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# Modulation of Notch in an Animal Model of Multiple Sclerosis

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**Modulation of disease in an animal model of Multiple Sclerosis**

A Thesis Presented

by

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**ABSTRACT**

MODULATION OF DISEASE IN AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

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Multiple Sclerosis (MS) is a neurodegenerative autoimmune disease that affects millions of people worldwide. Although the exact cause of MS is unknown, it is clear that CD4+ T helper cells play a significant role, namely T helper 1 (Th1) and T helper 17 (Th17) cells. The Notch family of proteins plays a role in the development and differentiation of T helper cells. Previous data has shown that inhibition of Notch impairs the ability of T helper cell differentiation. Additionally specific inhibition of certain Notch members inhibits specific T helper cell differentiation, for example the inhibition of Notch 1 inhibits Th1 and iTreg polarization [Samon et al., 2008]. However, the effects of the other Notch family members on $CD4^+$ T cells are not fully studied. We propose that Notch 3 plays an extensive role in the regulation of Th1, Th2, Th17, and iTreg polarizations. In addition, we propose that Notch 3 regulates function of T helper cell function in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE). Data in this thesis show that Notch 3 plays a significant role in the polarization of Th1, Th17 and iTreg polarization [Karlsson et al., 2011]. We present evidence that the heterozygous and homozygous Notch 3 knockout exhibits a significant decrease in polarization toward Th1, Th17 and iTreg cell fates.

Exopolysaccharide (EPS) is a compound that has been previously shown to play a protective role in other inflammatory diseases. EPS has been shown to produce anti-inflammatory macrophages. We propose that a similar anti-inflammatory effect might be possible in EAE. We found that EPS had a significant effect on EAE induction, decreasing the onset and peak disease score. EPS also reduced the concentration of IFN-γ, IL17A, and GM-CSF in the supernatants of the splenocytes after restimulation with MOG. Further experimental data is needed to prove the effects of EPS on EAE and the method by which EPS function. These data indicate that Notch 3 could be crucial in regards to EAE due to the effects on Th1 and Th17 which are instrumental in EAE induction [Raphael et al., 2015].

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# CHAPTER 1

# INTRODUCTION

## 1.1. $CD4^+$ T cell development and function

The human immune system can be divided into two distinct arms, the innate and the adaptive immune system. The innate immune system is comprised of cells and mechanisms that are responsible for immediate defense against infection. However the adaptive immune system is a complex system of cells designed to react against specific pathogens and create a memory against that pathogen for future repeated exposures. The adaptive immune system is comprised of many cell types including B cells and T cells, of which there are two kinds; $CD8^+$ T cells are responsible for inducing the death of damaged, infected or dysfunctional cells, while $CD4^+$ T cells are a subset of lymphocytes which are responsible for helping or suppressing and regulating the immune system [Radtke et al., 2013].

$CD4^+$ T cells, also known as T helper cells, are crucial for a targeted immune response. As all T cells, T helper cells originate from the bone marrow as lymphoid progenitor cells. From there, they migrate to the thymus and expand creating a population of T-cell precursors. These precursors go through multiple processes such as beta selection, positive selection, and negative selection, which results in the production of T cells that have a functional T cell receptor (TCR) capable of recognizing and binding to peptides without binding strongly to self-antigens. These cells then migrate to the spleen where they can be activated by antigen presenting cells (APCs). This is done by the MHC class II molecules on APCs in addition to a co-stimulatory signal by B7 through the TCR and CD28 receptor on the T cell respectively. These

two signals, accompanied by certain cytokines, result in the differentiation and proliferation of a specific T helper cells. These cells are Th1, Th2, Th17, Treg as well as other, less researched, subtypes. These T helper cells not only range in the cytokines they are able to produce but also the surface markers they express as well as being capable of re-differentiating into other T helper subtypes [Mitchell et al., 2001].

### 1.2. Th1 cell development and function

T helper 1 (Th1) cells play an important role in the defense of intracellular pathogens. Th1 cells produce IL-2, IL-10, interferon-gamma (IFN-γ) and Tumor Necrosis Factor-alpha (TNF-α) [Asseman et al., 1999]. Th1 cells are driven by the transcription factors T-bet and STAT-4, as well as STAT-1 and STAT-5 [Grifka-Walk et al., 2013, Zhou et al., 2009]. IFN-γ producing Th1 cells are important for $CD8^+$ T cell activation via MHC class II molecules as well as for activation of macrophages. IL-2 is a key cytokine for the proliferation of T cells and B cells. It also plays an important role in T cell memory. IL-10 acts as a negative feedback loop, suppressing excessive Th1 activity [Asseman et al., 1999].

With IFN-γ producing cells being found in the central nervous system (CNS) of MS patients, Th1 cells are thought to one of the more prominent inducers of MS. Along with Th17 cells, Th1 cells are responsible for the inflammation as well as the degeneration of neurons in the CNS [Jager et al., 2009]. Th1 cells are therefore another primary target of MS therapy along with Th17 cells. Although thought to have a prominent role due to the presence of T-bet and IFN-γ in the CNS of MS patients, the neutralization of IFN-γ does not result in a reduction in MS

score while inhibition of T-bet results in both a decrease in Th1 and Th17 induction and improves EAE in mice [Grifka-Walk et al., 2013, Jager et al., 2009].

### 1.3. Th2 cell development and function

T helper 2 (Th2) cells are important in the defense against extracellular parasites as well as play a role in allergies. Th2 cells are known for the production of the IL-4, IL-5, IL-9, IL-10, IL-13, and IL-25 cytokines [Zhu et al., 2001]. The key transcription factor for Th2 cells is GATA-3 in addition to other factors such as STAT6. These transcriptions factors play an important role in T cell development and the promotion and selection of the previously mentioned cytokines. Th2 cells are known to associate with B-cells, eosinophils and mast cells [Zhu et al., 2001]. Through IL-5, IL-9, Th2 cells promote the activation of these cells. IL-4 has many roles, including an effect on B cells resulting in class switching to produce IgE antibodies, as a positive feedback for Th2 cell differentiation, and possibly in the activation of macrophages against parasites [Cote-Sierra et al. 2004]. IL-10 is thought to play a role as a suppressor of Th1 differentiation and activity, thereby promoting increased Th2 differentiation. In addition to IL-4, IL-2 also plays an important role in Th2 differentiation [Cote-Sierra et al. 2004].

In Multiple Sclerosis (MS), Th2 cells are thought to be involved through B cells by the promotion of antibody production and class switching which can result in autoimmunity. However, Th2 cells are also thought to inhibit macrophages and suppress inflammatory cytokines, such as those produced by Th1 and Th17 cells, which are thought to be directly involved in MS induction [Sun et al., 2008]. It is therefore difficult to determine whether Th2

cells are involved positively in disease by the direct suppression of inflammatory cells or whether they are involved negatively by the induction of antibody mediated autoimmunity.

### 1.4. Th17 cell development and function

T helper 17 (Th17) cells are important in the defense against extracellular bacteria and fungi. Primary cytokines of Th17 cells include IL-17A, IL-17F, IL-21 and IL-22 [Ivanov et al., 2007]. Th17 cell transcription factors include STAT-3 and retinoic acid receptor related orphan receptor gamma T (RORyT) [Ivanov et al. 2006, 16]. Th17 cell polarization can be induced in the presence of IL-6, IL-21, IL-23 and TGFβ [Mangan et al., 2006, Zhou et al., 2007, Zhou et al., 2008]. IL-17 is a pro-inflammatory cytokine which can cause the production of multiple other cytokines, including IL-6, GM-CSF, and TNF-α, as well as the chemokine CXCL-8 [Sheng et al., 2014]. In addition, IL-17A and IL-17F are capable of recruiting and activating neutrophils, which are essential for the bacterial and fungal immune responses. IL-21 acts as a positive feedback loop, to further amplify Th17 polarization as well as activate dendritic cells (DCs), $CD8^+$ T cells, B cells, and natural killer (NK) cells [Zhou et al., 2007].

Th17 cells are thought to be a contributing factor to the inflammatory profile of the CNS in MS patients [Grifka-Walk et al., 2013]. IL-23 generated Th17 cells are very prominent in the development of MS and neutralization of IL-17 has shown a dramatic decrease in the severity of MS induction in mice [Manel et al., 2008]. In addition to Th1 cells, the pro-inflammatory abilities and neutrophil recruitment capability of Th17 cells are key reasons why they are thought to be a critical target in MS treatment.

### 1.5. Treg cell development and function

T regulatory (Treg) cells, as the name would suggest, are responsible for the regulation of the immune system, as well as protecting against autoimmune disease and reactivity towards self-antigens. There are two types of Treg cells, natural Treg (nTreg) and induced Treg (iTreg) [Curotto de Lafaille et al., 2009]. Treg cells that developed in the thymus are known as nTregs, while those that differentiate in the lymph nodes and are induced by cytokines are iTregs [Liu et al., 2008]. iTreg cells can be induced both in vivo outside of the thyus as well as in vitro, however nTreg cells can only be produced inside the thymus in vivo. Treg cells produce the anti-inflammatory cytokines transforming growth factor β (TGFβ), IL-10, and IL-35 [Curotto de Lafaille et al., 2009]. Treg cells express the Foxp3 transcription factor as well as STAT-5 [Fontenot et al., 2003]. The production of TGFβ allows Treg cells to both produce more iTregs as well as act as a suppressive cytokine [Li et al., 2007]. IL-10 is an anti-inflammatory cytokine and IL-35 acts as an immune suppressor [Asseman et al., 1999]. These cytokines help build the suppressive and regulatory profile of Treg cells in vitro [Karlsson et al., 2011]. iTreg cell polarization has been shown to be regulated by Notch 1 but could also be regulated by another Notch family member [Palaga et al., 2003, Samon et al., 2008].

Relating to MS, Treg cells are very important in reducing inflammation and reinforcing tolerance of immune cells towards self antigens. The neurodegenerative effects of Th1 and Th17 cells thought to be responsible for the symptoms of MS may be counteracted as well as possibly prevented by Treg induction [Karlsson et al., 2011]. However, as a drawback, the suppressive effect of Treg cells on autoimmunity, without control, could lead to over-suppression of the immune system.

### 1.6. Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disease that afflicts millions of people worldwide. Many risk factors exist which can make a person more susceptible to develop MS, including gender, race, life style, dietary and environmental factors, however the initial events that can cause development to occur are yet unknown. Certain genetic risk factors also exist, including polymorphism in MHC class II molecules as well as the IL-2 receptor α chain, both of which are important receptors in T cell development. Although not fatal, the disease is characterized by inflammation of the central nervous system and destruction of myelin sheaths of neurons, as well as oligodendrocytes responsible for re-myelination (Figure 2)[Jurynczyk et al., 2008]. In this way, this debilitating disease causes muscle weakness, muscle spasms, difficulty moving, ataxia, and many other neurological symptoms [Jurynczyk et al., 2010]. Early on, the lymphocytes responsible for MS breach the blood-brain barrier, forming lesions called plaques. These plaques contain $CD4^+$ and $CD8^+$ lymphocytes and macrophages, which destroy myelin. During late stage of MS, the plaques are reduced to demyelinated or destroyed axons and scar tissue, which can prevent remyelination or recovery [Jurynczyk et al., 2005].

Experimental Autoimmune Encephalomyelitis (EAE) is the most commonly used mouse model for MS in research. It mimics many of the neurological and muscular symptoms however over a quicker time course. The one drawback is that EAE can be fatal. Studies in EAE have revealed the critical role that $CD4^+$ T cells play in MS induction. From neutralization of specific $CD4^+$ T cells to the adoptive transfer of T cell immunized with EAE specific peptides, the role of various $CD4^+$ T cells have been identified and analyzed. Although not completely understood,

data have shown that various $CD4^+$ T cell cytokines play a significant role in MS development [Jurynczyk et al., 2005]. Specifically, it is known that both Th1 and Th17 cells play a role in MS induction, although the exact role that those cells play is not completely clear due to the paradoxical nature of Th1 involvement [Jager et al., 2009, Stoolman et al., 2014]. Data has shown that both reducing IFN-γ as well as overexpressing INF-γ results in exacerbated EAE severity [Raphael et al., 2015]. Th17 cells are shown to play a role by the blockage of IL17 resulting in reduced EAE severity [Raphael et al., 2015]. These reports seem to point to either an undefined relationship between Th1 and Th17 cells in the induction of EAE, or another indirect pathway through a different cell type. Another important consideration is the presence of the cytokine granulocyte-monocyte colony stimulating factor (GM-CSF), which is crucial in the maturation of monocytes into macrophages and the activation of those macrophages [Raphael et al., 2015, Sheng et al., 2014]. It is shown that reduction of GM-CSF in mice can lead to reduced EAE severity, even with similar IFN-γ and IL-17A production as normal WT mice [Sheng et al., 2014]. Therefore it is important to consider not only the cytokines that play a pivotal role in the disease, but also the cells which produce these cytokines since they provide multiple targets for therapy.

### 1.7. Notch signaling

Notch is a protein receptor that initiates key signaling pathways. It has previously been shown to be essential in development as well as play a significant role in various diseases, including modulation of MS [Arboleda-Velasquez et al., 2008]. There are four different isoforms of Notch (1-4), which vary in the number of EGF repeats in the extracellular region, the

possession of a trans-activation domain, the number of nuclear localization signal domains and the transcriptional targets (Figure 1). Notch signaling occurs when a ligand, either Delta-like 1-4 or Jagged 1-2, on the surface of another cell interacts with Notch. This results in an initial extracellular cleavage by an ADAM protease followed by a transmembrane cleavage by gamma secretase [Radtke et al., 2010]. Once free of the membrane, the intracellular domain of Notch is able to translocate to the nucleus where it is able to interact with RBPJk/CSL, removing the transcriptional repressor and initiating the activation of other targets [Palaga et al., 2003, Radtke et al., 2013, Tsukomo et al., 2004]. This process, known as canonical Notch signaling, is not the only process by which Notch signaling occurs. Another process by which Notch activation occurs, termed non-canonical Notch signaling, is less well characterized.

Notch signaling plays a crucial role in the development of T helper cells [Palaga et al., 2003, Radtke et al., 2004]. For example, T helper cells can be polarized toward Th1 by the overexpression of the Notch 1 as well as the Notch 3 isoform, or by the activation of the cell by the Delta-like ligand 1 (DLL1) [Palaga et al., 2003, Radtke et al., 2004]. In addition, it has been shown that the reduction of Notch signaling by gamma secretase inhibition, results in reduced Th1 polarization [Palaga et al., 2003]. Similarly, Jagged 1 signaling of T helper cells increases the polarization towards Th2 cells, as well as overexpression of Notch 1 or Notch 2 [Radtke et al., 2010, Radtke et al., 2004]. It is also suggested in a recent study by our group that Notch 1 plays an important role in Treg polarization [Palaga et al., 2003, Samon et al., 2008]. Due to effect of Notch on the polarization of T helper cells, and the effect of these cells in MS, it is possible that Notch could be a target for MS therapy. It has been shown that the using gamma secretase inhibitors decreased EAE scores compared to control groups [Jurynczyk et al., 2008]. In

addition, it has been shown that antibody-blocked Notch 1 has no effect on EAE scores and that a Notch 3 blocking antibody lessens EAE severity [Jurynczyk et al., 2010]. Although the authentication of the effectiveness and precision of these antibodies is unclear, these data suggest that Notch 3 may be an effective target for a new method of EAE therapy. In addition, a variety of Notch ligands have been shown to have effects on EAE severity, both being shown to ameliorate (DLL1 and DLL4) and exacerbate (Jagged 1) disease [Arboleda-Velasquez et al., 2008].

**1.8. Exopolysaccharide**

Through collaboration with Katherine L. Knight of Loyola University in Chicago, it has been determined that Exopolysaccharide (EPS) could play an important role in EAE suppression [Jones et al., 2014]. EPS is a secreted polysaccharide that has been shown to play a role in protection against intestinal inflammation. As EAE is an inflammatory disease of the CNS, we hypothesized that there could be a role for inflammation suppression of EPS in EAE. EPS is composed primarily of carbohydrates, namely mannose and glucose.

Using the inflammatory pathogen *Citrobacter rodentium* as a model for colitis, the Knight lab showed that the bacterium, *Bacillus subtilis*, protects mice from *C. rodentium* [Jones et al., 2014]. It was shown that no symptoms, including diarrhea, cytokine levels, and changes in colonic architecture, were present with mice that were treated with *B. subtilis*. In a previous study, Knight showed that EPS mutant *B. subtilis* failed to prevent disease from *C. rodentium*. Using EPS overexpressing strain of B. subtilis, it was shown that EPS was, by itself, sufficient enough to abrogate disease. In addition to showing the anti-inflammatory effects of EPS, they

also looked at the pathways through which EPS may work specifically focusing on the MyD88 pathways and Toll-like receptors (TLRs). Looking at MyD88, they were able to determine that MyD88 knock out mice displayed severe disease scores and the use of *B. subtilis* was ineffective. This showed a clear role for MyD88 in EPS-mediated prevention of colitis. To see which MyD88-dependent TLR was needed for protection against colitis, they looked at different TLR knockouts, (TLR4 and TLR2 specifically). They showed that when TLR4 was knockout out, disease was not prevented in the presence of EPS. However, they also showed that using just a TLR4 agonist does not have the same result on colitis protection. This data proves that EPS does affect TLR4 through MyD88.

Finally the Knight lab examined cells which bind to EPS [Jones et al., 2014]. EPS was injected peritoneally and it was observed that almost all the EPS was bound to peritoneal $F4/80^{+}$ $CD11b^{+}$ macrophages. In addition, they found that EPS bound to these peritoneal macrophages even in TLR4 knock-out mice, showing that EPS does not rely on TLR4 to bind to the macrophages and may bind to another receptor. In order to show the effects on cytokines related to colitis, the group incubated TLR4 knock-out and WT cells with EPS and observed no difference. In addition, they looked at the effects of TLR4 agonist (LPS) and a TLR2 agonist (Pam3Cys4) and also on WT and TLR4KO and noticed that no difference in the knock out cells, while WT cells expressed various inflammatory cytokines in response to LPS and Pam3Cys4. This experiment concluded that EPS requires TLR4 signaling, but may not bind directly to TLR4. In addition, EPS does not produce or directly cause the production of pro-inflammatory cytokines.

The Knight lab then looked at the effects of EPS on epithelial cells versus myeloid cells and noticed that there was no change, if not an increase, in disease score in myeloid knock out mice versus the expected decrease in disease in epithelial knock out mice [Jones et al., 2014]. Finally they looked at the ability of EPS protection to transfer from one mouse to another by adoptive transfer. In these experiments, they found that not only does the protective ability transfer, but it transfers from WT mice to TLR4 knock-out mice as well. In addition, it does not transfer from TLR4 knock-out mice to WT. They showed that epithelial myeloid cells require TLR4 and MyD88 to mediate protection, but that this protection can be transferred to other mice without TLR4. The results suggest that protection by EPS does require MyD88 signaling in myeloid cells, but is not as necessary in peritoneal cells.

The data from the Knight lab suggests that EPS could play a role in the suppression of inflammation, as in the colitis model used. They have shown that EPS can induce anti-inflammatory macrophages, which can be essential for and could alleviate, if not prevent, EAE [Jones et al., 2014]. Since EAE is an autoimmune disease which causes inflammation of the CNS, it is possible that these anti-inflammatory macrophages will have an effect on the Th1 and Th17 cells which are thought to be responsible for EAE. We tested this hypothesis and, in preliminary studies, have seen reduced EAE scores in EPS treated mice. However, further experimentation is required to confirm our initial observation.

## 1.9. Specific aims and significance

### 1.9.1. Aims

**Specific aim 1**: Determine which Notch family member is involved in development of EAE by determining the effect on T helper cell fate.

A. Determine the effects of Notch 3 on Th1 polarization. These experiments were done by Furkan Ayaz
B. Determine the effects of Notch 3 on Th2 cells by Gata3 levels using flow cytometry and IL4 and IL5 levels by ELISA.
C. Determine the effects of Notch 3 on Th17 cell polarization by IL17A levels using ELISA
D. Determine the effects of Notch 3 on iTreg polarization of $CD4^+CD25^-$ splenic cells to $CD4^+CD25^+$ Treg cells
E. Determine the effects of Notch 3 on nTreg cells by $CD4^+CD25^+$ cells in the thymus.

**Specific aim 2:** Determine whether EPS blocks Th1/Th17 response or expands Treg cells in EAE inducing conditions.

A. Determine the effect of EPS on in vivo EAE response
B. Identify the cell type that is affected by EPS in EAE response by observation of cytokines found in infected mice, namely: GM-CSF, IFN-γ, IL-2, and IL-17A.

Note: Much of the studies reported here were done in collaboration with Furkan Ayaz. Experiments conducted solely by Furkan are noted in the figure legends.

### 1.9.2. Significance

Multiple Sclerosis (MS) affects millions of people worldwide. The goal of these experiments is to further understand and make steps towards developing new treatments based on information from T cell experiments and EPS studies. Notch is known to play a large role in a number of cells and therefore has been linked to a variety of diseases. Knowing the role it plays in different cell types, in the context of EAE, could not only help develop therapies for MS patients, but also help advance our understanding of Notch in other autoimmune

diseases, as well as cancers. Based on previous data showing Notch 1 is important in Th1 polarization and not effective in EAE modulation as well as previous data showing Notch 3 to significantly reduce EAE, we expect to see Notch 3 regulate Th1 and Th17 cells. Based on preliminary data we expect these experiments to make large strides towards better understanding the changes caused by Notch 3 in mice immunized with EAE.

EPS has previously been shown to reduce the severity of another inflammatory disease in immunized mice. It is likely EPS has an effect on cytokine production or translocation of immune cells to the CNS. We expect a decrease in IL-17A, INF-y, and GM-CSF in in vitro re-stimulation of immunized T helper cells. These experiments in addition to understanding the effect EPS has on EAE immunization will help in beginning to develop new treatments as well as better understanding of the causes of MS, which are currently poorly understood

# CHAPTER 2

# RESULTS/DISCUSSION

## 2.1. Aim 1 results

### 2.1.1. Aim 1A

We examined the role of Notch 3 on Th1 cell polarization by looking at concentration of Th1 cytokine, IFN-γ. To achieve this, we looked at IFN-γ levels of $CD4^+$ cells from the spleen of wild-type (WT) versus Notch 3 heterozygous knockout (N3hetKO) as well as WT versus Notch 3 knockout (N3KO) that have been incubated under Th1 polarizing conditions. We noticed, that when under Th1 polarizing conditions, there is a significant decrease in IFN-γ levels in both N3hetKO and N3KO (Figure 3a,c). In addition, we looked at the IL-2 concentration in the same samples as a control since IL-2 plays an important role in the differentiation and regulation of $CD4^+$ cells. We noticed that there was virtually no difference in IL-2 concentration (Figure 3b,d) in either N3hetKO or N3KO samples.

### 2.1.2. Aim 1B

We hypothesized that Notch 3 may have an effect on Th2 cells. We looked at IL-4 and IL-5 concentrations in supernatents after polarizing splenocytes from WT, N3hetKO and N3KO mice toward the Th2 cell fate. First, in Figure 4a, we looked at polarized versus unpolarized cells for IL-5 expression. We found there is a difference between WT and N3KO in both polarized and unpolarized conditions and although not significant, the increase in N3KO Th2 population is clear. However IL-4 concentrations, as shown in Figure 4b, show no difference between N3KO and WT in either polarized or unpolarized conditions. In addition, we looked at the expressions of GATA-3 transcription factor in these conditions. We noticed that N3KO showed a significantly

lower Gata-3 concentration in both N3KO polarized and unpolarized conditions compared to the WT (Figure 4c). These data suggest that Notch 3 does not play a critical role in Th2 polarization or Th2 regulation.

### 2.1.3. Aim 1C

In Figure 5, we examined the effects of Notch 3 on Th17 cells. In order to determine the effect of Notch 3, we looked at IL-17A as an indicator of Th17 polarization as well as IL-2 as a control for $CD4^+$ T cell proliferation. In Figure 5a and Figure 5c, we showed that under polarizing conditions, the absence of Notch 3 significantly reduced concentration of IL-17A. In addition, we see that Notch 3 has little to no effect on IL-2. Therefore, it is likely that Notch 3 plays a significant role in Th17 polarization.

### 2.1.4. Aim 1D

In Figure 6a and 6b, we examined iTreg polarization in WT versus N3hetKO. We analyzed the difference between both the unpolarized and polarized groups to determine the effects of Notch3 on iTreg polarization. In order to determine the polarization Tregs, we look at Foxp3, which is the master regulator of Treg cells as well as CD25, which is found on the surface of activated T cells and we use to compare activated $CD4^+Foxp3^+$ cells to inactive $CD4^+Foxp3^+$ cells. We, therefore, define $CD25^+Foxp3^+$ cells as iTregs. Initially, we looked at the $CD4^+$ $Foxp3^+$ population to establish the effect of Notch3 on baseline $Foxp3^+$ cells as a control. However, we notice in Figure 6a that there is virtually no difference between any of the conditions for the $CD4^+$ $Foxp3^+$ populations. Next, in Figure 6b, we noticed that unpolarized N3hetKO iTreg population was significantly higher than the WT. Conversely, when under polarizing conditions, we noticed that the N3hetKO iTreg population was significantly lower than WT. This data shows

that in N3hetKO cells, polarizing cytokines have no effect and the percent of iTreg cells after polarization remains approximately the same. In contrast, the low levels of iTreg cells in WT mice are drastically different than the significantly higher percentage of iTreg cells in the polarized population.

We then looked at the iTreg polarization in WT versus (N3KO) mice. We once again compared unpolarized versus polarized conditions. In Figure 6c, we look at $CD4^+Foxp3^+$ as a control for $FoxP3^+$ cells and see that although N3KO has slight decrease in $CD4^+Foxp3^+$ the differences are insignificant. Figure 6d shows the iTreg polarization between WT and N3KO. Similar to N3hetKO, we see a significant decrease in iTreg polarization compared to WT when under polarizing conditions. The only difference we notice between N3KO and N3hetKO is a lower percentage of iTreg cells in N3KO compared to WT in non-polarizing conditions.

### 2.1.5. Aim 1E

In Figure 7 we hoped to examine the effect of Notch 3 on nTreg populations in WT vs N3KO lymph nodes. These nTreg cells are taken straight from lymph nodes, stained and read by flow cytometry. Due to the natural polarization of this population, there is no polarization step, so we only looked at differences between WT vs. N3KO. Figure 7a shows there is a significant difference in the percentage of nTreg cells present in the lymph node. N3KO mice has a significantly smaller nTreg cell population compared to WT. Figure7b-7e also shows other populations of the lymph node, including $CD4^+Foxp3^+$, $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ cell populations. These populations show that there is no significant difference in the other major cell populations in the lymph nodes. This control is done so that we know changes in other populations are not the cause of the difference in nTreg percentage.

**2.2. Aim 1 discussion**

Previous data done by our lab has shown there to be a strong correlation between Notch 3 and T helper cell types relevant to EAE (Ayaz et al. 2016, Suleiman et al. 2013). We know the effects of Th1 and Th17 cells have on EAE induction, but we also know that other cells may play a role, such as iTreg and nTreg cells in the regulation of the other population of T helper cells and cytotoxic T cell populations. Therefore, in order to study this, we need to look at Th1, Th17, iTreg and nTreg polarization in Notch 3 knockouts (N3KO) versus wild-type (WT) mice. It is also important to see the effects on populations we think may not play a large role in EAE, such as Th2 cells.

Treg polarization is a very interesting target for MS therapy as it targets and regulates many other cell types. Initially, we wanted to look at the effects of Notch 3 on the inducible Treg population. What we found was that Treg polarizing conditions in both N3KO and N3hetKO mice were far less effective than on the WT cells. This observation indicates that Notch 3 plays a significant and necessary role in the polarization of iTreg cells. In addition, we also determined that the N3hetKO group displays a significant decrease in the polarization into iTregs under Treg polarizing conditions. One caveat to the data is the inexplicable increase in $CD25^{+}Foxp3^{+}$ cells in the N3hetKO unpolarized condition. However since it is clear that under polarizing conditions the iTreg population is reduced, we feel confident that Notch 3 influences iTreg polarization.

Examining the natural Treg population also gives us information on the natural production of Treg cells in an environment without Notch 3. Since there is no polarization step,

we only looked at the N3KO versus WT conditions. From this data, we can also say that there is a significant decrease in the percentage of nTreg in N3KO animals compared to WT. Even though the total percentage is small, this is approximately the size of the population we would expect to see for nTreg in the lymph node. In addition, to control for differing cell populations, we looked at $CD4^+$ and $CD8^+$ as well as double positive populations and noticed no difference in the numbers, showing us that there is no difference in the overall cell population, such as higher $CD8^+$ percentage, leading to the lower nTreg population.

We also examined Th2 polarization in WT and N3KO conditions. Since Th2 is not a major player in MS pathology, we expected there to be either an increase or no difference with lack of Notch 3, as we expected a decrease of iTreg, Th1 and Th17 polarizations. To this end we analyzed the concentrations of IL-5 and IL-4 in the supernatants of the cells after polarization. What we noticed was a clear increase in the concentrations of IL-5. However with IL-4, we saw no difference at all. This disparity could be for a number of reasons, such as the IL-4 may be consumed more rapidly than the IL-5 for the polarization of additional Th2 cells. Despite this disparity, the data suggests that Notch 3 either has no effect or a negative effect on Th2 polarization.

I then looked at Th17 cells and the effect of N3hetKO and N3KO on Th17 polarization. Since the literature suggests an important role for Notch 3 driving Th17 polarization, we expected to see a dramatic decrease in IL-17A without Notch 3. With no polarizing cytokines for both WT and N3hetKO, we see almost no IL-17A at all. When under polarizing conditions, there is a significant decrease in Th17 polarization (Figure 5). In addition, we see almost the exact

same results for N3KO as N3hetKO, suggesting hemizygous deletion of Notch 3 is as effective as homozygous deletion. This data suggests a clear effect of Notch 3 on Th17 polarization.

Finally, I looked at the effect of Notch3 on Th1 polarization. Similar to Th17 polarization, there was no difference in IFN-γ levels between WT and N3KO or N3hetKO in the unpolarized conditions (Figure 3). However, there was a significant decrease in IFN-γ concentration in N3KO and N3hetKO conditions compared to WT, suggesting that Notch 3 plays a critical role in the polarization of Th1 cells.

Through these experiments, I determined what role Notch 3 plays on the T cells that are relevant to EAE induction. We determined that Notch 3 plays an important role on polarization of Th1 and Th17 cells and the lack of Notch 3 resulted in a reduction in the polarization of Th1 and Th17 cells, even in specific polarizing conditions. However, surprisingly Notch 3 also played a critical role in Treg polarization and natural Treg production. This is unfortunate because since all three T cells groups are so critically affected by Notch 3, targeting Notch 3 would result in decreased Th1 and Th17, but also Treg polarization. Although this is not ideal, our data indicates that Notch 3 could play an important role in EAE and MS therapy, such as Notch 3 blocking antibodies.

### 2.3. Aim 2 results

#### 2.3.1. Aim 2A

Our preliminary results show a clear difference between EPS treated mice and untreated EAE induced control. Figure 8 shows that although normal EAE induction reaches a mean of about three, when treated with EPS, mean disease score is reduced to two.

We next looked to see if pretreating EAE induction with EPS would have a greater effect than treating with EPS during induction. The disease scores (Figure 9) showed a similar significant pattern between normal EAE induction and EAE induction with EPS treatment. However in mice that were pretreated with EPS on day -3, no signs of disease developed.

### 2.3.2. Aim 2B

The supernatents from the previous experiment (not shown) have decreased IFN-γ levels in the EPS treated samples, however no real difference between the levels for IL17A and GM-CSF were shown between control, EAE induction and EAE induction with EPS treatment.

The ELISAs of the supernatents from the experiment shown in Figure 8 show that there is a significant decrease in IFN-γ concentration in EPS treated and EPS pretreated, with a slightly greater decrease in the EPS pretreated (Figure 10). However this is the only similarity to our preliminary results. We also see in Figure 10 that there is a significant decrease in both GM-CSF and IL17A and that pretreatment of EPS increases IL17A concentration significantly compared to regular treatment of EPS.

## 2.4. Aim 2 discussion

Based on previous data and preliminary results, we thought we would see a significant decrease in EAE induction consistently as well as an even greater decrease in both the onset of disease as well as peak score. Our first findings showed a highly significant decrease in score and a similar pattern in cytokine profile. Initially, we only saw a decrease in IFN-γ levels and similar levels of IL17A and GM-CSF for EAE induction with or without EPS treatment. However

upon further investigation, we noticed a significant difference in both GM-CSF and IL17A concentrations in the supernatents. In addition, we saw a slight increase in concentrations of both GM-CSF and IL17A when pretreated with EPS compared to regular EPS treatment. This disparity could be due to the pretreatment preventing disease earlier and by the time point of peak disease for normal EAE induction, the mouse could be already returning to homeostasis. However, since the difference isn't significant, it is also very likely that there is actually no difference and the disparity is just coincidence.

Due to technical problems with EPS purity and EAE induction the experiment could not be replicated. However there is clearly enough evidence here to continue this experiment and try to confirm the results shown in Figures 7-9.

**2.5. Summary/future directions**

Based on outside data as well as preliminary results by other members of the Osborne lab, I was interested in seeing the effects of Notch 3 on T helper cell fate and the effects that it could have on the mouse model of MS, EAE, as well as the effects of EPS on specific cell fates in respect to EAE. In collaboration with Furkan Ayaz, I looked at the effects of Notch 3 on Th1, Th2, Th17, and iTreg induction in vitro using Notch 3 knockouts and wild-type mice as a control. My results were obtained by looking at the cell populations by flow cytometry, as was the case for iTreg polarization, as well as by ELISA assay of premier cytokines for each cell type, such as IFN-γ for Th1, IL-4 and IL-5 for Th2, and IL17A for Th17 cells.

I first began by looking at Treg polarization, both nTreg and iTreg. In these experiments, I was able to determine that Notch 3 had no significant effect on the total $CD4^+$ T cell

population (Figure 6a, c and Figure 7b). There is a clear difference in the heterozygous Notch 3 knockout with unpolarized cells having a significantly highly portion of Tregs. However under polarizing conditions, there are a significantly smaller percentage of $CD25^{+}Foxp3^{+}$ cells. In Notch 3 knockouts however, we see no difference in the unpolarized cells, and a greater decrease in the Notch 3 knockout polarized group versus the polarized wild-type control group. We then looked at nTreg cells which occur naturally in the thymus and discovered that there was a significant decrease in Notch 3 knockout mice compared to the wild-type. I also found a very similar decrease in Th17 and Th1 polarized cells with Notch 3 knocked-out. With Th2, we saw a significant increase in Th2 polarization compared to wild-type when under polarizing conditions. These data point to a significant role that Notch 3 plays in the polarization of Th1, Th17 and Treg cells. Although this may not be a possible target for treatment of MS by itself due to the effect on Treg cells, it is possible that in conjunction with another treatment that may boost regulatory T cell production, this could be a very viable target as the lack of Notch 3 greatly reduces both Th1 and Th17 cells. It will also be important in the future to see what effects Notch 3 has directly on EAE induction and what the cytokine profile of Notch 3 knockout mice is after EAE induction.

From experiments done by our collaborator, Katherine Knight, as well as previous data, we have determined that EPS could be a treatment for EAE. It is unclear how EPS may protect mice from EAE induction. However it is thought that EPS may induce anti-inflammatory macrophages, which have been shown to reduce EAE disease scores. Through our experiment, it is clear that EPS could significantly reduce EAE through reduction of Th1, Th17 and GM-CSF producing T cells. Not much is known about how EPS could confer protection against these

inflammatory diseases, but there is clear evidence that EPS reduces disease peak and affects onset of severe symptoms. In addition, mice pretreated with EPS develop no symptoms of the disease at all and both pretreated and regularly treated mice have far lower concentrations of IFN-γ, GM-CSF, and IL17A in the supernatents of the splenocytes treated with MOG. Based on these results, we can say that it is likely EPS somehow decreases the polarization of Th1 and Th17 cells or Th1 and Th17 cytokine production, which greatly reduces disease onset of EAE. These experiments need to be replicated and further study needs to go into the method by which EPS reduces disease. The pathway by which EPS functions could make it clearer as to how disease is being protected and to the means by which prevention occurs and can be used for medical uses. Despite the drawbacks, this data is very promising and if it can be replicated, it could be a very big leap for MS research and treatment.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1. Mice

C57BL/6 and Notch3 knockout mice were purchased from Jackson Laboratory and bred in our mouse facilities and were used for $CD4^+$ T cell polarizations. C57BL/6 mice were purchased from Taconic and used for EAE induction experiments. Mice used for experiments were ages 10-13 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.

## 3.2. $CD4^+$ T cell *in vitro* polarizations

$CD4^+$ T cells are isolated from splenocytes using Anti-CD4 magnetic beads (BD Pharmingen). Cells are plated on a 12 well plate coated with 1ug/ml of anti-CD3 (BD Pharmingen) and 1ug/ml of anti-CD28 (BD pharmingen) at a concentration of $3x10^6$ cells/ml. Th1 polarization is done by adding 10ug/ml of anti-IL-4 and 1ng/ml recombinant mouse IL-12 (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). 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). The media used, RDGS, 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. After 72h of polarization incubation, cytokine levels in the supernatants are determined by an ELISA assay (BD Pharmingen). All antibodies are purchased from eBioscience and intracellular staining for Foxp3 is done by following the instructions on eBioscience Foxp3 intracellular staining kit.

**3.3 EAE induction and re-stimulation**

This protocol is described in the Hooke's Kit (EK-2110) and is done on female C57BL/6 mice aged 10+ weeks. Mice are injected subcutaneously on the flank with $MOG_{35-55}$/CFA Emulsion. After 2 hours and 24 hours from $MOG_{35-55}$ injection, pertussis toxin is injected intraperitoneally. Pertussis toxin was dissolved in PBS and injected at a concentration of 2ug/ml at 100 ul/injection. Disease onset is approximately 9-14 days after initial immunization. Disease progression is scored on a scale from 0-5 as such: 0-no signs of disease, 1- completely limp tail, 2- weakness and mild paralysis of hind legs, 3- Paralysis of hind legs and torso flattening, 4- Complete paralysis of hind and fore limbs and full torso flattening, 5- Death. At peak disease at

day 15, mice are sacrificed and splenocytes are isolated from spleens of EAE induced mice for re-stimulation. Splenocytes are plated in RDGS for 5 days at a concentration of $5x10^6$ cells/ml in the presence of $MOG_{35-55}$ antigen (Hooke's kit DS-0111) at 0, 10 and 20ug/ml concentrations. After 5 days, supernatents are collected from cultures for analysis of IFN-γ , GM-CSF and IL-17A cytokines by ELISA.

**3.4. EAE adoptive transfer**

Adoptive transfer protocol follows the same EAE induction stated above for donor mice. Due to the unpredictability of harvesting splenocytes, two donor mice were often used per recipient mouse. After induction of EAE in donor mice and isolation of splenocytes, cells were plated on round tissue culture dishes at a concentration of $3x10^6$ cells/ml at 10 mls per dish. Cells were plated in RDGS in the presence of 20 ug/ml of $MOG_{35-55}$ antigen (Hooke's kit DS-0111) and 20 ng/ml IL-23 (R&D systems) and incubated for 72 hours. After 72 hours, cells were harvested and resuspended in sterile PBS at a concentration of $4x10^7$ cells/ml. Recipient mice were then injection intraperitoneally with 1 ml of cells followed by intraperitoneal injection of pertussis toxin at 2hrs and 24hrs after induction. In addition, i.v. injections are also possible and done injecting half the concentration of cells into recipient mice ($2x10^7$ cells/ml).

**3.5. ELISA**

Cytokine secretion was measured in supernatants following incubation for polarization experiments and following restimulation for EAE experiments. Concentration of IFN-γ, IL-2, IL-4, IL-5, IL-17A, and GM-CSF were measured using antibodies described below in a sandwich ELSIA.

96 well flat-bottom plates were coated with capture antibody as instructed by supplier and incubated at 4°C overnight. Following day, coating was removed and plates were washed with PBS-T (0.05% Tween-20). Plates were then blocked for 2 hours in 200ul/well of 5% non-fat dry milk in PBS at room temperature. Plates were washed again using PBS-T and samples were added 100ul/well. Samples were diluted based on previous results such that the resulting concentrations fell within the range of detection. Samples were incubated at 4°C overnight. The following day, plates were washed again with PBS-T. Plates were then coated with Avidin Horseradish Peroxidase (HRP) for 2 hours at room temperature. Following another wash in PBS-T, plates were coated with matching biotinylated antibody (listed below) for 1 hour. Plates were then washed again with PBS-T followed by PBS. Plates were then read on a plate reader at 450nm and analyzed.

**3.6. Flow cytometry**

A Becton Dickinson LSR II was used for Flow Analysis. Samples were stained for CD4, CD8a, CD25, and intracellular Foxp3.

Cells were collected from plate and supernatents were removes. Cells were extracellularly stained in 50 ul of PBS with 2% BSA at 4°C for 30 minutes. After removing staining, cells that were to be intracellularly stained for Foxp3 were plating in 200 ul of fixation/permeabilization buffer (listed below) for 30 minutes at 4°C. After decanting supernatant, plates were coated in 50ul/well of permeabilization buffer and 2 ul of rat anti-mouse Foxp3 and incubated in 4°C for 30 minutes. Supernatents were then decanted again and cells were removed into FACS tubes,

resuspended in perm buffer and read on the LSR II. Cells not requiring intracellular staining were resuspended in perm buffer after extracellular staining was complete and read on LSR II.

### 3.7. Statistical analysis

Graphpad Prism Software (v. 5) is used to perform statistical analysis of data from ELISA assay, specifically unpaired two tailed t-test for cell polarizations. In addition, FlowJo (v. 7.6.5) is used for analysis of data collected from flow cytometry. $P > 0.05$ is considered significant.

**Table 1. Reagent for ELISAs, Antibodies and Growth Factors Used**

| Reagent Name | Vendor | Catalog Number |
|---|---|---|
| Mouse CD4 T Lymphocyte Enrichment Set-DM | BD Imag | 558131 |
| Recombinant Human TGF-β1 | R and D Systems | 240-B-002 |
| Recombinant Mouse GMCSF | eBioscience | 14-8331 |
| Recombinant Mouse IL2 | BD Pharmingen | 550069 |
| Recombinant Mouse IL3 | R and D Systems | 403-ML-010 |
| Recombinant Mouse IL4 | R and D Systems | 404-ML-010 |
| Recombinant Mouse IL6 | R and D Systems | 406-ML-005/CF |
| Recombinant Mouse IL23 | R and D Systems | 1887-ML-010 |
| Avidin Horseradish Peroxidase (HRP) | BD Pharmingen | 554058 |
| Purified Rat Anti mouse IFNγ | BD Pharmingen | 554409 |
| Biotin Rat Anti-mouse IFNγ | BD Pharmingen | 554410 |
| Purified Rat Anti-mouse IL-2 | BD Pharmingen | 554424 |
| Biotin Rat Anti-mouse IL-2 | BD Pharmingen | 554426 |
| Purified Rat Anti-mouse IL-4 | BD Pharmingen | 559062 |
| Biotin Rat Anti-mouse IL-4 | BD Pharmingen | 554390 |
| Purified Rat Anti-mouse IL-5 Clone: TRFK5 | eBioscience | 14-7052-85 |

| | | |
|---|---|---|
| Biotin Rat Anti-mouse IL-5 Clone: TRFK5 | eBioscience | 13-7051-85 |
| Purified Rat Anti-mouse IL17A | BD Pharmingen | 555068 |
| Biotin Rat Anti-mouse IL17A | BD Pharmingen | 555067 |
| Purified Rat Anti-mouse GMCSF Clone: MP1-22E9 | eBioscience | 14-7331-81 |
| Biotin Rat Anti-mouse GMCSF Clone: Mp1-3166 | eBioscience | 13-7332-81 |
| Anti-mouse CD4 Magnetic Particles DM | BD Pharmingen | 551539 |
| PE Rat Anti-mouse CD4 Clone: RM4-5 | BD Pharmingen | 553049 |
| PerCP Rat Anti-mouse CD4 Clone: RM4-5 | BD Pharmingen | 553052 |
| PE-Cy7 Rat Anti-mouse CD8a Clone: 53-6.7 | eBioscience | 25-0081-82 |
| APC Rat Anti-mouse CD25 Clone: PC61 | BD Pharmingen | 557192 |
| PE Rat Anti-mouse Foxp3 Clone: FJK-16s | eBioscience | 12-5773-82 |
| Foxp3 Staining Buffer Set | eBioscience | 00-5523-00 |

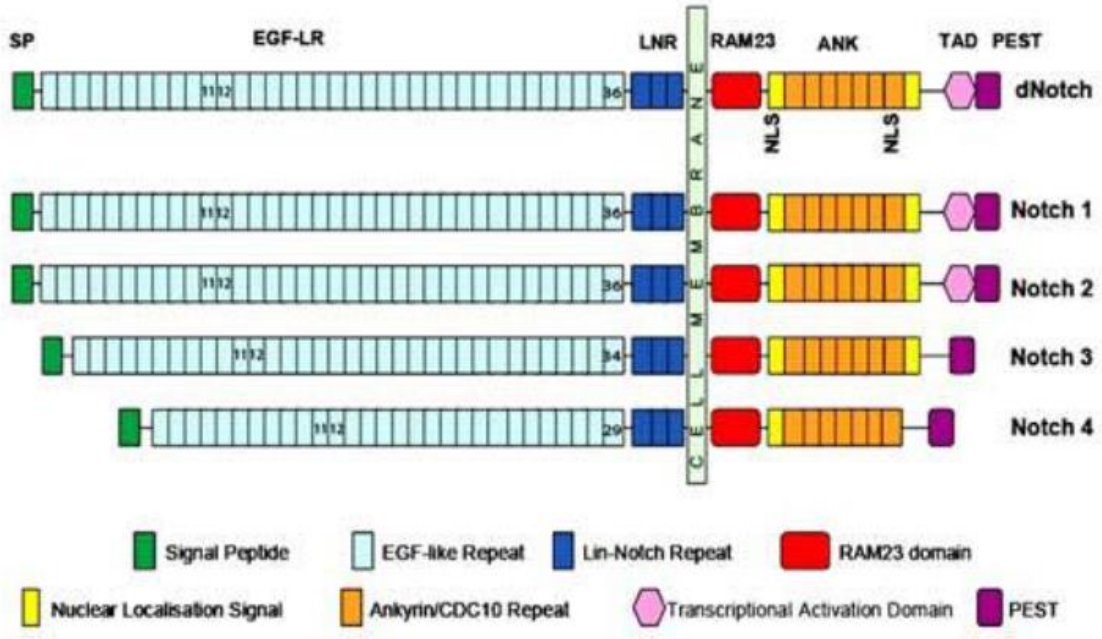



**Figure 1: Structure of Notch family members.** Notch 1, 2, 3 and 4 all have minor differences that vary between the number of EGF-like repeats, to the presence of a transcriptional activation domain. These differences change the behavior and function of each of the Notch members. Specifically Notch 3 has fewer EGF-like repeats than Notch 1 and 2 and does not have a transcription activation domain. Image from: Trojantec. 2007-2012. http://trojantec.com/site.80.articles.en.html.

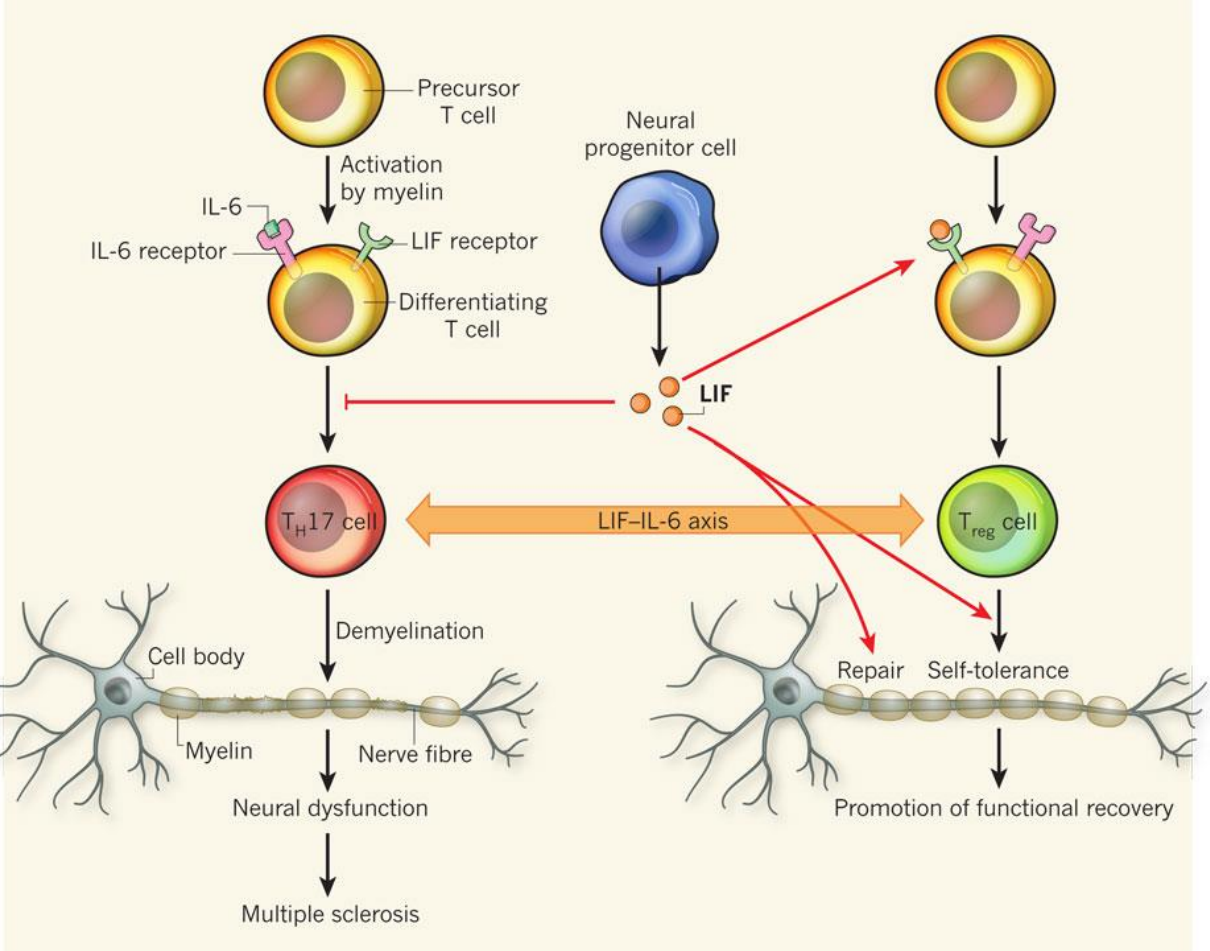



**Figure 2: Multiple Sclerosis function and overview.** Demyelination occurs by the activation and immunization of cells by myelin and neural degeneration of myelin sheaths of nerve cells. Repair of these cells is possible by oligodendrocytes (not shown) however multiple sclerosis prevents production of these cells. In addition Treg cells can promote self-tolerance but are also prevented by the activation of Th1 and Th17 cells. Image from: Metcalfe, Su M. (2011). Multiple sclerosis: One protein, two healing properties, Nature, 477, 287–288. doi:10.1038/477287a

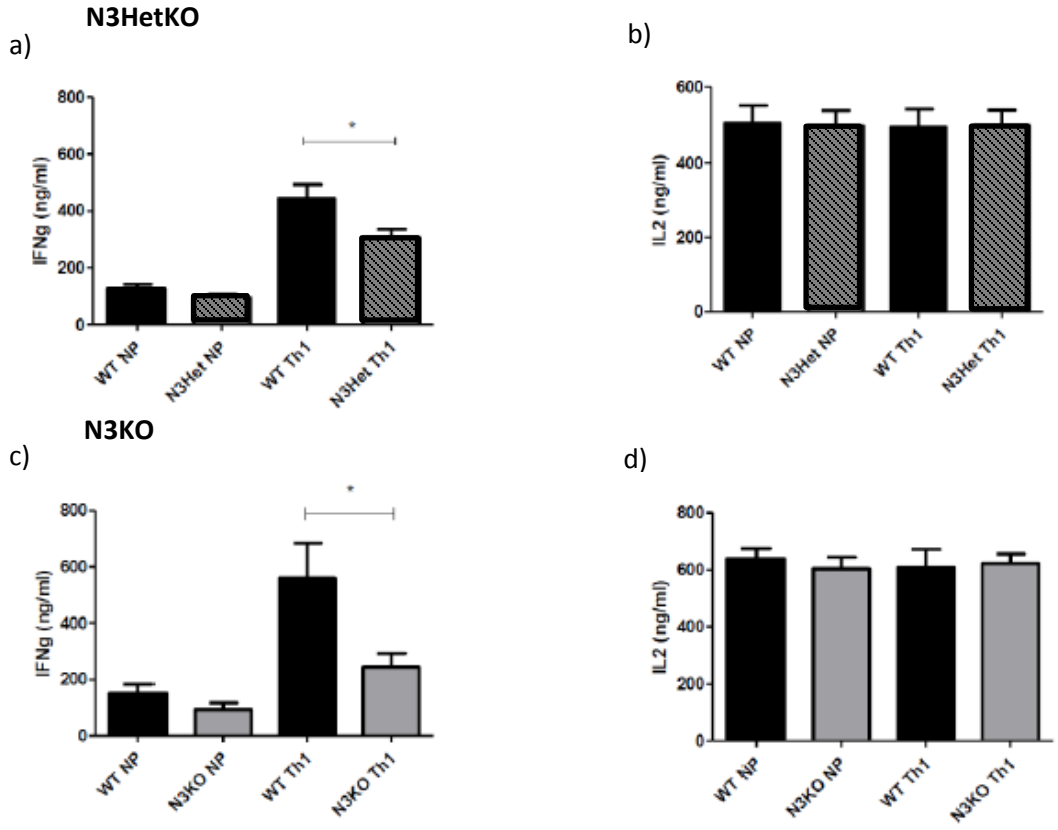



**Figure 3: Absence of Notch 3 results in decreased IFN-γ levels in cells polarized toward Th1 cell fate from CD4⁺ splenocytes.** $CD4^+$ splenocytes were isolated from wild-type (WT), heterozygous Notch 3 knockout (N3hetKO), and Notch 3 knockout mice (N3KO). Cells were plated in polarizing conditions for 72 hours, harvested and supernatents were analyzed by ELISA for IFN-γ (IFNg on axis). Conditions for both experiments include an unpolarized control group (NP), which was plated without any supplemental polarizing cytokines, and a Th1 polarized group which was plated with anti-IL-4 as well as recombinant IL-12. (a,d) IL-2 was used as a proliferation control and the ELISAs are shown. (b,c) Concentration of IFN-γ was used to determine Th1 polarization. Data represents the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$

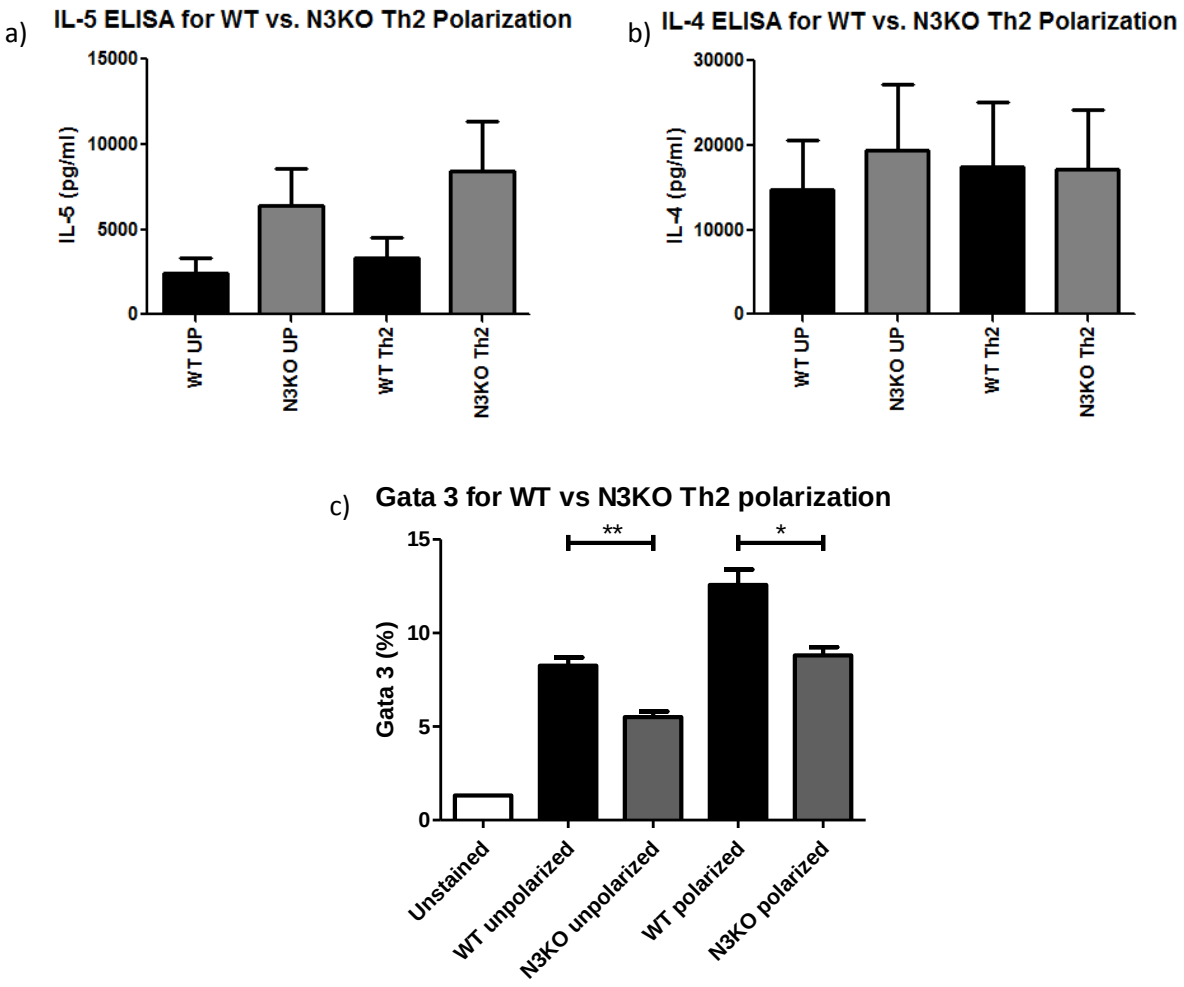



**Figure 4: Notch 3 knockouts decrease expression of Gata 3 as well as increase IL-5 concentration without having an effect on IL-4.** $CD4^+$ splenocytes were isolated from wild-type (WT) and Notch 3 knockout (N3KO) mice. Cells were isolated, incubated under polarizing conditions for 72 hours, harvested and then analyzed by ELISA. Conditions in which cells were played include an unpolarized control group (UP), which contained no supplemental polarizing cytokines, and a Th2 polarized group which was supplemented with anti-IFN-γ and IL-4. (a) IL-5 ELISA (b) results of IL-4 ELISA and (c) results of Gata-3 flow cytometry were done to determine effects on Th2 polarization. Data represents the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$

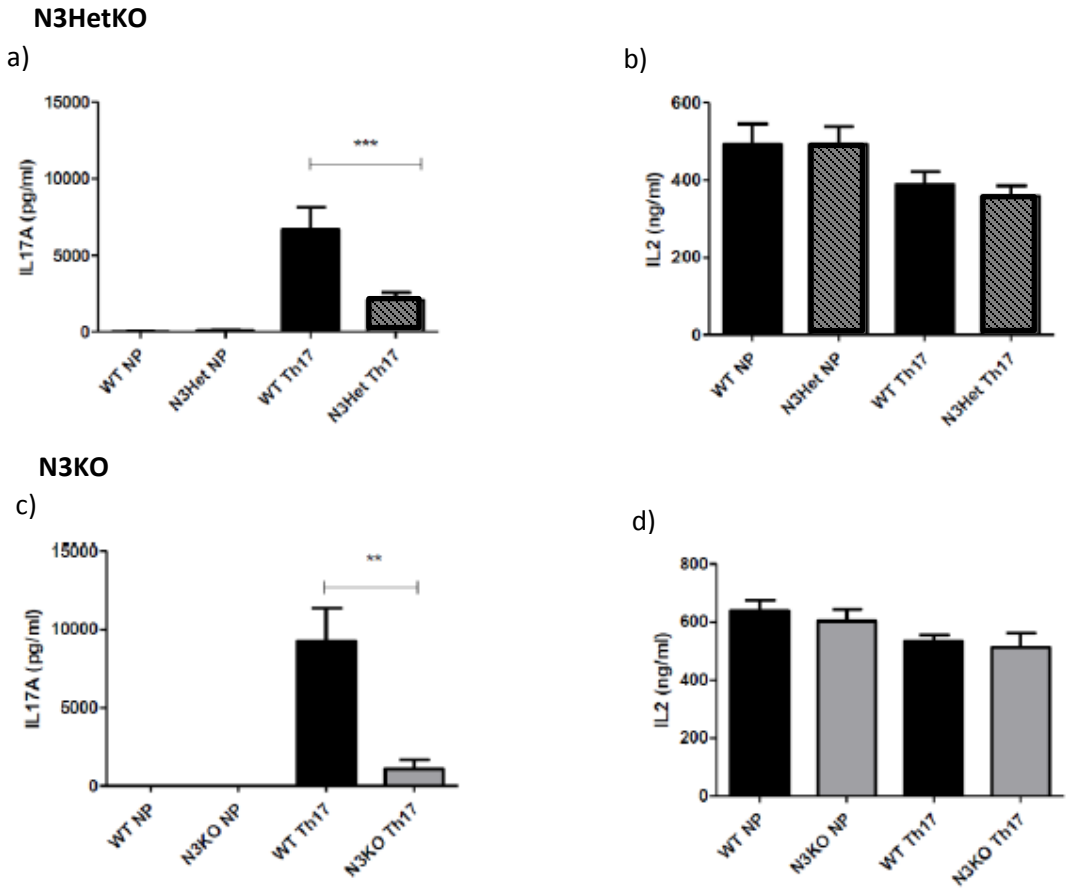



**Figure 5: Absence of Notch 3 results in decreased IL-17A levels in cells polarized toward Th17 cell fate from CD4$^+$ splenocytes.** CD4$^+$ splenocytes were isolated from wild-type (WT), heterozygous Notch 3 knockout (N3hetKO), and Notch 3 knockout mice (N3KO). Cells were plated in polarizing conditions for 72 hours, harvested and supernatents were analyzed by ELISA for IL-17A. Conditions for both experiments include an unpolarized control group (NP), which was plated without any supplemental polarizing cytokines, and a Th17 polarized group which was plated with anti-IFNγ, anti-IL4, recombinant IL6 and recombinant TGFβ1. (a,d) IL-2 was used as a proliferation control and the ELISAs are shown. (b,c) Concentration of IL-17A was used to determine Th17 polarization. Data represents the mean ± SEM, *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$

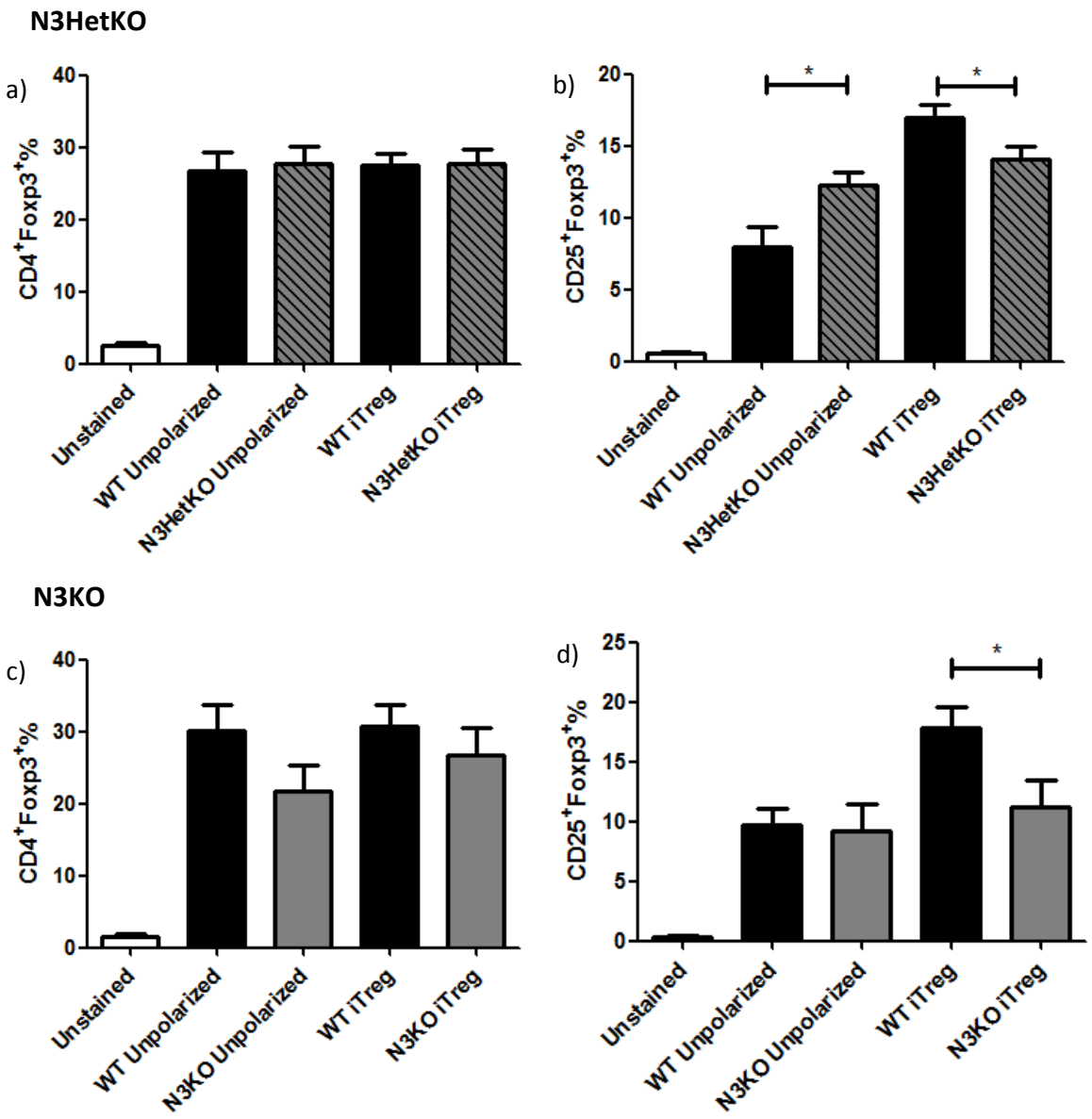



**Figure 6: Absence of Notch 3 reduces polarization of cells toward induced Treg cell fate.** $CD4^+CD25^-$ cells were isolated from the spleens of Wild-Type (WT), Notch 3 heterozygous knockout (N3HetKO) and Notch 3 knockout (N3KO) mice. Cells were then either plated with no supplemental cytokines (unpolarized) or were activated using iTreg polarizing cytokines (TGFβ1) for 72 hours. Groups were then stained for CD4, CD25 and intracellularly stained for Foxp3 and then analyzed by flow cytometry. (a,c) Control group showing the percentage of $CD4^+$ $Foxp3^+$ cells before and after polarization. (b,d) Experimental group showing the percentage of $CD25^+$ $Foxp3^+$ before and after polarization. Data represents the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$

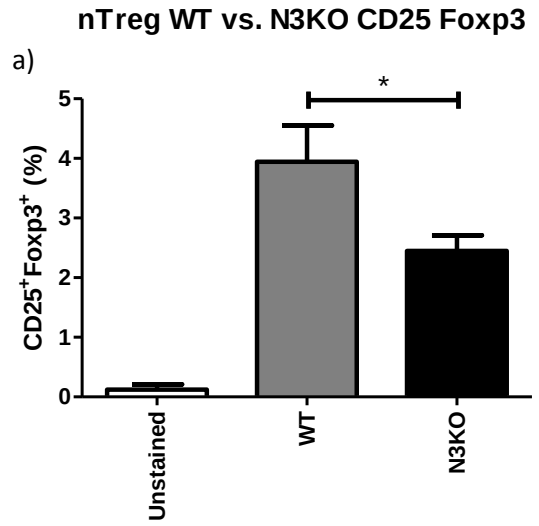
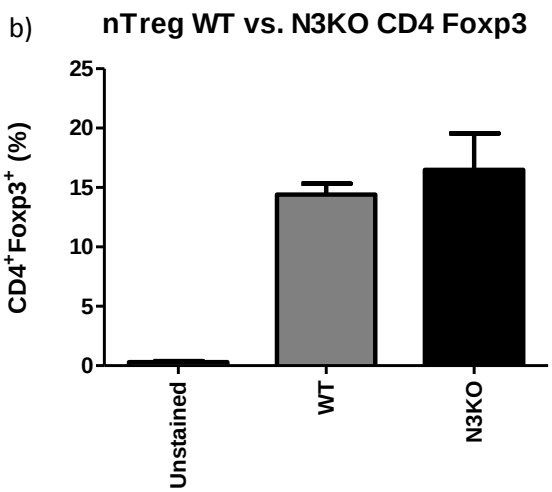
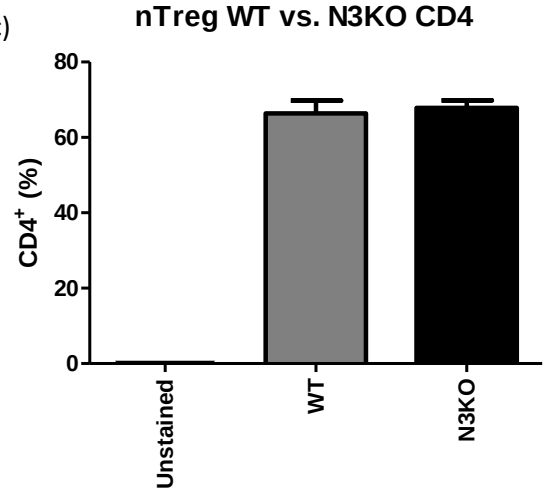
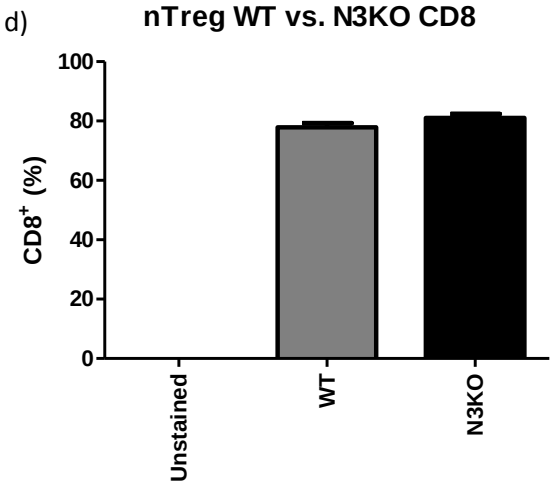
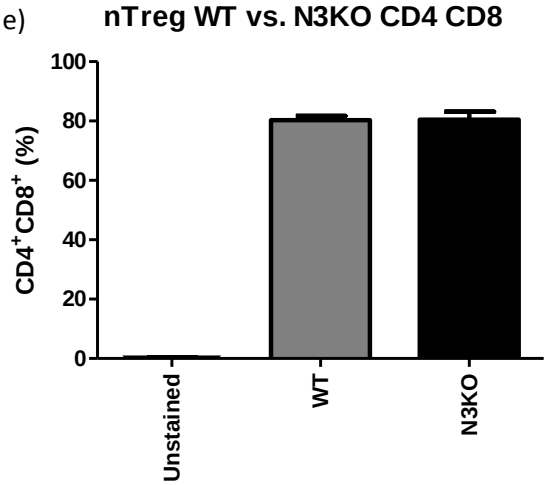



**Figure 7: Absence of Notch 3 reduces the percent of natural Tregs found in lymph nodes.** Cells were isolated from the lymph nodes of Wild-Type (WT) and Notch 3 knockout (N3KO) mice. Cells are isolated, stained for CD4, Cd8, Cd25 and intracellular Foxp3 and then analyzed by flow cytometry. (a) nTreg (CD25+Foxp3+) population. (b) control (CD4+Foxp3+) population (c) CD4+ control population. (d) CD8+ control population. (e) CD4+ CD8+ control population. Data represents the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$



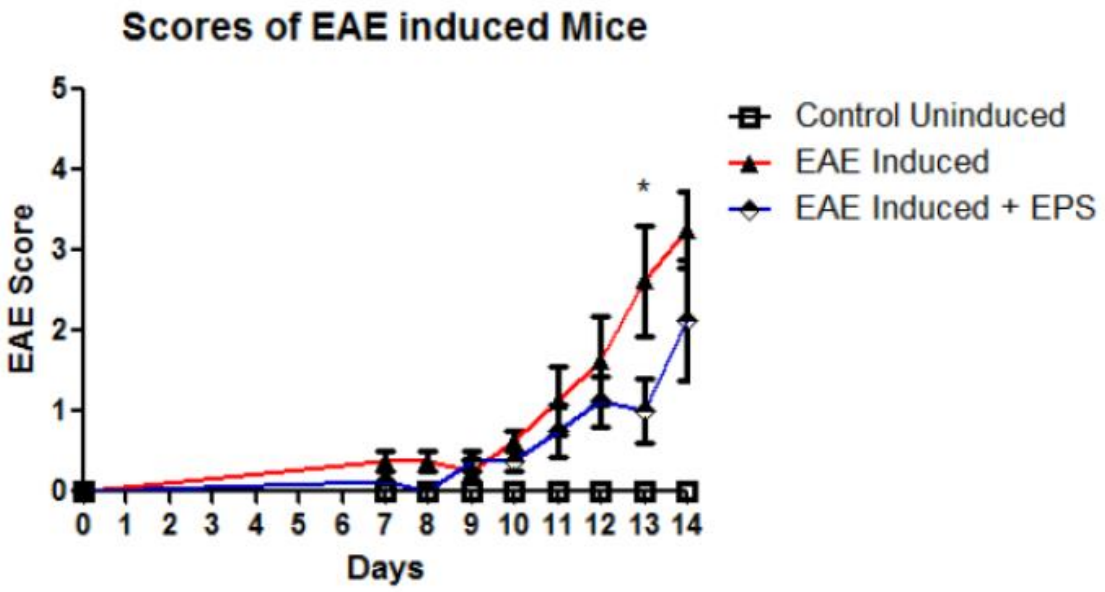



**Figure 8: Preliminary EAE induction with EPS treatment** of WT mice done by Furkan Ayaz. The EPS treatment was done at day 0, and disease score was noticed on a scale of 1-5 up to day 14. Data represents the mean ± SEM, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$

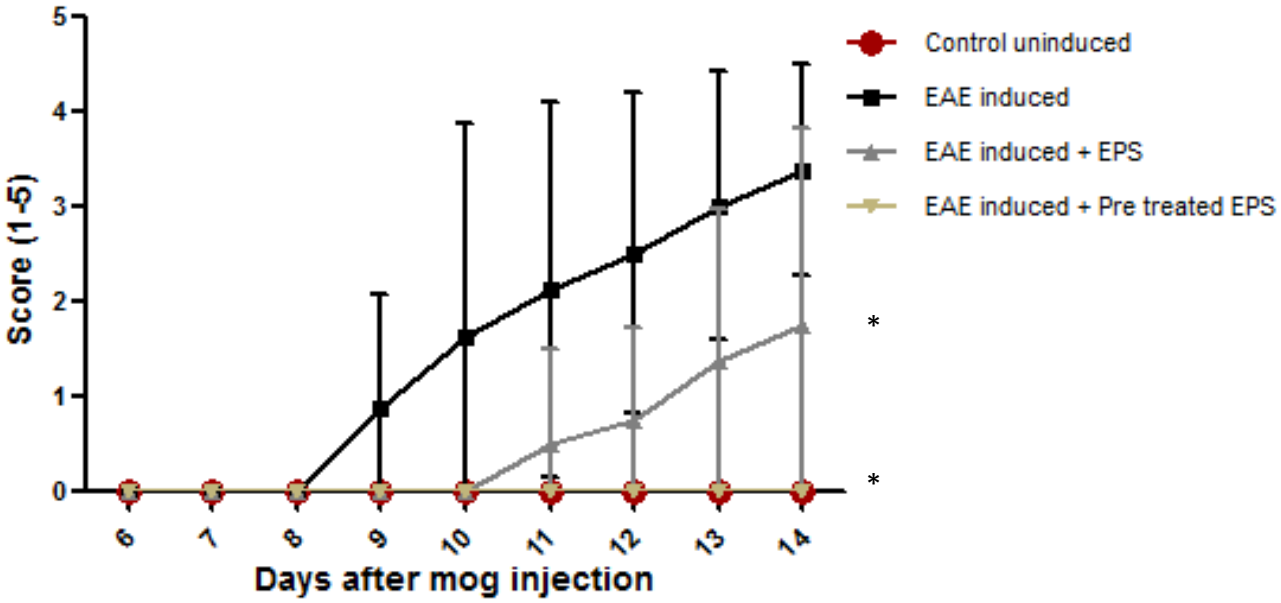



**Figure 9: Administration of EPS during EAE induction significantly reduces EAE severity and administration before EAE induction reduces EAE severity to zero.** Mice were either treated with just PBS (EAE induced), with EPS at day 0 (EAE induced + EPS), or with EPS at day -3 (EAE induced + pretreated EPS) from disease induction. Disease score was determined on a scale of 1-5 and presented here. N=4 for each group. Data represents the mean ± SEM, *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$

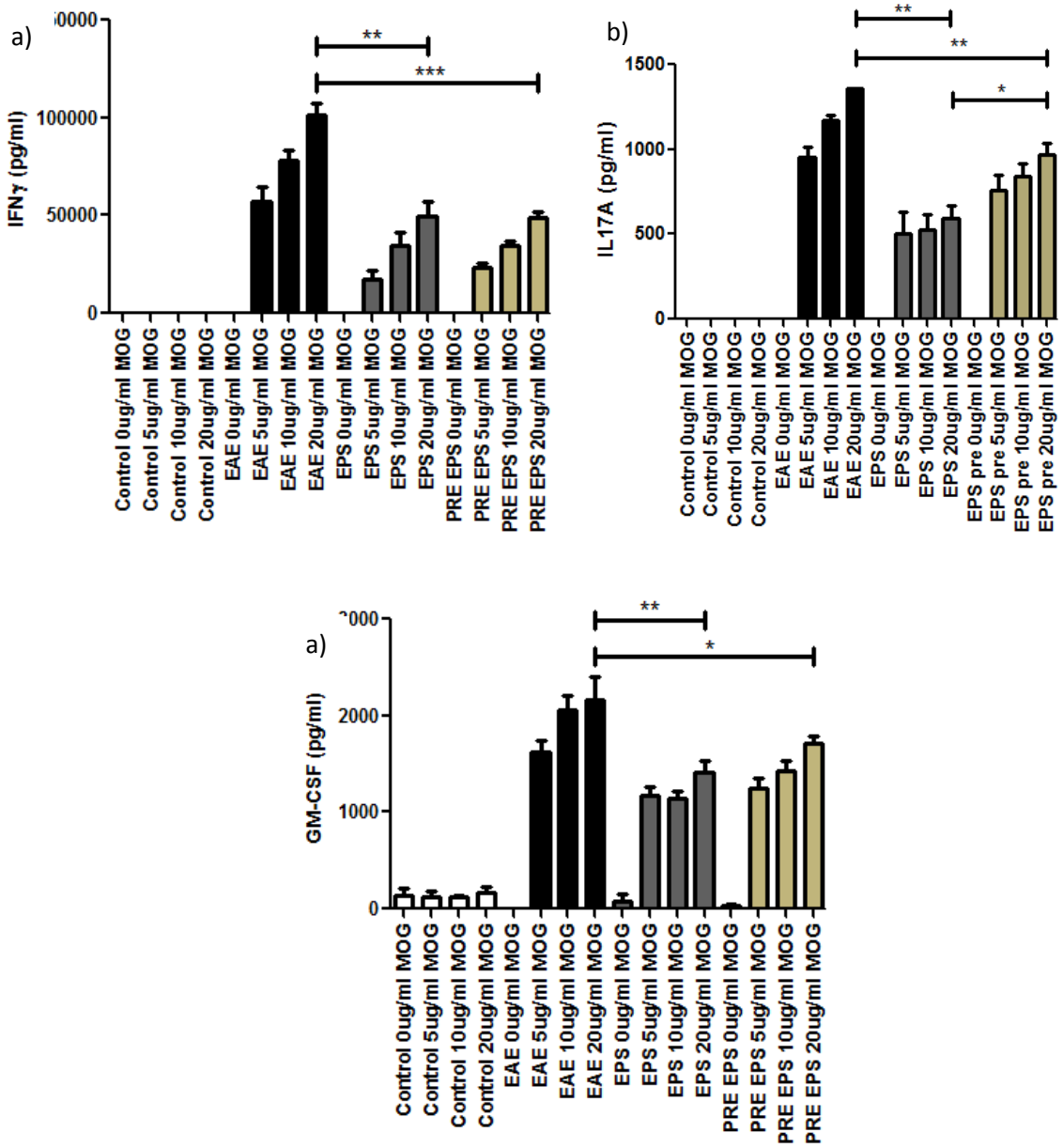



**Figure 10: EPS treated samples all significantly lower concentrations of IFN-γ, IL17A and GMCSF compared to control.** PBS treated vs EPS treated (EPS) and EPS pretreated (PRE EPS) samples were compared. After EAE induction (described in methods) splenocytes were isolated and incubated for 120 hours with different concentrations of MOG peptide (0, 5, 10, and 20 ug/ml). ELISAs were done for (a) IFNγ, (b) IL17A, and (c) GM-CSF.

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