The Role Of Notch In Th17 Differentiation

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THE ROLE OF NOTCH IN TH17 DIFFERENTATION

A Dissertation Presented

by

REEM SULEIMAN

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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September 2013

Program in Animal Biotechnology and Biomedical sciences
THE ROLE OF NOTCH IN TH17 DIFFERENTATION

A Dissertation Presented

by

REEM SULEIMAN

Approved as to style and content by:

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Department of Veterinary and Animal Sciences
DEDICATION

To my parents; Badreldin Suleiman and Soad Eltigani Elmahi;

Thank you for being such great role models, for teaching me to reach for the stars and helping me realize my dreams, for encouraging me to embrace the world while arming me with a sense of pride, but most of all, thank you for your unconditional love and support. Any success I achieve is because of you and any failure is completely my own.

To my husband Ammar;

Thank you for your constant support, encouragement and for always believing in me

To my children, Mahmoud and Suaad;

Thank you for bringing so much joy into my life; you make my world a brighter place
ACKNOWLEDGEMENTS

I would like to start by thanking my advisor Dr. Barbara A. Osborne. Being Barbara’s student has been both a privilege and a pleasure for she is not just a great scientist; she is also a wonderful supervisor, mentor and a genuinely nice person. I especially want to thank her for her grace, compassion, wisdom and unwavering support during a very difficult time in my personal life. I will be forever in her debt.

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I am indebted to Lisa Minter for all her help and support throughout the years. She has truly been a valuable resource! Thanks to all the past and present members of the “Osguiter labs” (Osborne, Anguita, and Minter). Our quadrant has truly been a stimulating and fun environment to work in. I am very grateful to all the faculty, staff and students at the VASCI Department and would also like to extend my thanks to the MCB community.

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ABSTRACT

THE ROLE OF NOTCH IN TH17 DIFFERENTIATION

SEPTEMBER 2013

REEM SULEIMAN, B.S., UNIVERSITY OF KHARTOUM SUDAN
Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Barbara A. Osborne

Th17 cells are pro-inflammatory cells that are characterized by the production of their signature cytokine, IL-17. Although they are thought to have arisen to protect against extracellular bacteria and fungi they have been shown to mediate autoimmune diseases such as EAE and psoriasis. Notch protein is a cell-surface receptor that has been widely conserved among species. It plays an essential role in determining multiple cell fates. More recently, it has been implicated in regulating peripheral CD4\(^+\) T-cell responses. In these studies, we report that blockade of Notch signaling significantly down-regulates the production of IL-17 and associated cytokines in both mouse and human in-vitro polarized Th17 cells, suggesting an intrinsic requirement for Notch during Th17 differentiation in both species. We also present evidence, using promoter reporter assays, knockdown studies as well as chromatin immunoprecipitation, that IL-17 and ROR\(\gamma\)t are direct transcriptional targets of Notch signaling in Th17 cells, with Notch 1 being the responsible Notch family member important in regulating the differentiation of human Th17 cells. In-vivo
inhibition of Notch signaling reduced IL-17 production and Th17 mediated disease progression in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis. In addition, by using Notch1 and Notch3 knockout mice, we have shown that Notch 3 is the Notch family member that is essential for murine Th17 differentiation. We have also investigated non-canonical Notch signaling in Th17 cells by using CD4⁺ T-cells from CSL/RBP-Jk knockout mice. Based on data obtained, we have concluded that canonical Notch signaling is dispensable in Th17 responses. Thus, this study highlights the importance of different Notch family members in Th17 differentiation and indicates that selective targeted therapy against Notch may be an important tool to treat autoimmune disorders, including multiple sclerosis.
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<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 Cell</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood–Brain Barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</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>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSL</td>
<td>CBF-1, Suppressor of Hairless, Lag-1</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>Acronym</td>
<td>Definition</td>
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<tr>
<td>DLL</td>
<td>Delta Like</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental Autoimmune Encephalomyelitis</td>
</tr>
<tr>
<td>FOX P3</td>
<td>Forkhead Box P3</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal centre</td>
</tr>
<tr>
<td>GSF</td>
<td>Granulocyte Stimulating Factor</td>
</tr>
<tr>
<td>GSI</td>
<td>Gamma Secretase Inhibitor</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILCHO</td>
<td>Z-Ile-Leu-Aldehyde</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>iTreg</td>
<td>Inducible regulatory T cells</td>
</tr>
<tr>
<td>kDa</td>
<td>KiloDalton</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin Oligodendrocyte Glycoprotein</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>NEC</td>
<td>Notch Extracellular</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>Notch IC</td>
<td>Notch Intracellular</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>NTM</td>
<td>Notch transmembrane</td>
</tr>
<tr>
<td>nTreg</td>
<td>Natural Regulatory T cell</td>
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<tr>
<td>OPC</td>
<td>Oligodendrocyte Precursor Cells</td>
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<tr>
<td>PEST</td>
<td>Proline-Glutamate-Serine-Threonine rich</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid Peptide</td>
</tr>
<tr>
<td>Poly IC</td>
<td>Polyinosinic: Polycytidylic acid</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>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>TCR</td>
<td>T cell Receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
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CHAPTER 1

INTRODUCTION

1.1. Overview of CD4+ T cells

CD4+ T cells are a principle component of adaptive immune responses which are vital for the efficient eradication of infectious agents (Harrington et al., 2006). Upon meeting cognate antigen, naive CD4 Th cells bind to complexes of foreign peptide and major histocompatibility complex class II (pMHCII) on antigen-presenting cells (APC) through their T cell antigen receptors (TCRs). Signals through the TCR and APC–derived costimulatory molecules such as CD28 trigger the naive cells to divide and become effector cell lymphoblasts. Depending on the nature of cytokines produced by the innate immune system, these effector cells undergo a differentiation process that involves the expression of specific transcription factors that control the ability to produce a certain cytokine profile. This differentiation also involves the expression of homing receptors that facilitate the migration of effector cells to non-lymphoid sites of inflammation, where these cells produce their cytokines to aid in antigen clearance (Pepper et al., 2011). Mossman and Coffman (1996) showed that Th cells can differentiate into Th1 and Th2 cell. Since then and over the last couple of years, several new classes of Th cells have been discovered. These new lineages include T-reg, Th17, Th22, Th9 and Tfh cells.

1.2. CD4+ T helper cell lineages
The major determinant for Th cell differentiation is the cytokine milieu at the time of antigen encounter, although the nature of cognate antigen and its affinity to the TCR as well as the available costimulants, many of which regulate initial cytokine production, can influence Th cell fate. It has been shown that the strength of TCR signaling regulates initial cytokine production since low concentrations of cognate peptide induce IL-4-independent IL-4 production during the first 24 h after T-cell engagement, whereas stimulation with high concentration of peptide suppresses ‘early’ IL-4 and induces IFN\(\gamma\) (Zhu and Paul, 2010).

Transcription factors are critical for Th cell differentiation and cytokine production. Cell fate determination in each lineage requires at least two types of transcription factors, the master regulators and the signal transducer and activator of transcription (STAT) proteins. STAT proteins are activated by cytokines through post-transcriptional modifications such as phosphorylation. Some STAT proteins are responsible for inducing the expression of master regulators.

Th1 cells are characterized by the production of their signature cytokine, interferon-\(\gamma\) (IFN-\(\gamma\)). T-bet is regarded as the master regulator for Th1 cell differentiation. IFN-\(\gamma\) activates STAT1 which has been shown to induce T-bet expression during Th1 differentiation in vitro. Therefore, the IFN-\(\gamma\)/STAT1/T-bet/IFN-\(\gamma\) pathway serves as a powerful amplification mechanism for in for vitro Th1 differentiation. T-bet potentiates expression of the ifng gene and upregulates the inducible chain of the IL-12 receptor (IL-12R\(\beta\)2) while suppressing Th2–associated factors. Induction of a competent IL-12 receptor on developing Th1 cells licenses IL-12
signaling through Stat 4, which further potentiates IFN-γ and upregulates T-bet expression. STAT4 activation by IL-12 is critical for Th1 responses both in vitro and in vivo (Zhu and Paul 2010; Weaver et al., 2006).

IFN-γ augments the antigen-processing and antigen-presenting ability of APCs, stimulates IgG2a production by B cells, induces the expression of cytokines and chemokines required for the recruitment of myeloid cells to the site of inflammation, and increases the expression of Toll-like receptors, nitric oxide synthase, and phagocyte oxidase by macrophages. Th1 cells possess anti-viral properties and are important in cellular immunity, where they aid in the clearance of intracellular organisms (Lazarevic et al., 2011). Th1 cells have also been implicated in a vast array of autoimmune diseases ranging from multiple sclerosis to type I diabetes.

Th2 effector differentiation depends on the presence of IL-4, STAT6 and is determined by expression of the GATA-3 transcription factor (Veldhoen et al., 2008). Th2 cells produce IL-4, IL-5 and IL-13. Like many T cell subsets, they can also produce IL-10. Th2 cells are mediators of humoral immunity and are important in the defense against parasites. They are also implicated in the pathogenesis of asthma and allergies. Th1 and Th2 cytokines are mutually exclusive and antagonistic to each other. Consequently, IFN-γ suppresses IL-4 production and IL-4 suppresses IFN-γ production.

Th17 cells are defined CD4+ effector T cells positive for the αβ T cell antigen receptor, which have high expression of the transcription factors RORα and RORγt, low expression of the transcription factors T-bet and GATA-3 and high surface expression of the chemokine receptor CCR6. They produce IL-17A-F and IL-22 cytokines, express the IL-23 surface receptor; and can produce the chemokine CCL20 (Flavell et al.,
2010). Signaling through STAT3 has been shown to be essential for Th17 differentiation (Yang et al., 2007). (Langrish et al., 2005; Park et al.; Betteli et al., 2006; Weaver et al., 2006; Harrington et al., 2006; Dong 2008).

While IL-17 and IL-17F induce neutrophilia, participate in immunity against certain bacterial and fungal infections, and are involved in the pathogenesis of multiple autoimmune diseases; IL-25(IL-17E) promotes eosinophilia and appears to play important roles in Th2-mediated host defense against helminthic parasite infection as well as in exacerbating allergic airway diseases.(Reynolds et al., 2010). IL-23 signaling is not required for Th17 commitment and early IL-17 production but instead appears to be important for amplifying and/or stabilizing the Th17 phenotype (Dong 2008, Weaver et al., 2006). Nevertheless, McGeachy et al., 2009 found that IL-23 was necessary for the full differentiation of activated T cells into effector Th17 cells. IL-23 also promotes T cell production of GM-CSF, a cytokine that is essential for the ability of Th17 cells to drive inflammation in the central nervous system (CNS). GM-CSF acts as a proinflammatory cytokine mainly by inducing the activation, maturation and differentiation of macrophages and dendritic cells, which are essential for the initiation and propagation of cell-mediated immune responses (McGeachey et al., 2011).

In mice, Th17 cells arise from naive CD4+ T cells when activated in the presence of transforming growth factor-β (TGF-β) and IL-6. But, a study by Das et al., 2009 shows that TGF-β does not directly promote Th17 cell differentiation but instead acts indirectly by blocking expression of the transcription factors STAT 4 and GATA-3, thus preventing Th1 and Th2 cell differentiation. IFN-γ and IL-4, the signature cytokines of Th1 and Th2 respectively, negatively regulate Th17 cells (Dong 2008).
The primary function of Th17 cells appears to be the clearance of pathogens that are not adequately handled by Th1 or Th2 cells, such as mediating immunity against extra-cellular bacteria and fungi (Dong 2008). IL-17 has diverse biological functions, but the best characterized relate to its proinflammatory effects. Specifically, IL-17 recruits neutrophils via effects on granulopoiesis and CXC chemokine induction, acts on macrophages to promote their recruitment and survival and stimulates the production of proinflammatory cytokines and anti-microbial peptides from a variety of immune and non-immune cells. However, Th17 cells are potent inducers of tissue inflammation and have been associated with the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions, including Crohn’s disease, systemic lupus erythematosus (SLE), rheumatoid arthritis and psoriasis (Kebir et al., 2007; Kuchroo et al., 2009).

The AHR is a ligand-activated transcription factor from the Per-Arnt-Sim (PAS) superfamily of proteins. AHR has been studied as a receptor for environmental contaminants and as a mediator of chemical toxicity (Stevens et al., 2009). Recently, it has been found that AHR is a regulator of Treg and Th17 cell differentiation in mice. AHR activation by its ligand 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin induces functional Treg cells that suppresses experimental autoimmune encephalomyelitis while AHR activation by 6-formylindolo (3, 2-b)carbazole interfered with Treg cell development, boosted Th17 cell differentiation and increased the severity of experimental autoimmune encephalomyelitis in mice. Thus, AHR regulates both Treg and Th17 cell differentiation in a ligand-specific fashion (Quintana et al., 2008).
Human Th17 cells are currently defined as cells that produce IL-17A and F, but not IFN-γ or IL-4. Th17 cells are also capable of producing tumour necrosis factor (TNF)-a, IL-6, IL-22, as well as IL-21 and IL-26. They express CCR4, CCR6 and IL-23R, but not CXCR3. In addition CD161, the human orthologue of NK1•1, is associated with human Th17 cells.

CD4+ regulatory T cells (Treg cells) are part of a regulatory mechanism that seeks to keep in check an overactive or dysregulated helper T cell response that may result in immune-mediated damage to the host. (O’Garra et al., 2011). Naturally occurring Treg cells occur in the thymus, however, in the presence of TGF-B, naive T cells in the periphery can commit to the T-reg lineage, these cells are called iTreg (inducible Treg cells). Treg cell differentiation and function are driven by the transcription factor Foxp3. Mice carrying a mutant Foxp3 show impaired Treg cell activity and succumb to a fatal lymphoproliferative disorder, which can be prevented by the transgenic expression of wild-type Foxp3 + Treg cells.

Treg cell development is closely related to the generation of IL-17-producing T cells (Th17). Large amounts of TGF-β in the absence of inflammatory mediators will result in the development of Foxp3+ Treg cells, whereas lower concentrations of TGF-β in the presence of inflammatory mediators such as IL-6, IL-1 and IL-23 produced in the context of infection mediates the development of Th17 cells (Zhou et al., 2008).

Follicular helper T (Tfh) cells reside in B cell follicles and are essential for the generation of high-affinity isotype switched antibodies and B cell memory (Stockinger et al., 2010). They are also important for the formation and maintenance of germinal
centers (GC). Tfh cells are characterized by the production of IL-21, BCL6 is the unique transcription factor required for their differentiation.

Th9 cells constitute a distinct population of helper–effector T cells that promote tissue inflammation and are derived in culture with a combination of TGF-β and IL-4 (Veldhoen et al., 2008). These cells are related to Th2 cells in that they require STAT6 and GATA-3 for development, but they have lower expression of Th2 cytokines. PU.1 has been identified as the transcription factor that induces the Th9 phenotype by promoting the expression of IL-9 and proallergic chemokines. (Chang et al., 2010). IL-9 is a pleiotropic cytokine with high expression in the lungs of asthmatic patients. In lung physiology, it has been proposed to induce mucous production, goblet cell hyperplasia and other features of airway remodeling. IL-9 is also involved in protective immunity to helminth infections, as indicated by enhanced kinetics of worm expulsion in IL-9 transgenic mice and susceptibility to helminth infection upon IL-9 depletion (Wilhelm et al., 2011).

IL-9-induced cell activation is mediated by a specific IL-9 receptor chain that forms a heterodimeric receptor with the common gamma chain (γc). Activating either STAT5, or STAT1 and STAT3, is required for IL-9-induced proliferation (Li and Rostami 2010).

Th22 cells are characterized by the production of IL-22 and have been found in humans but so far not in mice. Th17 cells are major producers of IL-22 in mice and RORγt, which controls the generation of Th17 cells, also seems critical for IL-22 production (Trifari et al., 2009). IL-22 can act synergistically or additively with IL-17A, IL-17F or tumor necrosis factor (TNF) to promote the expression of many of the genes
that encode molecules involved in host defense in the skin, airway or intestine (Sonnenberg et al., 2011). A substantial proportion of Th22 cells recognize lipid antigens presented by CD1a expressed on Langerhan’s cells, some dermal DCs and macrophages. Tumor necrosis factor and IL-6 favor the development of Th22 cells from naive cells in vitro (O’Garra 2011). It has been found to have critical roles in regulating host defense, tissue homeostasis and inflammation, in particular at barrier surfaces.

1.3. T helper cell plasticity

T-helper cell lineage commitment was originally viewed as a unidirectional process with nonreversible terminal differentiation of cells. With the discovery of new T-helper cell lineages, it has been recognized that the commitment of T-helper cell lineage is more plastic than previously appreciated and several modes of plasticity of T cell subsets have recently been described (Zhou et al., 2009, Murphy et al., 2010).

Th17 and iTreg cells show effector differentiation plasticity. TGF-β induces both Foxp3 and RORγt in naive T cells, but Foxp3 is dominant and antagonizes RORγt function unless IL-6 is present. Thus, an inflammatory environment tilts the balance between iTreg and Th17 differentiation (Yang et al., 2008).

Although Th17 is a distinct lineage, it has been shown that fully committed Th17 cells can revert and adopt a Th1 phenotype in the absence of Th17 polarizing cytokines (Guangpu et al., 2008). Acquisition of IFN-γ-producing potential by Th17 cells, particularly the simultaneous production of IL-17 and IFN-γ, is a common occurrence, especially in vivo. Th17 cells can even extinguish production of their
cytokine signature, becoming selective IFN-γ producers (O’ Shea 2010). TGF-β, a cytokine with a wide range of functions in the immune system, could alter Th2 cells to the extent that they lost their previous characteristics, including expression of GATA-3 and the ‘signature’ cytokines IL-4, IL-5 and IL-13, and ‘switch on’ IL-9 (Veldhoen et al., 2008, O’ Shea 2010).

T-cell plasticity has important implications in the treatment of Th17 mediated diseases, since any manipulation of cytokine concentrations can cause the Th17 lineage to switch production to an alternative cytokine which could potentially mediate different effects than those initially seen.

1.4 Notch

“If one was asked to choose the single, most important genetic variation concerned with the expression of the genome during embryogenesis in Drosophila melanogaster, the answer would have to be the Notch locus.”(Ted Wright, 1970). So begins the first review to emphasize Notch and today, some 42 years later and with the explosion of data regarding Notch biology, one could say with certainty that Notch has not just lived up to that inspiring sentence but with the discovery of the full extent of its effects throughout the animal kingdom and man, has actually surpassed it.

Notch is a transmembrane molecular receptor that is widely conserved among species. It was first discovered in Drosophilla melanogaster, where loss of one of two copies of the gene led to the appearance of Notches at the wing margins, giving rise to
the name “Notch”. Notch signals influence a wide spectrum of cell fate decisions, both
during development and in the adult organism.

Mammals have four Notch receptors (Notch 1, 2, 3 & 4) and five ligands, Delta-like 1, 3 & 4 (DLL1, 3 and 4) and Jagged 1 & 2 (J1 & J2) (Osborne et al. 2006; Bray 2006). Notch proteins are receptors for ligands of the DSL family (Delta-like/Jagged in mammals, Delta/Serrate in Drosophila melanogaster, Lag-2 in Caenorhabditis elegans).

Notch signaling was first implicated in T cell activation when it was discovered that intracellular Notch1 was upregulated in T cells upon TCR stimulation (Palaga et al., 2003; Adler et al., 2003). In the immune system, Notch has been shown to favor the development of the T cell lineage rather than B cell lineage. αβ T cell development requires continuous Notch signaling up to the DN3 stage, where cells have to pass a critical checkpoint known as β-selection (Wolfer et al., 2002). Another well-characterized role for Notch signaling in the lymphoid system involves the specification of marginal zone (MZ) versus follicular B cell fate in the spleen where MZB cell fate specification in the spleen depends upon nonredundant interaction between Notch2 and Dll1, (Pillai and Cariappa, 2009; Radkte et al., 2009). Notch signaling has also been implicated as a key regulator of peripheral T cell activation and effector cell differentiation (Yuan et al., 2010; Radtke et al., 2009).

Drosophila and mammalian Notch receptors are first synthesized in precursor form as 300-350 kD type 1 single-pass transmembrane glycoproteins. During maturation, mammalian Notch precursor polypeptides are proteolytically processed by a furin-like convertase at a site called S1, yielding two non-covalently associated subunits. The resulting two associated subunits, termed extracellular Notch (NEC) and
transmembrane Notch (NTM), constitute the mature heterodimeric form of the protein present at the cell surface (Kovall and Blacklow, 2010). Notch receptors have a modular domain organization. The ectodomains of Notch receptors consist of a series of N-terminal epidermal growth factor (EGF)-like repeats that are responsible for ligand binding. The number of EGF-like repeats varies by species and receptor subtype. O-linked glycosylation of these EGF repeats, including modification by O-fucose, Fringe, and Rumi glycosyltransferases, modulates the activity of Notch receptors in response to different ligand subtypes. The EGF repeats are followed by three LIN-12/Notch repeat (LNR) modules, which are unique to Notch receptors and participate in preventing premature receptor activation. The heterodimerization (HD) domain of Notch 1 is divided by furin cleavage, so that its N-terminal part (HD-N), terminates the NEC subunit, and its C-terminal half (HD-C) constitutes the beginning of the NTM subunit. Following the extracellular HD-C region, NEC has a transmembrane segment and an intracellular region (ICN). The intracellular region is composed of an RBPJk-association module (RAM) domain, seven ankyrin (ANK) repeats flanked by two nuclear localisation signals (NLS), a transactivation domain (TAD) and a PEST domain that harbours degradation signals necessary for the eventual deactivation of the NICD by proteolysis (Schmidt et al., 2010; Kovall and Blacklow, 2010).

Dysregulation of Notch signaling has been implicated in a wide range of human diseases like developmental disorders such as familial aortic valve disease caused by mutations within the Notch1 receptor or adult onset diseases such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), where patients suffer from stroke and dementia due to mutations in the
Notch3 receptor (Kopan and Llagan *et al.*, 2009). Furthermore, the deregulation of Notch has been implicated in neoplastic diseases such as breast cancer, adenocarcinoma, medulloblastoma and glioma (Koch and Radtke, 2010) A truncated, constitutively active form of mammalian Notch1, termed TAN-1, due to a (7; 9) chromosomal translocation has been implicated in human T-ALL (Ellison *et al.*, 1991) where it is seen in more than 50% of all cases (Weng *et al.*, 2004).

### 1.5 Notch Ligands

Canonical Notch ligands are type I transmembrane proteins belonging to two related families, Delta-like (DLL) and Jagged (Delta and Serrate in Drosophila). Both families contain highly conserved N-terminal DSL domains and varying numbers of EGF repeats in their extracellular domains (Yuan *et al.*, 2010). DLL 3 appears unable to activate Notch in vitro or to bind Notch receptors on hematopoietic cells (D’Souza *et al.*, 2010)

Most interestingly, Notch ligands are regulated by intracellular trafficking, as DSL ligands accumulate at the cell surface in the absence of endocytosis and do not further activate Notch signaling. The intracellular domains of DLL 1, DLL 4, Jagged1 and Jagged2 contain multiple ubiquitination sites that are targeted by E3 ligases such as Neuralised (Neur) and Mind bomb (Mib). However, only Mib1 acts in the ligand-presenting cell and is therefore essential for the regulation of Notch signaling.

Expression of Jagged ligands, but not Dll, on the surface of APCs was shown to induce Th2 cell differentiation in vitro (Amsen *et al.*, 2004). Furthermore, Benedito *et
al., showed that Jagged 1 is a potent proangiogenic regulator in mice that antagonizes Dll4-Notch signaling in cells expressing Fringe family glycotransferases. Mukherjee et al., 2009 showed that in the presence of skewing cytokines, DLL4 influences the generation of IL-17-producing T cells. In the absence of Notch signals, IL-17 production was significantly inhibited even under specific skewing conditions. These studies demonstrate that DLL4 up-regulates Rorc expression in T cells.

DSL family members represent the classical Notch ligands. But expanding repertoires of non-canonical ligands have been identified. Unlike the canonical ligands, these ligands lack the DSL domain required to interact with Notch and comprise a group of structurally diverse proteins that include integral and glycosylphosphatidylinositol(GPI)-linked membrane as well as secreted proteins. Membrane-tethered non-canonical ligands include Delta-like 1(Dlk-1) which is one of the first reported non canonical ligands for Notch and is best known for its role in preventing adipogenesis(D’Souza et al., 2010). Dlk-1 is structurally similar to Delta-like proteins even though it lacks a DSL domain. It is cleaved by ADAMs and is negatively regulated by Notch signaling.

1.6 Canonical Notch signaling

Upon binding Delta-like or Jagged ligands, Notch undergoes two proteolytic cleavages. The first cleavage is catalyzed by Adam-family proteases while the second cleavage is mediated by the gamma secretase complex (an enzyme complex that contains presenilin, nicastrin, PEN2 and APH1) leading to the translocation of the
Notch intracellular domain (N-ICD) into the nucleus. Canonically Notch interacts with the transcriptional repressor CSL (CBF-1, Suppressor of Hairless, Lag-1). N-ICD interaction with CSL displaces transcriptional corepressors from CSL and also recruits Mastermind (MAML) protein. The new transcriptional complex of N-ICD-CSL--MAML converts CSL from a repressor to a transcriptional activator leading to the activation of target genes (Osborne et al., 2006; Bray et al., 2006).

1.7 Non-canonical Notch signaling

The term non-canonical Notch signaling was originally used to describe signaling events that are Notch dependent but do not rely on CSL (Minter and Osborne, in press). In some instances it has become apparent that non-canonical Notch signaling may occur in the cytosol giving rise to the name non-canonical, non-nuclear Notch signaling (Minter and Osborne, in press). An example of non-canonical, non-nuclear Notch signaling is shown in data by Sarin et al., 2009 where they describe a signaling cascade activated by Notch, which inhibits apoptosis triggered by neglect in mammalian cells. In this pathway, the Notch intracellular domain (NICD), which is released after interaction with ligand, converges on the kinase mammalian target of rapamycin (mTOR) and the substrate-defining protein rapamycin independent companion of mTOR (Rictor), culminating in the activation of the kinase Akt/PKB. Akt regulates processes related to proliferation, metabolism, growth, and survival. Data from Sarin et al., 2009 identified AktS473 as a key downstream target in the antiapoptotic pathway activated by NICD.
Data from our lab also points to the existence of non-canonical nuclear Notch signaling since Notch has been found to be associated with the NF-κβ proteins, p50 or c-rel (Shin et al., 2006). Recently, ligand-independent Notch signaling has been shown to occur in circulating blood cells in drosophila, where in the absence of Notch ligand provided by neighbouring cells, Notch signaling is activated through stabilization with the transcription factor HIF-1α (Mukherjee et al., 2011). The study shows that HIF-1α and Notch colocalize in endocytic vesicles, where HIF-1α stabilizes Notch and allows a HIF-1α-NICD to transcriptionally activate target genes.

### 1.8 Pharmacological inhibition of Notch signaling

Blocking the Notch pathway can be achieved by using small compounds that target the activity of the gamma-secretase-presenilin complex that releases the intracellular NICD upon Notch receptor activation. Two commonly used GSIs (gamma-secretase inhibitors) are ILCHO and Compound E ((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).

These inhibitors were originally developed to block the proteolysis of the amyloid precursor protein (APP) thus preventing the accumulation of Aβ peptide during progression of Alzheimer’s disease. Unfortunately, GSIs affect a broad spectrum of pathways and are not specific to Notch signaling, thus confounding results and producing unintended consequences. Nevertheless, GSIs are approved in for use in clinic trials and are currently being used in the treatment of Alzheimer and several types

Notch signaling regulates cell renewal and binary fate decisions in the adult intestine. Ablation of Notch signaling in the intestine, using RBPjk conditional knockout mice, GSI, dibenzazepine, or double knockout of Notch1/Notch2 specifically increases the number of goblet cells at the expense of ISCs (intestinal stem cells) and absorptive enterocytes (Riccio et al., 2008). Thus, an unfortunate side effect following GSI treatment is severe gut toxicity. However, a recent report has shown that combination therapy employing GSI and glucocorticoids reduced GSI-associated intestinal toxicity and improved its antileukemic effects (Real et al., 2009).

1.9 Experimental Autoimmune Encephalomyelitis (EAE)

EAE was the first identified and is the best characterized animal model of human autoimmune disease. This model has had a major role in identifying and characterizing aspects of immune surveillance, inflammation and immune-mediated tissue injury, (Baxter 2007). It is characterized histologically by the infiltration of inflammatory cells, axonal damage and demyelination of nerve fibers in the CNS. The symptoms are characterized by an ascending paralysis and neurological signs. It shares clinical and pathological features with Multiple Sclerosis (MS) and is used as a model for the human disease (Mills et al., 2010).

A variety of methods are used to induce EAE including the injection of an encephalitogenic peptide of a myelin protein, or injection of spinal cord homogenate.
Adoptive (or passive transfer) of spleen and lymph node cells from an immunized animal into a naive animal can also induce disease. (Emerson et al., 2009). EAE is induced in SJL mice by injecting PLP 139-151 antigen, this model is characterized by the relapsing remitting form of EAE. The injection of MOG peptide induces EAE in the C57Bl6 mouse model, producing a chronic progressive form of the disease.

EAE is mediated by myelin-specific T cells, which are activated in the periphery and translocate into the CNS following permeabilization of the BBB. Upon entering the CNS, the T cells are reactivated by local and infiltrating activated antigen-presenting cells (APC), which present major histocompatibility complex (MHC) class II-associated peptides, resulting in subsequent inflammatory processes and eventually in demyelination and axonal damage (Fletcher et al., 2010). The importance of CNS homing in the development of EAE was confirmed by the fact that deficiency in C-C type chemokine receptor 6 (CCR6), a chemokine receptor expressed on Th17 cells, conferred resistance to EAE, although CCR6−/− mice still developed peripheral Th17 responses (Reboldi et al., 2009).

Previously, EAE was thought to be a Th1 mediated disease but now it is believed to be mediated by both Th17 and Th1 cells. In fact, a study done by Kebir et al., 2009 showed that a subpopulation of Th17 lymphocytes that coexpressed IFN-γ and IL-17 preferentially crossed the human BBB and that this same subpopulation accumulated in the CNS of mice during the effector phase of EAE. It seems that Th1 and Th17 play slightly different roles in EAE since IL-12-polarized T cells promote expression of monocyte attracting chemokines and macrophage-rich infiltrates into the spinal cord, whereas IL-23-polarized T cells activate neutrophil-attracting chemokines.
and promoted neutrophils, especially in the brain (Kroenke et al., 2008). A direct interaction of Th17 cells with neurons resulting in neuronal dysfunction in both axons and neuronal cell bodies been actually demonstrated by Siffrin et al., 2010. Th9 cells have also been found to induce EAE (Jager et al., 2009).

1.10 Multiple Sclerosis

MS is a chronic, progressive inflammatory disorder of the brain and spinal cord. The inflammatory plaque, whether determined histopathologically or by using magnetic resonance imaging (MRI), is the pathological hallmark of MS. Like EAE, it is thought that the disease is mediated by pathogenic T cell responses against myelin antigens, followed by a broader neurodegenerative process. The key morphological feature of MS is primary demyelination of nerve axons leading to signal conduction block or conduction slowing at the site of demyelination. Neurological symptoms develop when conduction block occurs simultaneously in a significant proportion of fibers within a given pathway (Fletcher et al., 2010). Patients usually present in early adulthood with a relapsing/ remitting form of the disease that over time develops into a chronic progressive state with increasing disability (Brosnan et al., 2009).

Currently available therapies for MS are aimed primarily at reducing the number of relapses and slowing the progression of disability. Options include beta-interferons, glatiramer acetate, mitoxantrone, and natalizumab. IFN-β drugs likely have multiple effects on the immune system. The effects in the periphery include inhibition of antigen presentation, and at the blood-brain barrier there is down-regulation of adhesion
molecules and decreased production of matrix metalloproteinases. This limits the entry of T cells into CNS (Derwenskus et al., 2011). Glatiramer Acetate (GA) (Copaxone Teva Pharmaceutical Industries Ltd) is a random mixture of polypeptides containing alanine, glutamic acid, lysine and tyrosine. GA was found to suppress MBP-induced EAE (Teitelbaum et al., 2004). Its mode of action is unclear but it has been shown to compete with MHC binding of MBP and limit activation of MBP reactive T-cells. GA also leads to a shift in the T-cell population toward a Th2 cytokine response profile (Kim et al., 2004). It has been proposed that these Th2 cells migrate through the BBB into the brain parenchyma, where they are activated. As a result, they produce immunomodulatory cytokines that counteract the proinflammatory Th1 response, an effect called "bystander suppression." GA reactive T-cells have also been shown to secrete brain-derived neurotrophic factor (BDNF), which has anti-inflammatory and neuroprotective functions (Ziemssen et al., 2002).

Natalizumab (Tysabri[R], Biogen-Idec/Elan) is a humanized monoclonal antibody (mAb) that targets alpha4beta1-integrin, acting as a selective adhesion molecule inhibitor. The glycoprotein alpha4beta1 integrin is also known as very late antigen 4 (VLA4). It is expressed on the surface of lymphocytes and monocytes and plays an important role in cell adhesion and trafficking across the blood-brain and other endothelial barriers. It is not clear exactly how natalizumab exerts its clinical effect but is thought to be due to the significantly reduced migration of leukocytes into the CNS parenchyma (Farrell and Giovannoni, 2010). Mitoxantrone (Novantrone[R] Serono) was originally designed as a chemotherapeutic agent and is most commonly used in treating breast cancer. It is an anthracenedione that inhibits topoisomerase-2, preventing
the successful unwinding of DNA. It is currently licensed for use in patients with aggressive relapsing MS who have failed first-line therapy. Mitoxantrone has an immunosuppressive action, inhibiting the proliferation of T-cells, B-cells, and monocytes and reducing secretion of proinflammatory cytokines (Farrell and Giovannon, 2010).

1.11 Notch and Multiple Sclerosis

MS is an inflammatory, demyelinating disease of the CNS that is thought to be mediated by an immune attack directed against oligodendrocytes and myelin. Activation of the Notch pathway has been shown to inhibit oligodendrocyte precursor cells (OPC) differentiation and to hamper their ability to remyelinate damaged fibers. Blocking the Notch pathway activation in oligodendrocytes promotes remyelination, reduces axonal damage and significantly enhances clinical recovery from the disease (Selmaj et al., 2008). In addition, the IL-17/IL-23 axis is clearly an important drug target for the treatment of MS (Mills et al., 2008), and hence any protein that regulates IL-17 becomes of therapeutic interest.

1.12 Rationale

Th1 cells were originally thought to be the main pathogenic T cells in EAE and MS. This conclusion was based partly on the observation that IL-12p40-defective (IL-12p40−/−) mice were resistant to EAE (Langrish et al., 2005; Matusevicus et al., 1999).
Since IL-12 is required for differentiation of Th1 cells, it was concluded that Th1 cells were the essential pathogenic cells. Similarly, adoptive transfer of Th1 cells caused the induction of disease in mice and treatment of MS patients with IFN-γ exacerbated disease.

However, vast amounts of confusing data emerged. IFN-γ−/− or STAT1−/− mice lacking Th1 cells were found to develop more severe EAE. In addition, Langrish et al., 2005 demonstrated that PLP-specific T cells cultured in the presence of IL-23 generated Th17 cell lines induced EAE following passive transfer into naive SJL mice, whereas Th1 cells lines generated by in vitro culture with IL-12 failed to induce EAE. Furthermore administration of neutralizing anti-IL-17 antibody reduced the severity of EAE, while blocking IFN-γ exacerbated disease.

These conflicting data were resolved partly following the discovery of IL-23, which is related structurally to IL-12. IL-23 shares the p40 chain with IL-12, which is associated with either a separate p19 or p35 chain for IL-23 and IL-12, respectively. IL-23p19−/− like the IL-12p40−/−, mice were found to be resistant to EAE, whereas IL-12p35−/− mice were susceptible. Since the IL-23 cytokine is essential for the maintenance and expansion of the Th17 lineage. (Fletcher et al., 2010), it was concluded that Th17 cells played a major role in the pathogenesis of MS and EAE.

Previous work in our lab (Minter et al., 2005) showed that inhibiting Notch signaling relieved symptoms of EAE through the inhibition of the transcription factor T-bet and consequently through the inhibition of IFN. With the advent of the new information concerning Th17 cells, the possibility arose that inhibiting Notch signaling had a negative effect on Th17 cells. My thesis was designed to ask whether Th17 cells
also required Notch signaling and whether blockade of Notch has any effect on Th17 mediated disease.

1.13 AIMS

The aims of my research were as follows:

**Aim 1:** Does Notch play a role in the development / differentiation of Th17 cells in vitro?

1.1 Does Notch signaling regulate Th17 cell differentiation in vitro?

1.2 What is the mechanism by which down regulation of Th17 cytokines occurs?

1.3 To genetically validate results seen by the use of Conditional Notch1 knockout mice

1.4 Does Notch signaling regulate Th17 cell differentiation in knockout mice?

**Aim 2:** Does Notch play a role in the differentiation of Th17 cells in vivo?

2.1 Does Notch inhibition prevent the development of EAE?

2.2 Does Notch inhibition prevent the differentiation of PLP-specific Th17 cells?

2.3 Use of Conditional Notch1 knockout mice in EAE experiments

**Aim 3:** Does Notch 3 play a role in differentiation of Th17 cells in vitro?

3.1 Does Notch 3 signaling regulate Th17 cell differentiation in vitro?

**Aim 4:** To investigate the role of non-canonical Notch signaling in Th17 development
FIGURE 1: CD4⁺ T helper lineages.

Each lineage is induced by specific cytokines that induce specific transcription factors that result in a certain cytokine profile.
Figure 2: Notch Receptors.

The extracellular portions of all four mammalian Notch receptors contain epidermal growth-factor-like repeats, which bind to ligands. These are followed by three LIN repeats. The intracellular portion contains a RAM domain (RBP-J-associated molecule) that binds CSL and 7 Ankyrin repeats that are used for protein binding. At either end of the Ankyrin repeats are nuclear localization signals. The transcriptional activation domain (TAD) activates downstream targets. Notch 3 and 4 do not have a TAD domain. The PEST domain regulates the degradation of Notch proteins.
After engagement with one of its ligands, Notch undergoes cleavage events mediated through ADAM proteases and gamma-secretase, as designated by the lighting symbol. Notch then translocates to the nucleus where it binds to CSL to activate downstream targets.
Figure 4: Summary of possible canonical and non-canonical roles for Notch signaling in T cells.

A: Canonical Notch signaling; NICD – CSL complex binds to DNA. B: Non-canonical nuclear Notch signaling; NICD is associated with NFκB units. C: Non-canonical nonnuclear Notch signaling; NICD is associated with AKT.
Figure 5: Migration and effector function of T cells in the CNS during EAE.

After immunization with myelin antigens, DCs are activated in the lymph nodes and present myelin antigen to naive T cells. The activated myelin-specific T cells enter the bloodstream and traffic to and enter the CNS. Breakdown of the BBB occurs, allowing recruitment of other inflammatory cells into the CNS. T cells expand and release inflammatory mediators which help recruit other immune cells to the site of inflammation. Activation of local microglial cells and infiltrating cells results in production of cytotoxic agents which promote myelin breakdown. Damage to the myelin sheath surrounding axons is followed by axonal damage and neurological impairment. (Fletcher et al., 2010)
CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

C57B6 mice for in-vitro experiments were obtained from Jackson laboratories. SJL/J mice used in EAE experiments were purchased from Charles River Laboratory (Wilmington, MA). Notch1 conditional knock-out mice were obtained from our collaborators in Umass, Amherst. They obtained Notch1 conditional floxed mice by crossing Notch1fl/fl (Notch1tm2Rko/GridJ) to MxCre+/- (B6.Cg-Tg (Mx1-cre) 1Cgn/J) from Jackson Laboratory (Bar Harbor, ME.). Homozygous Notch-1 fl/fl females were mated to Notch1fl/fl x MxCre+/- male mice to maintain breeding pairs.

CSL knock-out mice were similarly obtained by crossing homozygous female CSL fl/fl (kindly provided by V. Shapiro, Mayo Clinic) with MxCre +/- males. Notch3 knock-out spleens were kindly provided Spyros Artavanis-Tsakonas, Harvard University. All mice were housed in the Animal Care Facility at the University in accordance with the Institutional Animal Care and Use Committee guidelines.

2.2 In Vivo Floxing of Notch-1 and CSL mice

Notch1 fl/fl x MxCre +/- mice, Notch1 fl/fl x MxCre +/- mice, CSL fl/fl x MxCre +/- and CSL fl/fl x MxCre +/- mice were given 5 IP injections of Poly(I)-Poly(C), Amersham Biosciences (Piscataway, NJ), at a dose of 12-15 μg per gram of weight every other day.
Notch1 and CSL \(^{fl/fl}\) x MxCre\(^{+/-}\) control mice were given equal amounts of PBS vehicle. Mice were then rested for 3 weeks after the last injection before being used in experiments.

### 2.3 Antibodies

Anti-Notch 3(AF1308) and anti-Notch 1 (AF5267) antibody were obtained from R&D systems. For detection of mouse activated Notch1, anti-Notch 1 (ebioscience, mN1A clone) was used. β-actin antibody (Sigma Aldrich, St Louis) was used as a loading control.

### 2.4 Drugs and chemicals:

γ-secretase inhibitors Compound E (Alexis Biochemicals, San Diego, CA), and ILCHO (Palaga \textit{et al.}, 2003) were resuspended in DMSO and used in concentrations as indicated in figure legends.

### 2.5 Cell culture and in-vitro polarization

For in-vitro polarization assays CD4 T cells were cultured and polarized according to Keervisthan \textit{et al.}, 2011.Briefly cells (2.5- 3 106/ml) were pretreated in vitro at 37°C for 30 min with 10mg/ml anti-Notch3 or with anti-Notch1 antibody, 0.1% DMSO,GSI (25 μM ILCHO or 4 μM Compound E) or JLK6 inhibitor (7-Amino-4-chloro-3-methoxy-1H-2-benzopyran) (Tocris Bioscience) and then plated onto 12- or 6-well plates precoated with 1 mg/ml each anti-CD3 and anti-CD28. To polarize CD4\(^+\) T cells to a Th17 phenotype, 20 ng IL-6 (R&D Systems, Minneapolis,MN), 5 ng TGF-b...
(R&D Systems), and 10 mg both anti–IFN-g (BD Pharmingen) and anti–IL-4 (BD Pharmingen) were used per milliliter of cells. Cells were polarized for 24, 48, or 72 h. The activation supernatants were evaluated for IL-17A (BD Biosciences).

2.6 Intracellular and cell surface staining

Mouse CD4⁺ T cells were polarized toward a Th17 phenotype as described above. After 72 h, the cells were stimulated by adding 80 nM PMA and 2.5mM ionomycin in addition to brefeldin A for 5 h. Cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions. Fluorescent Abs (anti-mouse CD4-FITC, anti-mouse IL-17A-allophycocyanin, and anti-mouse IFN-γ-PE) were obtained from BD Biosciences. Anti-mouse Notch1-PE was obtained from eBioscience. Cells were analyzed on a FACS LSR II (Becton Dickinson).

2.7 Real time PCR

CD4⁺ T cells cultured as above were harvested and total RNA was isolated using the RNAqueous kit (Ambion, Austin, TX). Total RNA samples were subjected to treatment with DNase using the TURBO DNA-free kit (Ambion), cDNA was synthesized, and transcripts were amplified by quantitative real time polymerase (Q-PCR Stratagene MX3000P). Primer sequences for IL-17 were: (Forward): 5’-CTC CAG AAG GCC CTC AGA CTA C-3’, (Reverse): 5’-AGC TTT CCC TCC GCA TTG ACA CAG-3’ (1). RORγt (Forward): 5’-TTT GGA ACT GGC TTT CCA TC-3’, (Reverse):
5′-AAG ATC TGC AGC TTT TCC ACA-3′. The expression of each gene was normalized to the expression of β-actin by the \(2^{\Delta\Delta CT}\) method (2).

### 2.8 In-vivo GSI treatment

For the EAE experiments, 8-12 weeks old female SJL/J 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 then fed 2.5mg/kg LY chow for a week, immunized at that point, and after a total of two weeks on the 2.5mg/kg LY chow, they were returned to the 5mg/kg LY chow until the end of the experiment.

### 2.9 EAE Induction and Evaluation

EAE was induced by immunizing mice in the flank with 50μg PLP (139-151) (Invitrogen, Carlsbad, CA) supplemented with 400μg Mycobacterium tuberculosis H37RA (Difco, Detroit, MI). Pertussis toxin (Ptx; Sigma 200ng) was injected interaperitoneally 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 (3-5).
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\textsubscript{(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\textsubscript{(139-151)} antigen for 5 days. Cells from the spinal cord were stained for CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. IL-17 and IFN\textgamma ELISAs were then performed on supernatants from all re-stimulated cells. Splenocytes were intracellularly stained for IL-17.

2.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 4.0. Unpaired t-test (α = 0.05) were used when comparing two conditions.
CHAPTER 3

NOTCH SIGNALING REGULATES MOUSE AND HUMAN TH17 DIFFERENTIATION


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3.1 Introduction

The regulation of Th17 differentiation from naïve CD4⁺ T cells is an area of active investigation. Th17 cells and the pro-inflammatory cytokines (IL-17A, IL-17F, IL-21 and IL-22) produced by these cells, have been implicated in several autoimmune and inflammatory disorders (Bettelli et al., 2007; Weaver et al., 2007). The importance of this subset in autoimmune diseases was first recognized when mice lacking expression of the p19 subunit of IL-23, a cytokine involved in differentiation and expansion of Th17 cells, failed to develop certain autoimmune disorders (Cua et al., 2003; Langrish et al., 2005). The pathogenic role of IL-17 as well as Th17 cells has now been documented in numerous autoimmune diseases including multiple sclerosis (Matusevicius e et al., 1999), rheumatoid arthritis (Chabaud et al., 1999), psoriasis (Kagami et al., 2009), Crohn’s disease (Sakuraba et al., 2009) and systemic lupus erythematosus (Nalbandian et al., 2009).

Several factors are known to affect Th17 differentiation including antigenic stimuli (Mukherjee et al., 2009), expression of particular transcription factors (Zho et al., 2009) and epigenetic changes in the IL-17 gene locus (Akimzhanov et al., 2007). The cytokine milieu leading to Th17 differentiation is the most carefully studied factor. In mice, it is known that IL-6 along with proinflammatory cytokines TGF-β and IL-21, promotes differentiation of naïve CD4⁺ T cells into the Th17 lineage (Ivanov et al., 2007). (6). Manel et al., 2008 have similarly shown that human Th17 differentiation requires exposure to low doses of TGFβ in concert with IL-1β, IL-6, IL-21 and/or IL-23. In addition to the cytokine environment, transcription factors are important determinants
of Th17 differentiation (Ivanov et al., 2007; Egwuagu et al., 2009; Ziegler et al., 2009). The transcription factor retinoic acid receptor related orphan receptor γt (RORγt), in cooperation with RORA controls Th17 differentiation (Yang et al., 2008). Th17 differentiation also is regulated by histone-3 acetylation and H3Lys-4 methylation in both the IL-17A and the IL-17F promoters in a lineage dependent manner (Akimzhanov et al., 2007). Despite great progress in understanding the molecular mechanism of Th17 differentiation, the contribution of cell surface proteins found on CD4+ T cells is not well understood.

Notch proteins, are type 1 transmembrane proteins known to play a crucial role in cell fate determination in many cell lineages including early T cell development in the thymus (Deftos et al., 2000). Four Notch receptors (Notch1, 2, 3 and 4) are found in mammals. In developing T cells, Notch1 has been reported to regulate αβ versus γδ T cell differentiation (Garbe et al., 2007). T versus B cell fate determination (Radtke et al., 2004) and CD4+ versus CD8+ T lineage decision (Germain et al., 2002). Notch1 is also present on naïve (Amsen et al., 2004) and activated CD4+ T cells (Adler et al., 2003). Additionally, we and others have shown that Notch1 signaling is activated upon crosslinking of the T cell receptor (TCR) (Adler et al., 2003; Palaga et al., 2003).

Canonical Notch signaling is induced when one of the four mammalian Notch receptors (Notch1, 2, 3 or 4) encounter one of the five known ligands (Jagged 1, 2 or 3 or Delta like-1 or 2) on a neighboring cell. This interaction initiates a proteolytic cleavage of the transmembrane Notch peptide near the extracellular surface by an ADAM protease, which, in turn, induces a conformational change that allows access and cleavage of the Notch transmembrane domain by the γ-secretase complex. Cleavage of Notch
receptors by γ-secretase results in the release of an intracellular Notch domain (NICD), which rapidly translocates to the nucleus where it interacts with the DNA binding protein known as CSL (CBF-1, Suppressor of Hairless, Lag-1). In the absence of Notch signaling, CSL is bound to DNA in a complex with several repressor proteins. NICD translocation to the nucleus and binding to CSL results in disruption of the repressor complex followed by recruitment of several co-activator proteins resulting in the initiation of transcription of genes located downstream of Notch/CSL complexes (Osborne et al., 2006; Miele et al., 2006).

Notch is reported to play a critical role in Th1 (Minter et al., 2005; Maekawa et al., 2003) and/or Th2 (Fang et al., 2007; Amsen et al., 2007) mediated immune responses. Data from several laboratories suggest that antigen presenting cells (APCs) expressing Delta like-4 (DLL-4) drive the differentiation of Th1 cells (Skokos et al., 2007; Kassner et al., 2010) while APCs expressing Jagged1 promote differentiation of Th2 cells (Amsen et al., 2004; Liotta et al., 2008).

In this study, we examined the role of Notch signaling in Th17 polarization. We used pharmacologic inhibitors as well as knockdown approaches to establish a role for Notch signaling in Th17 polarization. Promoter analysis and chromatin immunoprecipitation assays demonstrated regulation of both the IL-17 and RORγt promoters by Notch1. Lastly, we present in-vivo data demonstrating that inhibition of Notch signaling ameliorates the severity of EAE, a murine autoimmune disease that displays several characteristics of human multiple sclerosis. These data provide further understanding of the Th17 differentiation pathway and suggest opportunities for exploiting the Notch signaling pathway to treat Th17 mediated autoimmune disorders.
3.2 Materials and Methods

3.2.1. Drugs and chemicals

\(\gamma\)-secretase inhibitors Compound E (Alexis Biochemicals, San Diego, CA), and ILCHO (Palaga et al., 2003) were resuspended in DMSO and used in concentrations as indicated in figure legends.

3.2.2 Antibodies

For detection of human Notch 1, anti-Notch1 (C20) (Santa Cruz) and anti-activated Notch 1 (Rockland, Gilbertsville, PA) antibodies were used. Anti-Notch2 (Abcam, Cambridge MA), anti-Notch3 (M134) (Santa Cruz, CA) and anti-Notch4 (H225) (Santa Cruz, CA) were also used. For detection of mouse activated Notch1, anti-Notch 1 (ebioscience, mN1A clone) was used. \(\beta\)-actin antibody (Sigma Aldrich, St Louis) was used as a loading control.

3.2.3 Cell culture and mouse in-vitro polarization

For mouse in-vitro polarization assays, naïve CD4\(^+\) T cells were isolated from C57BL/6 (Jackson Laboratories) using the IMag magnetic system (BD Pharmingen, San Jose, CA), according to the manufacturer’s protocol. Cells (2.5 x10\(^6%/ml\)) were pretreated in-vitro at 37\(^0\)C for 30 min with 0.1% DMSO or with GSI (25 \(\mu M\) ILCHO or 4 \(\mu M\) Compound E) and then were plated onto 12 or 6 well plates precoated with 1\(\mu g/ml\) each of \(\alpha\)CD3 and \(\alpha\)CD28. To polarize CD4\(^+\) T cells to a Th17 phenotype 20 ng of IL-6 (R&D Systems), 5 ng of TGF-\(\beta\) (R&D Systems) and 10 \(\mu g\) of both anti IFN\(\gamma\) (BD
Pharmingen) and anti-IL-4 (BD Pharmingen) were used per ml of cells (Betteli et al., 2006; Quintant et al., 2008). Cells were polarized for 24, 48 or 72 h. The activation supernatants were evaluated for IL-17A (BD Biosciences), IL-17F (R&D) and IL-21 (R&D) by ELISA. To study the effect of Notch inhibition on fully differentiated Th17 cells, naïve CD4+ T cells were differentiated towards the Th17 lineage for 4 days. These cells were then treated with either DMSO or ILCHO followed by culturing in α-CD3 coated plates. After 24 hours, supernatants were collected and analyzed for IL-17 cytokine by ELISA (BD Biosciences).

3.2.4 Cell culture and human in-vitro polarization

Human in-vitro Th17 polarization was performed using a modified protocol from Manel et al., 2008. Naïve CD4+ T cells were purified from peripheral blood mononuclear cells by negative selection using MACS separation according to the manufacturer’s instructions (Miltenyi Biotech, Sunnyvale, CA) and were cultured in a 37°C at 5% CO$_2$ in serum free X-VIVO 10 media (BioWhittaker, Maryland). Naïve cells (2 x 10$^6$ per ml) were plated in 24 well plates with beads coated with αCD3 and αCD28 (Dynabeads, Invitrogen, Norway) at a concentration of 1 bead per cell. Antibodies and cytokines were added at the time of plating at the following concentrations: 10 U/ml IL-2, 5 ng/ml TGF-β1, 10 ng/ml IL-6, 10 ng/ml IL-23, 10 ng/ml IL-21, 10 ng/ml IL-1β, anti-IL-4 (10μg/ml) and anti IFNγ (10μg/ml). All antibodies and recombinant cytokines used in polarization were purchased from R&D systems, MN. IL-17A, IL-17F, IL-21 and IL-22 protein levels in the activation supernatants were quantified by ELISA (eBioscience, San Diego, CA). To evaluate the effect of Notch inhibition on differentiated Th17 cells, naïve CD4+ T cells were cultured in Th17 polarizing conditions for 4 days followed by
treatment with either DMSO or ILCHO. Supernatants were collected after 24 hours and analyzed for IL-17 and IL-22 cytokines (eBioscience, San Diego, CA)

3.2.5 Cell lines and constructs

HEK 293T cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM medium (Mediatech, Inc, Manassas, VA) supplemented with 10% FBS (Cellgro, Mediatech, Manassa, VA), 2 mM glutamine, and 1 mM pyruvate. (Supplements are from Lonza, Walkersville, MD). The Notch1IC encoding plasmid construct was generated by cloning Notch 1IC cDNA into BamH1 and EcoRI sites of pcDNA3.0 (Hao et al., 2010).

3.2.6 Retroviral expression vector and transduction

The sequence encoding N1IC was subcloned into the retroviral vector LZRS and viral particles were produced as described previously (Curry et al., 2006). For transduction of virus, naïve human CD4+ T cells were isolated and stimulated with αCD3/αCD28-coated beads for 24h and transduced with retroviral supernatant in the presence of 8μg polybrene as described before (Curry et al., 2006). Transduced cells were then differentiated to Th0 or Th17 conditions. The cells were transduced again the following day with retroviral supernatants and cultured for an additional 48h.

3.2.7 Dual Luciferase assay

HEK 293 T cells were plated on 60 mm dishes and co-transfected with Notch1IC (1μg) expression vector constructs cloned into pcDNA 3.0 along with a human IL-17 (-
1125bp) promoter luciferase construct (1μg) kindly provided by Dr Sarah Gaffen (University of Pittsburg) (Liu et al., 2004) and 0.1 μg Renilla luciferase construct as the internal transfection control. Luciferase assays (Dual Luciferase assay system, Promega, Madison, WI) were performed according to the manufacturer’s instructions.

### 3.2.8 Intracellular staining and cell surface staining

Mouse CD4⁺ T cells were polarized towards a Th17 phenotype as described above. After 72 hours, the cells were stimulated by adding 80 nM PMA (phorbol myristate acetate) and 2.5 μM ionomycin for 1h in addition to Brefeldin A for 5 hours. Cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions. Fluorescent antibodies (anti mouse CD4-FITC, anti-mouse IL-17A-APC and anti-mouse IFNγ-PE) were obtained from BD Biosciences. Anti-mouse Notch 1-PE was obtained from e-Bioscience. Cells were analyzed on a FACS LSR II (BD Biosciences).

### 3.2.9 Real time PCR

Human naïve CD4⁺ T cells were polarized to Th17 as described above. RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA). The RNA was then DNAase I-treated (Qiagen, Valencia, CA) and cDNA was synthesized using Superscript III (Invitrogen, Carlsbad, CA). Quantitative real time PCR was then performed using 18S rRNA to normalize following the $2^{-ΔΔCT}$ method (2). The primer sequences used were: Notch1 (Forward): GTC AAC GCC GTA GAT GAC C, (Reverse): TTG TTA GCC CCG TTC TTC AG; RORγt (7) (Forward): TTT TCC GAG GAT GAG ATT GC,
CTT TCC ACA TGC TGG CTA CA; 18SrRNA (Forward): GGC GCC CCC TCG ATG CTC TTA G, (Reverse): GCT CGG GCC TGC TTT GAA CAC TCT.

Mouse CD4\(^+\) T cells cultured as above were harvested and total RNA was isolated using the RNAqueous kit (Ambion, Austin, TX). Total RNA samples were subjected to treatment with DNase using the TURBO DNA-free kit (Ambion), cDNA was synthesized, and transcripts were amplified by quantitative real time polymerase (Q-PCR Stratagene MX3000P). Primer sequences for IL-17 were: (Forward): 5′-CTC CAG AAG GCC CTC AGA CTA C-3′, (Reverse): 5′-AGC TTT CCC TCC GCA TTG ACA CAG-3′ (1). ROR\(\gamma\)t (Forward): 5′-TTT GGA ACT GGC TTT CCA TC-3′, (Reverse): 5′-AAG ATC TGC AGC TTT TCC ACA TC-3′. The expression of each gene was normalized to the expression of β-actin by the \(2^{-\Delta\Delta CT}\) method (Livak et al., 2009).

3.2.10 RNA- interference

To knock down the expression of Notch 1, CD4\(^+\) T cells were purified and nucleoporated with siRNA specific for Notch1 or scrambled siRNA (Santa Cruz Biotechnology, Santa Cruz, CA), using an Amaxa Nucleoporator system. Briefly, 5-10 x 10\(^6\) CD4\(^+\) T cells were resuspended in 100 μl of nucleofector solution and transfected with 100 nM siRNA using the U-014 Amaxa nucleofector program (Lonza, Switzerland). After transfection, the cells were incubated for 6h at 37\(^{\circ}\)C, and stimulated with αCD3/CD28 coated magnetic beads under Th17 polarizing conditions for 48 h.

3.2.11 MTS assay

Cytotoxicity assay was performed using CellTiter 96 AQ\(\text{ueous}\) one solution reagent (Promega) as per the manufacturer’s instructions.
3.2.12 Chromatin immunoprecipitation assay (ChIP)

ChIP assays (Upstate Cell Signaling Solutions) were performed using $1 \times 10^7$ naïve CD4$^+$ T cells stimulated with αCD3/CD28-coated magnetic beads (1 bead/cell) under Th0 (no cytokines) or Th17 conditions and pretreated with DMSO or GSI (ILCH0) for 24 h. The following primers were used for quantitative as well as standard PCR. IL-17 primer sets were: 17 CSL1 (Forward): 5’-TTG ACC CAT ATC GAT GCA GC-3’, (Reverse): 5’-TTC AGG GGT GAC ACC ATT TT-3’; 17CSL2 (Forward): 5’-GAA AAT CTC GTG TCT CTT GAA CC-3’, (Reverse): 5’-TTC CTC ACA GAT TCC TTG GC-3’; 17CSL3 (Forward): 5’-TTC CAC TTT CCA CTT CCC AC-3’, (Reverse): 5’-TTC CTC CCT GTC CTG CTC TA-3’; 17CSL4 (Forward): 5’-CAA TTG GGA AAA GCA AGC AT-3’, (Reverse): 5’-CCC TAC TGC CCC TCC TCT AC-3’. RORγt primer sets were: RCBF1 (Forward): 5’-ATC TCC AGC CTC AGC TTT GA-3’, (Reverse): 5’-GAT GCC CCT GTT TTC TTG AG-3’; RCBF2 (Forward): 5’-AGA GGG ACT CCT TGC CTC TC-3’, (Reverse): 5’-TCA AAG CTG AGG CTG GAG AT-3’. Antibodies used were rabbit anti Notch1 or normal rabbit IgG (Santa Cruz Biotechnology). Conditions for real-time PCR were 50°C 2min, 95°C 10min, 95°C 15sec, 60°C 1min (40 cycles); conditions for semiquantitative PCR were 95°C 5 min, 95°C 30s, 55°C 1min, 72°C 30s (35 cycles), 72°C for 2min.

3.2.13 In-vivo GSI treatment

For the EAE experiments, 8-12 weeks old female SJL/J 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 then fed 2.5mg/kg LY chow for a week, immunized at that point, and after a total of two weeks on the 2.5mg/kg LY chow, they were returned to the 5mg/kg LY chow until the end of the experiment.

3.2.14 EAE Evaluation

EAE was induced by immunizing mice in the flank with 50μg PLP\textsubscript{(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 (Serada et al., 2008; Hostetter et al., 2007; Jurynczyk 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\textsubscript{(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\textsubscript{(139-151)} antigen for 5 days. Cells from the spinal cord were stained for
CD4+ and CD8+ T cells. IL-17 and IFNγ ELISAs were then performed on supernatants from all re-stimulated cells. Splenocytes were intracellularly stained for IL-17.

3.2.15 Statistical analysis:

Statistical analyses were performed using GraphPad Prism version 4.0. Unpaired t-test (α =0.05) were used when comparing two conditions.

3.3 Results

3.3.1 Gamma secretase inhibition (GSI) during murine Th17 polarization results in reduced Th17 associated cytokines production

The effect of GSI on murine production of IL-17A, IL-17F and IL-21 was tested by treating Th17 polarized cells with ILCHO and Compound E, two chemically distinct GSI that block γ-secretase by different mechanisms. ILCHO 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. Naive CD4+ T cells were isolated from spleens of 8-12 weeks old C57/BL6 mice, pre-treated with GSI or DMSO control for 30 min at 37°C and cultured in Th17 polarizing conditions for 24, 48 or 72 hours and IL-17A, IL-17F and IL-21 cytokine levels were assessed. The level of IL-17A produced by Th17 cells treated with GSI was significantly reduced in comparison to DMSO treated Th17 polarized cells (Figure 6A). Similarly, a reduction in IL-17F and IL-21 cytokine levels were observed after GSI treatment as compared to DMSO (Figure 6A). The observed cytokine profiles
demonstrate that GSI reduced Th17 associated cytokines from in-vitro differentiated Th17 cells. Interestingly we also observed that Notch1 is upregulated in Th17 polarized cells as compared to Th0 conditions (Figure6B).

Notch is a primary target of GSI in CD4+ T cells and to ensure that GSI was effective at reducing Notch1 activation, intracellular levels of Notch1 were assessed by immunoblot (Figure 6B) and intracellular staining (Figure6C). These data revealed that Notch1 protein expression was reduced in Th17 polarized murine CD4+ T cells treated with GSI.

To determine the effect of GSI on IL-17A production on a per cell basis, intracellular staining of IL-17A was also performed in Th17 differentiated cells pre-treated with either DMSO or GSI (ILCHO). We observed a reduction in intracellular IL-17 levels in GSI treated Th17 cells as compared to DMSO (Figure6D). Additionally, the effect of Notch inhibition on already differentiated Th17 cells was assessed. Naïve CD4+ T cells were cultured in Th17 polarizing conditions for 4 days followed by treatment with either DMSO or GSI. Interestingly no changes in IL-17 levels were detected (Figure6E).

3.3.2 The inhibition of gamma secretase during human Th17 polarization results in decreased Th17 associated cytokine levels

To determine whether Notch signaling also plays a role in human Th17 differentiation, we treated in-vitro human Th17 polarized cells two different GSI (ILCHO and compound E). Naïve CD4+ T cells (CD4+CD45RA+) were purified from peripheral blood mononuclear cells, pretreated with either GSI or DMSO for 30 min and cultured
in Th17 polarization conditions for 24, 48 and 72 h. IL-17A, IL-17 F and IL-22 secreted by human Th17 cells was significantly reduced in the presence of GSI compared to DMSO (Figure 7A). Surprisingly we did not detect significant levels of IL-21 in human *in-vitro* differentiated Th17 cells (data not shown). Consistent with the murine data, Th17 polarization of human CD4⁺ T cells resulted in increased levels of activated Notch1, compared to those activated under neutral conditions (Figure 7B). An MTS assay was performed to confirm the decrease in IL-17 secretion by GSI was not due to an effect on cell proliferation (data not shown). Next we assessed the effect of Notch inhibition in fully differentiated Th17 cells. Naïve CD4⁺ T cells were differentiated in Th17 conditions for 4 days followed by treatment with either DMSO or GSI. As seen in murine cells no change in IL-17 A and IL-22 cytokines were observed (Figure 7C). Taken together, data in Figures 1 and 2 show that GSI treatment blocks the differentiation of naïve CD4⁺ T cells into Th17 cells. Moreover treatment with GSI affects Th17 differentiation at earlier time points, but not in cells already committed to the Th17 lineage, suggesting a requirement for Notch signaling at early stages of Th17 differentiation.

### 3.3.3 Delivery of Notch1 siRNA to human naïve CD4⁺ T cells leads to decreased IL-17 secretion

GSI blocks targets of γ-secretase including all members of the Notch family of proteins. To determine whether Notch1 is a functional target of GSI during Th17 polarization, expression of Notch1 was reduced by delivery of siRNA to naïve CD4⁺T cells. Naïve CD4⁺ T cells were nucleoporated with Notch1 specific siRNA and 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 (Figure8A). Western blot of Notch 2, 3 and 4 was also performed to confirm the specificity of Notch1 siRNA. Notch1 knockdown significantly inhibited IL-17A and IL-17F production under Th17 polarizing conditions (Figure8B). Surprisingly we did not observe a significant reduction in IL-22 production upon Notch1 knockdown (Figure8B). An MTS assay was performed to check whether the reduction in IL-17 in Notch1 siRNA-treated cells was due to differential cell survival revealed no change between scrambled siRNA and Notch1 siRNA.

The role of Notch1 in 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. An immunoblot for Notch1 confirmed over-expression (Figure8C). Naïve CD4⁺ T cells overexpressing Notch1IC LZRS produced higher levels of IL-17 compared to control cells (Figure 8D). Interestingly Notch1 overexpression also increased IL-17 secretion in cells activated under Th0 conditions.

3.3.4 Notch1 binds to the ROR gamma T promoter

The orphan nuclear receptor RORγt is a key transcription factor that regulates the differentiation of the Th17 effector cell lineage. Thus, we explored whether Notch may regulate its expression. RNA was isolated from mouse CD4⁺ T cells polarized under Th17 conditions. cDNA was then synthesized to perform quantitative RT-PCR. RORγt mRNA expression was reduced by two-fold in Th17 polarized cells treated with GSI compared to DMSO-treated cells (Figure 9A). To determine whether Notch1 influences
human Th17 polarization by regulating RORγt expression, naïve human CD4+ T cells were purified, and nucleoporated with Notch1 specific siRNA, followed by culture under Th17 polarizing conditions. Quantitative RT-PCR of RORγt demonstrated that Notch1 knockdown resulted in decreased levels of RORγt transcripts (Figure 9B). Taken together, these data indicate that Notch1 regulates the expression of RORγt.

We then explored the possibility that Notch1 may directly regulate the human RORγt promoter. Analysis of this promoter revealed two potential CSL sites within the proximal 3kb promoter upstream of the RORγt transcriptional start site (Figure9C). Chromatin immunoprecipitation analysis (ChIP) using an anti-Notch1 antibody was then performed to determine whether Notch1 binds directly to the RORγt promoter. The data presented in Figure 4D indicate that Notch1 binds directly to putative CSL binding sites in the human RORγt promoter (Figure9D). In particular, Notch1 bound at the CSL1 site, which could be inhibited by treatment with GSI.

3.3.5 Notch 1 regulates IL-17 promoter activity

Since Notch has been reported to regulate and bind directly to the IFN-γ and IL-4 promoters, it is possible that Notch may also regulate the IL-17 promoter in addition to the RORγt promoter. Mouse CD4+ T cells were differentiated in-vitro towards the Th17 lineage in the presence of either DMSO or GSI. Transcript levels of IL-17 were reduced by 9 fold in GSI treated Th17 cells as detected by quantitative real time PCR (Figure9A), suggesting Notch may directly regulate IL-17 promoter. Further, co-transfection of 293T cells with a human IL-17 promoter luciferase construct in combination with an activated Notch1 expression vector construct (N1IC) revealed that
Notch1 expression significantly increased IL-17 promoter activity (Figure 10A). This suggests that Notch1 regulates the IL-17 promoter. The human IL-17 promoter (3kb) upstream of the transcription start site (TSS) was therefore analyzed for putative CSL binding sites (Figure 5B). We found four putative CSL sites within this region (Figure 10B). ChIP analysis of cells polarized under Th17 conditions, showed that Notch1 binds to putative CSL binding sites in the human IL-17 promoter, particularly CSL1 and 4 (Figure 10C, 10D), but not CSL 2 and 3 (data not shown). The binding was inhibited by the pretreatment with GSI (Figure 10C). Thus Notch1 directly binds to both RORγt and IL-17 promoters and regulates Th17 differentiation.

3.3.6 GSI ameliorates the severity of EAE–induced inflammation and Th17 differentiation in-vivo

*In-vitro* experiments demonstrate that reducing Notch activation causes a significant decrease in IL-17 levels. To investigate if the *in-vitro* observations seen could be replicated in an *in-vivo* setting, an EAE model was used in this study. SJL/J mice were fed control chow or GSI (LY) chow. LY is an orally active GSI that is chemically similar to compound E. We have previously used LY incorporated into chow in mouse models of immune disorders and determined that doses between 2.5 and 5 mg/kg/day are safe and effective in reducing Notch activity systemically. Higher doses cause the well-known secretory diarrhea due to goblet cell metaplasia of the intestine. We have previously reported that GSI treatment ameliorates EAE progression (8). However, the role of Th17 cells in this model was not understood at the time, and in that report we explored exclusively Th1 responses. To induce EAE, mice were treated with PLP peptide emulsified in CFA and injected with pertussis toxin. The initial signs of EAE
were observed eight days after immunization of the control group and ten days after immunization for the GSI treated group. At the peak of disease the clinical mean score for the control group was 2, while it was 0.8 for the GSI treated group (Figure 11A). Therefore GSI treatment significantly delayed the disease progression as well as reduced the severity of EAE symptoms, as previously shown (Minter et al., 2005).

To determine whether Th17 responses were affected by GSI in-vivo, IL-17 levels in supernatents of peptide-stimulated splenocytes cultured from GSI- or control- treated mice were measured by ELISA. The GSI treated group showed significantly lower IL-17 levels than the control group (Figure 11B). 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 (Figure 11C). We also detected lower levels of IFNγ in peptide-restimulated splenocytes from GSI fed mice as compared to control mice (Figure 11D). To determine whether the effect of GSI mediated inhibition of IL-17 cytokines in-vivo is due to overall decrease in T cells number rather than Th17 cell differentiation, we determined the number of CD4+ and CD8+ T cells in spinal cord infiltrates. We found there were no significant differences in the number of cells infiltrating the spinal cord between the GSI or control chow fed mice (Figure 11E). Additionally, no significant differences in CD4+ and CD8+ cells were observed in the spleens of GSI-fed and control mice (data not shown). Indeed, we have maintained animals on GSI chow for as long as 6 months and not observed differences in CD4+ or CD8+ cell numbers (data not shown). This suggests that the decrease in IL-17 in the group of mice fed with GSI was not due to a difference in infiltrating cell numbers, but is more likely due to the effects of GSI on Th17 differentiation. Additionally, we
performed intracellular staining of IL-17 in CD4$^+$ T cells of splenocytes treated in vivo and observed significant decrease in mean fluorescent intensity of IL-17 in GSI fed mice as compared with control mice (Figure11F). Interestingly, we did not observe a decrease in the percentage of CD4$^+$ T cells producing IL-17, suggesting that inhibiting Notch signaling does not affect the number of CD4$^+$ T cells producing IL-17 but rather their inherent ability to produce Th17 associated cytokines.

3.4 Discussion

In this study, we addressed the role of Notch signaling in the development of a Th17 response in human and mouse CD4$^+$ T cells. We employed several strategies to investigate the function of Notch in driving a Th17 response. Our data demonstrates that treatment with GSI, compounds known to block $\gamma$-secretase function, also decreases Th17 differentiation and Th17 associated cytokines secretion. Additionally, we have shown that specific inhibition of Notch1 expression through the use of Notch1 siRNA abrogates IL-17 A and F production in polarized human Th17 cells. Surprisingly we do not observe a significant decrease in IL-22 cytokine levels upon Notch1 knockdown. Comparing our GSI data and specific Notch1 siRNA data, it may be possible that IL-22 is regulated by other downstream targets of $\gamma$-secretase. Alternatively, IL-22 may be regulated by other Notch family members, particularly Notch 2 as reported before (Alam et al., 2010). We also provide further insights into the role of Notch in Th17 induction by demonstrating that blockade of Notch, either through inhibition of $\gamma$-secretase or through siRNA mediated knockdown, results in reduced expression of
RORγt, the transcription factor known to be required for effective induction of Th17 cells. These data, coupled with experiments showing Notch1 binding to both the RORγt and the IL-17 promoters, suggest that Notch1 directly regulates the development of the Th17 subset of cells, at least in part, through the regulation of these two promoters. The biological consequences of Notch1 effects on Th17 development are highlighted in our in-vivo EAE experiments where GSI-mediated blockade of Notch activation results in reduced clinical disease as well as reduced levels of IL-17 produced by restimulated CD4+ T cells isolated from EAE-induced animals treated with GSI. Taken together, these data provide compelling evidence for a key role for Notch signaling in the development of an effective in-vitro and in-vivo Th17 response. We have also provide evidence that Notch signaling plays a role in early stages of Th17 differentiation as blocking Notch after 4 days of activation has no significant effect on Th17 associated cytokines in both mouse and human cells.

Notch1 has been implicated in the induction of both the Th1 and Th2 subsets of CD4+ T cells (Minter et al., 2005; Fang et al., 2007; Amsen et al., 2007. Amsen et al., 2004 suggested that different Notch ligands expressed on APCs drive differing T cell responses In particular, this group provided evidence that DLL ligands preferentially drive a Th1 cell fate while Jagged ligands drive a Th2 fate. 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 RORc and expansion of Th17 CD4+ T cells. The role of Notch signaling in mutually exclusive Th1, Th2 and Th17 differentiation may be mediated by different Notch ligands. Alternatively it may be due to upregulation of
different Notch family members or by differential expression of the same Notch paralog (with Notch1 being the most likely candidate). Notch signaling has been studied extensively but most experimental systems interrogate the conventional Notch signaling pathway, where activation of Notch leads to the production of intracellular Notch (NICD), which translocates to the nucleus and drives CSL-dependent transcription. More recent data indicate that activation of Notch also influences NF-κB signaling (Shin et al., 2006; Bellavia et al., 2000) suggesting cross talk between these two signaling pathways in T cells. Additionally, evidence from several groups in a variety of vertebrate and invertebrate systems reveal a role for Notch in the cytosol and point toward a non-nuclear role for Notch in activation of cell survival pathways (Perumalsamy et al., 2009; Perumalsamy et al., 2010). Therefore it is possible that different ligands activate different Notch signaling pathways, which, in turn, drive different outcomes that influence T helper differentiation and development. For example, the number of NICD molecules generated after activation and/or the duration of activation may dictate whether the canonical pathway or combinations of nuclear and cytoplasmic pathways are activated. Further experimentation is required to test this hypothesis.

In summary, in this report, we describe a role for Notch signaling in the development of both human and murine Th17 responses. A broad range of diseases require an active Th17 response, from multiple sclerosis to solid tumors. Our data suggest that Notch signaling inhibitors may act in-vivo at least by suppressing the Th17 response and may be useful in a variety of clinical situations where Th17 responses are required for disease pathogenesis.
FIGURE 6. GSIs significantly downregulate Th17-associated cytokine levels in murine Th17 in vitro polarization assays.

A, ELISA for IL-17A, IL-17F, and IL-21 in supernatants of activated CD4$^+$ T cells from C57BL/6 mice. Cells were pretreated in vitro with GSI (25 mM IL-CHO and 4 mM compound E) or with 0.1% DMSO (as a vehicle control) before 24, 48, and 72 h culture in Th17 polarizing conditions. Cells were then lifted, recouned, and cultured overnight. B, Notch1 expression in cells pretreated with or without ILCHO was evaluated by immunoblotting using Abs that recognized the cleaved active Notch1 Ab specific for actin was used to control for loading. C, Evaluation of Notch1 expression in cells pretreated with or without IL-CHO by flowcymetry using Abs specific for CD4+ cells and Notch1IC. D, Intracellular staining of IL-17 and IFN-$\gamma$ in Th17-differentiated cells treated with either DMSO or GSI. E, Naive CD4$^+$ T cells were differentiated towards the Th17 subset for 4 d followed by treatment with either DMSO or GSI. Supernatants were collected after 24 h and IL-17A ELISA was performed. Data shown represent one of at least three independent experiments done in triplicates. *p # 0.05, **p # 0.001, ***p # 0.0001.
FIGURE 7. GSIs significantly reduce Th17 cytokine levels in human in vitro Th17 polarization assays.

A, ELISA of IL-17A, IL-17F, and IL-22 in supernatants of Th17-polarized naive human CD4+ T cells treated with GSIs or DMSO as a vehicle control. Purified human CD4+ T cells were pretreated with GSIs (2 mM IL-CHO and 5 mM compound E) or DMSO as a vehicle control and then cultured in Th0 and Th17 polarizing conditions. Supernatants were collected at 24, 48, and 72 h and were analyzed for IL-17A, IL-17F, and IL-22. B, Whole-cell lysates were prepared from naive CD4+ T cells unstimulated (US) or differentiated under Th0 and Th17 conditions and immunoblotted for active Notch1IC. β-actin was used to confirm equal loading. C, Naive CD4+ T cells were activated in vitro under Th17 polarizing conditions for 4 d, followed by treatment with either DMSO or GSI. Supernatants were collected after 24 h and IL-17A and IL-22 ELISAs were performed. Data shown are representative of three independent experiments done in triplicates. *p # 0.05, **p # 0.001.
FIGURE 8. Notch 1 controls human Th17 polarization.

Purified human naïve CD4+ T cells (1 x 10^7) were nucleoporated with Notch1-specific siRNA or control 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 b-actin (loading control). B, ELISA of IL-17A, IL-17F, and IL-22 were performed on the supernatants of naïve CD4+ T cells nucleoporated with control siRNA and Notch1 siRNA followed by in vitro Th17 polarization. C, Immunoblot of Notch1IC after transduction of naive human CD4+ T cells with Notch1IC LZRS followed by Th17 differentiation. D, ELISA of IL-17 performed after naïve CD4+ T cells transduced with control LZRS and intercellular Notch LZRS followed by Th17 differentiation. The data are representative of three independent experiments done in triplicates. *p # 0.05.
FIGURE 9. Notch1 regulates RORγt promoter activity.

A, In vitro ILCHO treatment downregulates RORγt and IL-17 mRNA expression. Total RNA was isolated from CD4+ T cells pretreated with 25 mM IL-CHO or DMSO as a vehicle control and cultured in Th17 polarizing conditions and analyzed by quantitative real-time PCR. B, Human naive CD4+ T cells (1 x 10^7) were nucleoporated with Notch1-specific siRNA or scrambled siRNA followed by in vitro Th17 polarization. Cells were harvested and RORγt expression was determined by quantitative RT-PCR. Transcript abundance was normalized to 18S rRNA expression. C, Schematic representation of putative CSL binding sites in human RORγt promoter. D, Specific primers were used to amplify putative CSL binding sites on human RORγt promoter. A ChIP assay was performed to determine recruitment of Notch1 on RORγt promoter. Data shown represent fold recruitment of Notch1 on CSL binding sites on human RORγt promoter with respect to control IgG normalized with 1% input DNA. Semiquantitative PCR was also performed using 2 ml DNA eluates using specific primers against CSL sites in RORγt promoter to confirm transcript size. Data represent mean 6 SD of three independent experiments done in triplicates. *p # 0.05, **p # 0.01. US, unstimulated
FIGURE 10. Notch1 regulates human IL-17 promoter activity.

A. HEK 293T cells were cotransfected with intracellular activated Notch expression vector construct (Notch1IC) along with a human IL-17 promoter construct cloned upstream of firefly luciferase gene. A luciferase assay was performed and data were normalized to Renilla luciferase depicted as relative luciferase units (RLU). B, Schematic representation of putative CSL binding sites in human IL-17 promoter. C, A ChIP assay was performed to determine the recruitment of Notch1 on human IL-17 promoter. Data shown represents fold recruitment of Notch1 on human IL-17 promoter with respect to isotype control IgG normalized to input DNA. Semiquantitative PCR was also performed (2 ml DNA eluates) using specific primers against different putative CSL binding sites in human IL-17 promoter to confirm transcript size. Data shown represent the mean 6 SD of three independent experiments done in triplicates.
FIGURE 11. GSI treatment reduces EAE-induced inflammation and the development of PLP139–151-specific Th17 responses.

A, Clinical scores of SJL/J mice given GSI formulated chow, at 2.5 mg/kg alternated with 5 mg/kg for 4 wk. Control mice were given regular chow (n = 5 mice/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 PLP139–151 at increasing concentrations and cultured for 5 d. The restimulation supernatants were then analyzed for IL-17 by ELISA. D, ELISA of IFN-g was performed on PLP139–151-restimulated splenocytes. E, Total spinal cord cells were stained with CD4 and CD8 Abs and analyzed by flow cytometry. F, Intracellular staining of IL-17A in splenocytes of SJL/J mice fed with GSI or control chow. Data shown represent the mean fluorescence intensity of IL-17 in CD4+ T cells.
CHAPTER 4

TH17 SIGNALING REQUIRES NOTCH3 AND IS CSL/RBP-JK INDEPENDENT.

4.1 Introduction

Th17 cells are a distinct lineage of CD4+ helper T cells that are characterized by the production of IL-17, IL-22 and IL-21 as well as the expression of the transcription factors RORγt and RORα. Th17 cells play a role in the protection against extracellular bacteria and fungi. Notch is a type I transmembrane, heterodimeric cell surface receptor that is widely conserved among species. It is responsible for determining numerous cell fates and decisions (Osborne and Minter 2006, Amsen et al., 2009). In the immune system, Notch signaling is best characterized for its role in promoting T lineage commitment and maturation (Yuan et al., 2010). It has also been shown to regulate peripheral T cell responses, such as in Th1, Th17 and Treg cells (Minter et al., 200; Keerthivasan et al., 2011; Samon et al., 2008). However, dysregulated signaling has also been implicated in a number of different human diseases such as autoimmunity, neurodegeneration and cancer (Koch and Radtke, 2010).

There are four members of the Notch family protein, Notch1, Notch 2, Notch 3, and Notch 4. Canonical Notch ligands are type I transmembrane proteins belonging to two related families, Delta-like (Dll) and Jagged (Delta and Serrate in Drosophila). Both families contain highly conserved N-terminal DSL (Delta/Serrate/Lag-2) domains and varying numbers of EGF repeats in their extracellular domains (Yuan et al., 2010). Notch
1 and Notch 2 are robust activators of target genes while Notch3 is a weak activator since it lacks a transactivation domain. Nevertheless, some target genes of N3 include traditional Notch targets such as Hes-1, and Notch 3 is actually considered to be a strong activator of Hey-1. Other Notch 3 targets include Pbx1, a proto-oncogene, recently identified in ovarian cancer that is essential for cell proliferation and tumorigenicity. Pbx1 expression is transcriptionally regulated by Notch3 activation, and Notch3/CSL protein complex directly binds to the Pbx1 promoter segment harboring the CSL binding sequence (Park et al., 2008). Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is an inherited, dominant, late-onset syndrome that has been associated with mutations in Notch 3. Patients with CADASIL develop widespread arteriopathy that manifests itself most severely in brain vessels, eventually causing chronic ischemic degeneration of neurons and glia.

Activation of canonical Notch signaling requires interactions between a DSL ligand expressed on the surface of one cell (signal-sending cell) and a Notch receptor expressed on the surface of an opposing cell (signal-receiving cell). Notch is presented to ligand as a heterodimer produced as a result of processing by a furin-like protease during transit to the plasma membrane. Ligand binding triggers additional proteolytic cleavages of Notch, first by A-Disintegrin-And-Metalloproteases (ADAM) within the juxtamembrane region followed by γ-secretase within the transmembrane domain resulting in the release of the Notch intracellular domain (NICD) from the membrane. NICD translocates to the nucleus where it directly interacts with the CSL (CBF1, Su(H), LAG1) transcription factor and recruits coactivators including Mastermind to turn on expression of Notch target genes. (D'souza 2008).
The term non-canonical Notch signaling was originally coined to describe signaling events that are Notch dependent but do not rely on CSL and was first observed in Drosophila using a genetic approach to examine the precise requirements of various components of the Notch signaling pathway (Minter and Osborne, unpublished data). In some instances it has become apparent that non-canonical Notch signaling may occur in the cytosol giving rise to the name non-canonical, non-nuclear Notch signaling (Minter and Osborne, unpublished data). Data from our lab also points to the existence of non-canonical nuclear Notch signaling since Notch has been found to be associated with the NF-κB proteins, p50 or c-rel (Shin et al., 2006). We still have much to learn concerning the role of non-canonical Notch signaling in T cell development and function.

EAE is an autoimmune disease in mice, characterized by ascending paralysis and is used as a model to study Multiple Sclerosis (MS) in humans. It was previously thought to be completely mediated by Th1 cells and their signature cytokine, IFN-γ. But with the discovery of Th17 cells, it was found that IL-17 cytokines play an essential role in the pathogenesis of EAE. Consequently, EAE is considered to be a good model to study Th17 responses.

We and others have shown that inhibiting Notch by the use of GSI causes a significant decrease in IL-17 levels produced from CD4⁺ T cells polarized to Th17 in vitro (Keerthivasan et al., 2011, Jurynczyk et al., 2008). Also, the use of GSI significantly improved clinical scores in an EAE model where it was shown that IL-17 levels were reduced compared to controls. GSI are non-selective Notch inhibitors, inhibiting Notch 1 through Notch 4. We have recently shown that Notch 1 is essential for the regulation of
human Th17 cells and so we hypothesized that Notch1 is also responsible for the regulation of Th17 responses in mice. In this study, by using a conditional Notch 1 knock-out mouse we have shown that there are no significant differences in IL-17 levels produced by Notch 1 knock-out and control Th17 polarized CD4⁺ T-cells. Similarly, in an EAE model, clinical scores as well as IL-17 levels were similar between the Notch-1 knock-outs as well as the controls. This led us to the conclusion that Notch 1 is not required for Th17 differentiation in the mouse.

A previous report had showed that selective inhibition of Notch3, but not Notch1 receptor, abrogated Th1 and Th17-type responses of PLP-reactive T cells (Jurynczyk et al., 2008). We therefore hypothesized that Notch3 could be the Notch family member responsible for the regulation of Th17 responses. In this study, we use Notch3 knockout mice to show that Notch 3 is the Notch family member that is essential for Th17 differentiation.

Since mouse models are used to study human diseases, it becomes essential to understand differences in physiology and biology of cells between man and mouse. Therapeutic targets have been identified in mice and used to devise therapies in humans, making it essential to highlight differences between them. We have previously shown that Notch1 is responsible for Th17 differentiation in humans and in this study we provide evidence to show that Notch3 is responsible for regulating Th17 responses in mice.
4.2 Materials and Methods

4.2.1 Animals
Notch1 conditional knock-out mice were obtained from our collaborator, Lisa Minter in Umass, Amherst. They obtained Notch1 conditional floxed mice by crossing Notch1 \textsuperscript{fl/fl} (Notch1tm2Rko/GridJ) to MxCre\textsuperscript{+/−} (B6.Cg-Tg (Mx1-cre) 1Cgn/J) from Jackson Laboratory (Bar Harbor, ME.). Similarly, CSL knock-out mice were obtained by crossing homozygous CSL \textsuperscript{fl/fl} females to MxCre\textsuperscript{+/−} male mice. The homozygous female CSL floxed mice were obtained from our collaborators in Mayo clinic. Notch3 knock-out spleens were kindly provided by Spyrros Artavanis-Tsakonas, Harvard University. Wild-type mice used as controls were C57B6 mice that are bred and kept in our mouse colonies at the University of Massachusetts, Amherst. All mice were housed in the Animal Care Facility at the University in accordance with the Institutional Animal Care and Use Committee guidelines.

4.2.2 In Vivo Floxing of Notch-1 and CSL
Notch1\textsuperscript{fl/fl} x MxCre\textsuperscript{+/−} mice and Notch1\textsuperscript{fl/fl} x MxCre\textsuperscript{−/−} mice were given 5 IP injections of Poly (I)-Poly(C), Amersham Biosciences (Piscataway, NJ), at a dose of 12-15 μg per gram of weight every other day. Notch1\textsuperscript{fl/fl} x MxCre\textsuperscript{+/−} control mice were given equal amounts of PBS vehicle. Mice were then rested for 3 weeks after the last injection before being used in experiments.

4.2.3 Antibodies
Anti-Notch 3 and anti-Notch 1 antibody were obtained from R&D systems.
4.2.4 Cell culture and in-vitro polarization

For in-vitro polarization assays CD4 T cells were cultured and polarized according to Sheeret al., 2011. Briefly, cells (2.5-3 10^6/ml) were pretreated in vitro at 37°C for 30 min with 10mg/ml anti-Notch3 or with anti-Notch1 antibody and then plated onto 12- or 6-well plates precoated with 1 mg/ml each anti-CD3 and anti-CD28. To polarize CD4+ T cells to a Th17 phenotype, 20 ng IL-6 (R&D Systems, Minneapolis,MN), 5 ng TGF-b (R&D Systems), and 10 mg both anti–IFN-g (BD Phamringen) and anti–IL-4 (BD Phamringen) were used per milliliter of cells. Cells were polarized for 24, 48, or 72 h. The activation supernatants were evaluated for IL-17A (BD Biosciences).

4.2.5 Intracellular staining and cell surface staining

Mouse CD4+ T cells were polarized toward a Th17 phenotype as described above. After 72 h, the cells were stimulated by adding 80 nM PMA and 2.5mM ionomycin in addition to brefeldin A for 5 h. Cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions. Fluorescent Abs (anti-mouse CD4-FITC, anti-mouse IL-17A- allophycocyanin, and anti-mouse IFN-g-PE) were obtained from BD Biosciences. Anti-mouse Notch1-PE was obtained from eBioscience. Cells were analyzed on a FACS LSR II (BD Biosciences).

4.2.6 EAE evaluation

EAE was induced by immunizing mice in the flank with 150 mg MOG(Invitrogen, Carlsbad, CA) supplemented with 400 mg Mycobacterium tuberculosis H37RA (Difco,
Detroit, MI). Pertussis toxin (200 ng; Sigma-Aldrich) was injected i.p. on the day of immunization. The progression and severity of disease was monitored and scored from 0 to 5 as follows: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb and forelimb paralysis; 5, morbidity and death. Data are reported as the mean daily clinical score (42–44). 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 MOG Ag for 5 d. To prepare a single-cell suspension, spinal cords were cut into pieces and the tissues were mashed and passed through a 70-mm mesh. Mononuclear cells were isolated over a Percoll gradient and were then cultured with MOG Ag for 5 d. IL-17 ELISAs were then performed on supernatants from all restimulated cells.

4.3 Results

4.3.1 Notch-sparing GSIs added during Th17 polarization, does not result in reduced IL-17 cytokine production

GSIs have been widely used to block the Notch pathway by us (Keerthivasan et al., 2011) and others (Tsoa et al., 2009). One caveat when dealing with GSIs is the fact that they have multiple targets in addition to the enzyme gamma-secretase. GSIs are consistently used in Alzheimer disease studies since they prevent the cleavage of βAPP (β-amyloid precursor protein). The main histological hallmark of Alzheimer’s disease is the senile plaque, a proteinaceous deposit that is probably a result of exacerbated production of Aβ-
related peptides derived from proteolytic cleavage of βAPP by β - and γ-secretases. It has been hypothesized that reducing the extent of secretase activity would diminish the whole Aβ load and hopefully benefit patients with Alzheimer’s disease. But due to GSI inhibitors lack of γ-secretase substrate selectivity; GSI affect the cleavage of transmembrane proteins that include, amongst others, Notch, ErbB-4, nectin 1α, and E-cadherin (Petit et al., 2001, 2003). Of specific importance is Notch, since its inhibition can cause severe side-effects in the recipients. Consequently, Alzheimer disease researchers have developed GSIs that do not inhibit Notch. JLK-6 (7-amino-4-chloro-3-methoxyisocoumarin), is a GSI that does not inhibit Notch as well as α and β secretases, the proteasome, or GSK3 β.

In this study, we used JLK-6, a Notch sparing GSI and compared it to ILCHO, a regular GSI. Naive CD4+ T cells were isolated from spleens of 8- to 12-wk-old C57BL/6 mice, pretreated with GSI, JLK-6 or DMSO control for 30 min at 37°C and cultured in Th17 polarizing conditions for 48, or 72 h. IL-17 cytokine levels were assessed at 48 h. The level of IL-17A produced by Th17 cells treated with JLK-6 was significantly increased in comparison with both ILCHO and control DMSO-treated Th17-polarized cells (Figure 12A). Intracellular staining of Th17 cells polarized for 72 h showed the same trend, with JLK-6 treated cells producing more IL-17 than either ILCHO or DMSO treated cells (Figure 12B,C,D). We had hypothesized that no differences would be seen between Th17 polarized cells treated with JLK-6 or DMSO. Nevertheless, our data supports the notion that the decrease in IL-17 levels in Th17 polarized cells is due to inhibition of the Notch pathway.
4.3.2 Murine Th17 differentiation is Notch-1 independent

Keerthivasan et al., 2001 showed that Notch 1 was essential in regulating human Th17 cells. We hypothesized that Notch1 would also be the Notch family member regulating murine Th17 responses. Notch 1 is essential to the viability of organisms and therefore deleting Notch1 is embryonically lethal. To get around this issue, we used conditional Notch 1 knock-out mice. These conditional Notch1- knockout mice were generated using the Cre/loxP system, in which the gene of interest (Notch 1) is flanked by loxP sites (‘floxed’). The floxed sequence can be removed using Cre recombinase, which recognizes the loxP sites. The cre recombinase is produced upon activation of cre promoters by PolyIC injections. Although, we expected Notch 1 to be knocked out completely, we found that Notch1 was significantly reduced but not absent completely.

Naive CD4⁺ T cells were isolated from spleens of 8- to 12-wk-old of Mxcre positive mice (where Notch 1 is reduced and are hence to be referred to as Notch 1 knockouts) and Mxcre negative mice (where Notch 1 is intact and hence will be referred to as controls) Notch 1 knock-out mice cultured in Th17 polarizing conditions for 24 h. IL-17 cytokine levels were assessed by ELISA. The level of IL-17 produced by Th17 cells from Notch 1 knockout mice was similar to control Th17-polarized cells (Figure 13A). To ensure that Notch1 was reduced in the knock-out mice, intracellular staining was carried out. Notch1 was found to be reduced in the Mxcre positive mice as compared to Mxcre negative mice (Figure 13B). We therefore concluded that Notch 1 is not the Notch family member responsible for murine Th17 differentiation.
To determine whether our in-vitro results would also be apparent in-vivo, EAE was induced in these mice. Since they are on a B6 background, MOG peptide was used to induce EAE. 150ug of MOG peptide emulsified in CFA together with \textit{m.tuberculosis} were subcutaneously injected on the side of each flank of Notch 1 knockout mice and controls. Pertusis toxin was injected inter peritonealy to open up the blood brain barrier. Clinical scores were recorded daily for each group of mice and it was noted that no differences in scores were seen between the Notch 1 knockouts and controls (Figure 14A). Therefore, inhibiting Notch 1 does not reduce EAE inflammation.

To determine whether Th17 responses were affected by reduction of Notch1 in vivo, IL-17 levels in supernatants of peptide-stimulated splenocytes cultured from Notch 1 knockouts or control-treated mice were measured by ELISA. No significant differences were seen in IL-17 levels between the two groups (Figure 14B). These data led us to conclude that Notch 1 does not affect the severity of clinical symptoms in EAE nor does it affect the levels of IL-17, an essential cytokine in the pathogenesis of EAE.

\textbf{4.3.3 Notch 3 is the Notch family member responsible for regulating the differentiation of Th17 cells}

Based on observations made by Jurynczyk \textit{et al.}, 2008, we hypothesized that Notch3 may be responsible for Th17 regulation. We repeated these experiments where naive CD4+ T cells were isolated from spleens of 8- to 12-wk-old C57BL/6 mice, pretreated with anti-Notch 3 antibody, anti-Notch 1 antibody, or DMSO control for 30 min at 37°C and cultured in Th17 polarizing conditions for 72 h. IL-17 cytokine levels were determined by ELISA. The level of IL-17 produced by Th17 cells treated with anti-Notch 3
antibodies was significantly decreased in comparison with control Th17-polarized cells (Figure 15). As we have shown previously, IL-17 levels produced by GSI-treated Th17 cells were significantly decreased in comparison with control Th17-polarized cells while there were no significant differences in IL-17 levels between, anti-Notch 1 treated Th17 cells and controls.

Therefore, Notch 3 appears to be the Notch family member responsible for Th17 differentiation. In order to more fully establish the role of Notch 3, we used spleens from Notch 3 knockout mice. Since Notch3 is not essential for survival, germline Notch 3 knockouts can readily be achieved (Krebs et al., 2003). Naive CD4+ T cells were isolated from spleens of Notch 3 knockout mice and cultured in Th17 polarizing conditions for 72 h. IL-17 cytokine levels were then determined by ELISA. The level of IL-17A produced by Th17 cells from Notch3 knockout cells was significantly decreased in comparison with control Th17-polarized cells (Figure 16A). This led to the conclusion that Notch 3 is responsible for regulating Th17 responses. We also examined IL-2 production in these knockouts (Figure 16B). One of the Notch3 knockout samples produced less IL-2 but we do not believe that this plays a role in the results seen.

**4.3.4 Notch signaling in Th17 cells proceeds through a non-canonical pathway**

Having examined canonical Notch signaling in Th17 cells, we were interested in asking whether non-canonical Notch signaling played a role in Th17 differentiation. To answer that question, we generated CSL knockout mice using the Cre/loxP system. Naive CD4+ T cells were isolated from spleens of CSL knockout mice and cultured in Th17 polarizing conditions for 72 h. IL-17 cytokine levels were then determined by ELISA. The level of
IL-17A produced by Th17 cells from CSL knockout cells was increased in comparison with control Th17-polarized cells (Figure 17). This indicated that Notch regulates Th17 in a CSL independent manner.

4.4 Discussion

The role of Notch in the differentiation of Th17 cells and EAE has been reported by us as well as by others. Inhibiting the Notch pathway by the use of GSI causes a significant improvement in EAE symptoms. This is partly due to decreasing the production of IL-17 and IFN from Th17 and Th1 cells respectively. (Sherr et al., 2011; Minter et al., 2005). γ-secretase is a common target of pharmacological inhibition by molecules such as DAPT and ILCH0. Such γ-secretase inhibitors are powerful blockers of Notch activity, with the important caveat that the γ-secretase complex cleaves around ninety different proteins (Haapasalo and Kovacs et al., 2011). Thus, although γ-secretase inhibitors may be a useful tool to screen for Notch pathway involvement, genetic approaches are necessary for more definitive conclusions regarding such involvement. Indeed, the lack of substrate specificity of γ-secretase inhibitors and the concomitant challenges in interpreting clinical data have hampered progress in the field of Alzheimer’s disease therapeutics. In order to resolve this issue, we have used a GSI, JLK-6, which does not inhibit Notch. Since GSI are considered as potential therapeutics for Alzheimer’s disease, these Notch-sparing GSI were produced to circumvent the side-effects produced by inhibiting Notch.

In this study we pre-incubated naïve CD4+ T cells with JLK-6 for thirty minutes before culturing them in Th17 polarization conditions. At 48h, supernatants were tested for IL-
17 by ELISA and intracellular staining for IL-17 cells was carried out at 72h. In both cases there was a significant increase in IL-17 produced by JLK-6 treated cells compared to ILCHO treated cells and DMSO treated control cells. These results support the idea that the effects seen are due to Notch inhibition by GSI since we usually see a significant reduction in IL-17 levels in GSI treated cells. Nevertheless, these results can be controversial since an effect, even though it was a different effect, was seen when using the Notch-sparing GSI.

In order to validate our results, we used conditional Notch 1 knockout mice, which utilize the Cre/loxP system. We did not see any differences in IL-17 production between Th17 polarized CD4+T cells obtained from Notch 1 knockouts or controls. This was a surprising result since we expected Notch 1 to be the Notch family member responsible for Th17 differentiation. We have previously reported that Notch1 is involved in human Th17 differentiation (Keerthivasan et al., 2011) and had hypothesized that Notch 1 would also be important in murine Th17 differentiation.

To ensure that our in-vitro data was physiologically relevant, we induced EAE in Notch 1 knockout mice and controls. There were no differences in clinical scores or IL-17 levels between Notch1 KO and controls. These results clearly demonstrate that Notch 1 does not play a role in murine Th17 differentiation. Of interest, was the observation that non-polarized naïve CD4+ T cells from these floxed mice secreted high levels of IL-17, at almost twice the amount secreted by the corresponding wild-type non-polarized cells. Similarly, Th17 polarized CD4+ T cells from the floxed mice had more than twice the amount of Th17 than their corresponding Th17 controls. This indicates that the genetic
manipulation of these floxed mice causes an inflammatory response as suggested by the increased levels of IL-17. Consequently, it is imperative to use Mxcre negative mice instead of wild-type mice as controls for experiments using these floxed animals.

It is important to bear in mind that redundancy exists between different family members of the Notch family. A recent study (Tacchini-Cottier et al., 2012) investigated the role of Notch in Th1 cell differentiation following parasite infection. Mice with T cell-specific gene ablation of N1, N2 or both (N1N2ΔCD4Cre) were infected with the protozoan parasite, *Leishmania major*. N1N2ΔCD4Cre mice, on the C57BL/6 *L. major*-resistant genetic background, developed unhealing lesions and uncontrolled parasitemia whereas mice with single inactivation of N1 or N2 in their T cells were resistant to infection and developed a protective Th1 immune response. This showed that CD4⁺ T cell expression of N1 or N2 is redundant in driving Th1 differentiation. It would be interesting to see if deleting both Notch 1 and Notch2 could bring about different results in our Notch 1 knockout studies. We hope to address this question in future studies.

Recent data has suggested that selective inhibition of Notch3, but not Notch1, receptor abrogated proliferation and Th1 and Th17-type responses of PLP-reactive T cells. In this study, we asked whether this observation could be replicated in our in-vitro setting. Naïve CD4⁺ T cells, pretreated with GSI or blocking antibodies to Notch 1 or Notch 3 were cultured in Th17 polarizing conditions. Th17 cells treated with anti-Notch 1 antibody secreted significantly less IL-17 than Th17 cells treated with anti-Notch 1 antibody or Th17 controls. Furthermore, CD4⁺ T cells from splenocytes of Notch 3 knockout mice blocking, cultured similarly in Th17 polarizing conditions produced significantly less IL-
This result clearly demonstrates that Notch 3 is responsible for murine Th17 differentiation.

To date, differences are known to exist between human and mouse Th17 differentiation. A good example is the generation of murine Th17 cells in-vitro, which requires the addition of TGF-β, IL-6, anti-IL-4 and anti-IFN-γ to naive CD4+ T cells. Of note is a recent study that has suggested that TGF-β is dispensable for the differentiation of murine Th17 cells and that its effect is mainly to neutralize the effects of IL-4 and IFN-γ which are known antagonists of IL-17. Nevertheless, TGF-β is still considered to be a necessary cytokine in murine Th17 polarization. Human Th17 cells are generated by the addition of IL-1β, IL-6, anti-IL-4 and anti-IFN-γ to naive CD4+ T cells. TGF-β is absolutely not needed for IL-17 production in human T cells; in fact, TGF-β seems to inhibit IL-17 production from activated human CD4+ T cells (Matsushita et al., 2008). It is therefore not completely surprising that different Notch members are responsible for the regulation of Th17 responses in humans and mice.

Cannonical Notch signaling is signaling that ensues through the Notch core pathway, mainly that of the DSL-Notch- CSL-MAML axis. Any deviation from that core pathway is referred to as non-canonical Notch signaling but there is little data concerning this mode of signaling. To investigate non-canonical Notch signaling, conditional CSL/RBPjK knockout mice using the Cre/loxP system were used. CD4+ T cells isolated from splenocytes of CSL/RBPjK knockout and wild-type control mice were cultured in Th17 polarizing conditions. After 48 hours, IL-17 levels in supernatants were determined by ELISA. There were no differences in IL-17 levels between CSL/RBPjK and wild-type
controls. This indicates that Notch signaling on Th17 cells can proceed through a CSL/RBPjK independent pathway. It could be speculated that crosstalk between other signaling pathways could provide alternative proteins that could function in a CSL-independent manner. Further studies are required to elucidate this signaling pathway.

Our study highlights differences in the Notch members that regulate Th17 responses in humans versus mice. Th17 cytokines are important proinflammatory mediators that are thought to mediate several autoimmune diseases and Notch inhibitors are promising therapeutic targets. Therefore, it is imperative to fully understand the differences between disease pathogenesis in mice versus humans, thus enabling the accurate translation of any therapeutic benefits seen in mouse models. The discovery that Th17 signaling can ensue in a CSL/RBPjK independent manner, reveals the exciting possibility of regulating Th17 responses via potential new targets that are waiting to be discovered.
Figure 12: Notch sparing GSI, unlike regular GSI, increases IL-17 production from Th17 polarized CD4 T cells.

A, ELISA for IL-17 in supernatants of activated CD4+ T cells from C57BL/6 mice. Cells were pretreated in vitro with GSI (25 mM IL-CHO), JLK-6 (10 um) or with 0.1% DMSO (as a vehicle control) before 48 h culture in Th17 polarizing conditions. Intracellular staining of IL-17 in Th17-differentiated cells treated with either B, DMSO; C, JLK-6 or D, GSI.
Figure 13: IL-17 levels were similar in Notch 1 knock-out and control mice.

A, ELISA for IL-17 in supernatants of activated CD4+ T cells from Notch 1 knock-out mice. Cells were cultured for 24 h in Th17 polarizing conditions. B, Intracellular staining of Notch 1 in Th17-differentiated cells showing that Notch1 was reduced in the knock-outs.
Figure 14: EAE-induced inflammation and the development of MOG-specific Th17 responses are not reduced in Notch 1 knock-out mice

A. Clinical scores of Notch 1 knock-out mice, (n = 5 mice/group). Results represent the mean disease score of each group. Splenocytes (B) of EAE-induced mice were restimulated ex vivo with MOG peptide at increasing concentrations and cultured for 5 d. The restimulation supernatants were then analyzed for IL-17 by ELISA.
Figure 15: Notch 3 blocking antibody significantly down regulates IL-17 cytokine levels in Th17 in vitro polarization assays.

A. ELISA for IL-17 were performed in supernatants of activated CD4+ T cells from C57BL/6 mice. Cells were preincubated in vitro with anti-Notch 3(10 uM/ml), anti-Notch 1(10 uM/ml) and GSI (25 mM ILCHO) before 72 h culture in Th17 polarizing conditions.
Figure 16: IL-17 levels are significantly reduced in Notch 3 knock-out mice.

A. ELISAs for IL-17 and B, IL-2 were performed in supernatants of activated CD4+ T cells from Notch 3 knock-out mice. Cells were cultured for 72 h in Th17 polarizing conditions.
Figure 17: CSL knockouts produce significantly more IL-17 than controls.

ELISAs for IL-17 were performed in supernatants of activated CD4+ T cells from CSL knock-out mice. Cells were cultured for 48 h in Th17 polarizing conditions.
Cells need to sense cues from their extracellular environment and integrate this information into appropriate developmental or physiological responses. Although there are a number of mechanisms that relay information from the exterior of the cell to the interior, a relatively small set of highly evolutionarily conserved signaling pathways stand out as playing particularly crucial roles in this transmission of information. In this roster of ‘elite’ intracellular signaling mechanisms lies the Notch signaling pathway (Andersson et al., 2011).

The Notch signaling pathway is one of the most important pathways during developmental processes. In the adult system it is of great significance for different biological processes such as the maintenance of stem cells or in angiogenic processes. Further, dysregulation of Notch has been observed in many types of diseases ranging from developmental disorders, autoimmunity and numerous cancer types (Dickie et al., 2010).

Our understanding of T-cell differentiation has grown enormously in the past two decades. Since the discovery by Mossman and Coffman that CD4+ T cells can be divided into Th1 and Th2 according to the cytokines they secrete, several more classes of T helper cells have been described. These classes include T-regs, Th17, Th9, Th22 and Tfh. The branch of Th cell that is activated dictates the type of immune response that is mounted by the body. So, for example Th1 activates a cell-mediated immune response to
protect against intracellular organisms whilst Th2 activates a humoral-mediated immune response that protects against extracellular organisms. Unfortunately, Th immune responses can sometimes go awry and cause substantial damage to the very host they are supposedly protecting. Thus, Th1 and Th17 responses may cause autoimmunity whilst Th2 response may cause asthma and allergy. Hence, it becomes vital to understand mechanisms by which these Th cells can be harnessed and regulated.

Previous work done by our group has shown that Notch can regulate Th1 and Treg responses as well as affect proliferation of T cells (Minter et al., 2005; Samon et al., 2008). In this current study, we demonstrate that Notch, specifically Notch3, can regulate Th17 responses. Using GSI and Notch 3 knockout mice, we show that inhibiting Notch causes a significant decrease in in-vitro IL-17 production from Th17 cells. Furthermore, inhibiting Notch by the use of GSI significantly alleviates clinical scores in an EAE model and that can be partially attributed to a decrease in Th17 and Th1 responses. These studies and several others have highlighted the importance of Notch-inhibiting compounds as attractive therapeutic agents.

GSIs that block Notch proteolysis have long been used in the laboratory to inhibit Notch and are now being applied in clinical trials on cancer patients. In general, such inhibitors may show a broad range of side effects due to their low specificity (Dickie et al., 2010). γ-secretase inhibitors cause severe intestinal secretory metaplasia as a result of a marked increase in goblet cell differentiation and arrested cell proliferation in the intestinal crypts. (Grosveld 2009). Clearly, selective and specific inhibitors of Notch receptors or ligands are needed.
It is still not understood exactly how Notch signaling can generate appropriate signaling outputs in a variety of cellular contexts. It is also puzzling how one signaling pathway seems to regulate opposing mechanisms. For example, Notch down regulates or inhibits both the Th17 and T-reg responses, even though Th17 cells are proinflammatory whilst T-reggs are antinflammatory. Similarly, Notch signaling has mostly been implicated in oncogenic effects with the exception of epidermal keratinocytes, where Notch1 acts as a tumour suppressor by impacting the stromal microenvironment (Dikic et al., 2010). This example highlights the importance of context in regard to signaling, thus Notch may affect certain mechanisms in some tissues but not in others. Notch1 and 2 contain a transactivation domain, a region which contains phosphorylation sites that may allow other signaling pathways to selectively modulate Notch activity.

A conspicuous feature of the core canonical Notch pathway is the lack of an amplification step during signal transduction. Each activated Notch receptor molecule is consumed during signaling, yielding one NICD, suggesting that Notch signaling exhibits a stoichiometric relationship between signaling input and output and that signaling strength is important for generating the appropriate cellular response. Indeed, the Notch pathway is very sensitive to gene dosage deviations. In human, haploinsufficiency of Notch2 or Jagged 1 (JAG1), which encodes a Notch ligand, is observed in Alagille syndrome (McDaniell et al., 2006), a broad-spectrum syndrome characterized by liver, heart and eye defects as well as vertebral malformations (Alagille et al., 1987; Alagille et al., 1975), and Notch1 haploinsufficiency is also seen in aortic valve disease (Garg et al., 2006). Thus, signal strength, whether it means the number of ligand-receptor interactions on a cell or gene aberrations maybe responsible for the type of response elicited.
The relative strength of receptor-ligand interactions can also be modulated by post-translational modifications of Notch receptors. The extracellular epidermal growth factor (EGF) repeats of Notch receptors can be modified by O-glucose or O-fucose additions, which are then subject to further modification (Stanley and Okajima, 2010). The addition of O-fucose to Notch receptors by protein O-fucosyltransferase 1 (Pofut1), is necessary for the subsequent glycosylation of Notch receptors by Fringe proteins (such as lunatic fringe, manic fringe and radical fringe in mammals) (Okajima et al., 2008). Fringe proteins can then add N-acetylglucosamine (GlcNAc) sugars to the O-fucose moiety. This glycosylation modulates the relative response of Notch receptors to ligands of the Delta versus Jagged/Serrate classes: Fringe potentiates interactions with Dll1 and reduces responsiveness to Jag1 (Hicks et al., 2000; Kato et al., 2010). The Fringe-mediated transcriptional changes reported thus far appear to be quantitative rather than qualitative in nature, i.e. the level of expression of the same set of downstream genes is modulated but the set of downstream genes that is activated or repressed is not. In most cellular contexts, ligands are not uniquely expressed on the signal-sending cell and, vice versa, receptors are not expressed only on the signal-receiving cell. Directionality of Notch signaling stems, at least in part, from the fact that ligands activate receptors on contacting cells (trans-activation), but generally inhibit receptors expressed in the same cell (cis-inhibition) (de Celis and Bray, 1997). Cis-inhibition has at times been reported to lead to a downregulation of Notch receptor at the cell surface, as well as to a cell-autonomous downregulation of Notch target genes.

It also appears that a specific course of T cell differentiation can be dictated depending upon what ligands on DCs (delta-like or jagged) are used to engage Notch receptors on T
cells during activation (Mukherjee et al., 2009). Dll3 might serve exclusively as a cis-inhibiting ligand, as it is incapable of activating receptors in trans (Ladi et al., 2005). In addition, a multitude of non-canonical ligands can activate or inhibit Notch signaling. Therefore, there are multiple mechanisms that fine-tune and regulate Notch signaling, some of which are still undiscovered. This versatility and diversity in signaling helps explain how a single pathway could affect so many biological processes in different tissues.

Non canonical Notch signaling differs from canonical signaling in that it can be initiated by a non-canonical ligand, or may not require cleavage of the Notch receptor. Alternatively, in some forms of non-canonical signaling there is no involvement of CSL, which may reflect interactions with other signaling pathways upstream of the Notch ICD-CSL interaction. We have shown that Notch signaling in Th17 cells can proceed in a CSL independent manner. There is very little data concerning non-canonical signaling and much work remains to be done. Nevertheless, this is an exciting finding that could possibly highlight redundancy between the two pathways and potentially provide extra therapeutic targets for regulating Notch.

Our studies demonstrate an important role for Notch in the regulation of Th17 signaling. This discovery is of two-fold importance. First, Th17 cells and their products are mediators of many autoimmune diseases such as MS and RA. Consequently, it is possible that regulating Th17 responses by targeting Notch may provide a way to control these responses and improve disease outcome. Indeed, we and others have shown this to be the case where inhibiting Notch in an EAE model, significantly improved clinical symptoms and down regulated Th17 responses (Keerthivasan et al., 2011, Jurynczyk et al., 2008).
Secondly, Notch signaling dysregulation has been implicated in a great many adult human diseases ranging from cancer to neurodegeneration. Notch is therefore an important therapeutic target and it is currently being tested in clinical trials. It is therefore essential to understand how Notch manipulation may affect other immune mechanisms and cell types, thus avoiding potentially damaging side-effects.


Skokos, D., and M. C. Nussenzweig. 2007. CD82 DCs induce IL-12-independent Th1 differentiation through Delta 4 Notch-like ligand in response to bacterial LPS. J. Exp. Med. 204: 1525–1531.


