THE ROLE OF NOTCH3 SIGNALING IN T HELPER CELL DIFFERENTIATION AND INDUCTION OF EAE

A Dissertation Presented

By

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ABSTRACT

THE ROLE OF NOTCH3 IN T HELPER CELL DIFFERENTIATION AND INDUCTION OF MOUSE MODEL OF MULTIPLE SCLEROSIS EAE:

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Th1 and Th17 are subsets of $CD4^+$ T cells or T helper cells (Th). Th cells are the major adaptive immune cells involved in inflammation during the development of Multiple Sclerosis (MS). MS is a neurodegenerative autoimmune disease and one mouse model of the disease is Experimental Autoimmune Encephalomyelitis (EAE). Development and differentiation of Th1 and Th17 cells are regulated by the Notch family of trans-membrane proteins (Notch1, 2, 3 and 4). We and others have shown that pharmacological inhibition of Notch activity impairs Th1 and Th17 differentiation as well as development of EAE. However, it is not known which Notch family member or members play a major role in this process. In this thesis, by using Notch3 knockout mice, we demonstrate that Notch3 is one of the major regulatory members of Notch signaling that is involved in regulation of Th1, Th2, iTreg and Th17 polarization as well as pro-inflammatory cytokine GMCSF production by Th cells. Impaired Notch3 signaling did not affect Th cell activation and proliferation. Our results demonstrate a previously unknown role of Notch3 in the development of pro-inflammatory Th cell types. We also report that non-canonical Notch signaling through PKCθ may play an important role in

Th17 differentiation. Development of EAE was not affected by impaired Notch3 signaling which suggests a compensation mechanism by other Notch protein(s) that regulate the development of EAE *in vivo*.

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CHAPTER I

1. INTRODUCTION:

1.1. $CD4^+$ T cells (Th cell) Development and Function and their role in autoimmune diseases, such as Multiple Sclerosis.

T helper (Th) cell precursors (naïve $CD4^+$ T cells) develop in thymus and migrate into lymph nodes after maturation. They are involved in tumor immunity, adaptive immunity to various pathogens, allergic responses, asthma and autoimmunity [1, 2]. Th cells help B cells to produce antibody, $CD8^+$ T cells to enhance and maintain their function. They stimulate macrophage activity and define the type of response to a certain danger signal, such as a foreign antigen or self-antigen indicating wound in the tissue or dead cells or cancerous cells [1, 2]. After danger is removed or if the threat detected is a "self" molecule then in a healthy individual Th cells adopt a suppressive phenotype. This suppressive Th cells prevent immune responses damaging the tissue and eventually start wound healing processes [1, 2].

These adaptive immune cells are activated specifically against an antigen presented via MHC class II molecules by antigen presenting cells (APCs) such as dendritic cells and macrophages [3, 4, 5, 6]. Antigen presentation to the T cell receptor (TCR) accompanied by a co-stimulatory signal from B7 molecules on APCs through the CD28 receptor, lead to activation of Th cells [5, 6]. Furthermore, the cytokine (small molecular weight secreted signaling molecules) environment helps to determine the fate of Th cells in terms of their function and activity [1, 2]. Activated Th cells have an increased rate of proliferation as well as increased expression of CD25 and CD69 surface

markers [7, 8]. CD25 is a high affinity IL-2 receptor that is crucial in IL-2 signaling which is one of the primary cytokines involved in immunity and tolerance by regulating immune cell activity [7, 8]. CD69 is a C-type lectin receptor that regulates T cell proliferation as well as signal transmission to intracellular environment. In our studies we measure CD25 and CD69 levels as indicator of Th cell activation *in vitro* [7, 8]. Depending on the cytokine profile in the macro-environment, Th cells differentiate into different sub-types: Th1, Th2, Th17, Treg and Th_{GMCSF} [1, 2, 9]. These different cell types are mostly differentiated by the types of cytokines they produce as well as by the differential expression of cell surface molecules. Differential function in these Th cell sub-types is established by different gene expression profiles for each fate. Cytokine signaling further enhance or inhibit certain gene expression programs and eventually define the type of Th cell and response that would be most effective according to the type of danger detected [1, 2, 9].

In the following sections, I will give an overview of Th cell types, their development, function and role in autoimmunity especially in Multiple Sclerosis (MS).

1.2. Th1 cell development and function:

In the presence of interleukin (IL)-12 and interferon (IFN)-γ cytokines, $CD4^+$ T cells acquire an IFN-γ producing Th1 fate and these cells are effective against intracellular pathogens [1, 2, 10]. Th1 cells also produce IL-2, lymphotoxin α (LT-α), TNF-α and interleukin-10 (IL10) [1, 2]. Master regulators of gene expression program in Th1 cells are T-bet and STAT-4 [11, 12]. IFN-γ produced by Th1 cells increases the microbicidal activity of macrophages as well as increases the activation of $CD8^+$ T cells [1, 2]. LT-α has been a disease progression marker in Multiple Sclerosis (MS) patients

and LT-α deficient mice have less induction of the animal model of MS, Experimental Autoimmune Encephalomyelitis (EAE). IL-2 produced by Th1 cells is imperative for memory formation especially in $CD8^+$ T cells the role of IL-2 in memory cell formation has been shown more elaborately [1, 2, 13].

Th1 cells are associated with autoimmune diseases due to increased inflammatory environment in the tissue [3, 4, 14]. In the case of MS, Th1 cells increase the activity of macrophages by IFN-γ that leads to increased production of radical oxygen species, phagocytic activity and production of inflammatory signaling molecules such as tumor necrosis factor (TNF)-α by macrophages [3, 4, 14, 15]. These activated macrophages lead to increased inflammation, axonal damage and eventually death of neurons in MS lesions [14, 15]. Furthermore, by regulating the activity of $CD8^+$ T cells and the memory formation by them, Th1 cells regulate the progression of MS at another level [1, 2, 14, 15]. $CD8^+$ T cells are found in the inflammatory plaques formed in the central nervous system (CNS) of MS patients [14, 15, 16, 17, 18]. They can induce the death of neurons through their cytolytic molecules to further cause neurodegeneration [14, 15, 16, 17, 18]. Therefore, Th1 cells are one of the major pro-inflammatory players that modulate the inflammatory environment in the CNS of MS patients.

1.3. Th2 cell development and function:

Interleukin (IL)-4 and IL-2 induce polarization towards IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and amphiregulin producing Th2 cells and these cells are effective against extracellular parasites such as helminths, and are associated with allergies and asthma [1, 2, 19]. The master gene expression regulators in Th2 cells are GATA-3 and STAT-5 [1, 2, 20]. IL-4 produced by Th2 cells have positive feedback effect on themselves as well as

class switching in B cells to produce IgE antibodies [1, 2, 21]. Multivalent ligand and immunoglobulin E (IgE) complex interacts with FcεRI receptors on mast cells and basophils leading the secretion of serotonin and histamine as well as production of cytokines such as IL-4, IL-13 and TNF-α [1, 2, 22, 23, 24, 25, 26]. Eosinophils are recruited to the tissue by IL-5 cytokines. IL-9 plays a role in activation of lymphocytes and mast cells as well as production of mucin by epithelial cells during an allergic reaction (27). IL-10 has suppressive function on Th1 differentiation and activity as well as dendritic cell activation [1, 2, 28]. IL-13 plays a role in the expulsion of helminths and induces airway hypersensitivity [22]. Amphiregulin is a signaling molecule in epidermal growth factor family, inducing the cell proliferation in epithelial cells. It has been shown that amphiregulin plays an important role in the expulsion of nematodes. There are studies suggesting a role for amphiregulin in airway hypersensitivity [1, 2, 29]. IL-25 (IL-17E) signals through IL-17 receptor B (IL17RB) and induces IL-4, IL-5 and IL-13 production by non-lymphocyte populations [30]. IL25 leads the further initiation and amplification of Th2 polarization. In response to allergens lung epithelial cells produce IL-25 to initiate a Th2 type of response. IL-25 is involved in the recruitment of eosinophils to the tissue by induction of CCL-5 (RANTES) and CCL-11 (eotaxin) production [1, 2, 30, 31].

In an MS setting, Th2 cells can be involved in B cell function by inducing antibody production and class switching but studies suggest that the induction of Th2 responses may decrease the severity of MS due to decreased Th1 and Th17 polarization, which are the pro-inflammatory cell types primarily involved in the induction of MS [14, 15].

1.4. Th17 cell development and function:

IL-6, IL-21, IL-23, and transforming growth factor β (TGF-β) or IL-1β induce the polarization of Th cells into Th17. IL-17A, IL-17F, IL-21 and IL-22 producing Th17 cells are effective against extracellular bacteria and fungi. They have been associated with autoimmune diseases [1, 2, 14, 15, 32, 33]. RORγT and STAT-3 are the master gene expression regulators for Th17 program [34, 35]. IL-17A and IL-17F are located in the same locus and hence are mostly regulated through similar mechanisms and are co-expressed in most of the cases. They both signal through IL17RA chain of the receptor which further suggests the similarity in their signaling. IL-17A has higher affinity to IL17RA than IL-17F [1, 2, 36, 37]. IL-17A plays an important role in the inflammatory responses by inducing the production of IL-6 cytokine and CXCL-8 (IL-8) chemokine [36]. Neutrophils play an important role against bacteria and fungi, both IL-17A and IL-17F recruit and activate neutrophils during infection [1, 2, 38]. Th17 cells produce IL-21 as their positive feedback stimulator for further amplification as in the case of IFN-γ for Th1 cells and IL-4 for Th2 cells. IL-21 also plays role in dendritic cell, $CD8^+$ T cell, B cell and natural killer cell activation [1, 2, 38, 39]. IL-6 or IL-23 leads to activation of STAT-3 pathway that enables production of IL-22 by Th17 cells [40]. The aryl hydrocarbon receptor (AHR) is a dioxin receptor and is expressed in Th17 cells in abundance to lead IL-22 production whereas TGF-β inhibits the expression of IL-22 by Th17 cells [41]. During acute liver inflammation IL-22 has a protective role [42]. IL-22 leads to dermal inflammation and acanthosis induced by IL-23 [40] . There are studies supporting the role of IL-22 in mediating response against bacterial infections but the source of IL-22 in this case can be other innate cells other than Th17 cells [1, 2, 43].

In an MS setting, Th17 cells positively contribute to inflammatory lesions in the CNS by recruiting more neutrophils to the region as well as by inducing activation of dendritic cells that can increase their pro-inflammatory and antigen presenting capacity. Along with Th1 cells, Th17 cells are one of the major contributors of neurodegenerative inflammation in the CNS during MS progression [14, 15].

1.5. Treg cell development and function:

In the presence of TGF-β and IL-2 Th cells acquire an anti-inflammatory Treg fate and these CD25 and FoxP3 double positive cells produce IL-10, IL-35 and TGF-β suppressive cytokines [44, 45, 46]. Foxp3 and STAT-5 are the master regulators of gene expression program in Treg cells [1, 2, 47]. If the $CD4^+$ T cells differentiate into Tregs in the lymph nodes depending on cytokine milieu then these cells are induced regulatory T cells (iTregs). There are also some Th cells that acquired Treg phenotype during development in thymus and these are known as natural Tregs (nTregs) [1, 2, 48]. The cytokines produced by activated Th cells modulate the immune response in a suppressive fashion and induce "self" tolerance. Increasing Treg cell numbers and efficiency is a potential treatment method for autoimmune diseases as well as prevention of allograft rejection. It has been shown that increasing Treg cell numbers and enhancing their function prevented the allograft rejection in mice [1, 2, 49]. One drawback of the approach is that increased Treg function would hamper tumor immunity as well as immune reactions against pathogens. IL-10, IL-35 and TGF-β produced by Tregs form the molecular basis of suppression. TGF-β is involved in positive feedback loop by producing more induced regulatory T cell (iTregs) from CD^+ T cells in the periphery [1, 2, 45]. *In vivo* studies suggest that TGF-β as a strong suppressive cytokine whereas *in*

vitro setting it does not seem to be required [45]. The role of IL-10 is well established in the suppression of inflammatory bowel disease. Treg specific deletion of IL-10 resulted in lung inflammation and spontaneous colitis [1, 2, 46]. It has been shown that IL-10 plays an important role to decrease the severity of EAE at later stages of the disease [50]. Treg produced IL-10 maintains a homeostasis between the host and bacteria during *Leishmenia* infection, and eventually prevents a disruptive inflammatory response which is protective for the host [50]. IL-35 contributes to suppressive immunity by Tregs and it shares a common subunit with IL-12, IL-12 p35 [1, 2, 51].

In an MS setting, Tregs play an important role to induce tolerance to self-antigens and to suppress the inflammation. It could be useful to develop treatment methods that would induce Treg cell differentiation over pro-inflammatory Th17 and Th1 cell differentiations in MS patients to suppress neurodegenerative inflammation and eventually establish tolerance to self-myelin peptides that are presented as antigen to T cells [14, 15].

1.6. Th_{GMCSF} cell development and function:

There is a recent study showing the importance of GMCSF producing Th cells a newly defined Th cell subset that is required for the induction of EAE. GMCSF is a cytokine involved in the monocyte maturation into macrophages and their further activation to give a pro-inflammatory response [9, 52]. Sheng et al. showed that impaired STAT-5 signaling caused a decrease in the induction of EAE. IFN-γ and IL-17A production was similar between STAT-5 knockout and WT EAE induced mice $CD4^+$ T cells whereas there was a significant decrease in GMCSF production by STAT-5 knockouts compared to WT. They also demonstrated that IL-7 signaling induced GMCSF

production by $CD4^+$ T cells by activation of STAT-5 pathway. The transcription factors and cytokine milieu required for Th cell differentiation into Th_{GMCSF} are yet to be defined but this study implied an important role for Th_{GMCSF} subset that can increase the activity of macrophages hence induce neurodegenerative inflammation in the CNS of MS patients. Therefore, further studies would be useful to better understand this Th cell type and their role in the induction of MS to find possible therapeutic approaches.

1.7. Transcription factors that affect Th cell differentiation and fate determination:

In order to better understand Th cell activation and differentiation, it is important to know the intracellular mechanisms dictating their phenotype. Studies dissecting intracellular signaling molecules such as transcription factors can be fruitful in terms of defining more and specific targets to alleviate tissue degenerative inflammation during infection as well as autoimmunity.

NFκB, NFAT and AP-1 are three transcription factors that are activated upon TCR signaling and by cytokine signaling. These transcription factors regulate the gene expression to determine cell fate into different Th subsets [2]. There is evidence supporting involvement of multiple transcription factors in the Th subset differentiation other than NFκB, NFAT and AP-1. Studies suggest multiple master regulators of gene expression for T helper cell subsets [2]. It is important to further understand the transcription regulation in Th cells since they can be potential targets to alleviate the severity of autoimmune diseases in clinical settings. We should also be careful about which transcription factor(s) or Th subsets that will be targeted since there is a fine balance between Th cell subsets in terms of fate determination. They mutually exclude each other's differentiation programs, so one should be cautious about which Th subsets

to target in a certain disease setting not to worsen inflammatory response by pushing Th cell differentiation to a more robust inflammatory cell program.

In the following sections, I will go into more details of signaling pathways and transcription factors that are involved in each Th cell subtype differentiation.

1.8. Signaling pathways and transcription factors involved in Th1 cell development and function:

IFN-γ signaling leads to activation of STAT-1 pathway which later on induces the activation of T-bet, a Tbox family member as the master regulator of Th1 differentiation [53, 54]. T-bet knockout cells have deficiency in Th1 polarization and T-bet knockout mice develop severe asthma symptoms [55]. There is still IFN-γ production in T-bet knockout cells due to the Eoemes (Eomesodermin) transcription factor expression [56]. Eoemes is another T-box family member and is involved in IFN-γ production by $CD8^+$ T cells [57]. During Th1 polarization there is upregulation of Eoemes which contributes to IFN-γ production. IL-21 partially decreases IFN-γ production by Th1 cells by decreasing Eoemes expression whereas T-bet expression remains intact [58]. This further supports the role of Eoemes in IFN-γ production during Th1 cell response and suggests that T-bet is still efficient enough in the absence of Eoemes for Th1 differentiation.

IL-12 signaling leads to activity of STAT-4 [59]. STAT-4 further amplifies Th1 polarization by directly inducing IFN-γ production to create a positive feedback loop for the Th1 program [60]. Independent of TCR activation, IL-12 and STAT-4 signaling axis together with and NFκB signal inducer can lead to IFN-γ production [61].

Runx3 is another transcription factor involved in IFN-γ production by Th1 cells [62]. It is involved in T cell development at early stages by inhibiting $CD4^+$ T cell development and leading to generation of $CD8^+$ T cells [63]. In Runx3 knockout cells IFN-γ production is substantially decreased compared to wild type ones under Th1 polarizing conditions [62]. When Runx3 is overexpressed in Th2 cells, it leads to IFN-γ production independent of T-bet expression [64].

Hlx is another transcription factor involved in Th1 polarization. Hlx transcription factor plays an important role in embryogenesis and hematopoiesis. T-bet leads to expression of Hlx, which then interacts with T-bet protein to enhance the expression of IFN-γ [65].

1.9. Signaling pathways and transcription factors involved in Th2 cell development and function:

IL-4 signaling leads to activation of STAT-6 which is the major Th2 inducing factor [66, 67]. *In vitro*, STAT-6 deficiency leads to impaired Th2 polarization whereas *in vivo* Th2 responses and differentiation are intact independent of STAT-6 [67, 68]. STAT-6 expression is sufficient and required for the expression of GATA-3, master regulator of Th2 polarization, under *in vitro* conditions [69].

When GATA-3 is overexpressed in Th1 cells, it leads to IL-4 production [70]. Impaired GATA-3 signaling totally abolishes Th2 polarization both under *in vivo* and *in vitro* conditions [71]. IL-5 and IL-13 promoters have GATA-3 binding sites whereas IL-4 does not [72]. When GATA-3 was deleted in fully differentiated Th2 cells it leads to decreased IL-5 and IL-13 production whereas it does not affect IL-4 production [73].

This observation is in line with the presence of GATA-3 binding sites on promoted regions of IL-5 and IL-13 but not on that of IL-4.

IL-2 leads to STAT-5 activity and there are two types of STAT-5, STAT-5a and STAT-5b [74]. STAT-5 transcription factors are involved in cell proliferation and survival [75]. Strong STAT-5 signaling is required for Th2 polarization [76]. Despite the presence of STAT-5b, there is a substantial decrease in Th2 polarization when STAT-5a is impaired both *in vivo* and *in vitro* conditions [77]. STAT-5 directly binds to the DNase I hypersensitive sites (HSII and HSIII) in the second intron of IL-4 locus [78].

IL-4 production also is enhanced by c-Maf, which is upregulated under Th2 polarizing conditions. c-Maf does not affect the production of other Th2 cytokines [79]. IRF-4 is another transcription factor required for Th2 polarization and its deficiency causes decreased IL-4 production [80]. GATA-3 overexpression rescues the decreased IL-4 production phenotype in IRF-4 deficient cells, suggesting that GATA-3 is upregulated by IRF-4 [81].

The IL-4 early inducible gene, Gfi-1, is also transiently activated by TCR signaling. Gfi-1 modulates upstream and downstream IL-2 signaling to select the growth of cells with high GATA-3 expression [82].

1.10. Signaling pathways and transcription factors involved in Th17 cell development and function:

RORγt is the master regulator of gene expression during Th17 polarization. It induces IL-17 production [83]. RORγt deficient mice have reduced EAE disease scores compared to wild type ones, due to decreased IL-17 production [84].

RORα is a nuclear receptor related to RORγt and is upregulated during Th17 polarization. It is not required for IL-17 production but when both RORγt and RORα were targeted, IL-17 production was completely abolished [85].

IL-6, IL-21 and IL-23 signaling leads to activation of STAT-3 and is required for IL-17 production, since deletion of it abolishes IL-17 producing cells. In order to create a positive feedback loop, STAT-3 increases the expression of IL-23 receptor [86].

IRF-4 is another transcription factor playing a crucial role in Th17 polarization. IL-17 production is substantially decreased in IRF-4 knockout cells [87]. IRF-4 regulates RORγt expression without affecting FoxP3 expression and EAE cannot be induced in IRF-4 knockout mice [88].

1.11. Signaling pathways and transcription factors involved in Treg cell development and function:

FoxP3 is the master transcriptional regulator for Treg cell fate [89]. TGF-β signaling leads to FoxP3 expression in Th cells [90]. Continuous FoxP3 expression is required for sustained Treg suppressive activity [89]. Overexpression of FoxP3 in Th cells creates anergic and suppressive phenotype [91]. IL-2 expression induces STAT-5 activity and it may play a role in FoxP3 production by binding to its promoter region. There is a close relationship between Th2 and Treg polarization due to overlapping STAT-5 activity in both lineages [92]. When FoxP3 expression diminishes in later stages of Treg activity they acquire a Th2 cell like phenotype [93].

My studies focus on development of T helper cells and their contribution to Multiple Sclerosis. In the context of MS, dendritic cells and macrophages present myelin

proteins and other molecules, and some others yet to be defined, as antigens to Th cells in the lymph nodes which then differentiate into Th1, Th2, Th17 and Th_{GMCSF} cells [9, 14, 15, 94]. Th1, Th2, Th17 and Th_{GMCSF} cells migrate into the spinal cord and brain regions in response to chemokines secreted from the central nervous system (CNS) [9, 14, 15, 94]. Once Th cells reach to CNS, they exacerbate inflammatory reaction by recruiting more innate immune cells to the neuronal areas and furthermore enabling activation of these innate immune cells [9, 94]. Th cells have certain plasticity in their differentiation program so in depending on the cytokine environment in the lymph nodes and later on at CNS their functional fates are determined [1, 2, 14, 15]. Under normal circumstances, eradication of danger by the inflammatory response is followed by immune suppression and wound healing. Induced regulatory Th cells (iTregs) and natural regulatory Th cells (nTregs) can suppress inflammation and, in MS, oppose the action of Th1, Th2, Th17 and Th_{GMCSF} cells [1, 2, 9, 14, 15, 94].

In order to identify more specific targets for therapeutic approach in MS setting, signaling pathways determining the specific sub-type differentiation of Th cells have been widely studied. One of these pathways is the Notch signaling pathway that has been studied extensively by our lab and others. The study presented here focuses on the role of Notch3 in the Th cell differentiation and in the induction of EAE. Notch signaling pathway and its relevance to MS and EAE will be described in detail in another section. First, an overview of Multiple Sclerosis and EAE will be given in the following section.

1.12. An overview of Multiple Sclerosis.

Multiple Sclerosis (MS) is a debilitating autoimmune disease associated with inflammation in the nervous system [14, 15]. It commonly begins in early adulthood and

nearly 2.5 million people are affected by it worldwide [95]. Northern European descents and women are more susceptible to the disease. Although genetic, life style, dietary and environmental risk factors, especially viral infections, have been implicated for the development of this autoimmune disease, the initiating event that leads to the development of the disease and immunological targets are not known [14, 15, 95]. The symptoms are highly heterogeneous between patients in the clinical setting. The most common form of the disease is the relapsing remitting MS (RRMS), where there is alternating periods of worsening in the disease symptoms and followed by the dissipation of the severity. Relapsing remitting MS is also followed by sustained deterioration during secondary progressive MS (SPMS). Due to numerous disease mechanisms MS is multifaceted syndrome. During remission the endogenous functional recovery mechanisms are activated in the nervous system [14, 15, 17, 18, 95, 96, 97].

There are studies supporting the role of genetic background on the induction of MS. There is 25% concordance level between identical twins to develop the disease, indicating the role of genetic factors on the induction of the disease at the same time suggesting involvement of other factors in the disease development [14, 15, 94, 95, 96, 97]. Clinical and epidemiological studies support role for Epstein-Barr virus infection, smoking and lack of vitamin D in the serum as possible causatives of MS. As preventive strategies decreasing the cigarette usage and increasing sunlight exposure to boost vitamin D levels in the serum could be used at population level [14, 15, 94, 95, 96, 97].

It is known that during disease progression the inflammation is sustained by reaction against myelin sheath proteins covering the neural cells in brain, optical nerve and spinal cord [14, 15, 95, 96, 97]. There have been studies stressing the importance of

T cells in the development of the disease [14, 15, 95, 96, 97]. T cells orchestrate the type of immune response manifested during a danger signal, which can be triggered by a foreign molecule as well as a truncated or mislocalized self-antigen [1, 2]. Follow up studies suggest that B cells have an important role in the disease induction especially since the most common diagnostic tool is detection of oligoclonal bands in the patients [14, 15, 94, 95, 96, 97]. Targeting B cells in MS setting seems to be the efficacious way according to recent studies. Detection of autoantibodies is further helpful in our understanding of the disease, one of which indicated potassium channel KIR4.1 as a target molecule in MS [94, 98].

Other than the role of inflammation and preventive therapies, studying the recovery phase of MS is fruitful to develop better therapy methods. During inflammation the oxidative damage and energy insufficiency lead to axonal damage [94, 99]. There are studies focusing more on functional recovery rather than cellular level recovery since overall result of therapy might be more efficient than focusing on just cellular level repair mechanisms that may not necessarily lead to a functional recovery [94, 100].

The primary progressive form of MS is manifested in 10-20% of patients and most patients with the relapsing remitting form have transition to the progressive type. This transition is due to exhaustion of functional compensation mechanisms as well as mitochondrial damage that lead to expansion of existing lesions and trapping of inflammation behind the blood brain barrier [3, 4, 14, 15, 94, 101]. In relapsing remitting model of MS, there are treatments available to slow the progress and improve the symptoms but there is not any effective treatment against primary progressive type [3, 4, 14, 15, 94, 101]. More studies are needed to delineate the mechanism of primary

progressive type and to find better treatment betters. One of the possibilities is that, for now, based on similarities in disease mechanisms, therapies used in Parkinson's and stroke disease can be tried in MS setting, especially for the progressive type [101].

During the early stages of the disease, lymphocytes migrate through blood-brain barrier (BBB) into the brain, spinal cord and optic nerves and lead to formation of the perivascular inflammatory lesions, plaques [14, 15, 18]. When these plaques are formed in the eloquent areas, it drives neurological deficits. The incidence of relapses and frequency of periodic breaches of BBB diminish with disease duration according to natural history studies [14, 15, 18]. So far there is no marker of peripheral blood mononuclear cells (PBMCs) associated with MS progression and activity and there is no immunological assay to predict the severity of the disease [14, 15, 18].

Ongoing pathogenic neuroinflammation is evident by the presence of microglial activation in the white matter that appears macroscopically normal as well as lymphoid follicles in the leptomeninges of SPMS brains [102, 103]. Perivascular inflammatory lymphocytes with adjacent demyelination and axonal transection are histological hallmarks of acute MS lesions [16, 17]. $CD4^+$ T cells, $CD8^+$ T cells and myeloid cells form the majority of these perivascular inflammatory infiltrates. Genetic polymorphisms associated with the development of MS include $CD4^+$ T cell associated molecules. Polymorphisms in major histocompatibility complex (MHC) class II molecules that govern $CD4^+$ T cell activation are genetic risk factors associated with MS. Other examples of genetic polymorphisms associated with MS are interleukin-2 (IL-2) receptor α chain and interleukin-7 (IL-7) receptor α chain, these receptors are involved in T cell development, survival and activation [104].

Studies in EAE, the animal model of MS, further support a critical role for $CD4^+$ T cells in autoimmune demyelination [14, 15, 105]. EAE and MS manifest similar clinical and histological symptoms or phenotypes. EAE can be induced in an array of mammalian species by vaccination against myelin peptide epitopes restricted to MHC class II molecules [105, 106]. Adoptive transfer of $CD4^+$ T cells from EAE induced mice to unvaccinated mice can induce EAE and these studies are useful to dissect $CD4^+$ T cell specific role of proteins associated with MS [14, 15, 105, 106]. T helper 1 (Th1) and T helper 17 (Th17) lineages are associated with encephalitogenic myelin specific $CD4^+$ T cells and they produce interferon γ (IFN-γ) and interleukin-17 (IL-17) cytokines, respectively [14, 15]. Recent studies show a correlation in MS severity or development with increased IL17A, tumor necrosis factor α (TNF-α), IFN-γ positive and $IL\text{-}17A^+$ IFN-γ^+ double positive PMBCs in RRMS patients [107, 108]. Chemokines involved in T cell as well as innate immune cells trafficking are higher in MS patients' cerebrospinal fluid compared to those of patients with non-inflammatory neurodegenerative diseases. Direct evidence for autoimmune characteristics of the disease comes from clinical studies showing decrease in the disease progression or severity by immunomodulatory molecules, or drugs targeting T cell trafficking, growth as well as survival [109-117].

The treatment of MS is limited to relieving the symptoms and suppressing the immune reactions non-specifically or blocking immune cell trafficking into the central nervous system through BBB, which compromise the host response to other infections and does not support the recovery of degenerated neurons [14, 15, 18, 94]. Therefore, it is important to further understand the disease mechanism at both immunological and neural recovery levels to develop better treatment options. In the following section, I will give

an overview of Notch signaling that is known to play an important role in T helper cell development as well as in the induction of EAE.

1.13. An overview of Notch signaling:

Notch is a trans-membrane protein with four isoforms; Notch (1, 2, 3, 4) [Figure 1]. It links the fate of neighboring cells through regulation of cell proliferation, apoptosis, survival and differentiation in multiple metazoan tissues [118-121]. There are structural and functional differences between the Notch family members. EGF repeats at their extracellular region varies between the members. This may enable differential affinity for the ligands. Notch1 and Notch2 family members have trans-activation domain (TAD) which enables their interaction with transcription regulators. Notch3 and Notch4 lack TAD domain [118-121]. All Notch family members have two nuclear localization signal domains except Notch4 which bares only one copy [118-121]. Notch family member specific transcriptional targets are yet to be determined. We cannot associate a specific member for the activation of a particular pathway or transcription of particular genes [118-121]. Mutations that perturb its function are associated with several different genetic disorders and cancers [118-121]. In canonical Notch signaling, interaction of Notch protein with cell bound ligand (Delta-like 1, 2, 3, 4 or Jagged 1, 2) on the cell surface results in cleavage of Notch, first by ADAM10 and ADAM17 proteases, followed by cleavage by the gamma secretase complex at the transmembrane region [118-121]. At the end of this process, the Notch intracellular domain (NICD) translocates to the nucleus and interacts with RBPJκ/CSL, a transcription repressor. Upon interaction with NICD, RBPJκ/CSL becomes a transcription activator for downstream target genes [118-121].

However, more recent studies reveal the existence of several other modes of Notch signaling generally referred to as non-canonical Notch signaling. Interestingly, many instances of non-canonical signaling are associated with potentially pathological conditions including cancer and activation of the immune system while many normal cellular processes require canonical Notch signaling. For example, it is likely that early development in the mammalian embryo requires canonical Notch signaling since deletion of RBP-Jk mimics deletion of Notch1. Several other physiological processes, such as maintenance of the intestinal epithelium, also require canonical Notch signaling. Therefore, it is possible that blockade of non-canonical Notch signaling may provide opportunities to inhibit some instances of pathological Notch signaling leaving many other normal physiological processes intact [122]. However, since non-canonical Notch signaling is not as well characterized as the canonical signaling pathway, more in-depth inquiries in this area are likely to reveal potential new targets to manipulate non-canonical Notch signaling [122].

1.14. The role of canonical versus non-canonical Notch signaling in Th cells:

Notch signaling regulates some lineage decisions of hematopoietic cells, and enables generation of T cells at the expense of B and myeloid cells in the early stages of hematopoietic cell development. At later time points, Notch plays a key role in the survival, proliferation, and differentiation of T cells. Notch signaling also regulates the development of some innate lymphoid cells, marginal zone B cells from precursor B cells, megakaryocytes, and cytotoxic (CD8) T-cell lymphocytes (CTLs) [123-128].

Notch is important in driving the differentiation of naïve CD4 T cells into specific T helper (Th) subsets and targeting Notch signaling in Th cells provides the opportunity

for immune modulation. Studies in our lab demonstrate that gamma secretase inhibitor (GSI) treatment, which prevents Notch activity by inhibiting the formation of intracellular cleaved Notch, significantly reduces Th1, Th17, and induced Treg (iTreg) polarization [129-131]. Studies by other labs using different methods to block Notch signaling showed that Th2 polarization is also driven by Notch signaling [132-136]. We demonstrated a significant decrease in Th1 and iTreg differentiation in conditional Notch1 knockout Th cells and through the use of conditional RBPJk knockout T cells, revealed that Notch regulates Th cell differentiation into different Th cell fates independent of RBPJk and hence is non-canonical [137]. Furthermore, our data showed that both activation and proliferation of CD4 T cells are not impaired by conditional deletion of RBPJk. Thus, CD4 T cell activation, proliferation, and differentiation all require non-canonical Notch signaling, and recent data from our lab suggest Notch, in conjunction with NF-kB, and regulate this non-canonical signaling in CD4 T cells [137]. The possibility that non-canonical Notch signaling may occur through activation of NF-kB is not surprising since links between Notch and NF-kB have been documented by several groups including our own [137-141]. In cells of the immune system, Notch3 in collaboration with NF-kB is reported to cooperatively regulate FoxP3 expression [139]. Additionally, we recently reported that Notch1can initiate NF-kB activation via cytosolic interactions with components of the T cell signalosome [140, 141]. In particular, cytosolic Notch1 drives the formation of the CARMA1, BCL10, and MALT1 (CBM) complex that is essential for NF-kB activation in T cells. These data demonstrated that cytosolic, rather than nuclear, Notch1 drives CBM complex formation emphasizing the non-canonical role of Notch1 in this process [140, 141].

In addition to Notch signaling through NF-kB, non-canonical Notch signaling is implicated in T cell metabolism and cell survival. Upon lymphocyte activation, there is an immense change in the metabolic activity of T cells to enable the production of building blocks for cell division and growth as well as ATP production [142-144]. This metabolic switch is closely linked with cell survival. As described above, Perumalsamy et al described a link between non-canonical Notch signaling and the mTORC2-Akt cascade [142]. In this report, they also provide evidence that cell survival of activated T cells is regulated by the interaction of cytoplasmic or membrane tethered NICD with the mTORC2-Akt cascade and this may also be involved in cell metabolism. The same group had previously demonstrated that interaction of Notch1and kinases involved in early T cell activation (PI3K and p56lck) regulates an anti-apoptotic program in T cells through Akt signaling [142]. Interestingly, another group has demonstrated mitochondrial localization of full length Notch1protein in neuronal cells providing additional evidence in another system for non-canonical Notch signaling in metabolism and cell survival [143].

Data also supports a role for PKCθ in non-canonical Notch signaling pathway. TCR activation in T cells leads to increased PKCθ activity which further activates downstream components: NFAT, AP-1 and NFκB to regulate gene expression [1, 2, 145]. Activation of these transcription factors enables cell survival and proliferation. PKCθ has been implicated in the downstream of Notch signaling pathway [1, 2, 145]. In my studies I performed some preliminary experiments by using pharmacological PKCθ and NFκB inhibitors to further examine the role of canonical versus non-canonical Notch signaling

in Th cell differentiation. In the following section, the role of Notch signaling in Th cell differentiation will be described based on recent studies.

1.15. The role of Notch signaling in the development of Multiple Sclerosis and its mouse model EAE:

1.15.1. The role of Notch signaling in Th cell differentiation:

There are studies suggesting an important role for Notch signaling Th1 cells. When Delta1 ligands were used to activate Th cells, the cells were polarized towards Th1 fate partly independent of IL-12 signaling [133, 134, 146-148]. Ectopic expression of Delta1 or Delta4 by bone marrow derived dendritic cells or APCs lead to increased Th1 polarization [133,149, 150]. Overexpression of Notch3 intracellular domain but not the Notch1 intracellular domain in activated $CD4^+$ T cells promoted T-bet expression and Th1 polarization. But further studies also suggested that overexpression of Notch1 causes increased T-bet expression and eventually increased Th1 polarization [129, 133, 146, 151]. Furthermore, there are several studies showing that GSI treatment caused decreased IL-12 induced Th1 polarization *in vitro*. It has been shown that Notch directly regulates *Tbx21* (T-bet) expression to promote Th1 polarization [129]. Studies have suggested a role for non-canonical Notch signaling through NFκB pathway on IFN-γ expression to drive Th1 polarization [137, 140, 141].

It was shown by the Amsen group that Delta1 expression by fibroblasts caused enhanced IFN-γ production by antigen stimulated Th cells [133]. The same group suggested that Notch1-Jagged1 signaling increased IL-4 production in Th cells, suggesting that Th2 polarization is enhanced through Notch1 and Jagged1 signaling

independent of IL-4/STAT-6 signaling [133]. They further expanded their studies by showing that Notch regulated Th2 polarization by inducing GATA-3 and enhancing IL-4 production [133]. Other studies have shown that Th2 polarization was abrogated in the absence of both Notch1 and Notch2 signaling but not when either of them was impaired alone. Perturbed canonical Notch signaling also prevented Th2 function both *in vivo* and *in vitro* settings [134, 151].

It has been shown that DLL4 expressing dendritic cells promote Th17 polarization by inducing RORγt expression [135, 152, 153]. It has been shown that in an EAE setting Th1 and Th17 cells are prominent in the central nervous system and GSI treatment reduced Th1 and Th17 associated cytokines in EAE model as well as in *in vitro* settings [129, 154]. There are studies showing that DLL1 blocking antibody attenuated the induction of EAE whereas JAG1 blocking antibodies had an opposite effect, suggesting ligand dependent Th cell fate determination through Notch signaling [147].

There is data suggesting that Jagged1 is involved in Treg polarization both in human and mouse cells [155]. Our recent paper has shown that Notch1 plays an important role in Th1 and Treg polarization independent of canonical Notch signaling [137]. Another study suggested that Jagged1 stimulation of Th cells in a mouse model resulted in increased Treg generation and decreased development of an experimental animal model of diabetes, an autoimmune disease [156]. According to literature none of the Notch signaling impairing mice had decreased natural Treg generation, suggesting a possible compensation mechanism between Notch family members or by other proteins [135]. Studies by Samon et al have shown that GSI treatment in Th cells decreased TGF-β mediated FoxP3 expression, development and suppressive function of iTregs *in vitro*

[130]. Furthermore, GSI treatment of mice caused reduced FoxP3 expression and lead to development of autoimmune hepatitis. It was shown previously that autoimmune hepatitis resulted from decreased TGF-β signaling and Treg generation in mice [130, 135]. It has been shown that Notch3 overexpression leads to increased FoxP3 levels in Th cells and increased Treg generation in mice that eventually impairs the induction of type 1 diabetes in a mouse model [136, 157]. There are also studies suggesting a role for Notch signaling in the generation of Th9 subset but these require further examination [136, 158].

Overall, it has been shown by a multitude of studies that Notch signaling plays a significant role in Th cell differentiation and fate determination. In my studies, I further evaluated the role of Notch1 and Notch3 family members on Th cell differentiation as well as on the induction of EAE. In the following section I will summarize the role of Notch signaling in the induction of EAE and MS.

1.15.2. The role of Notch signaling in the induction of EAE and MS:

There are studies suggesting the role of Notch signaling in MS and also its animal model EAE through two different angles. One way of Notch regulation of disease is by defining the cell proliferation, differentiation and survival of oligodendrocyte precursor cells which lead to generation of oligodendrocytes [159]. Oligodendrocytes are supportive cells of neurons and they are involved in generation of myelin proteins. Therefore, oligodendrocytes play a crucial role in the regeneration process of nervous system after MS lesions. It has been shown that Notch1 plays an essential role in oligodendrocyte precursor cell differentiation and maturation [159]. Depending on ligand type interacting with Notch1, the cell fate of oligodendrocyte precursor cells is

determined. If canonical Notch ligands are involved in the signaling process then target genes inhibit the maturation and differentiation of oligodendrocyte precursor cells. Whereas if non-canonical Notch ligands are the signal initiators then activated target genes lead to formation of oligodendrocytes from precursor cells to enable regeneration in the central nervous system [159].

Canonical Notch signaling ligands are Delta, Serate/Jagged2 and Lag2 proteins. Example of non-canonical Notch signaling ligands in the central nervous system is F3/Contactin [159]. When chronic MS patient's tissue samples were examined there was Jagged1 in abundance, which indicates the destructive face of Notch in the CNS [159]. Furthermore, aggregation of intracellular domain of Notch1 was detected in the oligodendrocyte precursor cells of patient samples. Instead of enabling the regenerative program, this aggregates seemed to start neurodegenerative program in these cells [159]. In summary canonical ligand induced Notch1 signaling in neurons has neurodegenerative role in MS setting which suggests that inhibition of non-canonical ligand induced Notch signaling might be a better treatment approach, but further studies are required to examine the role of other Notch family members in the process.

The other way of Notch regulation for the induction of EAE and MS is through its role in Th cell proliferation, differentiation and activity [160]. It has been shown that Th cells play a major role in orchestrating an inflammatory response to the CNS proteins, especially against the antigens presented on oligodendrocytes, in MS and EAE [14, 15, 160]. The role of Notch signaling in Th cell development and differentiation has been shown by a wide array of studies. In the light of those studies, GSI was used by Minter et al to observe its effect on the induction of EAE and there was decreased disease scores

compared to mock control treated groups [129]. Jurynczyk et al has shown that Notch3 blocking antibodies were effective in impairing the induction of EAE compared to control treated group as well as Notch1 blocking antibody treated group, which gave similar results as control group [154]. One caveat of this study was that blocking antibodies and their effectiveness was not validated. Several independent studies have shown that blockage of DLL4, a notch ligand highly expressed on dendritic cells during MS and EAE, caused decreased EAE scores, immune cell infiltration to the CNS and, IFNγ and IL17A production compared to control mock treated group [161, 162]. Studies by Elyaman et al showed that DLL1 was another ligand that was highly expressed on dendritic cells during EAE and its blockage decreased inflammatory responses in CNS as well as induction of EAE compared to control mock treated group [163]. Studies have shown that inhibition of Jagged1 exacerbated EAE, therefore it is important to delineate the signaling mechanisms before targeting a certain group of proteins in EAE and MS settings [163]. The timing of drug administration is also crucial, since inhibition of Jagged2 before induction of EAE prevents the disease onset whereas concurrent inhibition of Jagged2 with the disease induction leads to increased disease scores [158].

Overall, it is imperative to further study the role of Notch signaling, specific family members and ligands in T helper cell differentiation as well as induction of EAE, in order to design better therapy methods for MS patients. In the following section I will describe the rationale of our studies, specific aims and their significance for the field.

1.16. Rationale, Specific Aim and Significance of the Project:

As summarized in the above sections, previous studies demonstrated that when Notch activation was inhibited by a pharmacological gamma secretase inhibitor (GSI) in

mice, there was a decrease in the development of Experimental Autoimmune Encephalomyelitis (EAE), a mouse model of MS, compared to untreated control mice. Under *in vitro* Th1 and Th17 polarizing conditions, GSI treated $CD4^+$ T cells had impaired differentiation towards these fates compared to control $CD4^+$ T cells. GSI prevents the gamma secretase complex mediated cleavage of all type I trans-membrane proteins, including all 4 Notch family members. Furthermore, GSI treatment has detrimental side effects on tissues requiring high Notch activity such as gut, therefore there is need to understand the specific details of which Notch protein(s) and downstream components of the pathway that affect Th cell differentiation [122].

In light of studies suggesting the role of Notch1 in Th cell development, we used conditional Notch1 knockout mice to study the role of Notch1 in Th cell differentiation as well as in the induction of EAE. Here, we are showing that Th17 differentiation as well as induction of EAE was intact when Notch1 signaling was impaired. These observations and recent studies showing the role of Notch3 by blocking antibodies in the induction of EAE as well as in Th1 differentiation by using siRNA for Notch3, lead us to the usage of Notch3 knockout animals for our further studies.

Here, we used Notch3 Knockout (N3KO) mice from the Jackson Laboratory to study the effect of Notch3 on Th cell activation, proliferation and differentiation as well as on the induction of EAE [164]. As depicted in figure 2, these mice were generated by targeting exons 16 and 17 to prevent the generation of Notch3 protein. These mice don't have any major phenotype. Notch3 N terminus is expressed in these mice $CD4^+$ T cells whereas C terminus cannot be detected by q-RT-PCR. Under *in vitro* Th1, Th17 and iTreg polarizing conditions Notch3 was required for differentiation of $CD4^+$ T cells to

these cell types. Moreover, impaired Notch3 signaling caused an increase in Th2 polarization as well as GMCSF production under non-polarizing conditions. There was no difference in nTreg levels between wild type (WT) versus N3KO mice thymii.

When EAE was induced in N3KO and WT control mice the disease scores and cytokines produced from these mice splenocytes were similar, suggesting a compensation mechanism under physiological conditions for Th cell polarization and the induction of EAE. Here for the first time, we demonstrate the role of Notch3 in Th1, Th17, Th2, iTreg polarization and GMCSF production by Th cells *in vitro* by using Notch3 knockout animals.

Notch3 exerted its effect without influencing Th cell proliferation as well as activation *in vitro*. Our data suggest that inhibition of only one Notch family member *in vivo* was not sufficient to prevent the Th cell polarization towards pro-inflammatory type as well as the induction of EAE. Another significance of this study is that we suggest PKCθ as non-canonical target of Notch signaling to prevent Th17 polarization, major cell type involved in the induction of EAE. Combination therapy with GSI and PKCθ inhibitor, such as Rottlerin, might give promising results in prevention of EAE.

In addition, we also used another N3KO mice strain generated differentially by the William Skarnes group to study Th cell differentiation [165]. These mice were generated by gene trap method where the disrupted gene and its product were characterized [165]. Only Notch3 gene and its protein product were disrupted in these mice [165]. As depicted in figure 3 there is not any major developmental phenotype in these mice but they develop CADASIL, a brain stroke disease [165]. In the $CD4^+$ T cells

of these mice we can detect Notch3 N terminus whereas C terminus cannot be detected by q-RT-PCR. There was a tendency for increase in Th17 polarization based on IL17A production measured by ELISA. Since Th17 polarization results conflicted with previous studies showing importance of Notch signaling in Th17 polarization and the results of our siNotch3 knockdown and overexpression studies where impaired Notch3 signaling was decreasing Th17 polarization and overexpression of Notch3 caused significant increase in IL17A production, we did not further pursue the disease induction and other Th cell polarizations in this set of mice.

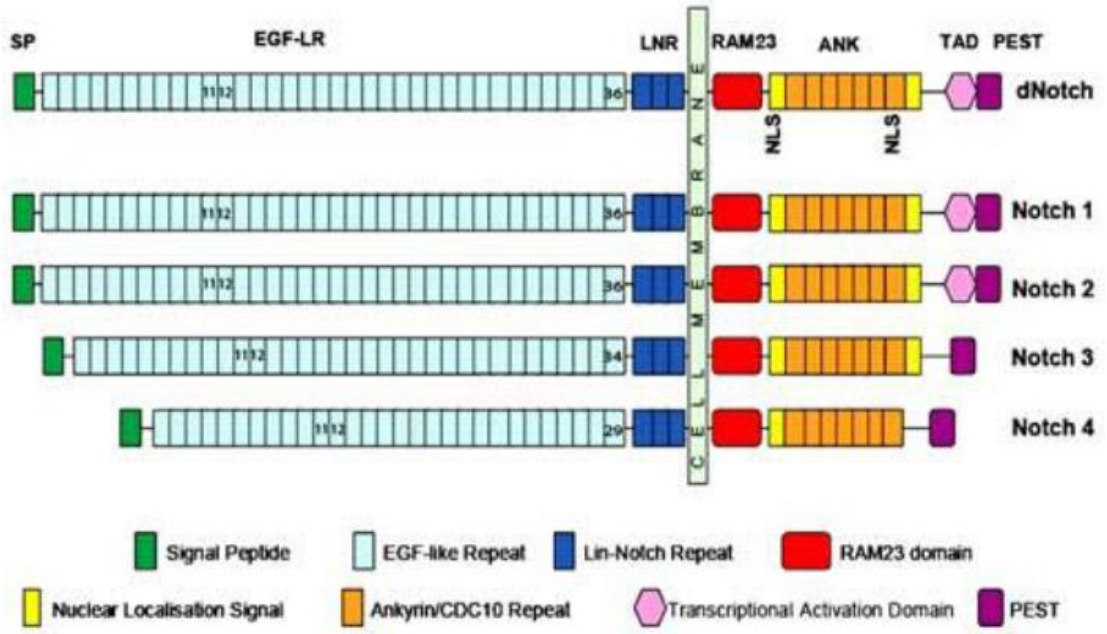

Figure 1. Notch family members 1, 2, 3 and 4:

There are structural and functional differences between the Notch family members. EGF repeats at their extracellular region varies between the members. This may enable differential affinity for the ligands. Notch1 and Notch2 family members have trans-activation domain (TAD) which enables their interaction with transcription regulators. Notch3 and Notch4 lack TAD domain. All Notch family members have two nuclear localization signal domains except Notch4 which bares only one copy

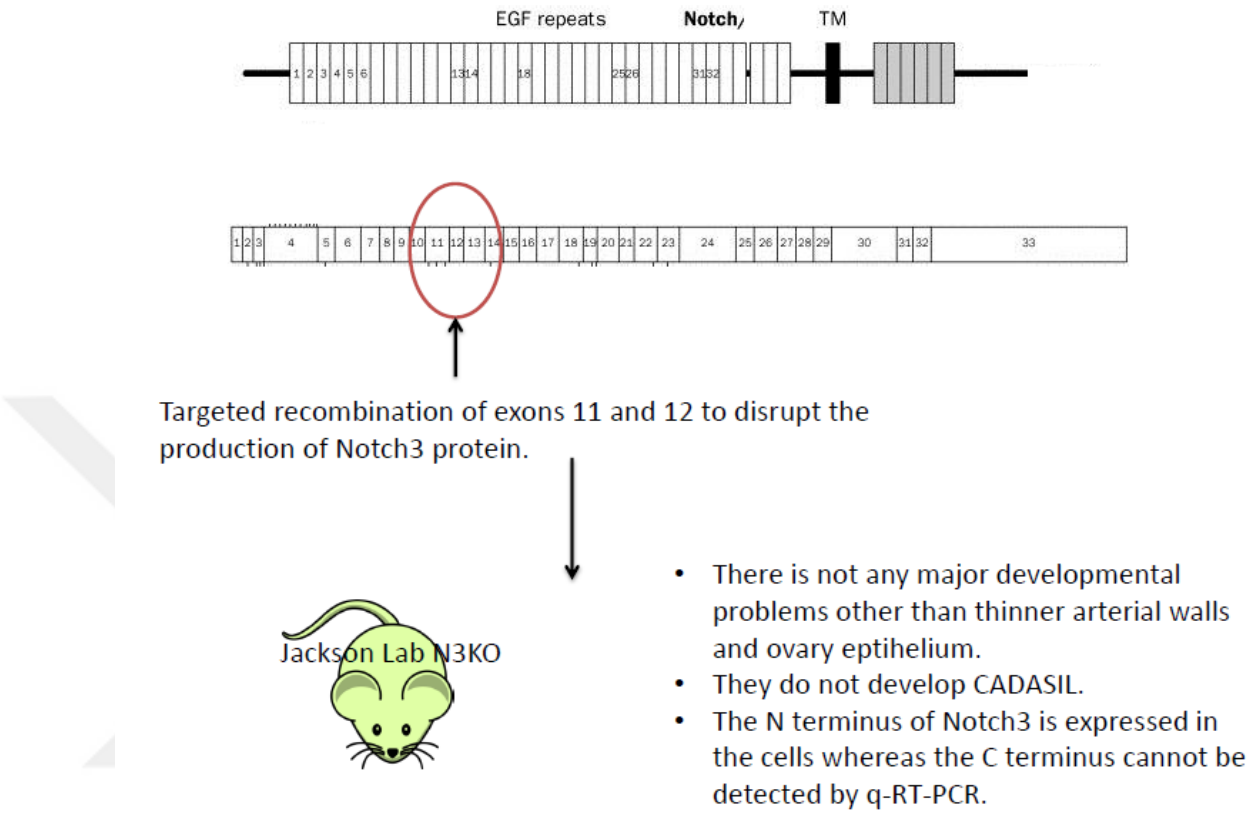

Figure 2. Generation of Jackson laboratory Notch3 knockout mice:

Exons 11 and 12 were specifically targeted to disrupt the production of Notch3 protein. There is not any major developmental problem other than thinner arterial walls and ovary eptihelium. They do not develop CADASIL spontaneously. The N terminus of Notch3 is expressed in the $CD4^{+}$ T cells whereas the C terminus cannot be detected by q-RT-PCR.

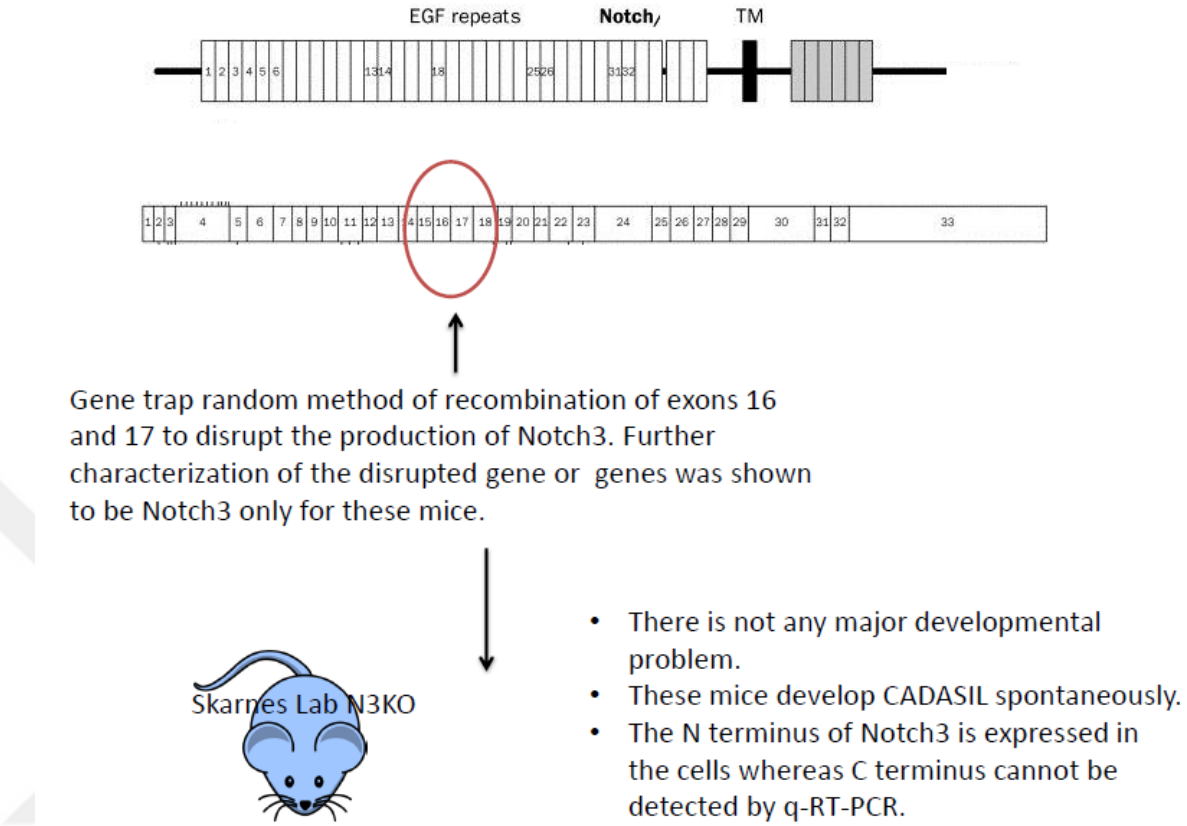

Figure 3. Generation of Skarnes Notch3 knockout mice:

Gene trap random method of recombination of exons 16 and 17 was used to disrupt the production of Notch3. Further characterization of the disrupted gene or genes was shown to be Notch3 only for these mice. There is not any major developmental problem and these mice develop CADASIL spontaneously. The N terminus of Notch3 is expressed in the cells whereas the C terminus cannot be detected by q-RT-PCR.

2. RESULTS:

2.1. Notch sparing GSI does not affect Th1 and Th17 polarization and, decreasing the expression of Notch1 does not prevent Th17 polarization as well as induction of EAE:

GSI targets all type I transmembrane proteins including Notch family members, in order to determine whether the effects we have observed with GSI on Th cell polarization were Notch signaling specific, we used a Notch sparing GSI, JLK6 [166]. JLK6 did not affect Th1 and Th17 polarization whereas GSI was able to inhibit differentiation of $CD4^+$ T cells into these fates (Figure 7). To further study the role of a specific Notch family member, Notch1, that has been shown to play an important role on $CD4^+$ T cell development, we used conditional Notch1 knockout mice. In these mice Notch1 gene was floxed with Cre binding sites and Cre was under control of Mx promoter, which is activated by IFN signaling. PolyIC injection of mice as described in materials and methods section, lead to acute floxing (deletion) of Notch1 in $CD4^+$ T cells. Under Th17 polarizing conditions there was no difference in IL-17A production between control WT and conditional Notch1 knockout $CD4^+$ T cells (Figure 7). When we induced EAE in the mice, the induction of the disease was similar between the groups (Figure 7). These suggested that Notch1 was not the primary signaling pathway in Th17 polarization as well as in the induction of EAE. Next, we focused on Notch3 whose role has been implicated in the induction of EAE by blocking antibodies as well as in Th1 polarization to further study Th polarization as well as the induction of EAE.

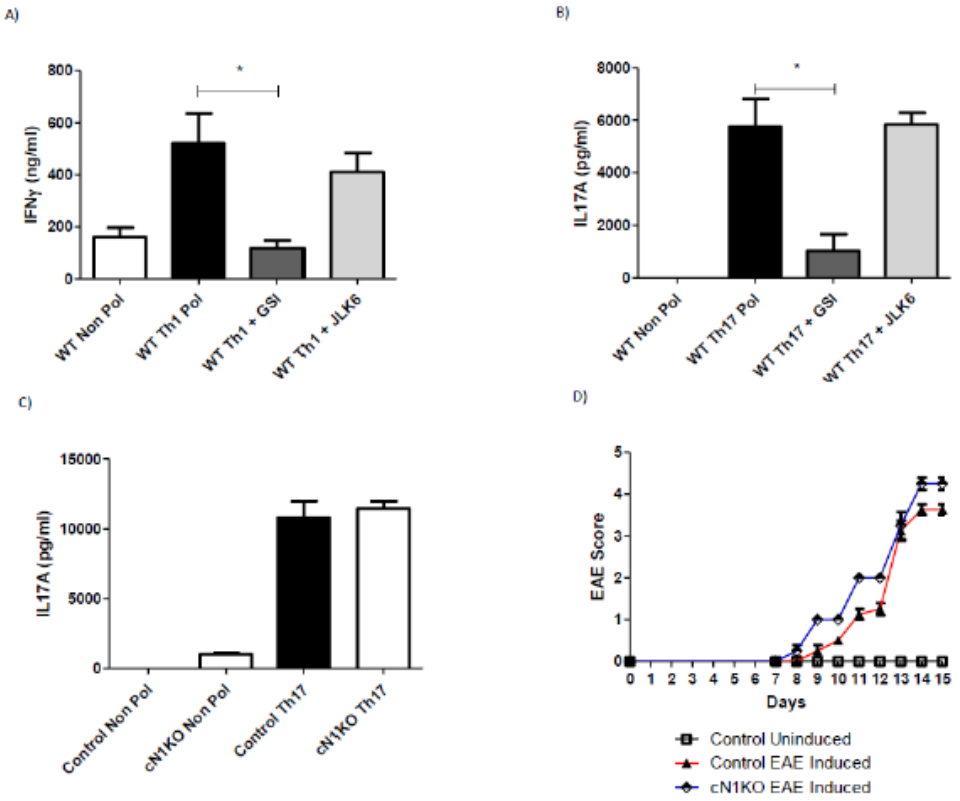

Figure 4. Notch sparing GSI does not prevent Th1 and Th17 polarization and, impaired Notch1 signaling does not affect Th17 polarization and the induction of EAE:

IFNγ and IL17A ELISAs for the supernatants of $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor under Non-Polarizing (NP), Th1 polarizing (Th1) and Th17 polarizing (Th17)conditions. **A,** IFNγ ELISA for WT cells incubated for 72 hours in the presence of GSI and Notch sparing GSI (JLK6). **B,** IL17A ELISA for WT cells incubated for 72 hours in the presence of GSI and Notch sparing GSI (JLK6). **C,** IL17A ELISA for control WT (control) versus conditional Notch1 Knockout (cN1KO) cells were incubated for 72 hours under Th17 polarizing conditions. **D,** EAE scores of Control versus conditional N1KO knockout (cN1KO) mice at day 15. All data represent at least three independent experiments. Data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

2.2. Notch3 is expressed in $CD4^+$ T cells after TCR activation and decreasing its expression impairs Th17 cell polarization:

In order to determine whether Notch3 was activated after T cell receptor (TCR) signaling, we did Q-RT-PCR for Notch3. According to our results Notch3 has the highest expression at 24 hour time point after TCR activation by plate bound anti-CD3 and anti-CD28 and its expression decreases at later time points (Figure 5). In order to examine the role of Notch3 in Th17 polarization, shRNA plasmids targeting Notch3 expression or Notch3 intracellular domain (N3ICD) overexpression construct were delivered by viral infection .We did Q-RT-PCR to show the decreased or increased Notch3 expression in infected WT $CD4^+$ T cells and our infection method was efficient enough to either decrease or increase Notch3 expression (Figure 6). When Notch3 was knocked down by lentiviral infection to deliver shRNA plasmid to the $CD4^+$ T cells, compared to control scrambled RNA plasmid infected cells there was a significant decrease in IL-17A production under Th17 polarizing conditions at 72 hour time point (Figure 6). We delivered (N3ICD) over-expression vectors through retroviral infection. Compared to cells infected with control scrambled RNA plasmid there was an increase in IL-17A production of N3ICD plasmid infected WT $CD4^+$ T cells under Th17 polarizing conditions (Figure 6). These results suggested an important role for Notch3 in $CD4^+$ T cells polarization of Th17 cells which play a major role in the induction of EAE and other autoimmune diseases. In order to further dissect the role of Notch3 in $CD4^+$ T cell activity and differentiation we used Notch3 knockout mice (N3KO) in our experiments.

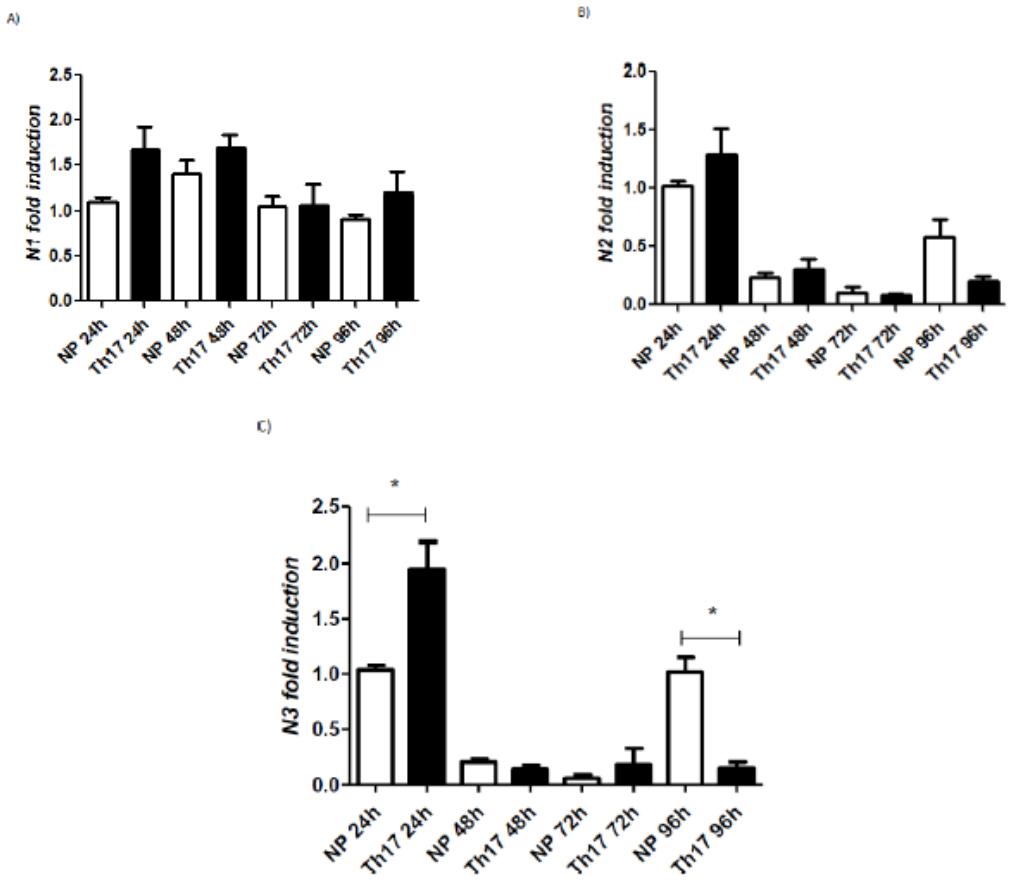

Figure 5. Notch1, Notch2 and Notch3 genes are expressed in CD4+ T cells throughout 96 hour time course:

A) Notch1 mRNA levels by q-RT-PCR in wild type (WT) $CD4^+$ T cells at 24, 48, 72 and 96 hour time points after incubation in anti-CD3 and anti-CD28 coated wells to activate T cell receptor. **B)** Notch2 mRNA levels for the samples in A **C)** Notch3 mRNA levels for the samples in A. All data represent at least three independent experiments. Data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

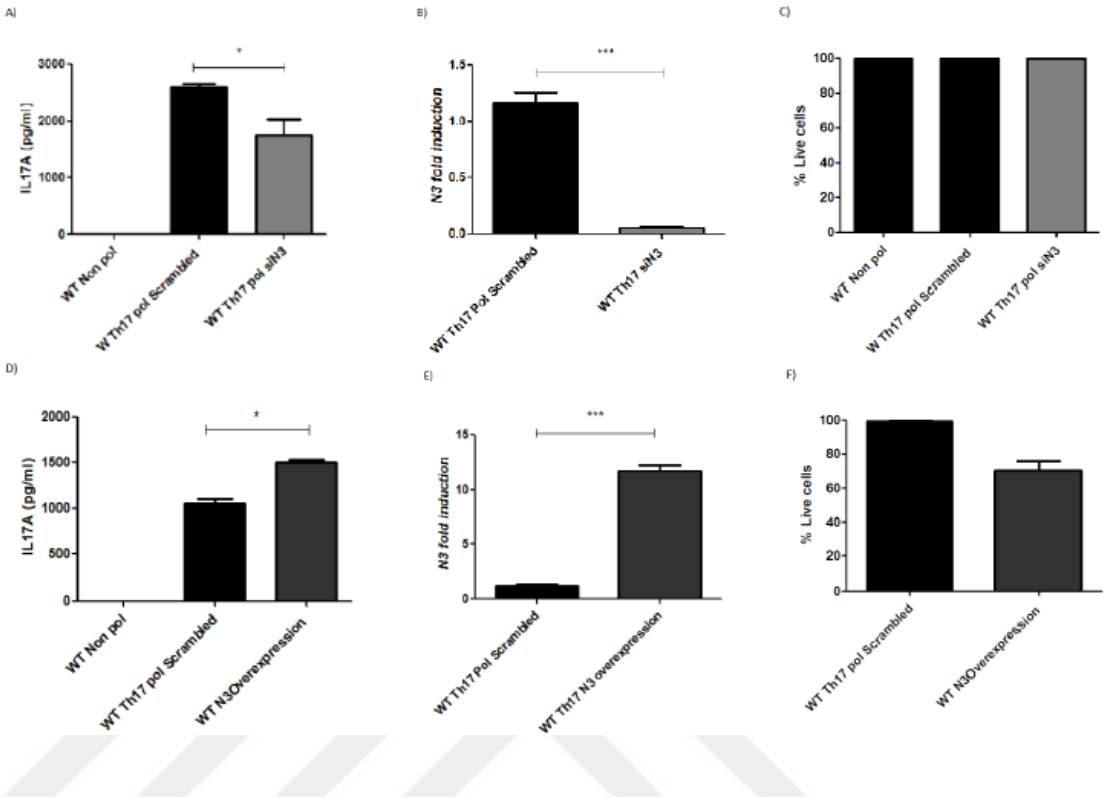

Figure 6. Knocking-down Notch3 expression causes decrease in IL17A production whereas over-expression of the intracellular domain of Notch3 increases IL17A production by Th17 polarized wild type (WT) CD4+ T cells:

IL17A ELISA for the supernatants of $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor under Non-Polarizing (NP) and Th17 polarizing (Th17) conditions. **A)** IL17A ELISA for scrambled control versus shNotch3 containing plasmid infected WT $CD4^+$ T cells were incubated for 72 hours. **B)** Notch3 q-RT-PCR for the samples from A. **C)** Cell viability for the samples from A and B. **D)** IL17A ELISA for scrambled control versus Notch3 intracellular domain containing plasmid infected WT $CD4^+$ T cells were incubated for 72 hours. **E)** Notch3 q-RT-PCR for the samples from C. **F)** Cell viability for the samples from D and E. All data represent at least three independent experiments. Data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

2.3. Impaired Notch3 signaling does not affect $CD4^+$ T cell development, proliferation and activation:

For our experiments, we used Tom Gridley N3KO animals and in order to make sure that phenotypes that we would observe were independent of the early development defects in Th cell generation as well as defects in Th cell proliferation and activation, we measured $CD4^+$ and $CD8^+$ T cell levels in thymus and the spleen of WT versus N3KO mice and, rate of Th cell proliferation and activation marker levels *in vitro* comparing two mice groups. There was not any observable impairment in the development of CD4 T cells since the percentages of $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ cells were similar in the spleen and thymus of WT and N3KO mice (Figure 7 and Figure 8). N3KO $CD4^+$ T cells did not have any Notch3 expression, whereas WT cells were expressing Notch3 gene at 72 hour time point (Figure 7). When we labeled the cells with CFSE to track the rate of cell proliferation there was no difference between WT and N3KO $CD4^+$ T cells after 72 hours of TCR activation (Figure 9). We measured the percentage and the expression levels of CD25 and CD69 surface markers as a way of examining the cell activation. There was no difference in the percentages and expression levels of CD25 and CD69 between WT and N3KO $CD4^+$ T cells after 72 hours of TCR activation (Figure 10). These results suggest that Notch3 did not affect CD4 and CD8 T cell development based on cell percentages and, the rate of cell proliferation and activation were not impaired when Notch3 signaling was abrogated. Next, we wanted to examine the role of Notch3 in T helper cell polarization by using these N3KO $CD4^+$ T cells.

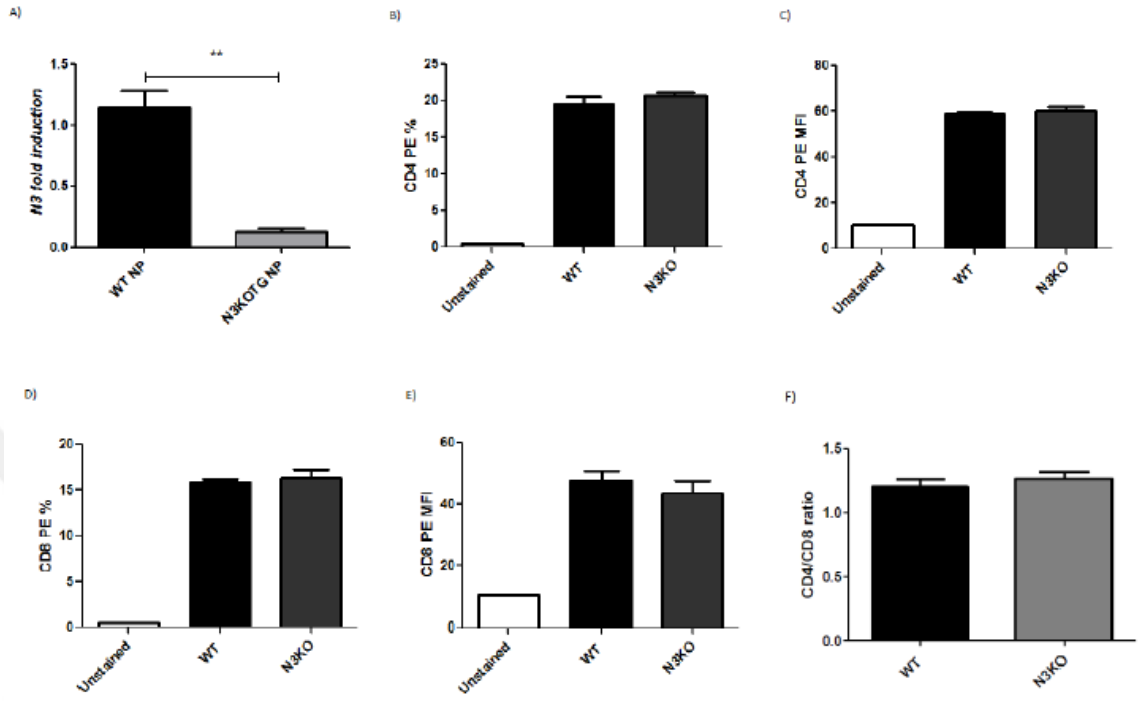

Figure 7. CD4+ T cells have reduced Notch3 expression in Notch3 homozygous knockout (N3KO) mice compared to wild type (WT) ones. CD4+ and CD8+ T cell numbers in the spleens of WT versus N3KO animals are similar:

A) Notch3 q-RT-PCR by using primers against C terminus of Notch3 in wild type WT versus Notch3 Homozygous Knockout (N3KO) $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor for 72 hours. **B, C)** CD4 staining and FACS analysis for wild type WT and Notch3 Homozygous Knockout (N3KO) splenocytes. **D, E)** CD8 staining and FACS analysis for wild type WT and Notch3 Homozygous Knockout (N3KO) splenocytes. **F)** CD4 to CD8 ratio for the samples from B and D. All data represent at least three independent experiments. Data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

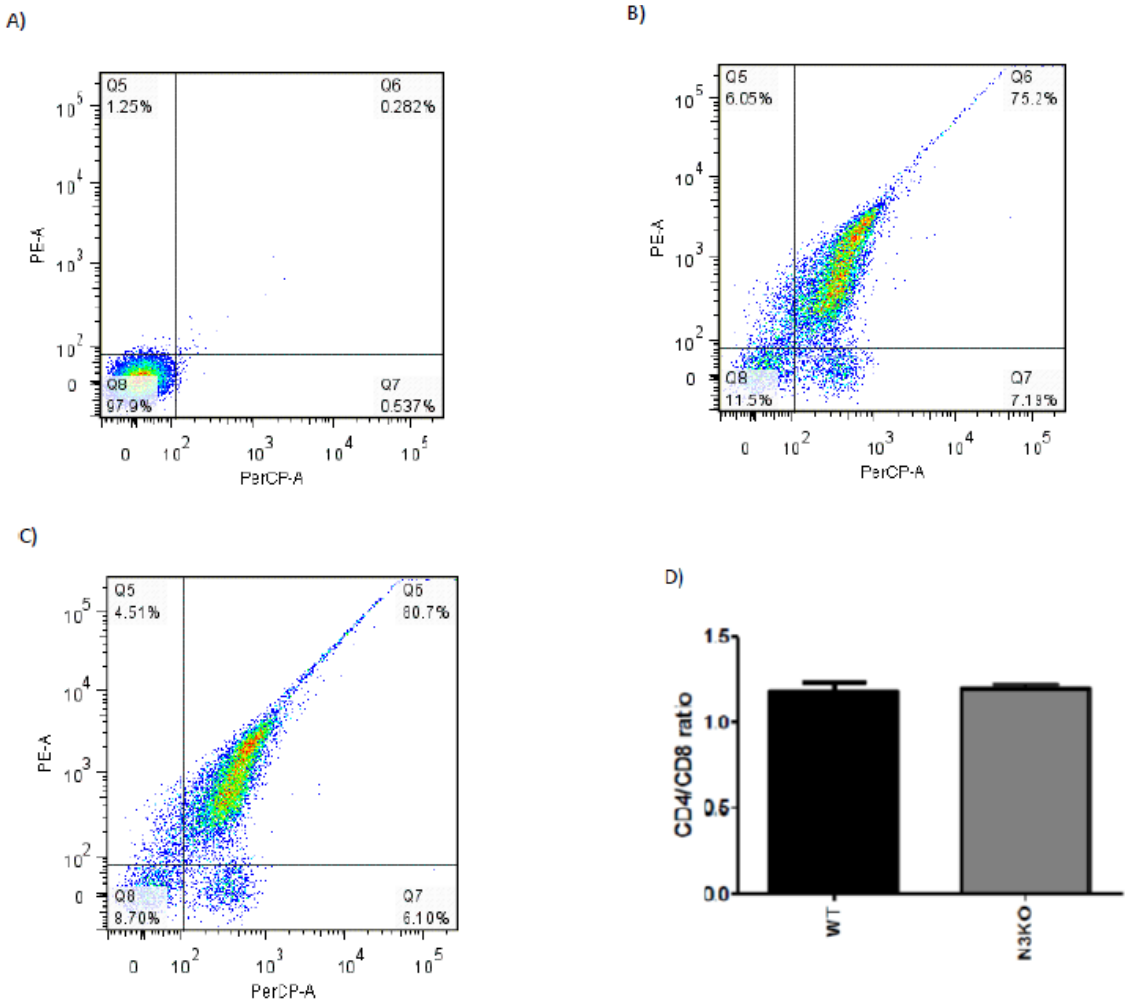

Figure 8. CD4+ and CD8+ cell numbers in the thymii of WT versus N3KO animals are similar:

A) CD4 staining and FACS analysis for wild type WT and Notch3 Homozygous Knockout (N3KO) thymocytes, unstained control CD8-PE and CD4-PerCp antibodies. **B)** CD4 staining and FACS analysis for wild type WT and Notch3 Homozygous Knockout (N3KO) thymocytes. **C)** CD8 staining and FACS analysis for wild type WT and Notch3 Homozygous Knockout (N3KO) thymocytes. **D)** CD4 to CD8 ratio for the samples from B and C.

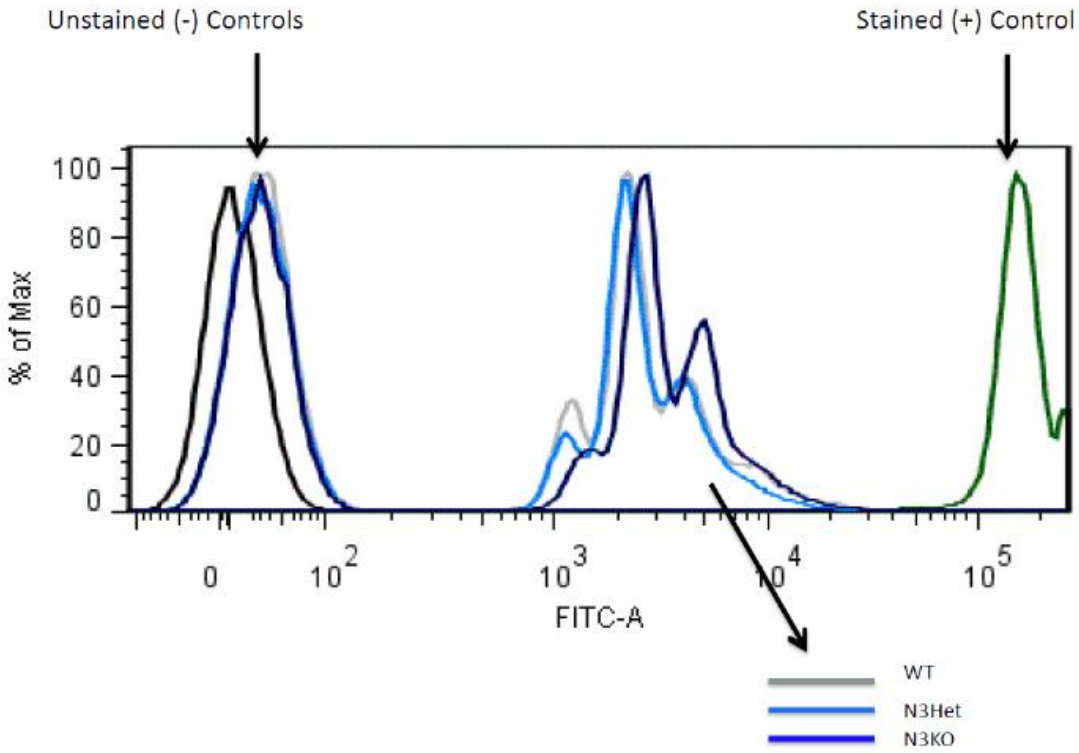

Figure 9. T cell receptor activation induced proliferation is similar between wild type (WT), Notch3 Heterozygous Knockout (N3Het), and Notch3 Homozygous Knockout (N3KO) CD4+ T cells:

CFSE labeling and FACS analysis for wild type WT, Notch3 Heterozygous Knockout (N3Het), and Notch3 Homozygous Knockout (N3KO) $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor for 72 hours.

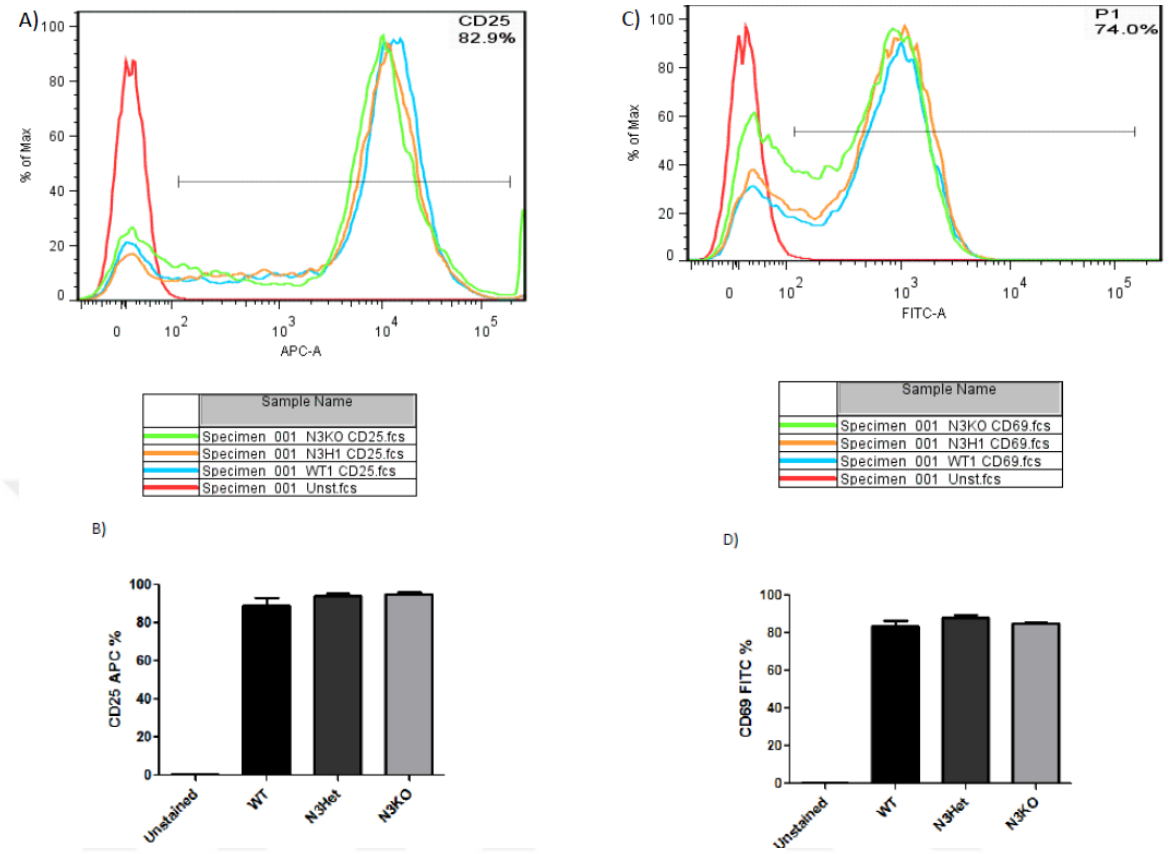

Figure 10. T cell receptor activation induced CD25 and CD69 surface expression is similar between wild type (WT), Notch3 Heterozygous Knockout (N3Het), and Notch3 Homozygous Knockout (N3KO) CD4+ T cells:

A, B) CD25 staining and FACS analysis for wild type WT, Notch3 Heterozygous Knockout (N3Het), and Notch3 Homozygous Knockout (N3KO) $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor for 72 hours. **C, D)** CD69 staining and FACS analysis for wild type WT, Notch3 Heterozygous Knockout (N3Het), and Notch3 Homozygous Knockout (N3KO) $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor for 72 hours.

2.4. Notch3 signaling regulates $CD4^+$ T cell polarization towards Th1, Th2, Th17 and iTreg fates:

In order to study the role of Notch3 in Th1 cell polarization, we polarized $CD4^+$ T cells towards Th1 and measured the Th1 signature cytokine IFN-γ levels in the supernatants at 72 hour time point. Compared to WT cells Notch3 heterozygous knockout (N3Het) and N3KO cells had significant decrease in IFN-γ production under Th1 polarizing conditions (Figure 11). To examine the role of Notch3 in Th17 differentiation, we measured IL-17A levels as the signature cytokine of Th17 cells. In N3Het and N3KO $CD4^+$ T cells there was a significant decrease in IL-17A levels compared to WT cells under Th17 polarizing conditions (Figure 12). Literature supports imperative role of Th1 and Th17 cells in the induction of EAE as they sustain inflammatory environment. To assess the role of Notch3 in iTreg polarization, we measured $CD25^+$ $FoxP3^+$ cell percentages. There was impaired iTreg polarization in N3KO $CD4^+$ T cells compared to WT cells (Figure 14). When we measured nTreg levels in the thymus of N3KO mice compared to WT mice, the $CD25^+FoxP3^+$ cell percentages were similar (Figure 14). In order to examine the role of Notch3 in Th2 polarization, we measured IL-4 and IL-5 levels as signature cytokine of Th2 polarization in the supernatants of Th2 polarized cells at 72 hour time point. There was increase in IL-4 and IL-5 levels between WT and N3KO $CD4^+$ T cells (Figure 13). To study the role of Notch3 in GMCSF production, we

measured GMCSF cytokine levels in the supernatants of non-polarized cells at 72 hour time point, there was an increase in GMCSF production by N3KO $CD4^+$ T cells compared to WT ones (Figure 13). Overall, there was a decrease in inflammatory Th1 and Th17 cell polarization *in vitro* in N3KO mouse $CD4^+$ T cells compared to WT ones. Whereas there was an increase in Th2 polarization and GMCSF production in N3KO cells compared to WT ones. Our next aim was examining the role of canonical versus non-canonical Notch signaling in Th17 polarization.

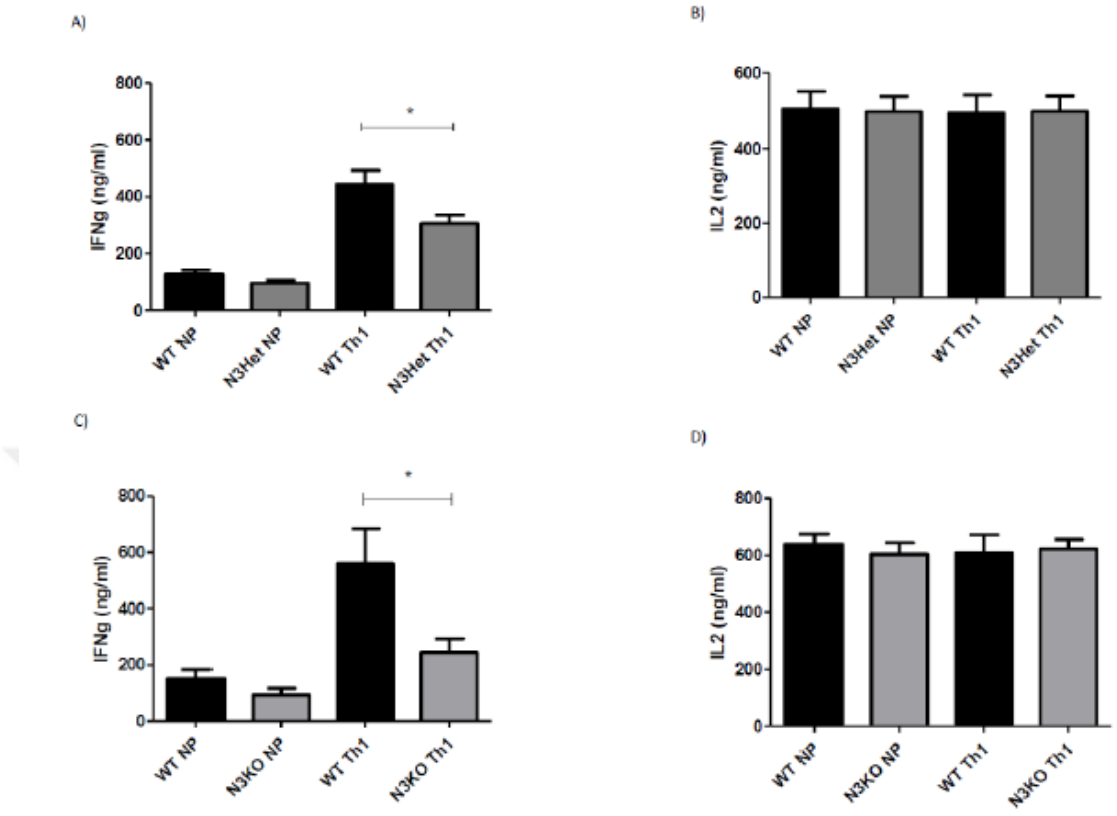

Figure 11. Notch3 regulates IFNγ protein levels in Th1 cells:

IFNγ and IL2 ELISAs for the supernatants of $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor under Non-Polarizing (NP) and Th1 polarizing (Th1) conditions. **A,** IFNγ ELISA for WT versus Notch3 Heterozygous Knockout (N3Het) cells were incubated for 72 hours. **B,** IL2 ELISA for the samples in A. **C,** IFNγ ELISA for WT versus Notch3 Homozygous Knockout (N3KO) cells were incubated for 72 hours. **D,** IL2 ELISA for the samples in C. All data represent at least three independent experiments. Data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

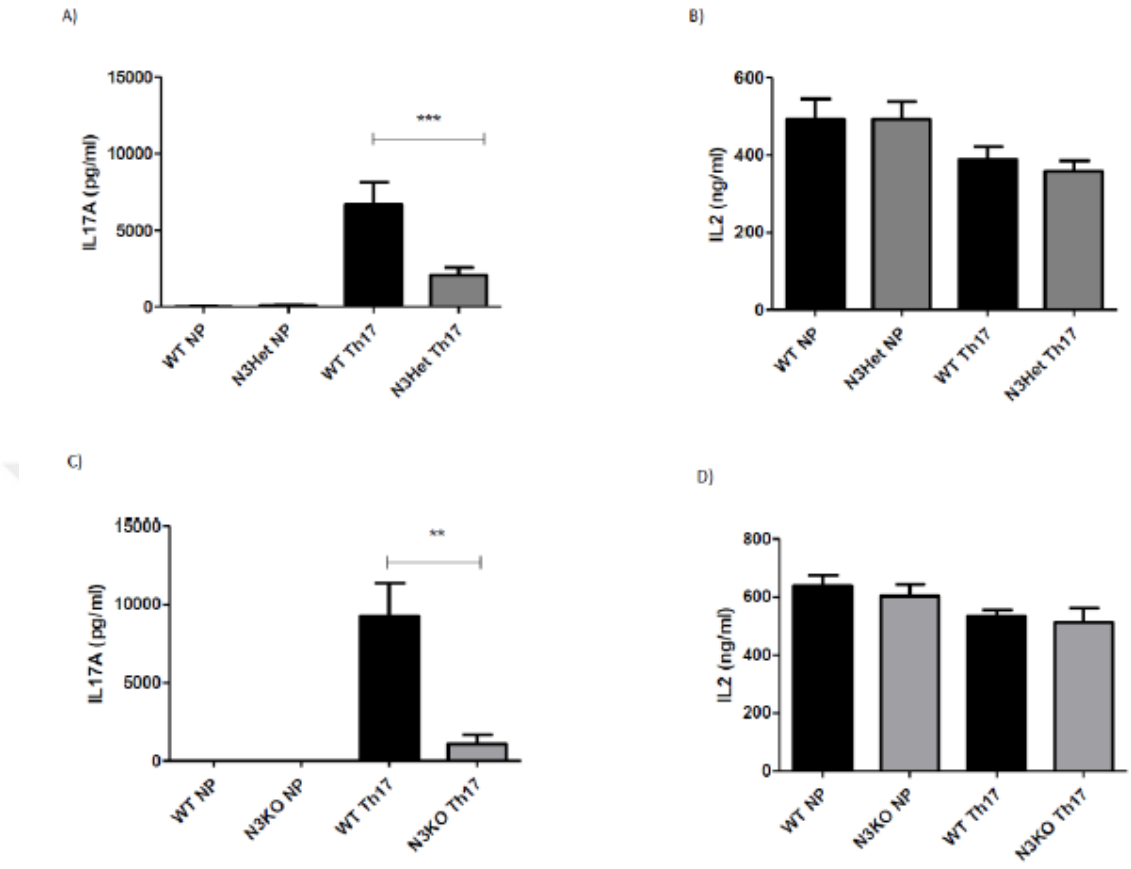

Figure 12. Notch3 regulates IL17A production by Th17 cells:

IL17A and IL2 ELISAs for the supernatants of $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor under Non-Polarizing (NP) and Th17 polarizing (Th17) conditions. **A,** IL17A ELISA for WT versus Notch3 Heterozygous Knockout (N3Het) cells were incubated for 72 hours. **B,** IL2 ELISA for the samples in A. **C,** IL17A ELISA for WT versus Notch3 Homozygous Knockout (N3KO) cells were incubated for 72 hours. **D,** IL2 ELISA for the samples in C. All data represent at least three independent experiments. Data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

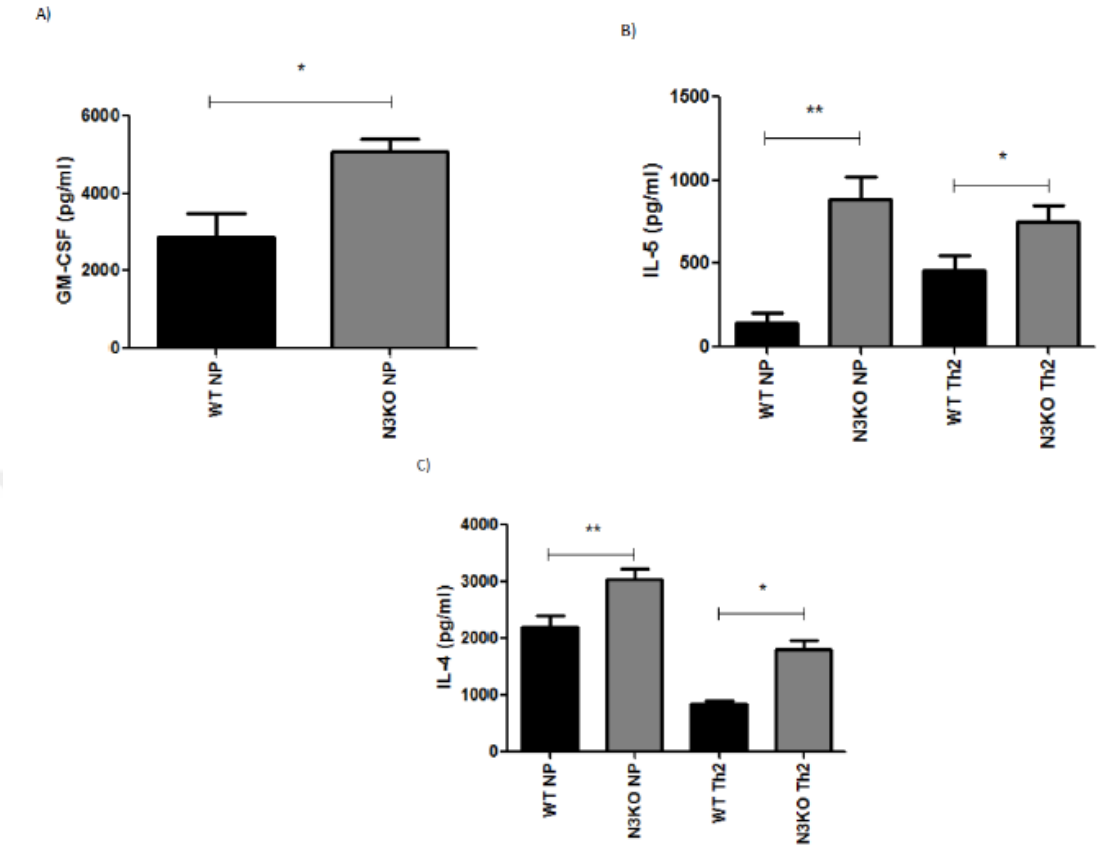

Figure 13. Notch3 regulates GMCSF, IL4 and IL5 production by CD4+ T cells:

GMCSF, IL4 and IL5 ELISAs for the supernatants of $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate wild type (WT) versus Notch3 Homozygous Knockout (N3KO) T cell receptor under Non-Polarizing (NP) and Th2 polarizing (Th2) conditions. **A,** GMCSF levels after 72 hours. **B,** IL5 levels after 72 hours. **C,** IL4 levels after 72 hours. All data represent at least three independent experiments. Data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

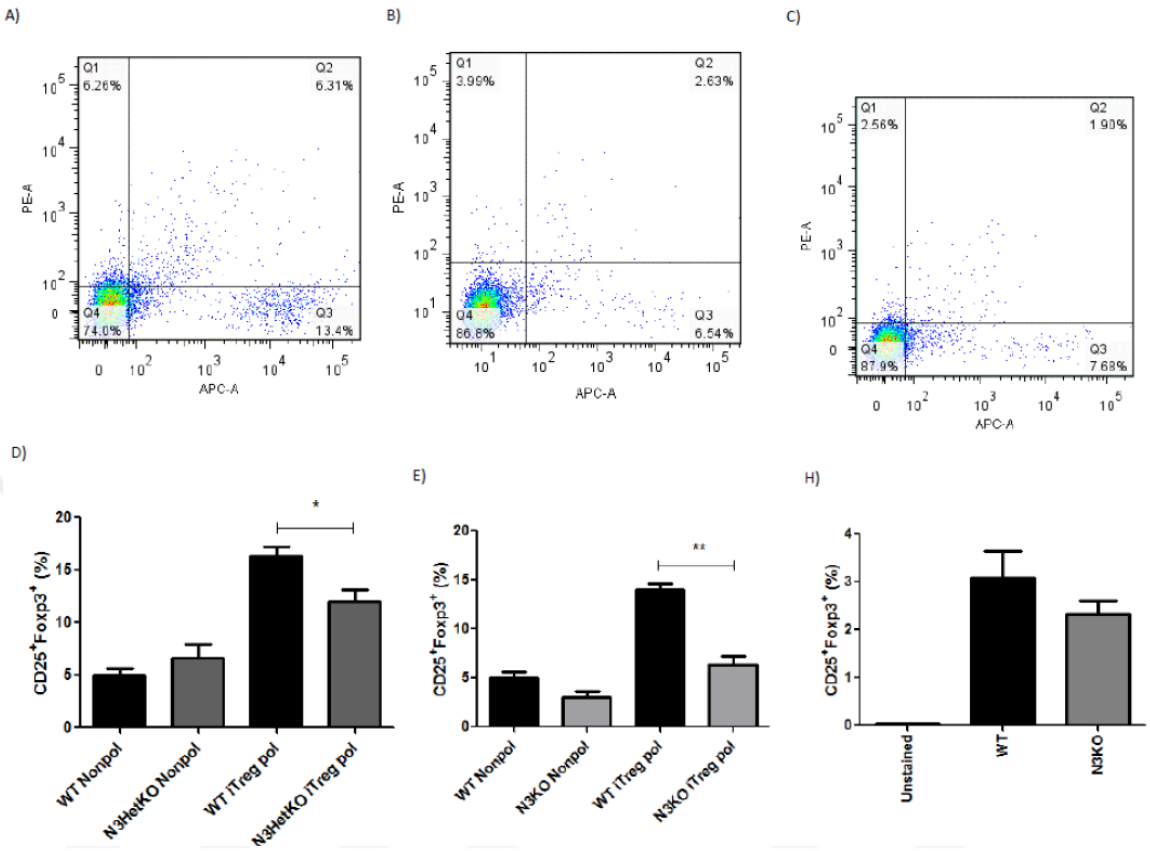

Figure 14. Notch3 regulates iTreg polarization of CD4+ T cells, whereas it does not affect nTreg levels in Thymii of mice:

CD25-APC and FoxP3-PE double staining and FACS analysis for wild type (WT), Notch3 Heterozygous Knockout (N3Het), and Notch3 Homozygous Knockout (N3KO) $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor for 96 hours. **A)** WT control representative data **B)** N3Het representative data, **C)** N3KO representative data. **D)** WT versus N3Het samples. **E)** WT versus N3KO samples. **H)** CD25 and FoxP3 double staining and FACS analysis for wild type (WT) and Notch3 Homozygous Knockout (N3KO) mice thymii. All data represent at least three independent experiments. Data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

2.5. Non-canonical Notch signaling PKCθ positively regulates Th17 polarization whereas NFκB has negative effect on Th17 differentiation and, RBPJκ dependent canonical Notch signaling is not required for Th17 polarization:

In order to examine the role of canonical versus non-canonical Notch signaling in Th17 cell polarization we used RBPJκ conditional knockout versus control WT mice as well as pharmacological inhibitors Rottlerin for PKCθ and Bay11 for NFκB signaling. When RBPJκ expression was decreased there was a tendency for increased IL-17A production in Th cell compared to WT ones under Th17 polarizing conditions (Figure 15). This suggests a de-repressive effect of impaired RBPJκ signaling on Th17 differentiation. In order to determine a non-canonical Notch signaling candidate required in Th17 polarization, we used NFκB inhibitor Bay11 since it has been shown that NFκB plays a role in Th1 polarization as well as in Notch signaling pathway. IL-17A production was significantly higher in Bay11 treated WT $CD4^+$ T cells compared to DMSO treated control ones under Th17 polarizing conditions (Figure 15). In order to further assess the role of non-canonical Notch signaling in Th17 polarization we used PKCθ inhibitor Rottlerin. Studies have shown the role of PKCθ in Th cell activation as well as its interaction with Notch signaling pathway. IL-17A production was significantly decreased in Rottlerin treated WT $CD4^+$ T cells compared to DMSO treated control ones under Th17 polarization conditions (Figure 15). Our results suggest that non-canonical Notch signaling through PKCθ positively regulates Th17 polarization whereas NFκB is a negative regulator of Th17 differentiation. Our next aim was examining the role of

Notch3 in the induction of an autoimmune disease model EAE by using WT versus N3KO mice.

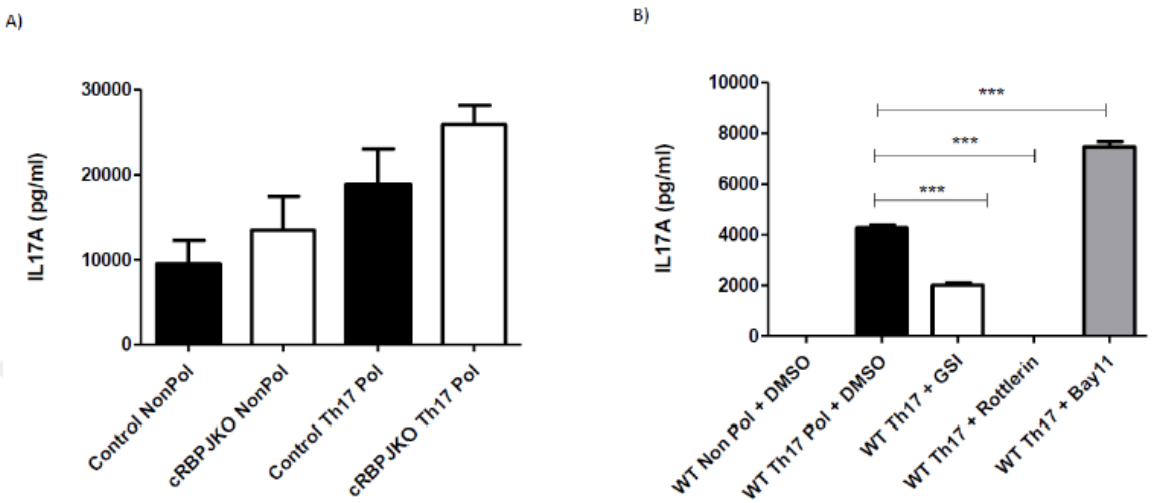

Figure 15. Non-canonical PKCθ dependent Notch signaling regulates Th17 polarization of CD4+ T cells:

IL17A ELISA for the supernatants of $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor under Non-Polarizing (NP) and Th17 polarizing (Th17) conditions. **A,** IL17A ELISA for control WT (control) versus conditional RBPJκ Knockout (cRPJKO) cells were incubated for 72 hours. **B,** IL17A ELISA for WT cells incubated for 72 hours in the presence of gamma secretase inhibitor (GSI), PKCθ inhibitor (Rottlerin) and NFκB inhibitor (Bay11). Data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

2.6. Impaired Notch3 signaling does not affect the induction of EAE:

Next, we were interested in whether the *in vitro* decrease in Th cell differentiation in Th1 and Th17 polarization would manifest itself in a physiologically relevant case, EAE. We used MOG_{35-55} peptide in complete Freund's adjuvant (CFA) to induce EAE. Pertussis toxin was used to breach the blood brain barrier. When we induced EAE, compared to WT mice N3KO mice had similar disease scores throughout the 28 day time period (Figure 16). At the peak of the disease (15 days), we isolated the splenocytes of EAE induced mice and re-stimulated them in the presence of MOG_{35-55} peptide for 5 days (Figure 17). Compared to WT control, the re-stimulated splenocytes of EAE induced N3KO mice had significantly higher IFN-γ production whereas IL-17A and GMCSF production was similar (Figure 18). Similar IL-17A, GMCSF and increased IFN-γ production in the supernatants of EAE induced N3KO splenocytes are not in line with our *in vitro* observations for the role of Notch3 in Th1 and Th17 polarization. This discrepancy might be due to other cellular mechanisms *in vivo* to compensate impaired Notch3 signaling.

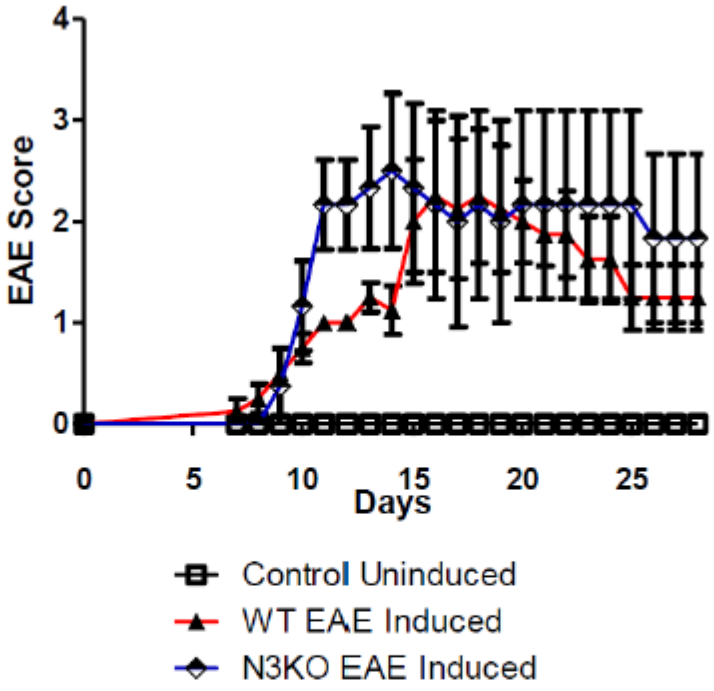

Figure 16. Non-canonical PKCθ dependent Notch signaling regulates Th17 polarization of CD4+ T cells:

A) EAE disease scores of WT versus N3KO mice during 28 day time course (N=12 for each group).

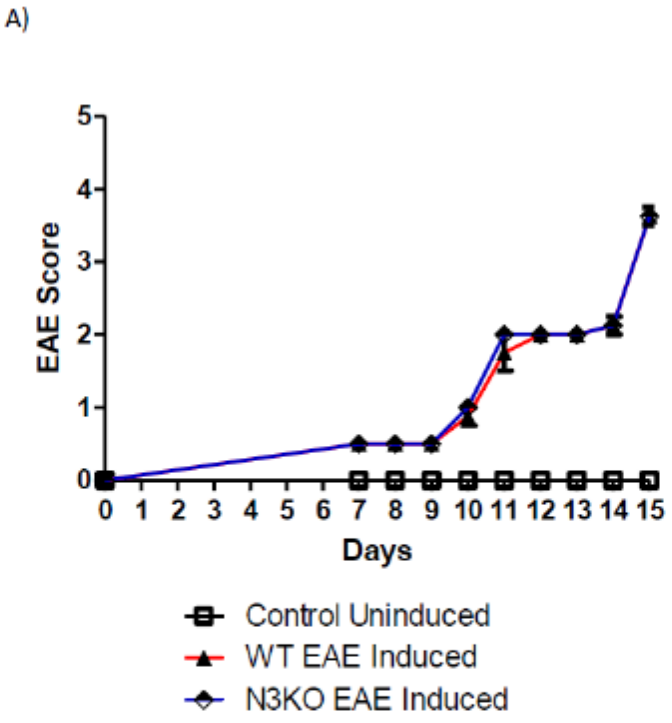

Figure 17. The induction of EAE is similar between wild type (WT) versus Notch3 Homozygous Knockout (N3KO) mice:

A) EAE disease scores of WT versus N3KO mice during 15 daytime course. (N=12 for each group)

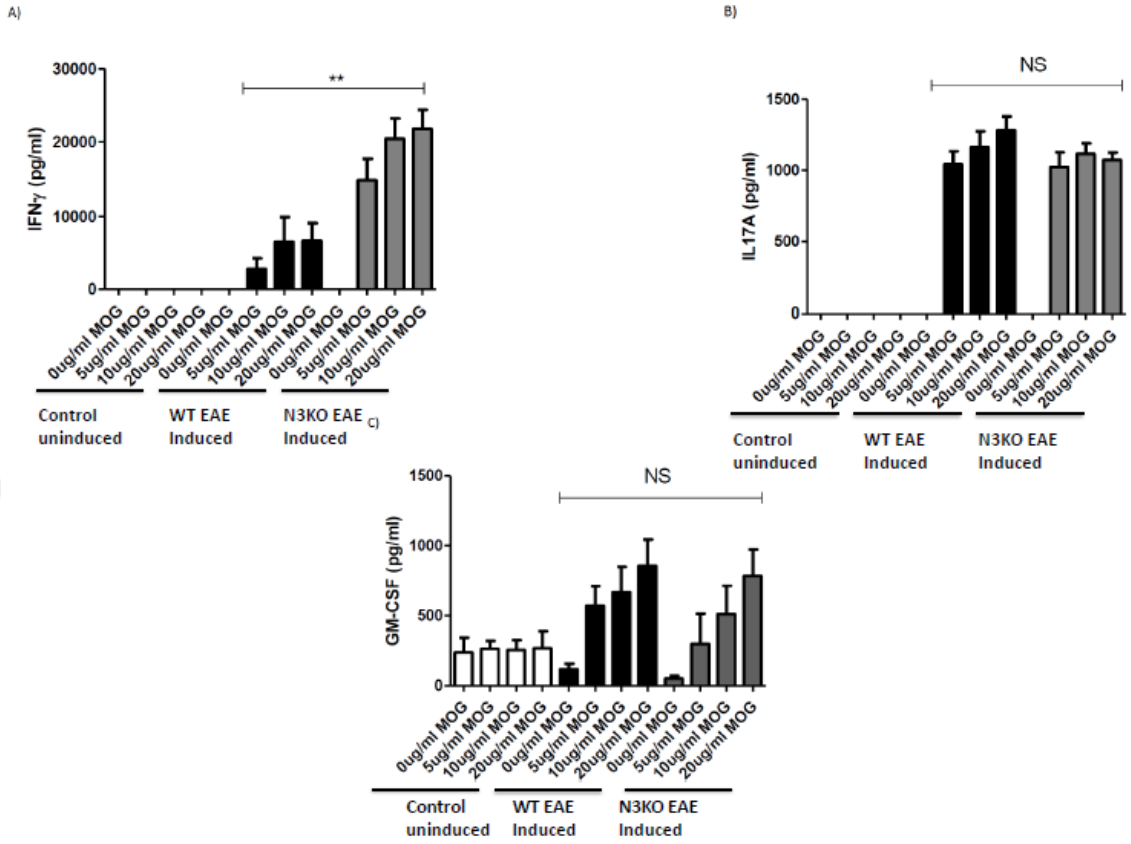

Figure 18. IFNγ production is higher by the MOG peptide restimulated splenocytes of EAE induced Notch3 Homozygous Knockout (N3KO) mice compared to wild type (WT) ones whereas IL17A and GMCSF levels are similar:

ELISAs for the supernatants of splenocytes incubated for 5 days with MOG peptide **A)** IFNγ ELISA. **B)** IL17A ELISA. **C)** GMCSF ELISA. N=8 for each group and data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

2.7. Differentially generated William Skarnes Notch3 knockout mice gave a different phenotype for Th17 polarization:

In order to further strengthen our observations we used a different N3KO mouse strain (Figure 19). There was no difference in CD4 and CD8 cell percentages as well as CD4 and CD8 double positive cell percentages in the splenocytes of WT versus N3KO mice (Figure 22). We polarized $CD4^+$ T cells towards Th17 and measured the IL-17A levels in the supernatants at 72 hour time point. Compared to WT cells, N3KO cells had higher tendency of IL-17A production under Th17 polarizing conditions (Figure 20). When we measured nTreg levels in the thymus of N3KO mice compared to WT mice, the $CD25^+FoxP3^+$ cell percentage was significantly higher in WT ones (Figure 21). When we labeled the cells with CFSE to track the rate of cell proliferation there was no difference between WT and N3KO $CD4^+$ T cells after 72 hours of TCR activation (Figure 24). We measured the percentage and the expression levels of CD25 and CD69 surface markers as a way of examining the cell activation. There was no difference in the percentages and expression levels of CD25 and CD69 between WT and N3KO $CD4^+$ T cells after 72 hours of TCR activation (Figure 23). Since the Th17 polarization results were in conflict with previous studies showing the role of Notch signaling in Th17 polarization and also our siNotch3 experiment results, we did not further examine this mouse line.

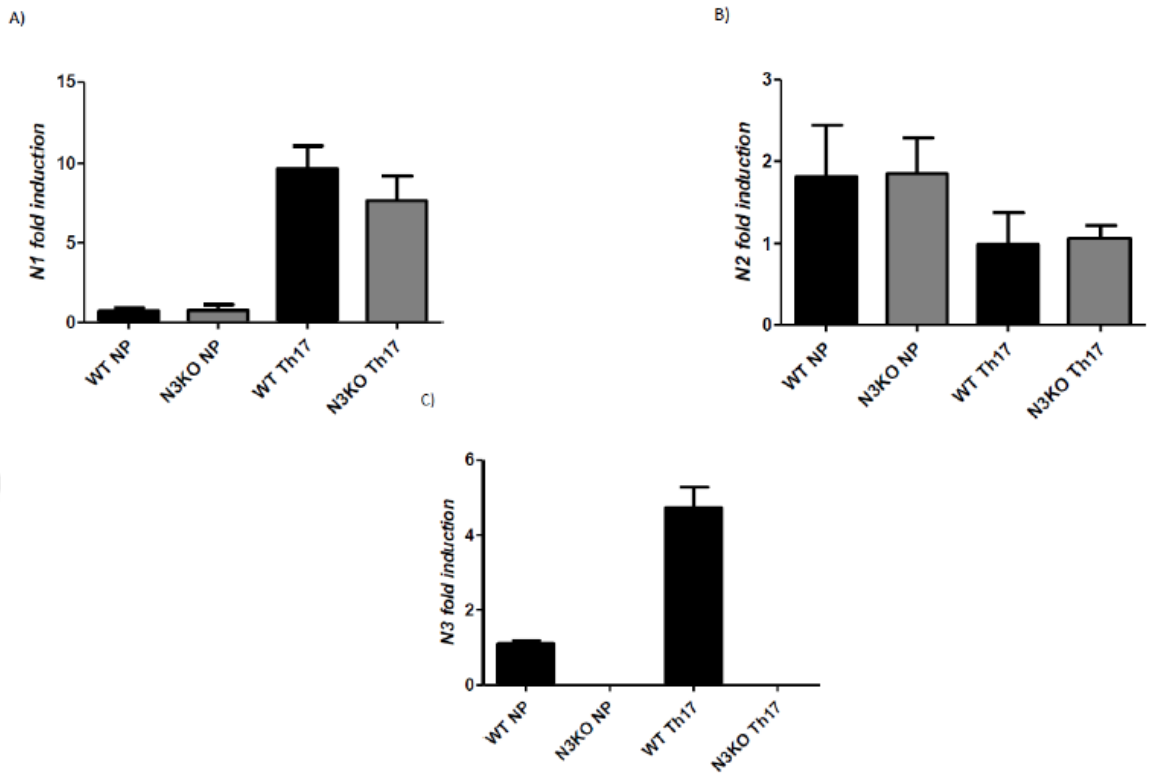

Figure 19. Notch1, Notch2 and Notch3 gene expression levels in N3KO William Skarnes Mice:

A) Notch1 mRNA levels by q-RT-PCR in wild type (WT) vs N3 homozygous Knockout William Skarnes Mice (N3KO) $CD4^+$ T cells at 72 hour time point after incubation in anti-CD3 and anti-CD28 coated wells to activate T cell receptor. **B)** Notch2 mRNA levels for the samples in A **C)** Notch3 mRNA levels for the samples in A. All data represent at least three independent experiments. Data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

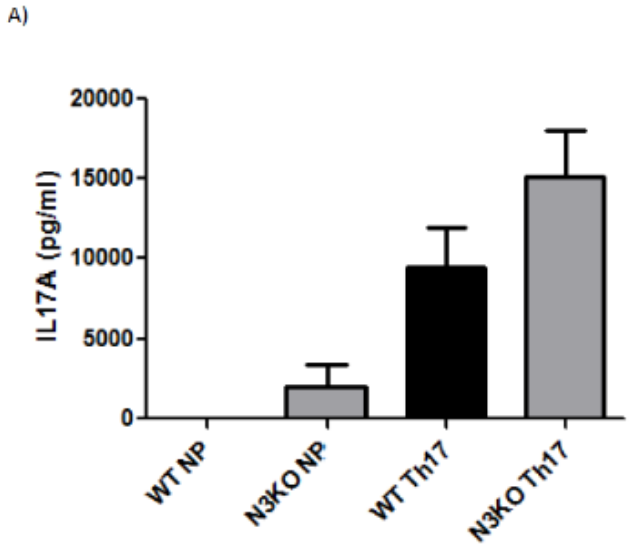

Figure 20. Notch3 regulates IL17A production by Th17 cells:

IL17A ELISA for the supernatants of $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor under Non-Polarizing (NP) and Th17 polarizing (Th17) conditions. **A,** IL17A ELISA for WT versus Notch3 Homozygous Knockout (N3KO) William Skarnes cells were incubated for 72 hours.

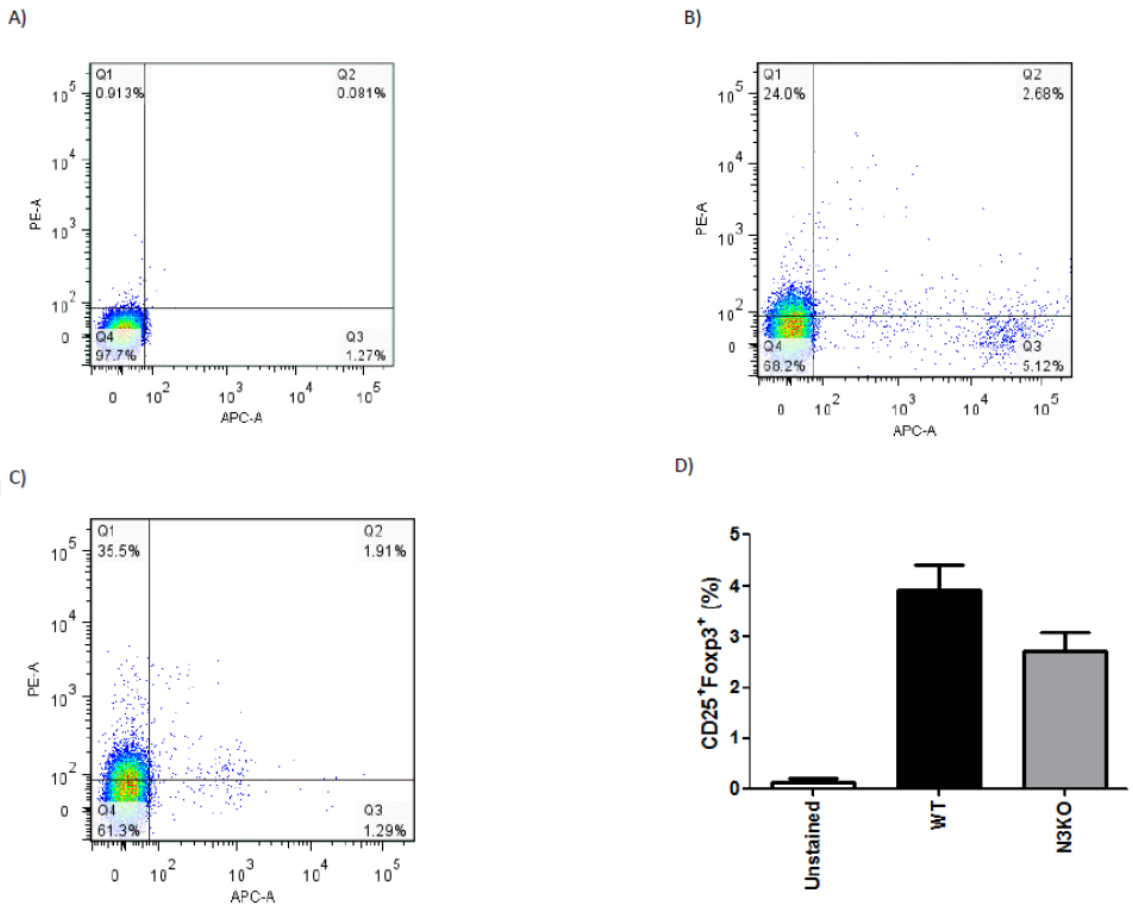

Figure 21. Notch3 regulates nTreg levels in Thymii of mice:

CD25-APC and FoxP3-PE double staining and FACS analysis for wild type (WT) and Notch3 Homozygous Knockout (N3KO) William Skarnes mice thymii. **A)** Unstained control, **B)** WT double stained sample, **C)** N3KO double stained sample. **D)** Data represents at least three independent experiments for nTreg levels in WT vs N3KO. Data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

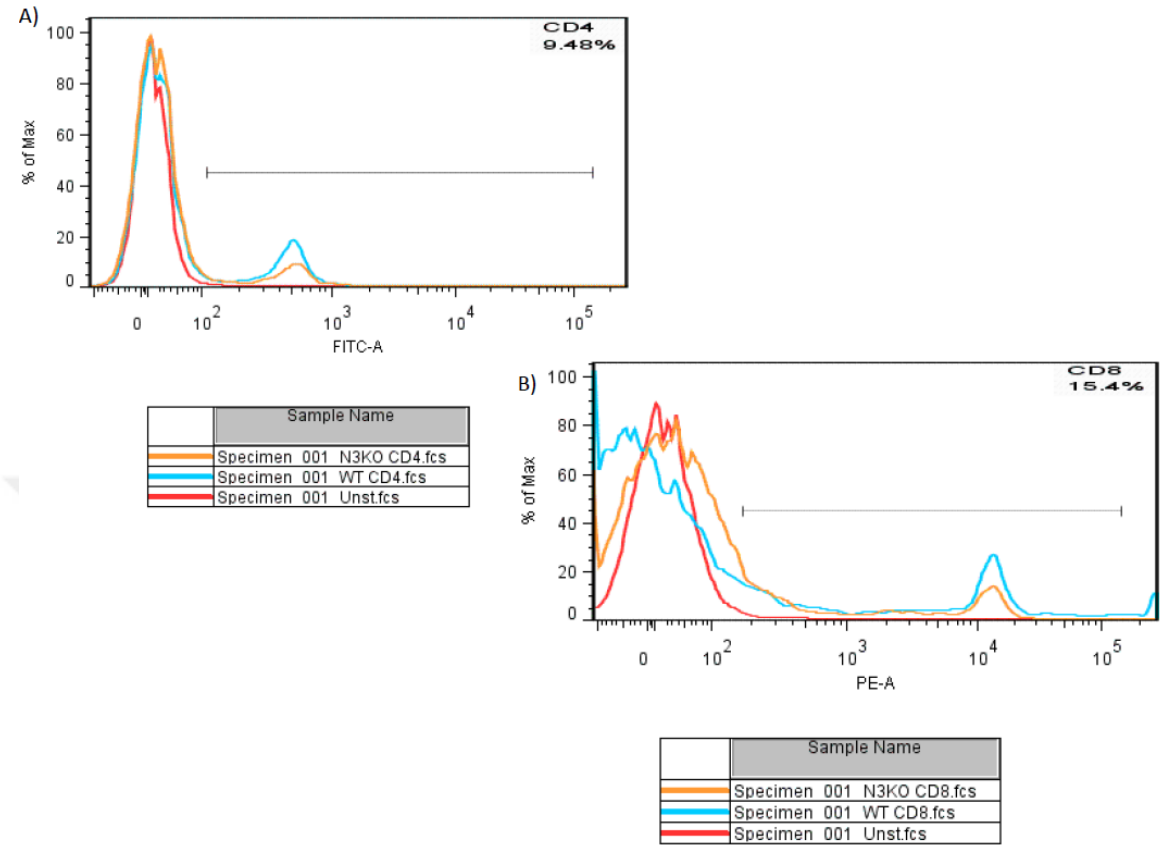

Figure 22. CD4+ and CD8+ T cells in the splenocytes of WT versus N3KO animals are similar:

CD4-FITC and CD8-PE staining and FACS analysis for wild type WT and Notch3 Homozygous Knockout (N3KO) William Skarnes splenocytes. **A)** CD4 staining and FACS analysis for wild type WT and Notch3 Homozygous Knockout (N3KO) William Skarnes splenocytes. **B)** CD8 staining and FACS analysis for wild type WT and Notch3 Homozygous Knockout (N3KO) William Skarnes splencoytes.

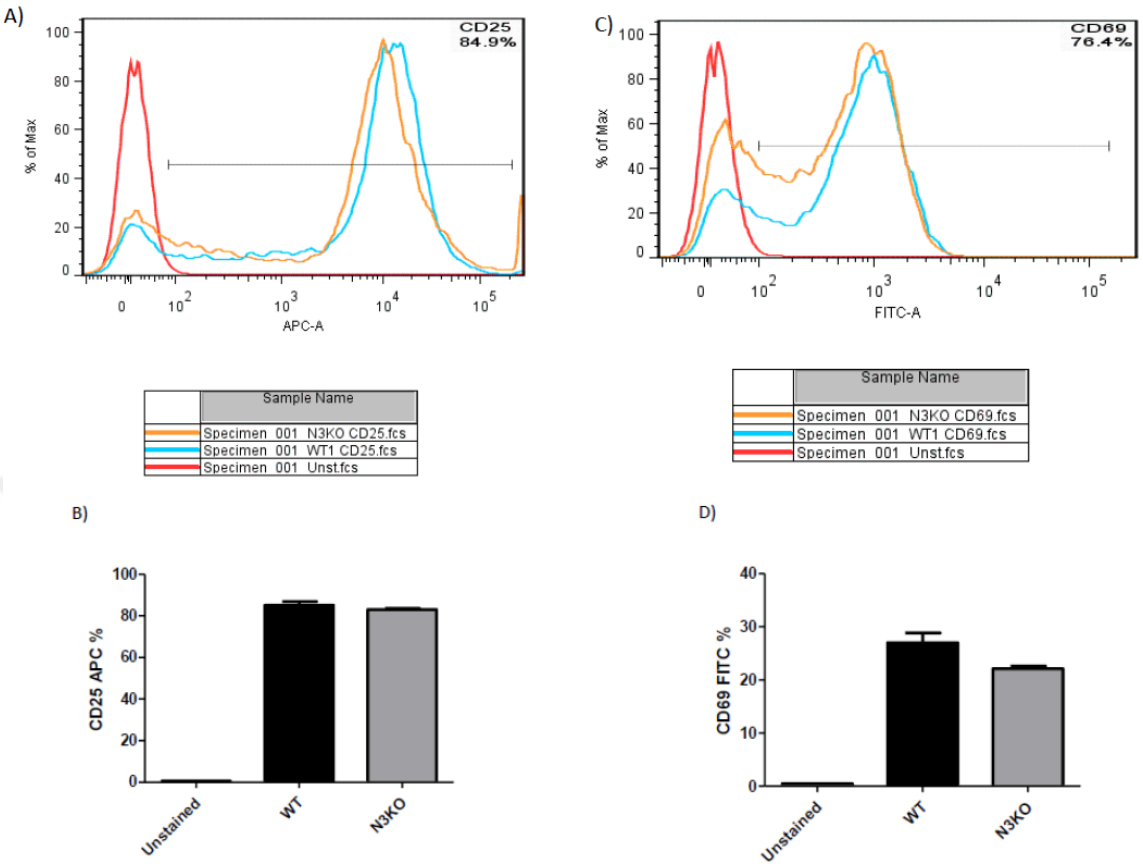

Figure 23. T cell receptor activation induced CD25 and CD69 surface expression is similar between wild type (WT) and Notch3 Homozygous Knockout (N3KO) CD4+ T cells:

A, B) CD25-APC staining and FACS analysis for wild type WT and Notch3 Homozygous Knockout William Skarnes (N3KO) $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor for 72 hours. **C, D)** CD69-FITC staining and FACS analysis for wild type WT and Notch3 Homozygous Knockout William Skarnes (N3KO) $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor for 72 hours.

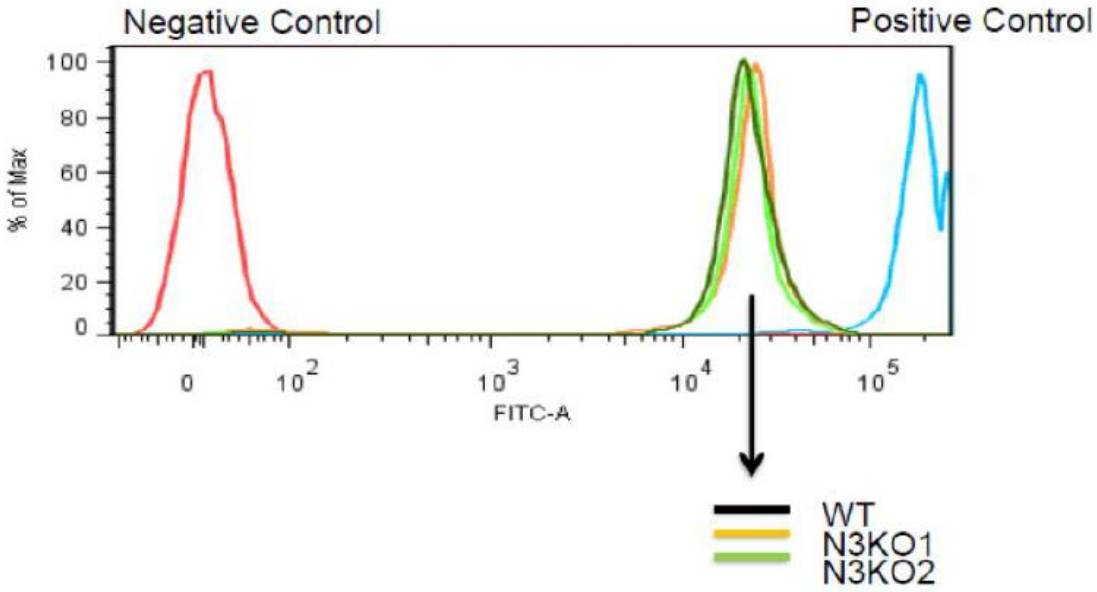

Figure 24. T cell receptor activation induced proliferation is similar between wild type (WT) and Notch3 Homozygous Knockout (N3KO) CD4+ T cells:

CFSE labeling and FACS analysis for wild type WT and Notch3 Homozygous Knockout William Skarnes (N3KO) $CD4^+$ T cells incubated in anti-CD3 and anti-CD28 coated wells to activate T cell receptor for 72 hours.

3. DISCUSSION:

It has been shown by our lab and others that Notch signaling plays an important role in the activation, proliferation and differentiation of Th cell sub-types [59, 60, 61, 62, 63]. These studies were conducted by using pharmacological inhibition of gamma secretase which significantly decreases polarization of Th cells towards Th1, Th2, Treg and Th17 fates as well as the induction of EAE [59-63, 65, 66]. There are studies showing the role of Notch signaling in direct regulation of GATA-3 expression, hence Th2 polarization [59-63]. Recently our lab showed that Notch1 regulates activation, proliferation of Th cells and their differentiation towards Th1 and iTreg fates [64].

In this study we have demonstrated that impaired Notch1 signaling did not affect the Th17 polarization of $CD4^+$ T cells as well as the induction of EAE. There are limitations in this kind of studies with Notch1. Since the deletion of Notch1 is embryonically lethal and its specific deletion in CD4 T cells affect Th cell development we used a mouse model in which we could conditionally knockdown Notch1. Residual Notch1 might be enough to drive Th17 polarization and also the induction of EAE in our knockdown model. Since previous studies with GSI treatment as well as DLL1 and DLL4 blocking antibody treatments already suggested that Notch signaling plays an important role in Th1 and Th17 polarization as well as the induction of EAE, we examined the role of another Notch family member in these processes [59-63, 65, 66, 68, 69].

There have been studies suggesting a role for Notch3 in T cell development, Th1 polarization as well as development of EAE [59-63, 65-69]. By siRNA targeting Notch3 it was shown that Th1 polarization was impaired *in vitro*. Notch3 overexpression caused increased Th1 polarization *in vitro* [59-63]. In another study, EAE was induced by adoptive transfer of lymphocytes and during *ex vivo* incubation of these lymphocytes cells were incubated either control solvent or Notch3 blocking antibody [67]. According to their observations there was decreased EAE scores when Notch3 blocking antibody was used compared to control groups. One caveat of the study was that blocking ability and the specificity of Notch3 blocking antibody was not validated. Furthermore, how the disease induction was impaired was not demonstrated through mechanistic details. In this study we used a Notch3 knockout mouse generated by Tom Gridley group to further expand our understanding for the role of Notch3 in Th cell differentiation as well as in the induction of EAE [70]. These mice are viable with no observable negative phenotype. First time to our knowledge in this study, we have demonstrated that Notch3 is crucial *in vitro* since its impaired activity abrogated pro-inflammatory phenotype of Th cells by decreasing Th1 and Th17 polarization. Absence of Notch3 signaling altered the balance of Th cell polarization since there was increased Th2 polarization as well as GMCSF production by Th cells from N3KO animals compared to WT ones *in vitro*. Our *in vivo* data suggests that Notch3 is not sufficient for the induction of EAE since the disease scores in N3KO mice compared to WT ones were similar. This discrepancy in the *in vitro* versus *in vivo* results might be because of other compensatory mechanisms for Th1 and Th17 polarization in the splenocytes. Therefore, blocking Notch3 signaling only is not a

promising candidate to impair pro-inflammatory Th cell differentiation in EAE setting as well as in MS.

Our data differs from previous studies showing that Notch3 blocking antibody decreased the EAE scores in an adoptive transfer model of EAE compared to control treated ones. Possible reasons for the different observations might be that in that study cells were treated *ex-vivo* for a certain period of time before adoptive transfer and that might lead to absence of compensatory mechanisms that would be effective *in vivo* in our setting where Notch3 is impaired from birth, hence resulted in decreased induction of EAE. In that study the role of Notch3 in Th cell differentiation was not delineated. In addition the Notch3 blocking antibody used in the study was not validated in terms of its blocking abilities and specificity (it might cross react with other Notch family members), which is a caveat of those studies. In this study we utilized $CD4^+$ T cells from a N3KO mouse strain by Tom Gridley to further dissect the role of Notch3 on Th cell differentiation as well as on the induction of EAE. In order to support our observations we overexpressed or knocked down Notch3 gene expression by viral infection of $CD4^+$ T cells and showed that Notch3 plays an important role in Th17 cell differentiation *in vitro*. There are studies demonstrating the role of Notch3 in Th1 cell polarization *in vitro* by knocking down or overexpressing Notch3 in mouse $CD4^+$ T cells.

Overall our results with this Notch3 knockout mouse strain from Gridley group show, that Notch3 positively regulates Th1 and Th17 polarization *in vitro.* We also unraveled a previously unknown role of Notch3 in negative regulation of both GMCSF production by Th cells and in Th2 polarization *in vitro*. Previously the role of Notch3 in nTreg development was shown by overexpression studies [72]. Our data supports the role

of Notch3 in iTreg polarization of Th cells *in vitro*, whereas it does not affect nTreg development. This impaired iTreg polarization might be another reason why EAE scores were similar between WT and N3KO mice. There was lack of immunosuppressive mechanisms and disease can be further induced by intact pro-inflammatory Th cell differentiation in N3KO mice.

Our data demonstrates that $CD4^+$ T cell activation based on the levels of CD25 and CD69 markers, as well as cell proliferation are not affected by the impaired Notch3 signaling. Therefore, Notch3 regulates Th1, Th2, Th17, iTreg development as well as GMCSF production by Th cells without affecting their rate of proliferation and level of activation. Based on our previous studies, GSI treatment and impaired Notch1 signaling perturbed Th cell activation and proliferation [57, 59-64]. We have also shown that impaired Notch3 signaling increased Th2 polarization and production of pro-inflammatory cytokine GMCSF. Intact Th cells activation and proliferation as well as increased Th2 polarization and GMCSF production might be other level of regulation that lead to our observations where the induction of EAE was similar between WT and N3KO Tom Gridley mice.

Moreover, our data is novel to show that Th17 polarization is not regulated by RBPJκ dependent canonical Notch signaling. Data from our previous publications suggest that Th1 and iTreg polarization were through RBPJκ independent NFκB dependent non-canonical Notch signaling [64]. When we inhibited NFκB in Th17 cells there was a significant increase in IL-17A production compared to control non-treated cells. These suggest that NFκB is more important for Th1 and iTreg fates while doing so it may have inhibitory role on Th17 polarization program. Pharmacological inhibition of

PKCθ suggests that Th17 polarization is positively regulated by non-canonical PKCθ dependent Notch signaling. Further examination of these pathways at transcriptional and phosphorylation level is required.

In order to further strengthen our observations about the role of Notch3 in Th cell differentiation and the induction of EAE, we also utilized from a mouse line generated by William Skarnes group to knockout Notch3 [71]. This mouse cells had Notch3 extracellular protein trapped inside the cells. We could also detect Notch3 mRNA when N-terminus was targeted for amplification by primers in q-RT-PCR. $CD4^+$ T cell activation as well as proliferation was similar between WT and this set of N3KO mice. nTreg cell percentage was significantly lower in N3KO mice thymii compared to WT ones. Th17 polarization of $CD4^+$ T cells from these mice showed a tendency of higher potential compared to WT ones. This conflicted with our siNotch3 data as well as previous studies showing the role of Notch signaling in Th17 polarization. These mice also develop CADASIL syndrome spontaneously which would complicate our observations about the induction of EAE. Hence, we did not further study these mice. Discrepancy between Tom Gridley and William Skarnes Notch3 knockout mouse lines further suggests that differentially generated knockout mouse strains can give different results due to differences in the function of truncated protein either stimulating or blocking the signaling pathway.

In conclusion, by using N3KO animals from Tom Gridley group we have revealed a previously unknown role of Notch3 in the regulation of Th1, Th2, Th17 and iTreg differentiation as well as GMCSF production by Th cells *in vitro*. This impaired pro-inflammatory Th cell differentiation in the absence of Notch3 was not reflected under

physiological conditions. EAE scores of WT versus N3KO mice were similar. Possible explanations might be compensatory signaling pathways that are missing *in vitro* conditions. Our data suggests that Th17 polarization is regulated by RBPJκ independent PKCθ dependent non-canonical Notch signaling. More studies should be done where combinatorial targeting of multiple Notch family members as well as other signaling molecules such as PKCθ is aimed to impair the induction of EAE. Pharmacological inhibition of NFκB significantly increased Th17 polarization *in vitro,* suggesting that we should be cautious about usage of NFκB inhibitors depending of disease context. Since there has been studies supporting the role of NFκB in Th1 polarization, inhibition of NFκB might be a better strategy for diseases lead by Th1 polarization but not by Th17 polarization.

4. CONCLUSIONS AND FUTURE DIRECTIONS:

The role of Notch signaling in T helper cell differentiation and in the induction of EAE have been shown and studied but the field is still far from total understanding of the mechanism how Notch signaling regulates Th cell differentiation as well as induction of EAE [1, 2, 4, 132-136]. Since Notch is involved in cell differentiation in many different types of cells and tissues, it is complicated to dissect its Th cell intrinsic or neuron cell intrinsic functions in EAE setting [3, 4, 167]. Another level of complexity comes from differential outcomes of different Notch ligand interactions to initiate the signaling event [167].

In order to further dissect the role of Notch signaling in the induction of EAE and Th cell differentiation, we studied first the role of Notch1 signaling in the induction of EAE and Th17 polarization, major Th cell subtype involved in the induction of EAE. It has been shown by our lab previously that GSI prevents Th1 and Th17 polarization as well as the induction of EAE [129, 130]. Our recent publication clearly demonstrates that in conditional Notch1 knockout $CD4^+$ T cells there was reduced Th1 polarization [159]. My data here shows that Th17 polarization as well as the induction of EAE was not affected in conditional Notch1 knockout mice. Possible reason might be residual Notch1 activity in these mice after induction of gene knock down.

Based on a study showing the preventive effect of Notch3 blocking antibody in mice that had EAE induction by adoptive transfer of lymphocytes, we have focused on the role of Notch3 in the induction of EAE as well as Th cell differentiation [154]. First

time in the literature, we are showing that Notch3 is indispensable for Th1, Th17 and iTreg polarizations *in vitro*, whereas it is inhibitory for Th2 polarization and GMCSF production by Th cells. Based on our *in vivo* data, Notch3 is not required for the induction of EAE and Th17 polarization as well as GMCSF production whereas its impaired activity lead to increased Th1 activity *in vivo*. A possible reason of this discrepancy is compensatory mechanisms that are effective *in vivo* system whereas are lacking *in vitro*. Another reason might be that Notch3 can be important for neural cell development. In future it would be informative to study the role of Notch3 in neurons and oligodendrocytes by isolating these cells from Notch3 knockout mice and further examining their characteristics to compare with those of wild type mice. Another future direction would be inducing EAE by adoptive transfer of lymphocytes into wild type mice from previously EAE induced Notch3 knockout and wild type mice to examine the disease scores and further focus on T helper cell intrinsic role of Notch3 in the induction of EAE. It would be useful to generate double knockouts of Notch1 and Notch3 to study the role of these two proteins in the induction of EAE, since our data suggest that Notch1 and Notch3 are differentially important for Th cell differentiation *in vitro*.

Based on our preliminary data NFκB is crucial for Th1 polarization whereas it inhibits Th17 polarization and PKCθ is crucial for Th17 polarization as well as Th1 based on other studies [1, 2, 159, 167]. Combination therapy targeting Notch signaling pathway and depending on disease type, Th1 or Th17 driven or both Th1 and Th17 driven, inhibitors of NFκB and PKCθ can be used in future studies. Furthermore, interplay between Notch signaling and PKCθ as well as NFκB signaling pathways can be further dissected.

Overall, our results show that Notch3 plays an important role in Th cell differentiation by its interplay with NFκB and PKCθ signaling pathways *in vitro*. Notch3 was not required for the induction of EAE *in vivo*, suggesting involvement of other protein families as well as other Notch proteins in the disease induction. Further studies delineating the role of Notch signaling and its partner signaling pathways in T helper cell differentiation and induction of EAE would be beneficial for finding new therapeutics in clinical settings.

5. MATERIALS AND METHODS:

5.1. Mice: C57Bl6 and Notch3 Knockout mice were purchased from Jackson Laboratory and bred in our mouse facilities. . cNotch1KO and cRBPJκKO mice were generated by crossing Notch1$^{fl/fl}$ or RBPJκ$^{fl/fl}$ mice to mx1Cre$^{+/-}$ mice from Jackson Laboratory. Gene floxing was induced by 12-15 ug/g body weight injection of PolyI:PolyC (Amersham, Imgenex) every other day for 5 days. Injected mice are rested for 3 weeks then they are used for experiments. Age of mice to be used for experiments ranged between 7-13 weeks old. All mice were housed at animal facilities according to guidelines of Institutional Animal Care and Use Committee at University of Massachusetts-Amherst. In order to, control the influence of microbiota on the induction of EAE cage beddings with excretions were exchanged between knockout and wild type control mice cages at least for one week while housing in the same animal facility.

5.2. *In vitro* CD4$^+$ T cell Culture and Polarizations: Anti-CD4 magnetic beads (BD Pharmingen) are used to isolate CD4$^+$ T cells from splenocytes. 3x10^6 cells/ml are plated on each well of 12 well plate coated with 1ug/ml of anti-CD3 (BD Pharmingen) and 1ug/ml of anti-CD28 (BD Pharmingen). RDG is used as media which consists of half and half mixture of RPMI and DMEM (LONZA) with 10% Fetal Bovine Serum (GIBCO), 5% L-Glutamine, 5% Na-Pyruvate, 5% Penicillin/Streptomycin (LONZA) and 0.1% β-mercaptoethanol. Th1 polarization is done by adding 10ug/ml of anti-IL4 and 1ng/ml recombinant mouse IL12 (BD Pharmingen) into the culture media. Th2 polarization is done by adding 10ug/ml of anti-IFNγ and 1ng/ml recombinant mouse IL4

(BD Pharmingen) into the culture media. Th17 polarization is done by adding 10ug/ml of anti-IL4, 10ug/ml of anti-IFNγ (BD Pharmingen), 20ng/ml IL6 and 5ng/ml TGFβ1 (R&D systems) into the culture media (In some conditions 5ng/ml IL23 was also added into culture media (R&D systems)). For iTreg polarization $CD4^+CD25^-$ cells from bulk splenocytes are enriched by CD4 T cell enrichment set with 2.5ug biotin conjugated anti-CD25 (BD Pharmingen). Cells are activated in the presence of 2ng/ml of TGFβ1 (R&D systems). Cytokine levels in the supernatants are determined by standard ELISA assay (BD Pharmingen) after 72h incubation of cells. nTreg levels are measured in the thymus of mice by isolating the thymocytes and then doing CD25 surface and FoxP3 intracellular staining. CD4, CD8, CD25, and CD69 surface staining as well as FoxP3 intracellular staining are done at 72 hour time point of cell activation and cells are analyzed on a FACS LSRII (Becton Dickinson). All antibodies are purchased from ebiosciences and intracellular staining for FoxP3 is done by following the instructions on ebiosciences FoxP3 intracellular staining kit.

5.3. *In vitro* $CD4^+$ T cell infection by lentivirus or retrovirus: Anti-CD4 magnetic beads (BD Pharmingen) are used to isolate $CD4^+$ T cells from splenocytes. $1x10^6$ cells/ml are plated on each well of 12 well plate coated with 1ug/ml of anti-CD3 (BD Pharmingen) and 1ug/ml of anti-CD28 (BD Pharmingen). Before polarization of cells, they are infected either with control (pLKO.1) or shNotch3 lentiviral (Dharmacon) supernatants or Notch3 intracellular over-expression construct (Addgene) containing retroviral supernatants. Viral supernatants are generated by transfecting 293T cells with 4ug viral construct plasmid and 4ug of coating vectors. Media of transfected 293T cells is used after 72 hours by filtering with 0.45uM filters. Spin infection of cells is done at $30^{\circ}C$

for 90 mins at 2500rpm in 500ul volume of viral or control supernatants in the presence of Polybrene. Then extra RDG is added into each well and plates are put in 37°C incubators for 72 hours. RDG is used as media which consists of half and half mixture of RPMI and DMEM (LONZA) with 10% Fetal Bovine Serum (GIBCO), 5% L-Glutamine, 5% Na-Pyruvate, 5% Penicillin/Streptomycin (LONZA) and 0.1% β-mercaptoethanol. Th17 polarization is done by adding 10ug/ml of anti-IL4, 10ug/ml of anti-IFNγ (BD Pharmingen), 20ng/ml IL6 and 5ng/ml TGFβ1 (R&D systems) into the culture media Cytokine levels in the supernatants are determined by standard ELISA assay (BD Pharmingen) after 72h incubation of cells.

5.4. Isolation of RNA and Q-RT-PCR analysis for Notch3: Activated $CD4^+$ T cells are washed in PBS and then cell pellet is lysed to isolate mRNA by using Ambion (Life Technologies) RNA extraction kit protocol and reagents. Reverse transcription is done by using PROMEGA reverse transcription reagents and following the manufacturer directions. TAKARA SYBR green is used to do quantitative PCR by targeting C-terminus of Notch3 (Exon 32 and exon 33) and for Q-PCR the manufacturer directions are followed.

5.5. Inhibitor Treatments of $CD4^+$ T cells: $3x10^6$ cells/ml are treated with 0.1% DMSO, 25uM ILCHO (Gamma secretase inhibitor (GSI)), 5uM JLK6 (Notch Sparing GSI), 1uM Bay11 (NFκB Inhibitor) and 3uM Rottlerin (PKCθ inhibitor) for 30 minutes in 37°C water bath then they are plated into anti-CD3 and anti-CD28 coated 12 well plates. *In vitro* polarizations are done as explained in section 2.2.

5.6. CFSE Proliferation Assay: Cells are counted and $2x10^6$ cells/ml are re-suspended in PBS 0.1% BSA. 5mM of CFSE is added to the cells after mixing well cells are incubated in 37°C water bath for 10 minutes. Then, 5x volume of ice-cold media is added onto cells and incubation is done on ice for 5 minutes. After centrifugation cells are washed in cold media then plate for activation or polarization. At the end of time 72 hour time point Flow Cytometry analysis is done for CFSE.

5.7. Induction of EAE and re-stimulation of EAE induced mice splenocytes: Hooke's Kit (EK-2110) and protocol are used on female mice with C57Bl6 background after 10 weeks of age. Disease progression is scored according to following scale: 0- No disease, 1- Limp Tail, 2- Hind Limb Weakness, 3- Hind Limb Paralysis, 4- Hind and Fore Limb Paralysis and 5- Morbidity and Death. Mice are anesthetized and splenocytes were isolated from the spleens of EAE induced mice and are re-stimulated in non-coated 24 well plates in RDG media for 5 days with $5x10^6$ cells/ml concentration, in the presence of MOG 35-55 antigen (Hooke's Kit DS-0111) with 0, 10 and 20ug/ml concentrations. Supernatants of cultures are collected for ELISA analysis of IFNγ, GMCSF and IL17A cytokines.

5.8. Statistical Analysis: GraphPad Prism Software version 5 is used to perform statistical analysis by unpaired two tailed student's t-test for cell polarizations and Q-RT-PCR results, and two-way ANOVA for EAE scores.

Table 5.1. List of Reagent Used as ELISA kits, Blocking antibodies and growth factors:

Reagent Name	Vendor	Catalog Number
Mouse IL17F DuoSet ELISA	R and D Systems	DY2057
Recombinant Human TGF-β1	R and D Systems	240-B-002
Anti-mouse GMCSF Purified	eBioscience	14-7331
Anti-mouse GMCSF Biotin	eBioscience	13-7332
Mouse GMCSF Recombinant Protein	eBioscience	14-8331
Purified Rat anti mouse IFNγ	BD Pharmingen	554409
Recombinant Mouse IL6	R and D Systems	406-ML-005/CF
Recombinant Mouse IL3	R and D Systems	403-ML-010

Recombinant Mouse SCF/c-kit Ligand	R and D Systems	455-MC010
PolyIC HMW VacciGrade	InvivoGen	Vac-pic
Recombinant Mouse IL23	R and D Systems	1887-ML-010
Recombinant Mouse IL4	R and D Systems	404-ML-010
Recombinant Mouse IL2	BD Pharmingen	550069
Anti-Mouse/Rat FoxP3-PE	eBioscience	12-5773
Avidin Horseradish Peroxidase (HRP)	BD Pharmingen	554058
Mouse CD4 T Lymphocyte Enrichment Set-DM	BD Imag	558131
Purified Rat Anti-mouse IL2	BD Pharmingen	554424

Purified Rat Anti-mouse IL17A	BD Pharmingen	555068
Biotin Rat Anti-mouse IL17A	BD Pharmingen	555067
Purified NA/LE Rat Anti-Mouse IL4	BD Pharmingen	554432
Purified NA/LE Rat Anti-Mouse IFNγ	BD Pharmingen	554408
Recombinant Mouse IL17F	R and D Systems	421-ML-025/CF
Anti-Mouse CD4 Magnetic Particles DM	BD Imag	551539

Table 5.2. List of Primers Used for Genotyping of Mice and Q-RT-PCR Experiments:

Primer Name	Usage	Sequence (5’ to 3’)
N3 Mutant Reverse Tom Gridley	Genotyping of Tom Gridley Mice	GGTACTGAGAACCAAACTCAG
N3 Mutant Forward Tom Gridley	Genotyping of Tom Gridley Mice	TCGCCTTCTATCGCCTTTCTTG
N3 Wild Type Reverse Tom Gridley	Genotyping of Tom Gridley Mice	CACATTGGCACAAGAATGAGCC
N3 Wild Type Forward Tom Gridley	Genotyping of Tom Gridley Mice	CGATGAGGATGCTATCTGTGAC
Notch3 Exon 16 Forward William Skarnes	Genotyping of William Skarnes Mice	GATCAAGACATTGACGACTGTGAC
Notch3 Exon 17 Reverse William Skarnes	Genotyping of William Skarnes Mice	GTCGAGGCAAGAACAGGAAAAG

Notch3 Trap Forward William Skarnes	Genotyping of William Skarnes Mice	GCAGGGAGAGTTGAGATGGAAGG
Notch3 Trap Reverse William Skarnes	Genotyping of William Skarnes Mice	CCGTCACTCCAACGCAGCACCATCAC
Notch3 Exon 32 Forward	Q-RT-PCR to detect C-Terminus of Notch3	TGGGAAATCTGCCTTACACTGG
Notch3 Exon 33 Reverse	Q-RT-PCR to detect C-Terminus of Notch3	AGCAGCTTGGCAGCCTCATAG
Actin Forward	Q-RT-PCR to detect Actin	GGCTGTATTCCCCTCCATCG
Actin Reverse	Q-RT-PCR to detect Actin	CCAGTTGGTAACAATGCCATGT
Notch1 Forward	Q-RT-PCR to detect Notch1	CCCTTGCTCTGCCTAACGC
Notch1 Reverse	Q-RT-PCR to detect Notch1	GGAGTCCTGGCATCGTTGG

Notch2 Forward	Q-RT-PCR to detect Notch2	ATGTGGACGAGTGTCTGTTGC
Notch2 Reverse	Q-RT-PCR to detect Notch2	GGAAGCATAGGCACAGTCATC

6. APPENDIX:

6.1. Immunomodulatory effects of nanoparticles in immune-challenged systems:

The ability of nanoparticle surface functionalities to regulate immune responses during an immunological challenge (i. e. inflammation) would open new doors for the therapeutics. We used functionalized 2nm core gold nanoparticles to control the innate immune responses of *in vitro* and *in vivo* systems activated with an inflammatory challenge. The results showed that hydrophobic zwitterionic functionalities dramatically boost inflammatory outcomes while hydrophilic zwitterionic structures generate minimal immunological responses. Surprisingly, tetra(ethylene glycol) headgroups generate a significant anti-inflammatory response both *in vitro* and *in vivo*. These results demonstrate the ability of simple surface ligands to provide immunomodulatory properties, making them promising leads for the therapeutic usage of nanomaterials in diseases involving inflammation.

6.2. Suppressive role of Exopoylsaccharide (EPS) in the induction of EAE:

Beneficial microbes modulate host immune responses, but in most cases, the mechanism by which bacterial molecules affect these responses is poorly understood. It has been demonstrated that a single oral dose of *Bacillus subtilis* prevents disease induced by the enteric pathogen *Citrobacter rodentium*. Protection was not due to reduced pathogen colonization or to changes in intestinal permeability. Instead, it appears that protection was mediated by immune modulation. Recently, exopolysaccharide (EPS) was identified as the protective molecule of *B. subtilis* [168].

EPS binds $F4/80^{+}CD11b^{+}$ peritoneal macrophages, and in preliminary studies, treatment of mice with clodronate-loaded liposomes to deplete macrophages, prevented EPS-mediated protection from *C. rodentium*- induced disease, suggesting that macrophages are required for protection. Intraperitoneal injection of EPS increases the number of peritoneal anti-inflammatory M2 macrophages (M2MΦ), and the protective effects of EPS can be adoptively transferred to naïve wildtype (WT) mice using macrophage-rich peritoneal cells from EPS-treated WT mice, hypothesized to be a co-receptor of TLR-4, and test if binding induces resident macrophages to become anti-inflammatory M2MΦ [Unpublished data]. M2MΦ are potent anti-inflammatory cells known to inhibit T cell activation, and much of the pathology observed during *C. rodentium* infection results from excessive Th1 and Th17 responses.

Our goal with the collaborators was to elucidate the role of EPS in prevention of experimental autoimmune encephalomyelitis (EAE) (Figure 25 and Figure 28). Our

preliminary data suggest that there is decrease in GMCSF production from MOG peptide restimulated splenocytes of EAE induced mice 3 days pre-treated with EPS compared to control animals (Figure 27). IL-17A production was similar between the groups but IFN-γ production was higher in PBS mock control treated group compared to 3 days EPS pre-treated group (Figure 27). These data suggest that EPS blocks GM-CSF production for pathogenic T cells and support the current hypothesis in the EAE field that GMCSF is the critical cytokine for the production of encephalitic T cells. According to our preliminary results, when EPS was injected the day of EAE induction the IL-17A and GMCSF production was similar between PBS injected control group and EPS injected groups (Figure 26). Whereas IFN-γ production was higher in the EPS treated group splenocytes compared to that of PBS treated group (Figure 26). Discrepancy between the results obtained between different experiments might be due to possible deviation encountered in the induction of EAE. Therefore, more experiments are required to done to reach statistically interpretable data.

Additionally, it is known that TGF-β can block the development of GMCSF producing Th17 cells [1, 2]. Since M2 MΦ produce TGF-β, these data drive our hypothesis that EPS treatment is protective against EAE through the development of M2 MΦ that produce TGF-β which, in turn, suppresses the formation of GMCSF producing encephalitic T cells. In future studies, we will investigate the mechanism by which EPS protects from and ameliorates inflammatory disease, EAE and also begin studies to determine if EPS functions in humans.

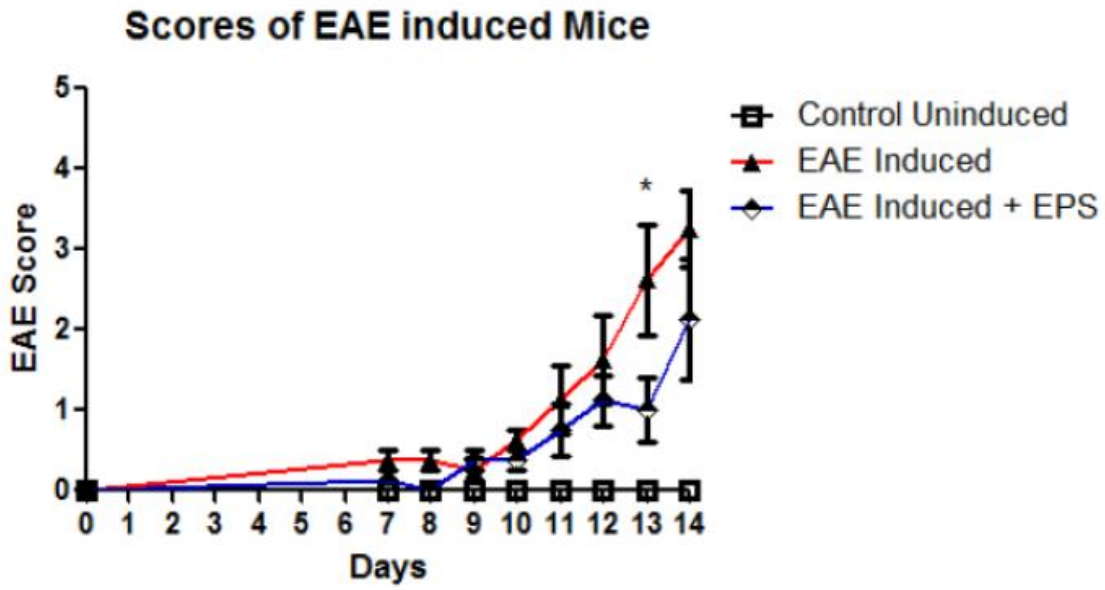

Figure 25. The induction of EAE is lower in EPS injected animals compared to control ones:

A) EAE disease scores of WT PBS injected control versus WT EPS injected group during 15 day time course. EPS injections started at day 0 of EAE induction. (N=8 for each group)

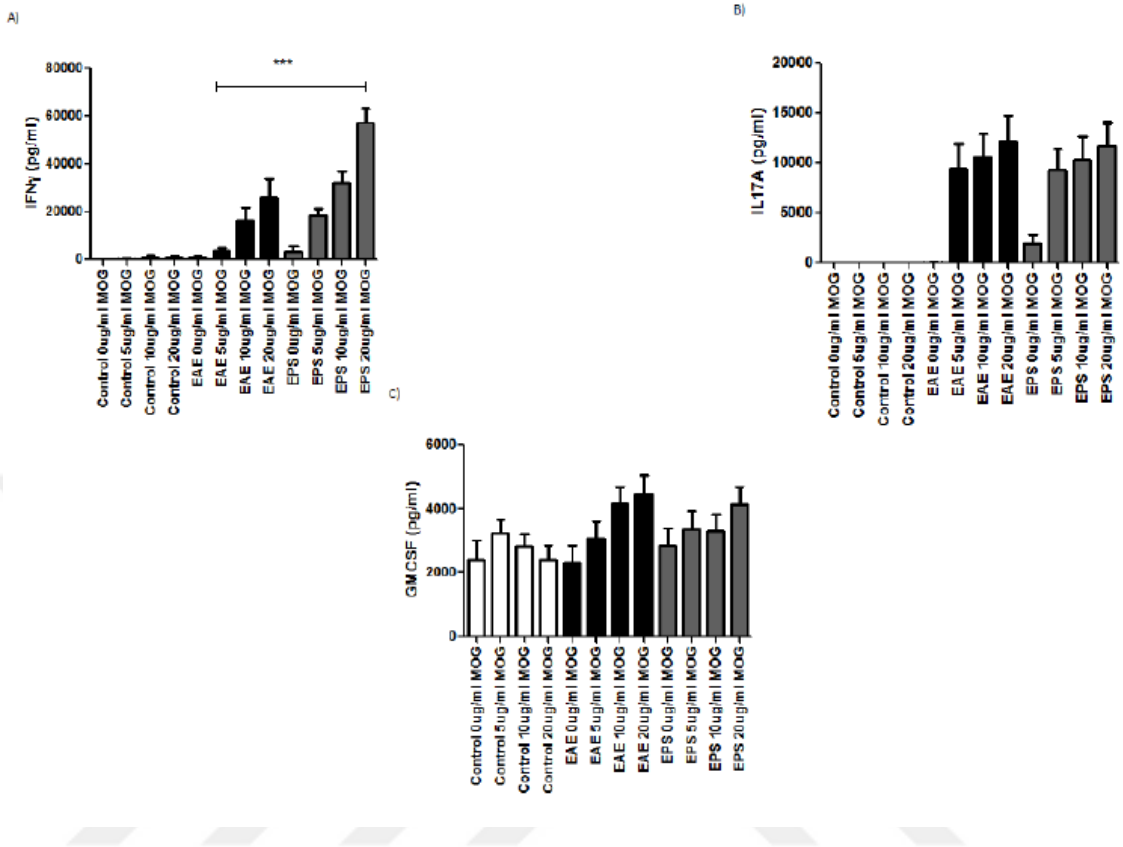

Figure 26. IFNγ production is higher by the MOG peptide restimulated splenocytes of EAE induced EPS treated mice compared to control group whereas IL17A and GMCSF levels are similar:

ELISAs for the supernatants of splenocytes incubated for 5 days with MOG peptide **A)** IFNγ ELISA. **B)** IL17A ELISA. **C)** GMCSF ELISA. N=8 for each group and data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

A)

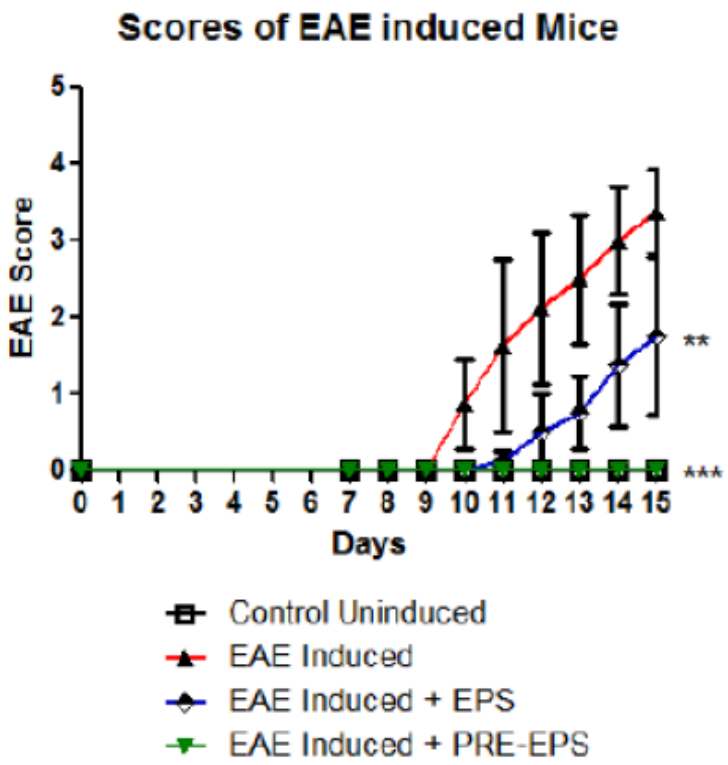

Figure 27. The induction of EAE is abolished in EPS pre-treated (day -3) animals and lower in EPS injected (day 0) animals compared to control ones:

A) EAE disease scores of WT PBS injected control versus WT EPS injected group at day 0 of disease induction and WT EPS injected group at day -3 of disease induction (PRE EPS) during 15 day time course. (N=4 for each group)

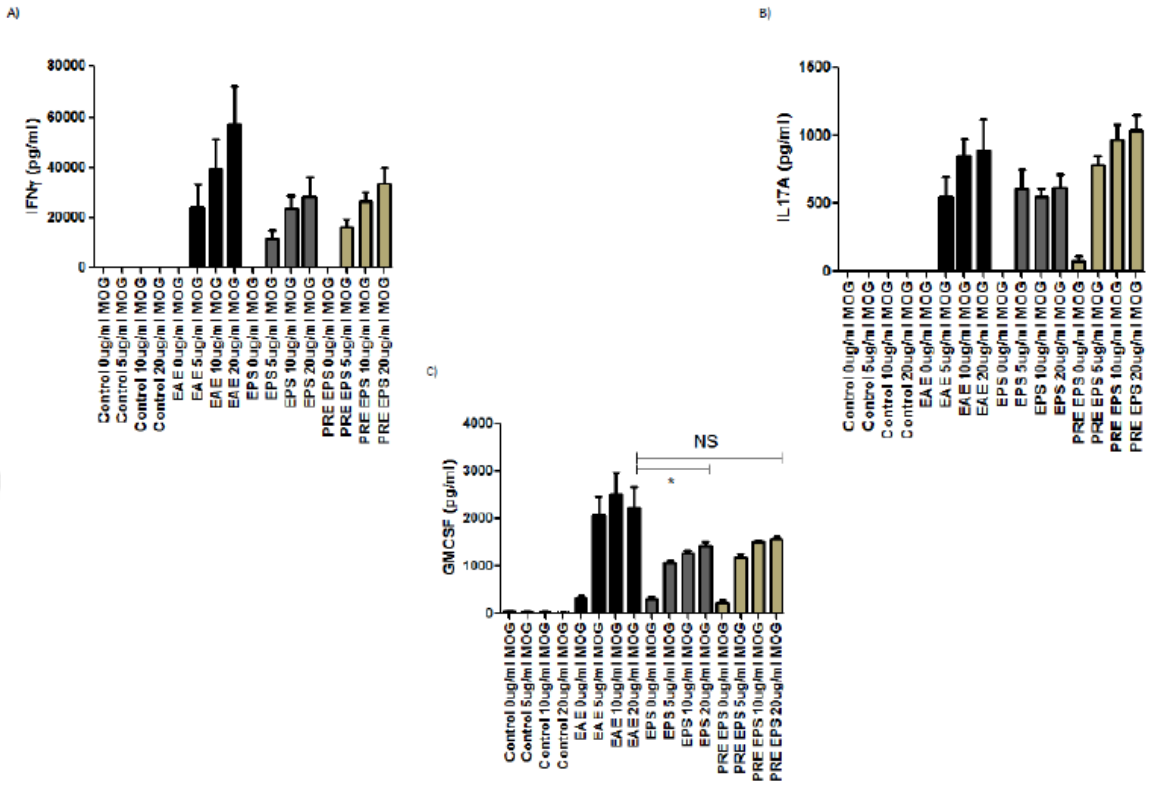

Figure 28. GMCSF production is higher by the MOG peptide restimulated splenocytes of EAE induced PBS treated animals compared to EPS treated mice whereas IL17A and IFNγ levels are similar:

ELISAs for the supernatants of splenocytes incubated for 5 days with MOG peptide **A)** IFNγ ELISA. **B)** IL17A ELISA. **C)** GMCSF ELISA. N=4 for each group and data represent the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

6.3. Differential effect of Notch1 localization in the induction of T-ALL, implications of non-canonical Notch signaling:

Canonical Notch signaling is initiated by γ-secretase mediated cleavage of the Notch receptor, leading to the release of the active intra-cellular domain of Notch that migrates to the nucleus and interacts with RBP-Jκ, resulting in the activation of downstream target genes. While canonical Notch signaling is well known to play an active role in several steps during development as well in multiple cell fate decisions, recent evidence from both invertebrate as well as vertebrate systems indicate non-canonical, RBP-Jκ independent signaling is important in several cellular processes [122]. These observations raise the possibility that, through an understanding of non-canonical Notch signaling, novel strategies for inhibiting Notch signaling may prove useful in the design of therapies targeted to block aberrant Notch activity.

The first indication for a role of Notch pathway in oncogenesis came from the studies of Aster and Pear in T-cell acute lymphoblastic lymphoma (T-ALL). Chromosomal translocation of the *Notch1* gene was identified as a cause of T cell oncogenesis [169, 170]. Subsequently, the Notch pathway has been associated with tumorigenesis and cancer progression in the other cancers including: breast, ovarian, cervical, lung, prostate carcinomas, gliomas, and mesotheliomas [171-179]. Notch

signaling regulates proliferation, differentiation and survival of tumor cells [180, 181] and is reported to be involved in maintaining the stem cell-like characteristics of cancer stem cells while giving rise to pluripotent neoplastic cells [182-184]. It is also required for further progression of differentiated cancer cells by regulating the metabolism, survival and transcription in these cells. In addition to its role in tumorigenesis, Notch also has been reported to act as a tumor suppressor in certain cell types such as skin epithelium [185]. This observation makes it quite clear that an understanding of Notch signaling pathways are essential if therapeutic manipulation of Notch is likely to be successful.

Inhibition of γ-secretase does not block all Notch related functions in tumor cells, suggesting a role for the non-canonical Notch signaling in transformed cells [171, 172, 174-176, 179]. Additionally, transformation of baby rat kidney cells through cooperation between adenoviral E1A protein and NICD does not require the RBPJκ/CSL binding domain of NICD, suggesting transformation in this system may be non-canonical. However, this non-canonical Notch signaling still required nuclear localization of NICD to be oncogenic [186, 187].

Studies in the Screpanti lab showed that non-canonical Notch3 signaling regulates T-cell development and leukemia through activation of the NFκB pathway. In their transgenic mouse model, Notch3 was overexpressed specifically in T cells and this led to development of leukemia [188]. This group showed that increased Notch3 expression enabled constitutive activation of NFκB and demonstrated that Notch3 interacts with IKKα to maintain NFκB activity [188]. Additionally, in breast cancer cells non-canonical Notch signaling is known to regulate IL-6 expression. IL-6 acts on tumor cells to further

increase their oncogenic potential [189]. Cytoplasmic NICD was sufficient to engage with the non-canonical NFκB pathway to induce IL-6 expression [189]. Taken together, these studies support a role for non-canonical Notch signaling via NFκB pathway in oncogenesis.

In human myelogenous leukemia cells, Notch1 directly interacts with the transcription factor, YY1, to drive expression of oncogenic transcription factor *c-myc* independent of CSL [190]. In HPV-driven human cervical cancer, non-canonical Notch signaling enables oncogenesis, independent of CSL, via PI3K pathway [191]. However little is known about how non-canonical Notch signaling drives transformation in these situations.

In this study we examined the effect of differential localization of Notch1 in the induction of T-ALL. Localization to the cytoplasm implicates non-canonical role of Notch1 in the disease induction. We used two different constructs previously generated in our lab. One of them has extra nuclear localization signals (NLS) and the other construct has extra nuclear export signals (NES). Bone marrow cells were infected with these two different constructs as well as a positive control plasmid to express Notch1 in the cells and eventually induce T-ALL. At day 40 mice were sacrificed to analyze thymii and spleens for CD4 and CD8 double positive cell percentages. Compared to control un-induced Bone marrow cells injected mice, both Notch1 NLS and Notch1 NES constructs infected cells injected mice had an increase in CD4 and CD8 double positive cell population (Figure 29). When we analyzed the thymii of these groups, Notch1 NES infected cell containing mice had more robust increase in CD4 and CD8 double positive cells compare to Notch1 NLS infected cells injected mice group (Figure 29). This

preliminary study suggests that cytoplasmic Notch1 was more potent to induce T-ALL, based on CD4 and CD8 double positive cell numbers in thymii, compared to nuclear Notch1. It supports that non-canonical cytoplasmic signaling of Notch1 is more crucial in the induction of T-ALL than canonical Notch1 signaling. It is worth to repeat these experiments and also to do further analysis in terms of number of cancer stem cells when T-ALL was induced by differentially localized Notch1 proteins. Furthermore, studying the signaling pathways differentially activated in cancerous cells from these T-ALL induced mice.

It is important to note that inhibitors are available for many of the signaling pathways involved in non-canonical Notch signaling (NF-κB, PI3K, AKT, mTOR, HIF-1α and β-catenin) and, in several instances, these inhibitors have passed through clinical trials [122]. Thus, in the near future, it should be feasible to test the possibility that combination therapy using Notch inhibitors, in addition to inhibitors of these other pathways, might prove more efficacious in the treatment of diseases regulated by Notch.

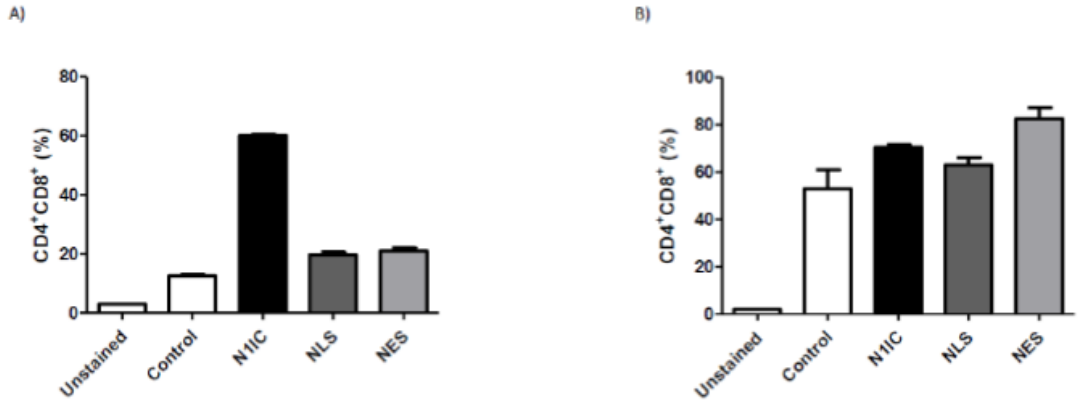

Figure 29. Nuclear versus Cytoplasmic localization of Notch1 regulates CD4 and CD8 double positive cell levels in Thymii and the spleen of mice:

A) CD4 and CD8 double staining and FACS analysis for the spleen of mice with bone marrow re-constituted by the cells infected with control plasmid, Notch1 intracellular domain (N1IC) positive control plasmid, Notch1 Nuclear Localization Signal (NLS) plasmid, Notch1 Nuclear Export Signal (NES) plasmid. **B)** CD4 and CD8 double staining and FACS analysis for the thymii of mice with bone marrow re-constituted by the cells infected with control plasmid, Notch1 intracellular domain (N1IC) positive control plasmid, Notch1 Nuclear Localization Signal (NLS) plasmid, Notch1 Nuclear Export Signal (NES) plasmid. N=3 for each group. At day 40 after bone marrow reconstitution mice were sacrificed for analysis.

7. BIBLIOGRAPHY:

1. Zhu J, Yamane H, Paul E W. Differentiation of effector CD4 T cell populations. Annual Review Immunology (2010) 28:445-489. Doi:10.1146/annurev-immunol-030409-101212

2. Zhu J, Paul E W. CD4 T cells: fates, functions and faults. Blood (2008) 112:1557-1569. Doi: 10.1182/blood-2008-05-078154.

3. Chastain M L E, Miller D S. Molecular mimicry as an inducing trigger for CNS autoimmune demyelinating disease. Immunological Reviews (2012) 245:227-238. Doi: 10.1111/j.1600-065X.2011.01076.x

4. Chastain M L E, Duncan S D, Rodgers M J, Miller D S. The role of antigen presenting cells in Multiple Sclerosis. Biochimica et Biophysica Acta (2011) 1812:265-274. Doi: 10.1016/j.bbadis.2010.07.008

5. S.L. Bailey, B. Schreiner, E.J. McMahon, S.D. Miller, CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4(+) T(H)-17 cells in relapsing EAE, Nat. Immunol. 8 (2007) 172–180.

6. P. Guermonprez, J. Valladeau, L. Zitvogel, C. Thery, S. Amigorena, Antigen presentation and T cell stimulation by dendritic cells, Annu. Rev. Immunol. 20 (2002) 621–667.

7. Janeway C A Jr, Travers P, Walport M. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science (2001).

8. Radulovic K, Rossini V, Manta C, Holzmann K, Kestler H A, Niess H J. The Early Activation Marker CD69 Regulates the Expression of Chemokines and CD4 T Cell Accumulation in Intestine. Plos One (2013). Doi: 10.1371/journal.pone.0065413

9. Shen W, Yang F, Zhou Y, Yang H, Low P Y, Kemeny D M, Tan P, Moh A, Kaplan M H, Zhang Y, Fu X. STAT5 programs a distinct subset of GMCSF producing T helper cells that is essential for autoimmune neuroinflammation. Cell Research (2014) 24:1387-1402. Doi: 10.1038/cr.2014.154

10. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. 1993. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 260:547–49

11. Mullen AC, High FA, Hutchins AS, Lee HW, Villarino AV, Livingston DM, Kung AL, Cereb N, Yao TP, Yang SY, Reiner SL. 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. Science 292:1907–10

12. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100:655–69

13. Williams, M. A., A. J. Tyznik, and M. J. Bevan. 2006. Interleukin-2 signals during priming are required for secondary expansion of CD8_ memory T cells. Nature 441: 890–893.

14. J.R. Podojil, S.D. Miller, Immunopathological mechanisms in multiple sclerosis, Drug Disc. Today: Dis. Mech. 3 (2006) 177–184.

15. S.L. Bailey, P.A. Carpentier, E.J. McMahon, W.S. Begolka, S.D. Miller. Innate and adaptive immune responses of the central nervous system, Crit. Rev. Immunol. 26 (2006) 149–188.

16. Lassmann H. 2010. Axonal and neuronal pathology in multiple sclerosis: what have we learnt from animal models. Exp Neurol 225:2–8.

17. Adams CW, Poston RN, Buk SJ. 1989. Pathology, histochemistry and immunocytochemistry of lesions in acute multiple sclerosis. J Neurol Sci 92:291-306.

18. Segal B M. 2014. Stage Specific Immune Dysregulation in Multiple Sclerosis. Journal of Interferon and Cytokine Research 34:633-639. Doi:10.1089/jir.2014.0025

19. Le Gros G, Ben-Sasson SZ, Seder R, Finkelman FD, Paul WE. 1990. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. J. Exp. Med. 172:921–29.

20. Zhen W P, Flavell R A. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell (1997) 89:587–96.

21. Noben-Trauth N, Hu-Li J, Paul WE. IL-4 secreted from individual naive CD4+ T cells acts in an autocrine manner to induce Th2 differentiation. Eur. J. Immunol. (2002) 32:1428–33.

22. Wynn TA. IL-13 effector functions. Annu Rev Immunol. (2003) 21:425-456.

23. Urban JF Jr, Noben-Trauth N, Donaldson DD, Madden KB, Morris SC, Collins M, Finkelman FD. IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis. Immunity. (1998) 8:255-264.

24. Mitsuyasu H, Izuhara K, Mao XQ, Gao PS, Arinobu Y, Enomoto T, Kawai M, Sasaki S, Dake Y, Hamasaki N, Shirakawa T, Hopkin JM. Ile50Val variant of IL4R alpha upregulates IgE synthesis and associates with atopic asthma. Nat Genet. 1998;19:119-120.

25. Mitsuyasu H, Yanagihara Y, Mao XQ, Gao PS, Arinobu Y, Ihara K, Takabayashi A, Hara T, Enomoto T, Sasaki S, Kawai M, Hamasaki N, Shirakawa T, Hopkin JM, Izuhara K. Cutting edge: dominant effect of Ile50Val variant of the human IL-4 receptor alpha-chain in IgE synthesis. J Immunol. 1999;162:1227-1231.

26. Pykalainen M, Kinos R, Valkonen S, Rydman P, Kilpalainen M, Laitinen LA, Karjalainen J, Nieminen M, Hurme M, Kere J, Laitinen T, Lahesmaa R. Association analysis of common variants of STAT6, GATA3, and STAT4 to asthma and high serum IgE phenotypes. J Allergy Clin Immunol. 2005; 115:80-87.

27. Longphre M, Li D, Gallup M, Drori E, Ordonez C L, Redman T, Wenzel S, Bice D E, Fahy J V, Basbaum C. Allergen induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. J Clin Invest. 1999;104:1375-1382.

28. Asseman C, Read S, Powrie F. Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by CD4_ regulatory T cells and IL-10. J Immunol. 2003;171:971-978.

29. Zaiss DM, Yang L, Shah PR, Kobie JJ, Urban JF, Mosmann TR. Amphiregulin, a TH2 cytokine enhancing resistance to nematodes. Science. 2006; 314:1746.

30. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, Menon S, Clifford T, Hunte B, Lesley R, Muchamuel T, Hurst SD, Zurawski G, Leach MW, Gorman DM, Rennick DM. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity. 2001;15:985-995.

31. Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB, Liu YJ, Zhu Z, Dong C. Interleukin 25 promotes the initiation of proallergic type 2 responses. J Exp Med. 2007;204:1509-1517.

32. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, Cua DJ. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell mediated pathology. Nat Immunol. 2007;8:1390-1397.

33. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity. 2006;24:677688.

34. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17_ T helper cells. Cell. 2006;126:1121-1133.

35. Mathur AN, Chang HC, Zisoulis DG, Stritesky GL, Yu Q, OMalley JT, Kapur R, Levy DE, Kansas GS, Kaplan MH. Stat3 and Stat4 direct development of IL-17-secreting Th cells. J Immunol. 2007;178:4901-4907.

36. Yao Z, Fanslow WC, Seldin MF, Rousseau AM, Painter SL, Comeau MR, Cohen JI, Springs MK. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. Immunity. 1995;3:811-821.

37. Hymowitz SG, Filvaroff EH, Yin JP, Lee J, Cai L, Risser P, Maruoka M, Mao W, Foster J, Kelley RF, Pan G, Gurney AL, de Vos AM, Starovasnik MA. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. EMBO J. 2001;20:5332-5341.

38. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, Kuchroo VK. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 2007;448:484-487.

39. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, Schluns K, Tian Q, Watowich SS, Jetten AM, Dong C. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature. 2007;448:480-483.

40. Zheng Y, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, Ouyang W. Interleukin-22, a T(H)17 cytokine, mediates IL-23- induced dermal inflammation and acanthosis. Nature. 2007;445:648-651.

41. Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, Stockinger B. The aryl hydrocarbon receptor links TH17-cellmediated autoimmunity to environmental toxins. Nature. 2008;453:106-109.

42. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Karow M, Flavell RA. Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. Immunity. 2007;27:647-659.

43. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, Ouyang W. Interleukin- 22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med. 2008;14:282-289.

44. Li MO, Sanjabi S, Flavell RA. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell dependent and -independent mechanisms. Immunity. 2006;25:455-471.

45. Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity. 2007;26:579-591.

46. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med. 1999;190:995-1004.

47. Yao Z, Kanno Y, Kerenyi M, Stephens G, Durant L, Watford WT, Laurence A, Robinson GW, Shevach EM, Moriggl R, Hennighausen L, Wu C, Oshea JJ. Nonredundant roles for Stat5a/b in directly regulating Foxp3. Blood. 2007;109:4368-4375.

48. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A critical function for TGF-beta signaling in the development of natural CD4_CD25_Foxp3_ regulatory T cells. Nat Immunol. 2008;9:632-640.

49. Joffre O, Santolaria T, Calise D, Al Saati T, Hudrisier D, Romagnoli P, van Meerwijk JP. Prevention of acute and chronic allograft rejection with CD4_CD25_Foxp3_ regulatory T lymphocytes. Nat Med. 2008;14:88-92.

50. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4_CD25_ regulatory T cells control Leishmania major persistence and immunity. Nature. 2002;420:502-507.

51. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DAA. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature. 2007;450:566-569.

52. Herndler-Brandsetter D, Flavell R A. Producing GMCSF: a unique T helper subset?. Cell Research (2014) 24:1379-1380. Doi: 10.1038/cr.2014.155

53. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman G, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000;100:655-669.

54. Lighvani AA, Frucht DM, Jankovic D, Yamane H, Aliberti J, Hissong BD, Nguyen BV, Gadina M, Sher A, Paul WE, Oshea JJ. T-bet is rapidly induced by interfere on-gamma in lymphoid and myeloid cells. Proc Natl Acad Sci (2001) 98:15137-15142.

55. Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH, Ackerman K, Haley K, Galle PR, Szabo SJ, Drazen JM, De Sanctis GT, Glimcher LH. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. Science (2002) 295:336-338.

56. Pearce EL, Mullen AC, Martins GA, Krawczyk CM, Hutchins AS, Zediak VP, Banica M, DiCioccio CB, Gross DA, Mao CA, Shen H, Cereb N, Yang SY, Lindsten T, Rossant J, Hunter CA, Reiner SL. Control of effector CD8 T cell function by the transcription factor Eomesodermin. Science (2003) 302:1041-1043.

57. Buggert M, Tauriainen J, Yamamoto T, Frederiksen J, Ivarsson MA, Michaelsson J, Lund O, Hejdeman B, Jansson M, Sonnerborg A, Koup RA, Betts MR, Karlsson AC. T-bet and Eomes are differentially linked to the exhausted phenotype of CD8 T cells in HIV infection. Plos Pathogens (2014) 10:e1004251. Doi: 10.1371/journal.ppat.1004251.

58. Suto A, Wurster AL, Reiner SL, Grusby MJ. IL-21 inhibits IFN-gamma production in developing Th1 cells through the repression of Eomesodermin expression. J Immunol. (2006) 177:3721-3727.

59. Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. Nature (1996) 382:174-177.

60. Thierfelder WE, van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, Sangster MY, Vignali DA, Doherty PC, Grosveld GC, Ihle JN. Requirement for Stat4 in interleukin-12 mediated responses of natural killer and T cells. Nature (1996) 382:171-174.

61. Robinson D, Shibuya K, Mui A, Zonin F, Murphy E, Sana T, Hartley SB, Menon S, Kastelein R, Bazan F, Ogarra A. IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB. Immunity (1997) 7:571-581.

62. Djuretic IM, Levanon D, Negreanu V, Groner Y, Rao A, Ansel KM. Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. Nat Immunol. (2007) 8:145-153.

63. Naoe Y, Setoguchi R, Akiyama K, Muroi S, Kuroda M, Hatam F, Littman DR, Taniuchi I. Repression of interleukin-4 in T helper type 1 cells by Runx/Cbf beta binding to the Il4 silencer. J Exp Med. (2007) 204:1749-1755.

64. Finkelman FD, Morris SC, Orekhova T, Mori M, Donaldson D, Reiner SL, Reilly NL, Schopf L, Urban JF Jr. Stat6 regulation of in vivo IL-4 responses. J Immunol. (2000) 164:2303-2310.

65. Mullen AC, Hutchins AS, High FA, Lee HW, Sykes KJ, Chodosh LA, Reiner SL. Hlx is induced by and genetically interacts with T-bet to promote heritable Th1 gene induction. Nat Immunol. (2002) 3:652-658.

66. Kaplan MH, Schindler U, Smiley ST, Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity (1996) 4:313-319.

67. Shimoda K, van Deursen J, Sangster MY, Sarawar SR, Carson RT, Tripp RA, Chu C, Quelle FW, Nosaka T, Vignali DA, Doherty PC, Grosveld G, Paul WE, Ihle JN. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. Nature (1996) 380:630-633.

68. Jankovic D, Kullberg MC, Noben-Trauth N, Caspar P, Paul WE, Sher A. Single cell analysis reveals that IL-4 receptor/Stat6 signaling is not required for the in vivo or in vitro development ofCD4 lymphocytes with a Th2 cytokine profile. J Immunol. (2000) 164:3047-3055.

69. Kurata H, Lee HJ, O'Garra A, Arai N. Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. Immunity (1999) 11:677-688.

70. Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, Murphy KM. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity (1998) 9:745-755.

71. Zhu J, Guo L, Watson CJ, Hu-Li J, Paul WE. Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. J Immunol. (2001) 166:7276-7281.

72. Pai SY, Truitt ML, Ho IC. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. Proc Natl Acad Sci. (2004) 101:1993-1998.

73. Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, Killeen N, Urban JF Jr, Guo L, Paul WE. Conditional deletion of Gata3 shows its essential function in Th1-Th2 responses. Nat Immunol. (2004) 5:1157-1165.

74. Lin JX, Leonard WJ. The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. Oncogene (2000) 19:2566-2576.

75. Cote-Sierra J, Foucras G, Guo L, Chiodetti L, Young HA, Hu-Li J, Zhu J, Paul WE. Interleukin 2 plays a central role in Th2 differentiation. Proc atl Acad Sci. (2004) 101:3880-3885.

76. Bell BD, Kitajima M, Larson RP, Stoklasek TA, Dang K, Sakamoto K, Wagner KU, Kaplan DH, Reizis B, Hennighausen L, Ziegler SF. The transcription factor STAT5 is critical in dendritic cells for the development of Th2 but not Th1 responses. Nat. Immunol. (2013) 14:364-371. Doi: 10.1038/ni.2541.

77. Takatori H, Nakajima H, Hirose K, Kagami S, Tamachi T, Suto A, Suzuki K, Saito Y, Iwamoto I. Indispensable role for STAT5a in STAT6-independent Th2 cell differentiation and allergic airway inflammation. Journal of Immunology (2005) 174:3734-3740.

78. Zhu J, Cote-Sierra J, Guo L, Paul WE. Stat5 activation plays a critical role in Th2 differentiation. Immunity (2003) 19:739-748.

79. Kim JI, Ho IC, Grusby MJ, Glimcher LH. The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. Immunity (1999) 10:745-751.

80. Lohoff M, Mittrucker HW, Prechtl S, Bischof S, Sommer F, Kock S, Ferrick DA, Duncan GS, Gessner A, Mak TW. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. Proc Natl Acad Sci (2002) 99:11808-11812.

81. Rengarajan J, Mowen KA, McBride KD, Smith ED, Singh H, Glimcher LH. Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. J Exp Med. (2002) 195:1003-1012.

82. Zhu J, Guo L, Min B, Watson CJ, Hu-Li J, Young HA, Tsichlis PN, Paul WE. Growth factor independent-1 induced by IL-4 regulates Th2 cell proliferation. Immunity (2002) 16:733-744.

83. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17 T helper cells. Cell (2006) 126:1121-1133.

84. Duhen R, Glatigny S, Arbelaez A C, Blari TC, Oukka M, Bettelli E. Cutting Edge: The pathogenicity of IFN gamma producing Th17 cells is independent of Tbet. The Journal of Immunology (2013). Doi: 10.4049/jimmunol.1203172.

85. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, Ma L, Shah B, Panopoulos AD, Schluns KS, Watowich SS, Tian Q, Jetten AM, Dong C. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity (2008) 28:29-39.

86. Harris TJ, Grosso JF, Yen HR, Xin H, Kortylewski M, Albesiano E, Hipkiss EL, Getnet D, Goldberg MV, Maris CH, Housseau F, Yu H, PArdoll DM, Drake CG. Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. J Immunol. (2007) 179:4313-4317.

87. Mathur AN, Chang HC, Zisoulis DG, Stritesky GL, Yu Q, Omalley JT, Kapur R, Levy DE, Kansas GS, Kaplan MH. Stat3 and Stat4 direct development of IL-17-secreting Th cells. J Immunol. (2007) 178:4901-4907.

88. Brustle A, Heink S, Huber M, Rosenplanter C, Stadelmann C, Yu P, Arpaia E, Mak TW, Kamradt T, Lohoff M. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. Nat Immunol. (2007) 8:958-966.

89. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol. (2007) 8:277-284.

90. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4_CD25- naive T cells to CD4 CD25 regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med. (2003) 198:1875-1886.

91. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4 CD25 regulatory T cells. Nat Immunol. (2003) 4:330-336.

92. Davidson TS, DiPaolo RJ, Andersson J, Shevach EM. Cutting Edge: IL-2 is essential for TGF-beta mediated induction of Foxp3 T regulatory cells. J Immunol. (2007) 178:4022-4026.

93. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature (2007) 445:766-770.

94. Goldenberg M M. Multiple Sclerosis Review. Pharmacy and Therapeutics (2012) 37:175-184.

95. Ebers, G.C. (2008) Environmental factors and multiple sclerosis, Lancet Neurol 7: 268–277.

96. S.L. Hauser, J.R. Oksenberg, The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration, Neuron 52 (2006) 61–76.

97. P.Y. Paterson, R.H. Swanborg, Demyelinating diseases of the central and peripheral nervous systems, in: M. Sampter, D.W. Talmage, M.M. Frank, K.F. Austen, H.N. Claman (Eds.), Immunological Diseases, 4, Little, Brown and Co, Boston, 1988, pp. 1877–1916.

98. Wekerle H, Meinl E. Multiple Sclerosis: An old drug plays a new trick. Nature (2013) 502:314-315. Doi: 10.1038/nature12694

99. Franklin R J M, French-Constant C. Remyelination in the CNS: from biology to therapy. Nature Reviews Neuroscience (2008) 9:839-855. Doi: 10.1038/nrn2480

100. Tomassini V, Matthews PM, Thompson AJ, Fuglo D, Geurts JJ, Johansen-Berg H, Jones DK, Rocca MA, Wise RG, Barkhof F, Palace J. Neuroplasticity and functional recovery in multiple sclerosis. Nature Reviews Neurology (2012) 8:635-647. Doi: 10.1038/nrneurol.2012.179

101. Lassmann H, van Horssen J, Mahad D. Progressive multiple sclerosis: pathology and pathogenesis. Nature Reviews Neurology (2012) 8:647-656. Doi: 10.1038/nrneurol.2012.168.

102. Kutzelnigg A, Lucchinetti CF, Stadelmann C, Bru¨ck W, Rauschka H, Bergmann M, and Schmidbauer M, Parisi JE, Lassmann H. Cortical demyelination and diffuse white matter injury in multiple sclerosis. Brain (2005) 128:2705–2712.

103. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Aloisi F. 2004. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. Brain Pathol 14:164–174.

104. Sawcer S, Hellenthal G, Pirinen M, Spencer CC, Donelly P, Patsopoulos NA, Moutsianas L, Alastair Compston. 2011. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature 476:214–219.

105. A. Denic, A.J. Johnson, A.J. Bieber, A.E. Warrington, M. Rodriguez, I. Pirko. The relevance of animal models in multiple sclerosis research, Pathophysiology (2010).

106. S.D. Miller, W.J. Karpus, Experimental autoimmune encephalomyelitis in the mouse, in: J.E. Coligan, B. Bierer, D.H. Margulies, E.M. Shevach, W. Strober, R.F. Coico (Eds.), Current Protocols in Immunology, Chapter 15, John Wiley & Sons, New York, 2007, pp. 15.11.11–15.11.18.

107. Kroenke MA, Carlson TJ, Andjelkovic AV, Segal BM. 2008. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. J Exp Med 205:1535– 1541.

108. Kebir H, Ifergan I, Alvarez JI, Bernard M, Poirier J, Arbour N, Duguette P, Prat A. 2009. Preferential recruitment of interferongamma- expressing TH17 cells in multiple sclerosis. Ann Neurol 66:390–402.

109. Krumbholz M, Theil D, Cepok S, Hemmer B, Kivisa¨kk P, Ransohoff RM, Hofbauer M, Farina C, Derfuss T, Hartle C, Newcombe J, Hohlfeld R, Meinl E. 2006. Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment. Brain 129:200–211.

110. Festa ED, Hankiewicz K, Kim S, Skurnick J, Wolansky LJ, Cook SD, Cadavid D. 2009. Serum levels of CXCL13 are elevated in active multiple sclerosis. Mult Scler 15:1271– 1279.

111. Sellebjerg F, Bo¨rnsen L, Khademi M, Krakauer M, Olsson T, Frederiksen JL, Sorensen PS. 2009. Increased cerebrospinal fluid concentrations of the chemokine CXCL13 in active MS. Neurology 73:2003–2010.

112. Piccio L, Naismith RT, Trinkaus K, Klein RS, Parks BJ, Lyons JA, Cross AH. 2010. Changes in B- and T-lymphocyte and chemokine levels with rituximab treatment in multiple sclerosis. Arch Neurol 67:707–714.

113. Khademi M, Kockum I, Andersson ML, Iacobaeus E, Brundin L, Sellebjerg F, Olsson T. 2011. Cerebrospinal fluid CXCL13 in multiple sclerosis: a suggestive prognostic marker for the disease course. Mult Scler 17:335–343.

114. Ragheb S, Li Y, Simon K, VanHaerents S, Galimberti D, De Riz M, Fenoglio C, Scarpini E, Lisak R. 2011. Multiple sclerosis: BAFF and CXCL13 in cerebrospinal fluid. Mult Scler 17:819–829.

115. Bielekova B, Komori M, Xu Q, Reich DS, Wu T. 2012. Cerebrospinal fluid IL-12p40, CXCL13 and IL-8 as a combinatorial biomarker of active intrathecal inflammation. PLoS One 7:e48370.

116. Alvarez E, Piccio L, Mikesell RJ, Klawiter EC, Parks BJ, Naismith RT, Cross AH. 2013. CXCL13 is a biomarker of inflammation in multiple sclerosis, neuromyelitis optica, and other neurological conditions. Mult Scler 19:1204–1208.

117. Romme Christensen J, Bo¨rnsen L, Khademi M, Olsson T, Jensen PE, Sørensen PS, Sellebjerg F. 2013. CSF inflammation and axonal damage are increased and correlate in progressive multiple sclerosis. Mult Scler 19:877–884.

118. Guruharsa G K, Kankel W M, Artavanis-Tsakonas Spyros. The Notch signaling system: recent insights into the complexity of a conserved pathway. Nature Reviews (2012) 13:654-666. Doi: 10.1038/nrg3272.

119. Andersson R E, Sandberg R, Lendahl U. Notch signaling: simplicity in design, versatility in function. Development Review (2011) 138:3593-3612. Doi: 10.1242/dev.063610.

120. Kopan R, Ilagan M X G. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell (2009) 137:216-233. Doi: 10.1016/j.cell.2009.03.045.

121. Baron M. An overview of Notch signaling. Seminars in Cell and Developmental Biology (2003) 14:113-119. Doi: 10.1016/S1084-9521(02)00179-9

122. Ayaz F, Osborne B A. Non canonical Notch signaling in cancer and immunity. Frontiers in Oncology (2014) 4:345. Doi: 10.3389/fonc.2014.00345.

123. Bell J J, Bhandoola A. The earliest thymic progenitors for T cells possess myeloid lineage potential. Nature (2008) 452:764 7.doi:10.1038/nature06840

124. Feyerabend T B, Terszowski G, Tietz A, Blum C, Luche H, Gossler A, Gale NW, Radtke F, Fehling HJ, Rodewald HR. Deletion of Notch1 converts pro-T Cells to dendritic cells and promotes thymic B cells by cell-extrinsic and cell-intrinsic mechanisms. Immunity (2009) 30:67–79. Doi: 10.1016/j.immuni.2008.10.016

125. Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. NatRevImmunol (2009) 9:767–77.doi:10.1038/nri2656

126. Wada H, Masuda K, Satoh R, Kakugawa K, Ikawa T, Katsura Y, Kawamoto H. Adult T-cell progenitors retain myeloid potential. Nature (2008) 452:768–72.doi:10.1038/ nature06839

127. Kwon C, Cheng P, King I N, Andersen P, Shenje L, Nigam V, Srivastava D. Notch post- translationally regulates b-catenin protein in stem and progenitor cells. NatCell Biol (2011) 13:1244–51.doi:10.1038/ncb2313

128. Cho O H, Shin H M, Miele L, Golde T E, Fauq A, Minter L M, Osborne BA. Notch regulates cytolytic effector function in CD8+ T cells. J Immunol (2009) 182:3380–9. doi:10.4049/jimmunol.0802598

129. Minter LM, Turley DM, Das P, Shin HM, Joshi I, Lawlor RG, Cho OH, Palaga T, Gottipati S, Telfer JC, Kostura L, Fauq AH, Simpson K, Such KA, Miele L, Golde TE, Miller SD, Osborne BA. Inhibitors of gamma-secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21. Nature Immunology (2005) 680-688.

130. Samon J B, Champhekar A, Minter L, Telfer JC, Miele L, Fauq A, Das P, Golde TE, Osborne BA. Notch1 and TGFβ1 cooperatively regulate Foxp3 expression and the maintenance of peripheral regulatory T cells. Blood (2008) 112:1813-1821. Doi: 10.1182/blood-2008-03-144980.

131. Keerthivasan S, Suleiman R, Lawlor R, Roderick J, Bates T, Minter L, Anguita J, Juncadella I, Nickoloff BJ, Le Poole IC, Miele L, Osborne BA. Notch signaling regulates mouse and human Th17 differentiation. J Immunol (2011) 187:692–701.doi:10.4049/jimmunol.1003658

132. Tsukomo S, Yasutomo K. Notch governing mature T cell differentiation. The Journal of Immunology (2004) 173:7109-7113. Doi: 10.4049/jimmunol.173.12.7109.

133. Amsen D, Blander J M, Lee G R, Tanigaki K, Honjo T, Flavell RA. Instruction of distinct CD4 T helper cell fates by different Notch ligands on antigen presenting cells. Cell (2004) 117:515-526.

134. Amsen D, Antov A, Flavel R A. The different faces of Notch in T helper cell differentiation. Nature Reviews (2009) 9:116-124. Doi: 10.1038/nri2488.

135. Radtke F, Fasnacht N, MacDonald H R. Notch signaling in the immune system. Cell (2010) 32:14-27. Doi: 10.1016/j.immuni.2010.01.004.

136. Radtke F, Macdonal H R, Tacchini-Cotier F. Regulation of innate and adaptive immunity by Notch. Nature Reviews (2013) 13:427-437. Doi: 10.1038/nri3445.

137. Dongre A, Surampudi L, Lawlor RG, Fauq AH, Miele L, Golde TE, Osborne BA. Non-canonical Notch signaling drives activation and differentiation of peripheral $CD4^+$ T cells. Front Immunol (2014) 54:1–14.10.3389/fimmu.2014.00054.

138. Vacca A, Felli M P, Palermo R, Di Mario G, Calce A, Di Giovine M, Frati L, Gulino A, Screpanti I. Notch3 and pre-TCR interaction unveils distinct NF-kB pathways in T-cell development and leukemia. EMBO J (2006) 25:1000–8.doi:10.1038/sj.emboj.7600996

139. Barbarulo A, Grazioli P, Campese A F, Bellavia D, DiMario G, Pelullo M, Ciufetta A, Colantoni S, Vacca A, Frati L, Gulino A, Felli MP, Screpanti I. Notch3 and canonical NF-kappa B signaling pathways cooperatively regulate Foxp3 transcription. J Immunol (2011) 186:6199–206.doi:10.4049/jimmunol. 1002136

140. Shin H M, Minter L M, Cho O H, Gottipati S, Fauq A H, Golde T E, Sonenshein GE, Osborne BA. Notch1 augments NF-kB activity by facilitating its nuclear retention. EMBO J (2006) 25:129 38. doi:10.1038/sj.emboj.7600902

141. Shin H M, Tilahun M E, Cho O H, Chandiran K, Kuksin C A, Keerthivasan S, Fauq AH, Golde TE, Miele L, Thome M, Osborne BA, Minter LM. NOTCH1 can initiate NF-kB activation via cytosolic interactions with components of the T cell signalosome. FrontImmunol (2014) 249:1–15. doi:10.3389/fimmu.2014.00249

142. Perumalsamy L R, Nagala M, Banerjee P, Sarin A. A hierarchical cascade activated by non-canonical Notch signaling and the mTOR-Rictor complex regulates neglect-induced death in mammalian cells. Cell Death Differ (2009) 16:879–89. doi:10.1038/cdd.2009.20

143. Lee K S, Wu Z, Song Y. Roles of PINK1, mTORC2, and mitochondria in preserving brain tumor-forming stem cells in a non-canonical Notch signaling pathway. Genes Dev (2013) 27:2642–7.doi:10.1101/gad.225169.113

144. Adler S H, Chiffoleau E, Xu L, Dalton N M, Burg J M, Wells A D, Wolfe M S, Turka L A, Pear W S. Notch signaling augments T cell responsiveness by enhancing CD25 expression. J Immunol (2003) 171:2896–903.doi:10.4049/jimmunol.171.6.2896

145. Felli MP, Vacca A, Calce A, Bellavia D, Campese AF, Grillo R, Di Giovine M, Checquolo S, Talora C, Palermo R, Di Mario G, Frati L, Gulino A, Screpanti I. PKC theta mediates pre-TCR signaling and contributes to Notch3-induced T-cell Leukemia. Oncogene (2005) 24:992-1000.

146. Maekawa Y, Tsukomo S, Chiba S, Hirai H, Hayashi Y, Okada H, Kishihara K, Yasutomo K. Delta-1 Notch3 interactions bias the functional differentiation of activated $CD4^+$ T cells. Immunity (2003) 19:549-559.

147. Elyaman W, Bradshaw EM, Wang Y, Oukka M, Kivisakk P, Chiba S, Yagita H, Khoury SJ. JAGGED1 and Delta1 differentially regulate the outcome of experimental autoimmune encephalomyelitis. J. Immunol. 179, 5990–5998 (2007).

148. Okamoto M, Takeda K, Joetham A, Ohnishi H, Matsuda H, Swasey CH, Swanson BJ, Yasutomo K, Dakhama A, Gelfand EW. Essential role of Notch signaling in effector memory CD8+ T cell‐mediated airway hyper responsiveness and inflammation. J. Exp. Med. 205, 1087–1097 (2008).

149. Skokos, D. & Nussenzweig, M. C. CD8– DCs induce IL‐12‐independent Th1 differentiation through Delta 4 Notch‐like ligand in response to bacterial LPS. J. Exp. Med. 204, 1525–1531 (2007).

150. Sun, J., Krawczyk, C. J. & Pearce, E. J. Suppression of Th2 cell development by Notch ligands Delta1 and Delta4. J. Immunol. 180, 1655–1661 (2008).

151. Amsen, D, Antov A, Jankovic D, Sher A, Radtke F, Souabni A, Busslinger M, McCright B, Gridley T, Flavell RA. Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. Immunity 27, 89–99 (2007).

152. Ito, T., Schaller, M., Hogaboam, C.M., Standiford, T.J., Sandor, M., lukacs, N.W., Chensue, S.W., and Kunkel, S.L. (2009). TLR9 regulates the mycobacteria-elicited pulmonary granulomatous immune response in mice through DC-derived Notch ligand delta-like 4. J. Clin. Invest. 119, 33–46.

153. Mukherjee, S., Schaller, M.A., Neupane, R., Kunkel, S.L., and Lukacs, N.W. (2009). Regulation of T cell activation by Notch ligand, DLL4, promotes IL-17 production and Rorc activation. J. Immunol. 182, 7381–7388.

154. Jurynczyk M, Jurewicz A, Raine CS, Selmaj K. Notch3 Inhibition in Myelin Reactive T cells Down-Regulates Protein Kinase C θ and Attenuates Experimental Autoimmune Encephalomyelitis. The Journal of Immunology (2008) 4:2634-2640. Doi: 10.4049/jimmunol.180.4.2634.

155. Vigouroux, S., Yvon, E., Wagner, H.J., Biagi, E., Dotti, G., Sili, U., Lira, C., Rooney, C.M., and Brenner, M.K. (2003). Induction of antigen-specific regulatory T cells following overexpression of a Notch ligand by human B lymphocytes. J. Virol. 77, 10872–10880.

156. Kared, H., Adle-Biassette, H., Foı¨s, E., Masson, A., Bach, J.-F., Chatenoud, L., Schneider, E., and Zavala, F. (2006). Jagged2-expressing hematopoietic progenitors promote regulatory T cell expansion in the periphery through notch signaling. Immunity 25, 823–834.

157. Anastasi E, Campese AF, Bellavia D, Bulotta A, Balestri A, Pascucci M, Checguolo S, Gradini R, Lendahl U, Frati L, Gulino A, Di Mario U, Screpanti I. Expression of activated Notch3 in transgenic mice enhances generation of T regulatory cells and protects against experimental autoimmune diabetes. J. Immunol. 171, 4504–4511 (2003).

158. Elyaman W, Bassil R, Bradshaw EM, Orent W, Lahoud Y, Zhu B, Radtke F, Yagita H, Khoury SJ. et al. Notch receptors and Smad3 signaling cooperate in the induction of interleukin- 9-producing T cells. Immunity 36, 623–634 (2012).

159. Brosnan C F, John G R. Revisiting Notch in remyelination of Multiple Sclerosis. The Journal of Clinical Investigation (2009) 119:10-13. Doi: 10.1172/JCI37786.

160. Bassil Ribal, William Orent, and Wassim Elyaman. Notch Signaling and T-Helper Cells in EAE/MS. Clinical and Developmental Immunology (2013) 1: 570731. Doi:10.1155/2013/570731. http://dx.doi.org/10.1155/2013/570731.

161. N. Takeichi, S. Yanagisawa, T. Kaneyama, H Yagita, YH Jin, BS Kim, CS Koh. Ameliorating effects of anti-Dll4 mAb on Theiler's murine encephalomyelitis virus-induced demyelinating disease. International Immunology, vol. 22, no. 9, pp. 729–738, 2010.

162. N.D. Reynolds, N.W. Lukacs, N. Long, and W. J. Karpus. "Deltalike ligand 4 regulates central nervous system T cell accumulation during experimental autoimmune encephalomyelitis," Journal of Immunology, vol. 187, no. 5, pp. 2803–2813, 2011.

163. W Elyaman, E M Bradshaw, Y Wang, M Oukka, P Kivisak, S Chiba, H Yagita, S J Khoury. "Jagged1 and delta1differentially regulate the outcome of experimental autoimmune encephalomyelitis," Journal of Immunology, vol. 179, no. 9, pp. 5990–5998, 2007.

164. Krebs L T, Xue Y, Norton C R. Characterization of Notch3 deficient mice: Normal embryonic development and absence of genetic interactions with a Notch1 mutation. Genesis (2003) 37:139-143. Doi: 10.1002/gene.10241.

165. Mitchell K J, Pinson K I, Kelly O G. Functional analysis of secreted and transmembrane proteins critical to mouse development. Nature Genetics (2001) 28:241-249.

166. Petit A, Pasini A, Alves Da Costa C, Ayral E, Hernandez J F, Dumanchin-Njock C, Phiel C J, Marambuad P, Wilk S, Farzan M, Fulcrand P, Martinez J, Andrau D, Checler F. JLK Isocoumarin inhibitiors: selective gamma-secretase inhibitors that do not interfere with notch pathway in vitro or in vivo. Journal of Neuroscience Research (2003) 74:370-7.

167. Wang M M. Notch signaling and Notch signaling modifiers. The International Journal of Biochemistry and Cell Biology (2011) 43:1550-1562. Doi: 10.1016/j.biocel.2011.08.005

168. Anastasi E, Campese A F, Bellavia D, Bulotta A, Balestri A, Pascucci M, Checquolo S, Gradini R, Lendahl U, Frati L, Gulino A, Di Mario U, Screpanti I. Expression of activated Notch3 in transgenic mice enhances generation of T regulatory cells and protects against experimental autoimmune diabetes. The Journal of Immunology (2003) 171:4503-4511. Doi:10.4049/jimmunol.171.9.4504.

169. Jone S E, Paynich M L, Kearns D B, Knight K L. Protection from intestinal inflammation by bacterial exopolysaccharides. The Journal of Immunology (2014) 192:000-000. Doi: 10.4049/jimmunol.1303369.

170. Aster J, Pear W, Hasserjian R, Erba H, Davi F, Luo B, Scott M, Baltimore D, Sklar J. Functional analysis of the TAN-1 gene, a human homolog of Drosophila Notch. Cold Spring Harb Symp Quant Biol (1994) 59:125–36.doi:10.1101/SQB.1994.059.01.016

171. Pear W S, Aster J C, Scott M L, Hasserjian R P, Soffer B, Sklar J, Baltimore D. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. J ExpMed (1996) 183:2283–91.doi:10.1084/jem.183.5. 2283

172. Swiatek P J, Lindsell C E, del Amo F F, Weinmaster G, Gridley T. Notch1is essential for post implantation development in mice. Genes Dev (1994) 15:707–19. doi:10.1101/gad.8.6.707

173. Aster J, Pear W, Hasserjian R, Erba H, Davi F, Luo B, Scott M, Baltimore D, Sklar J. Functional analysis of the TAN-1 gene, a human homolog of Drosophila Notch. ColdSpringHarb SympQuantBiol (1994) 59:125 36.doi:10.1101/SQB.1994.059.01.016

174. Miele L, Golde T, Osborne B A. Notch signaling in cancer. CurrMolMed (2006) 6:905–18.doi:10.2174/156652406779010830

175. Purow B. Notch inhibition as a promising new approach to cancer therapy. Adv ExpMedBiol (2012) 727:305–19.doi:10.1007/978-1-4614-0899-4_23

176. Ersvaer E, Hatfield K J, Reikvam H, Bruserud O. Future perspectives: therapeutic targeting of Notch signaling may become a strategy in patients receiving stem cell transplantation for hematologic malignancies. BoneMarrowRes (2011) 2011:1–15.doi:10.1155/2011/570796

177. Chen Y, DeMarco M A, Graziani I, Gazdar A F, Strack P R, Miele L, Bocchetta M. Oxygen concentration determines the biological effects of Notch1 signaling in adeno- carcinoma of the lung. CancerRes (2007) 67:7954–9.doi:10.1158/0008-5472.CAN-07-1229

178. Reedijk M, Odorcic S, Chang L, Zhang H, Miller N, McCready D R, Lockwood G, Egan SE. High- level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. CancerRes (2005) 65:8530–7. doi:10.1158/0008-5472.CAN-05-1069

179. Rizzo P, Osipo C, Foreman K, Golde T, Osborne B A, Miele L. Rational targeting of Notch signaling in cancer. Oncogene (2008) 27:5124–31.doi:10.1038/onc.2008.226

180. Jundt F, Anagnostopoulos I, Förster R, Mathas S, Stein H, Dörken B. Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. Blood (2002) 2002(99):3398–403. doi:10.1182/blood.V99.9.3398

181. Bellavia D, Campese A F, Checquolo S, Balestri A, Biondi A, Cazzaniga G, Lendahl U, Fehling HJ, Hayday AC, Frati L, von Boehmer H, Gulino A, Screpanti I. Combined expression of pTalpha and Notch3 in T cell leukemia identifies the requirement of pre-TCR for leukemogenesis. ProcNatlAcadSciUSA (2002) 99:3788–93.doi:10.1073/pnas.062050599

182. Lee K S, Wu Z, Song Y, Mitra S S, Feroze A H, Cheshier S H, Bingwei Lu. Roles of PINK1, mTORC2, and mitochondria in preserving brain tumor-forming stem cells in a noncanonical Notch signaling pathway. GenesDev (2013) 27:2642–7. doi:10.1101/gad.225169.113

183. Liu N, Zhang J, Ji C. The emerging roles of Notch signaling in leukemia and stem cells. BiomarkRes (2013) 1(1):1-23.doi:10.1186/2050-7771-1-23

184. Bolós V, Blanco M, Medina V, Aparicio G, Díaz-Prado S, Grande E. Notch signaling in cancer stem cells. Clin TranslOncol (2009) 11:11–9.doi:10.1007/ s12094-009-0305-2

185. Nicholas M, Wolfer A, Raj K, Kummer J A, Mill P, Noort M V, Hui CC, Clevers H, Dotto GP, Radtke F. Notch1 functions as a tumor suppressor in mouse skin. NatGenet (2003) 33:416–21. doi:10.1038/ng1099

186. Jeffries S, Capobianco A J. Neoplastic transformation by Notch requires nuclear localization. MolCellBiol (2000) 20:3928–41.doi:10.1128/MCB.20.11.3928-3941.2000

187. Dumont E, Fuchs K P, Bommer G, Christoph B, Kremmer E, Kempkes B. Neo- plastic transformation by Notch is independent of transcriptional activation by RBP-J signaling. Oncogene (2000) 19:556–61.doi:10.1038/sj.onc.1203352

188. Vacca A, Felli M P, Palermo R, DiMario G, Calce A, DiGiovine M, Frati L, Gulino A, Screpanti I. Notch3 and pre-TCR interaction unveils distinct NF-kB pathways in T-cell development and leukemia. EMBO J (2006) 25:1000–8.doi:10.1038/sj.emboj.7600996

189. Jin S, Mutvei A P, Chivukula I V, Andersson E R, Ramskold D, Sandberg R, Lee KL, Krongvist P, Mamaeva V, Ostling P, Mpindi J P, Kallioniemi O, Screpanti I, Poellinger L, Sahlgren C, Lendahl U. Non-canonical Notch signaling activates IL-6/JAK/STAT signaling in breast tumor cells and is controlled by p53 and IKKa/IKKb. Oncogene (2013) 32:4892–902.doi:10.1038/onc.2012.517

190. Liao W R, Hsieh R H, Hsu K W, Wu M Z, Tseng M J, Mai R T, Wu Lee YH, Yeh TS. The CBF1- independent Notch1signal pathway activates human c-myc expression partially via transcription factor YY1. Development (2007) 122:3765–73.doi:10.1093/ carcin/bgm092

191. Veeraraghavalu K, Subbaiah V K, Srivastava S, Charkrabarti O, Syal R, Krishna S. Complementation of human papilloma virus type 16 E6 and E7 by Jagged1-specific Notch1-phosphatidyl inositol 3-kinase signaling involves pleiotropic oncogenic functions independent of CBF1; Su(H); Lag-1 activation. J Virol (2005) 79:7889–98.doi:10.1128/JVI.79.12.7889-7898.2005