscript{(139-151)} (Invitrogen, Carlsbad, CA) supplemented with 400μg Mycobacterium tuberculosis H37RA (Difco, Detroit, MI). Pertussis toxin (Ptx; Sigma 200ng) was injected interaperitonially on the day of immunization. The progression and severity of disease was monitored and scored from 0-5 as follows: Score 0-no disease; 1-limp tail, 2-hind limb weakness; 3-hind limb paralysis; 4-hind and fore limb paralysis; 5-morbidity and death. Data is reported as the mean daily clinical score (Hofstetter et al., 2007; Jurynczyk et al., 2008a; Serada et al., 2008).

Mice were anesthetized and perfused through the left cardiac ventricle with PBS during the peak of disease (day 15 post immunization). Spinal cords and spleens were removed by dissection. Splenocytes were cultured at 37°C with medium alone or with different concentrations of PLP \textsuperscript{(139-151)} antigen for 5 days. To prepare a single cell suspension, spinal cords were cut into pieces and the tissues were mashed and passed through a 70 μm mesh. Mononuclear cells
were isolated over a Percoll gradient and were then cultured with PLP (139-151) antigen for 5 days. Cells from the spinal cord were stained for CD4+ and CD8+ T cells. IL-17 ELISAs were then performed on supernatants from all re-stimulated cells.
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VITA

Shilpa Keerthivasan was born in New Delhi, India on August 13, 1979 to Narender Singh and Jeewan lata. She received a Bachelor of Science degree with a Major in Human Biology from the All India Institute of Medical Sciences (New Delhi, India) in August 2001. Later she was selected as one of the only three candidates chosen in the all India competitive entrance examination selected for Master of Science in Biochemistry at All India Institute of Medical Sciences, New Delhi, India. As an undergraduate student, Shilpa studied molecular typing of Salmonella Typhi in typhoid patients. As a Masters student she prepared her thesis on “Transcriptional regulation of Cathepsin L promoter by VEGF” in the laboratory of Dr Shyam S Chauhan.

In August 2005, Shilpa joined the Molecular Biology Ph.D. Program at Loyola University Medical Center. She performed research in several areas ranging from apoptosis of keratinocytes by ionizing radiation, Graft vs Host disease and STAT signaling. As a PhD thesis topic, Shilpa studied the role of Notch signaling in T cell differentiation under Drs Lucio Miele and Caroline Le Poole. While at Loyola, Shilpa received best student presentation award (3rd) in St Albert’s Day and best poster award (1st) in Molecular Biology retreat. He got chances to present her work in the form of oral as well as poster presentation in local, regional as well as national conferences.
Shilpa is married to Dr. Ganesan Keerthivasan, MD, post-doctoral fellow in Northwestern University, Chicago. She has one beautiful daughter, Pragnya Keerthivasan. After completing her Ph.D., Shilpa will go onto a post-doctoral position in the laboratory of Dr. Fotini Gounari, Ph.D, at the University of Chicago. Her research interests are T cell differentiation and development.