The Role of Notch Signaling in Type-2 Immunity

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Immunology in the Graduate School
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2018

ABSTRACT

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Abstract

Over 2 billion individuals are infected with intestinal helminths worldwide, with a majority of those infected living in developing nations. In developed nations intestinal helminth infections are very rare, while incidences of allergy and asthma are common. The incidence of allergic afflictions is growing rapidly every year. Both clearance of helminth infections and propagation of allergic disease are mediated by type 2 immune responses. The cytokines interleukin-4 (IL-4) and interleukin-13 (IL-13) play major roles in the propagation of type 2 immune responses. IL-4 and IL-13 are produced by a number of immune cells, within both the innate and adaptive arms of immunity, that are important in driving allergic responses.

CD4+ T follicular helper (Tfh) cells reside in the B-cell follicle and specialize in aiding the maturation of germinal center (GC) B cells. IL-4 produced by Tfh cells is required for GC B cell Immunoglobulin (Ig) class switching to type-2 isotypes, IgE and IgG1. IgE serves as a critical mediator of type-2 immune responses. CD4+ T helper 2 (Th2) cells localize to the periphery at sites of infection and damage. Th2 cells make both IL-4 and IL-13 cytokines. Th2 cells, along with multiple innate cell types, are critical for driving the peripheral hallmarks of type-2 immune responses, including mucus production and smooth muscle contractility.

Elucidating the pathways that regulate the differentiation, function, and maintenance of Tfh and Th2 cells is critically important for discovering potential therapies for allergic disease and helminth infections. Notch signaling is capable of driving Th2 IL-4 production and differentiation *in vitro*. However, the *in vivo* role for Notch signaling in Th2 populations remains unclear. The mechanisms controlling Tfh IL-4 production are largely unknown. Given that Notch signaling is required for the differentiation of Tfh cells and is known to influence cytokine production in T cells, we hypothesized that Notch signaling also plays an important role in regulating the function of Tfh cells. *Nippostrongylus brasiliensis* infection drives a robust type-2 immune response and allows for analysis of both Tfh and Th2 cells. Here, infection with N. *brasiliensis* was used to characterize whether Notch signaling is required for Th2 and Tfh differentiation and function *in vivo.*

Deletion of Notch receptors on T cells of infected mice results in reduced IL-4 producing Tfh, but not Th2 cells. As a result, we saw impairments in overall Tfh functionality while peripheral Th2 immunity remained intact. Notch deficient T cells had major impairments in Tfh, but not Th2, cell differentiation. Overexpression of Notch signaling in CD4+ T cells leads to increased IL-4 production by Tfh cells, but not Th2 cells. Furthermore, we identified that conventional dendritic cells (cDCs) do play a role as sources for Notch ligand early during the immune response. However, neither cDCs

or follicular dendritic cells (FDCs) are essential sources of Notch ligand to drive Tfh cell differentiation.

While Notch signaling is critical for Tfh differentiation, it is not known if Notch signaling plays a continued role beyond Tfh differentiation. We used pharmacologic inhibition of Notch signaling to assess a role for Notch in Tfh maintenance. Here, we show that inhibition of Notch signaling after Tfh differentiation results in altered expression and activity of important trafficking receptors. This change was accompanied by aberrant localization of IL-4 expressing T cells in the lymph node. Additionally, late Notch inhibition resulted in an altered transcriptional program in Tfh cells. These findings suggest that Notch signaling plays a critical role in Tfh, but not Th2 driven immunity. In total, the data shown here demonstrate that Notch signaling is not only important for Tfh differentiation, but also for regulating Tfh cell fate, function, and maintenance.

Contents

Abstract iv

List of Figures xii

List of abbreviations xv

Acknowledgements xviii

1. Introduction 1

1.1 Type-2 immunity 1

1.1.1 Parasitic helminths 1

1.1.1.1 Helminths in human disease 1

1.1.1.2 *Nippostrongylus brasiliensis* infection model for type-2 inflammation 3

1.1.2 Allergic disease 5

1.2 Initiation of type-2 immunity: helminths and allergy 7

1.3 Innate immune response in type-2 immunity 13

1.3.1 Basophils 13

1.3.2 Eosinophils 17

1.3.3 Group 2 innate lymphoid cells 19

1.3.4 Mast cells 21

1.4 CD4+ T helper cell activation 22

1.5 CD4+ T helper 2 cells 24

1.5.1 Th2 cell differentiation 25

1.5.1.1 TCR signaling in Th1 vs Th2 differentiation 26

1.5.1.2 IL-4 signaling and GATA-3 in Th2 differentiation 29
1.5.1.3 Other factors important for Th2 differentiation 31
1.5.2 Cytokine regulation and function 32
1.5.2.1 Interleukin-4 32
1.5.2.2 Interleukin-5 35
1.5.2.3 Interleukin-13 35
1.6 Lymph node architecture and germinal centers 37
1.7 CD4+ T follicular helper cells 43
1.7.1 Tfh master regulator BCL6 44
1.7.2 Tfh differentiation 45
1.7.2.1 TCR signaling in Tfh vs peripheral Th differentiation 45
1.7.2.2 B cell help during Tfh differentiation 49
1.7.2.3 Other factors contributing to Tfh differentiation 51
1.7.3 Cytokine production and function 55
1.7.3.1 Interleukin-4 55
1.7.3.2 Interleukin-21 56
1.8 Cytokine-reporter mice 57
1.9 Notch receptor signaling pathway 59
1.9.1 Canonical Notch signaling 59
1.9.2 Non-canonical Notch signaling 62
1.9.3 Notch signaling in T cell development 63
1.9.4 Notch signaling in Th differentiation 64

1.9.4.1 Ligand-based and unbiased amplifier models 64

1.9.4.2 Notch signaling in Th1 differentiation and function 66

1.9.4.3 Notch signaling in Th17 differentiation and function 67

1.9.4.4 Notch signaling in Treg differentiation and function 68

1.9.4.5 Notch signaling in Th2 differentiation and function 68

1.9.4.6 Notch signaling in Tfh differentiation and function 71

2. Methods 75

2.1 Mice 75

2.2 Infections, worm counts, and immunizations 75

2.3 Notch inhibition studies 76

2.4 Tamoxifen injections 76

2.5 Immunofluorescence histology 76

2.6 Quantification of IL-4 competent CD4+ T cell accumulation in T cell zone 78

2.7 Flow cytometry 78

2.8 Transcription factor staining 80

2.9 FDC isolation 80

2.10 ELISA 81

2.11 Real-time PCR 82

2.12 Cell proliferation study 82

2.13 In vitro T cell activation studies 83

2.14 Statistics 84

3. Notch signaling represents an important checkpoint between Tfh and Th2 cell fate ... 85

3.1 Introduction 85

3.1 Deletion of Notch receptors in T cells results in abrogated humoral immunity but normal Th2-mediated immune hallmarks 86

3.2 T cell-specific deletion of Notch receptors leads to a selective reduction in IL-4 competent CD4+ T cells in the lymph nodes compared to the lungs after helminth infection 90

3.3 Tfh cell generation, IL-4 expression, and germinal center B cell numbers are significantly reduced in mice that lack Notch in T cells 95

3.4 Overexpression of Notch intracellular domain increases IL-4 production by Tfh but not Th2 cells 101

3.5 Functional Notch ligands on conventional dendritic cells can influence Tfh cell fate early during CD4+ T cell differentiation, but are not required for full Tfh commitment. 106

3.6 Functional Notch ligands on B cells and follicular dendritic cells are not required for Tfh cell commitment. 112

3.7 Discussion 117

4. Continuous Notch signaling is required to maintain Tfh fate and function 124

4.1 Introduction 124

4.2 Late inhibition of Notch signaling results in a reduction of Tfh fate and function 125

4.3 Persistent Notch signaling is required to maintain proper localization of IL-4 competent cells in the lymph node 128

4.4 Accumulation of IL-4 expressing CD4+ T cells in the paracortex of GSI-treated mice requires S1P1 136

4.5 Loss of Notch signaling in established Tfh cells results in a loss of Tfh transcriptional program 142

4.6 Temporal genetic ablation of Notch receptors results in altered profile of IL-4 producing CD4+ T cells in the lymph node 147

4.7 Genetic ablation of Notch receptors has a minor effect on markers of TCR-mediated activation 152

Discussion 156

5. Final conclusions and future directions 162

5.1 Concluding remarks: Notch in type-2 Immunity 162

5.2 Future directions: How does Notch signaling promote Tfh fate and function 165

5.2.1 Determine if Notch signaling regulates cellular localization of Tfh cells 165

5.2.2 Identify whether Notch signaling is important to initiate the Tfh transcriptional program 174

5.2.3 Assess if Notch signaling regulates TCR-mediated activation 176

5.3 Tfh cells and Notch signaling in humans and disease 179

Bibliography 185

Biography 228

List of Figures

Figure 1: Model of type-2 inflammation 12

Figure 2: TCR signaling helps to determine Th2 vs Th1 differentiation 28

Figure 3: TCR signaling in Tfh vs Th1 differentiation 48

Figure 4: Schematic of the Tfh transcriptional network 53

Figure 5: IL4^{4get} and IL4^{KN2} reporter mouse constructs 58

Figure 6: Canonical Notch signaling 61

Figure 7: Notch signaling on CD4+ T cells is required for humoral immunity but is dispensable for Th2-mediated type-2 immune hallmarks. 87

Figure 8: Increased CD4+ T cells in the lung and lymph node in mice with Notch deficient T cells. 89

Figure 9: Deletion of Notch receptors on T cells results in reduced IL-4 competency in CD4+ T cells residing in the lymph node but not lung. 91

Figure 10: Deletion of Notch1 and Notch2 on T cells results in impaired IL-4 production by LN-resident CD4+ T cells, but not lung resident CD4+ T cells. 92

Figure 11: Enhanced Tbet and FoxP3 expressing CD4+ T cell populations in mice with Notch deficient T cells 94

Figure 12: Notch receptors are highly expressed on Tfh cells during helminth infections. 95

Figure 13: Notch deficiency in T cells results in reduced Tfh cell numbers, Tfh IL-4 production, and germinal center B cells. 97

Figure 14: Deletion of Notch receptors on T cells results in a significant reduction in BCL6 expression. 98

Figure 15: Deletion of Notch1 alone results in an intermediate phenotype compared to T cells deficient in both Notch1 and Notch2. 100

Figure 16: Over-expression of NICD leads to increased IL-4 production by Tfh cells but not lung resident CD4+ T cells. 102

Figure 17: Over-expression of NICD leads to increased IL-4 production in lymph node non-Tfh cells. 103

Figure 18: Over-expression of NICD leads to increased expression of BCL6 and GATA-3 in lymph node, but not lung, CD4+ T cells. 105

Figure 19: Successful deletion of MIB1 in CD11c+ dendritic cells. 107

Figure 20: Deletion of MIB1 in conventional dendritic cells is important in early Tfh cell fate decision. 109

Figure 21: Deletion of MIB1 in conventional dendritic cells does not result in a general proliferation or activation defect in antigen specific T cells. 111

Figure 22: CD21 expression amongst lymph node stromal cell populations. 113

Figure 23: Deletion of MIB1 on FDCs and B cells is dispensable for Tfh differentiation and function. 115

Figure 24: MIB1 deficiency in CD4+ T cells has no effect on Tfh differentiation or Tfh IL-4 production. 116

Figure 25: GSI administration after Tfh differentiation leads to reduced Tfh differentiation and IL-4 production. 127

Figure 26: Reduced germinal center B cell population in mice treated with GSI 127

Figure 27: IL-4 expressing cells lose their Tfh phenotype after GSI treatment 129

Figure 28: Treatment with GSI leads to reduced expression of CXCR5 by IL-4 expressing CD4+ T cells 130

Figure 29: GSI-treatment results in aberrant localization of IL-4 expressing CD4+ T cells. 132

Figure 30: Release of GSI-treatment results in re-localization of IL-4 expressing cells back to the follicle. 134

Figure 31: Reduced expression of ASCL2 in Tfh cells from mice treated with GSI. 136

Figure 32: S1P1 inhibition restores normal localization of IL-4 expressing CD4+ T cells observed when administered GSI 138

Figure 33: CD69 expression is reduced in IL-4 expressing CD4+ T cells in mice treated with GSI. 140

Figure 34: KLF2 expression is enhanced in Tfh cells from mice administered GSI. 142

Figure 35: Mice treated with GSI have an altered transcriptional program in their Tfh cells. 144

Figure 36: GSI-treatment results in reduced expression of cMAF in Tfh cells. 146

Figure 37: Model for temporal genetic ablation of Notch receptors in transferred OT-II CD4+ T cells 148

Figure 38: Late temporal deletion of Notch receptors on transferred CD4+ T cells had no effect on Tfh differentiation 150

Figure 39: Temporal ablation of Notch signaling results in reduced expression of Tfh characteristic markers by IL-4 producing cells 151

Figure 40: Deletion of Notch receptors has no effect on early TCR-mediated activation 153

Figure 41: Notch deficient CD4+ T cells have reduced BATF and IRF4 expression upon TCR-mediated activation 155

Figure 42: Updated model for the role of Notch signaling in type-2 immunity and T helper cell differentiation 164

Figure 43: Model for Notch regulation of follicular localization 173

Figure 44: Updated model of Tfh transcriptional network 175

List of abbreviations

AHR	Airway hyperreactivity
APCs	Antigen presenting cells
ASCL2	Achaete-scute homolog 2
BATF	Basic leucine zipper ATF-like transcription factor
BCL6	B-cell lymphoma 6
BCL-xL	B-cell lymphoma-extra large
CD	Cluster of differentiation
cDC	Conventional dendritic cells
CXCR5	C-X-C receptor type 5
CCR7	C-C receptor type 7
CCL19	Chemokine (C-C motif) ligand 19
CCL21	Chemokine (C-C motif) ligand 21
CXCL13	Chemokine (C-X-C motif) ligand 13
FcR	Fragment crystallizable receptor
FDC	Follicular dendritic cell
GC	Germinal center
GFP	Green fluorescent protein
GSI	Gamma secretase inhibitor

HDM	House dust-mite
huCD2	human cluster of differentiation 2
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-2Rγc	IL-2R-common-gamma-chain
IL-4R	Interleukin-4 receptor
IL-13R	Interleukin-13 receptor
IL-21R	Interleukin-21 receptor
ILC	Innate lymphoid cell
ILC2	Group 2 innate lymphoid cell
IRES	Internal ribosomal entry site
IRF4	Interferon regulatory factor 4
KLH	Keyhole limpet hemocyanin
MAML1	Mastermind-like protein 1
MHC-II	Major histocompatibility complex-II
NICD	Notch intracellular domain
NECD	Notch extracellular domain
PAF	Platelet activating factor
PD-1	Programmed cell death protein-1

PFA	Paraformaldehyde
OVA	Ovalbumin
RBPJ	Recombination binding protein-J
RFP	Red fluorescent protein
S1P1	Sphingosine-1-phosphate receptor 1
SRBC	Sheep red blood cell
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
Tfh	T follicular helper
Treg	T regulatory cell
TSLP	Thymic stromal lymphopoietin
YFP	Yellow fluorescent protein

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1. Introduction

1.1 Type-2 immunity

Type-2 immunity is associated with allergic disease which afflicts roughly 1.5 billion people worldwide [1]. In addition, approximately 2 billion people suffer from soil-transmitted helminth infections each year, many of which are infected with multiple different helminths simultaneously [2]. The majority of these infected individuals reside in developing nations. In developed nations, type-2 inflammation more commonly presents as afflictions of allergy, asthma, and atopic dermatitis. Type-2 immune responses are mediated by cells of both the innate and adaptive arms of the immune system. Eosinophils, basophils, ILC2s, and mast cells are key mediators stemming from the innate arms of type-2 immunity; from the adaptive arm CD4+ follicular T helper (Tfh) cells and T helper type 2 (Th2) cells are critical modulators of type-2 responses [3,4]. Through cooperative action, these cells function to clear dangerous parasitic infections but they also develop the settings in which allergic disease can manifest.

1.1.1 Parasitic helminths

1.1.1.1 Helminths in human disease

Infections with parasitic helminths remains a significant health and financial burden in developing nations. There are two major classes of helminths: roundworms (which include soil-transmitted helminths and filarial worms) and flatworms (which include schistosomes and tapeworms). The most common helminth infections are from

soil-transmitted helminths which include: *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), and several species of hookworm including *Nectar americanus* and *Ancylostoma duodenale*. Soil-transmitted helminths colonize and attach at various places within the gastrointestinal (GI) tract depending on the infecting parasite. Within the GI tract, the parasites will reproduce and lay eggs, which are passed through the feces back into the environment. As a result, the main cause of these infections is poor hygiene coupled with a lack of access to clean food and water. The main routes of infection with soil-transmitted helminths are oral ingestion and through the skin.

In 2003, approximately 2 billion individuals were estimated to be infected with soil-transmitted helminths [5]. Soil-transmitted helminth infections are most common in young children and adolescents [2]. Infections from soil-transmitted helminths are rarely fatal, yet their effects on the host cannot be understated. Colonized parasitic helminths absorb host nutrients and negatively affect host metabolism. Intestinal bleeding induced by helminths leads to iron deficiency and malnutrition [6]. As a result, chronic helminth infections in children results in the anemia, stunting of growth, and impairments in memory and cognition [2,6]. Although most common in younger individuals, soil-transmitted helminth infections are also prevalent in the adult population of developing nations.

Schistosome infections (schistosomiasis) are far less common than soil-transmitted helminth infections but are perhaps the largest cause of helminth related

mortality. Schistosomiasis is estimated to affect approximately 200 million individuals worldwide [5]. Larval worms are released into water by freshwater snails. Infection begins when individuals come into contact with infested water and worms penetrate the skin. The primary cause of schistosomiasis disease is due to deposition of schistosome eggs at blood vessels in the bladder and intestines [5]. Chronic disease results from damage of blood vessels and host entrapment of schistosome eggs. The immune response to entrapped eggs results in granuloma formation [5]. The granuloma can lead to severe bladder ulceration as well as lesions to the spleen and liver. If left untreated, schistosomiasis can ultimately lead to bladder obstructions and end-stage renal failure [5]. Schistosomiasis is estimated to result in as many as 280,000 deaths annually [5,7].

Infections with intestinal helminths represent one of the most prevalent health afflictions in humans [8]. As described above, infections with parasitic helminths result in a wide-range of symptoms and outcomes depending on the infecting parasite. Although difficult to estimate, cumulative mortality attributed to helminth infections are estimated to be over 150,000 to 400,000 deaths annually [5,8,9]. For these reasons, continued investigation into the pathogenesis of intestinal parasites is of great importance.

1.1.1.2 *Nippostrongylus brasiliensis* infection model for type-2 inflammation

Nippostrongylus brasiliensis is a rodent specific helminth that is commonly used as an infection model to study type-2 inflammation. *N. brasiliensis* infection in rodents

closely resembles human hookworm infections and therefore serves as a strong model to study the immune response to helminths. Typically, *N. brasiliensis* L3 larvae are injected subcutaneously in the lower flank. One to two days post injection the worms will enter into the bloodstream and migrate to the lungs. A type-2 immune response is generated in the lungs and lung draining mediastinal lymph nodes (medLN). By day three to four, the worms are coughed up and swallowed and then travel to the intestines. Within the intestines, adult worms attach to the luminal wall and lay eggs. The immune response in the intestines will result in clearance of the adult worms and eggs through the GI tract into the feces. A healthy, immunocompetent animal will fully clear *N. brasiliensis* in eight to nine days.

Allergic inflammation in this model is initiated when exposure to the intestinal helminth results in tissue damage. Damaged epithelium in the lung and intestines releases alarmins and inflammatory molecules such as thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, and IL-33 [10-15]. The precise roles of these pro-inflammatory molecules will be discussed in another section. In brief, however, the presence of these molecules leads to the activation and mobilization of various immune cells to the sites of inflammation and initiates the processes that generate an adaptive immune response. Infected lungs are marked by a major increase in infiltrating immune cells including: eosinophils, basophils, type-2 innate lymphoid cells (ILC2s), and CD4+ T cells [16-23]. These cells are also heavily recruited to the intestines during infection.

During infection, Th2 cells are the predominant CD4+ T helper cell recruited to sites of inflammation [16,17,23,24]. Recruitment of Th2 cells is essential for the clearance of *N. brasiliensis*. Recombination activating gene (RAG) knockout and severe-combined immunodeficiency mice (SCID) mice, which do not have B or T cells, are incapable of clearing *N. brasiliensis* [20-22,25,26]. Furthermore, depletion of CD4+ T cells using a monoclonal antibody results in impaired worm clearance [25].

Th2 cells produce the cytokines IL-4, IL-5, and IL-13. IL-4 and IL-13 produced by Th2 cells serve partially redundant roles that are important for worm clearance. However, production of IL-13, but not IL-4, is essential for the clearance of *N. brasiliensis* [27,28]. In support, IL-13 deficient animals are incapable of clearing *N. brasiliensis* while IL-4 deficient animals have no major impairments in worm clearance [27,28]. Although not essential to clear *N. brasiliensis*, recombinant IL-4 administered in high doses restores worm clearance in immunocompromised mice [25]. The mechanistic roles of IL-4 and IL-13 during allergic inflammation will be discussed in greater detail in other sections.

1.1.2 Allergic disease

Allergic inflammation manifests as food allergy, allergic rhinitis, asthma, and atopic dermatitis. The incidence of allergic disease has increased dramatically and continues to do so each year. Currently, there are an estimated 1.5 billion people afflicted with some form of allergic disease and this number is expected to grow [1]. The most common allergic disease is allergic rhinitis which is estimated to affect as many as

500 million individuals worldwide [29]. Allergic rhinitis is characterized by inflammation in the nose induced by allergen exposure resulting in mucus production, sneezing, and itching. Atopic dermatitis is a chronic skin disease characterized by inflammation and itching of the skin and is very common in young children. Approximately 10% of children are affected by atopic dermatitis worldwide [30].

Food allergies result as an adverse reaction to food antigens and affects about 6% of children and 3-4% of adults worldwide [31]. In the United States, studies on self-reported peanut allergy have found that the incidence has doubled from the years 1997 to 2002 with overall prevalence reaching an estimated 1% in children [32]. Responses to food allergy vary significantly, and severe allergic reactions can cause anaphylaxis and be life threatening. Symptoms of anaphylaxis include: inflammation of the skin, nausea, vomiting, dizziness, swollen tongue and throat, and constriction of airways. These symptoms often result in difficult breathing and shortness of breath and can be fatal. However, deaths resulting from food related anaphylaxis are extremely rare [33]. In fact, accidental death is more common in the general populations than death from food-related anaphylaxis [33]. Anaphylaxis can also be induced by allergic responses to other innocuous environmental stimuli including: stings from various insects, latex, and some medications.

Asthma is caused by allergic inflammation in the airways that results in airflow obstruction, shortness of breath, wheezing, and coughing. The prevalence of asthma in

the United States doubled between the years of 1980 and 1990 [34,35] and recent projections estimate that over 300 million individuals are currently afflicted with asthma [36,37]. This number is expected to grow to 400 million by the year 2025 [36]. While many allergic diseases are unlikely to be fatal, asthma alone accounts for as many as 250,000 deaths per year worldwide [36,37].

Access to clean food and water, as well as widespread deworming programs, have led to major reductions in parasitic helminth infections in developed nations. And while infections with helminths have become far less common, allergic disease, as described above, has increased dramatically. The strong negative correlation that exists when assessing geographic incidences of allergic disease versus helminth infections is one of the phenomena contributing to the theory termed the "Old Friends" hypothesis [38,39]. The "Old Friends" hypothesis posits that infections with certain organisms, such as parasitic helminths, early in life plays an important role in training the type-2 immune response. In the absence of these anti-helminth responses, type-2 immunity manifests into a system that begins to increasingly target innocuous allergens and results in allergy.

1.2 Initiation of type-2 immunity: helminths and allergy

The type-2 immune response to helminths is initiated upon tissue damage elicited by the infecting parasite (Figure 1). Similar to helminth infections, allergic disease manifests when normally innocuous allergens breach the epithelial barrier.

Repeated exposure to these allergens could result in an inappropriate type-2 immune response. Damaged epithelium, either during allergic sensitization or helminth infection, releases the alarmins IL-25, IL-33, and TSLP [10-15]. These factors alert the immune system and mobilize the type-2 immune response. The outcome of this response ultimately leads to enhanced mucus production and smooth muscle contractility which is characteristic of allergic disease and is essential for the 'weep and sweep' of intestinal epithelium to clear parasitic helminths.

TSLP is constitutively expressed in the gut but its production is enhanced in the epithelium upon tissue damage [10,11]. Antigen presenting dendritic cells recruited to the site of inflammation and stimulated by TSLP are more likely to promote a Th2 phenotype in CD4+ T cells [10]. This is due in part to TSLP related signaling leading to inhibited IL-12 production by dendritic cells, a cytokine important for Th1 polarization [40]. TSLP is also capable of directly promoting Th2 differentiation and IL-4 production independent of antigen presenting cells (APCs) *in vitro* [41]. Additionally, TSLP activates the maturation and mobilization of a specific subset of basophils [42]. One study demonstrated that TSLP signals are required for the expulsion of *Trichuris muris* but are dispensable for controlling infection with *Heligmosomoides polygyrus* and *N. brasiliensis* [43]. Thus, the ultimate contributions of TSLP during a type-2 immune response may vary depending on the infecting parasite. TSLP activated mast cells and eosinophils enhance their type-2 cytokine production and play important roles in atopic dermatitis

and asthma [44-46]. Of relevance, keratinocyte-specific deletion of the Notch coactivator RBPJ enhanced TSLP production and promoted dermatitis [47].

IL-25 was originally discovered in 2001 as a member of the IL-17 family [12]. IL-25 (IL-17E) signals through the IL17 receptor (IL17R) and is produced by epithelial cells in the intestines, more specifically, by a specialized type of intestinal epithelial cells known as Tuft cells [13]. Administration of recombinant IL-25 enhances type-2 cytokine production and antibody generation [12]. The predominant role of IL-25 in type-2 immunity is to activate and expand group 2 innate lymphoid cell (ILC2s) populations. ILC2s stimulated by IL-25 will upregulate their production of the type-2 cytokines: IL4, IL-5, IL-9 and IL-13 [20,22,21]. IL-25 deficient mice have delayed Th2 responses and a notable defect in worm expulsion [26]. The IL25 receptor is also expressed highly on certain subtypes of dendritic cells, and IL-25 signaling results in enhanced expression of certain toll-like receptors (TLRS) [48]. These dendritic cells are present in the airway in increasing numbers upon allergen challenge [48].

IL-33 expression has been reported to be high in endothelial [49] and epithelial cells at mucosal sites [14]. IL-33 binds and signals through the cell surface receptor ST2; ST2 signals then induce type-2 cytokine production, activation, and accumulation of Th2 cells [50] and ILC2s [20,22,21,15]. IL-33 activated ILC2s are sufficient to induce intestinal parasite clearance [20,21,15]. IL-33 promotes wound healing by inducing regulatory T

cell function [51]. However, IL-33 is also implicated in driving pathogenic allergic inflammation in atopic dermatitis and asthma [15,52,53].

The adaptive immune response against infecting parasites and allergens begins when the parasite or allergen crosses a breached epithelial barrier. APCs, such as dendritic cells, then process and display the allergens or helminth antigens on major histocompatibility II (MHC-II) molecules on their cell surface. The APCs then traffic to the secondary lymphoid organs (spleen and lymph nodes) and present the antigen and activate naïve CD4+ T cells. The CD4+ T cells are activated when their T cell receptor (TCR) recognizes a specific peptide presented on an MHC molecule by APCs. Activation of the naïve CD4+ T cells sends signals to the cell that then begins the process of T helper cell differentiation. In the context of type-2 immunity, a naïve CD4+ T cell will undergo a fate decision to become one of two primary CD4+ T helper cells: the Th2 or Tfh cell. The processes of T helper cell activation and differentiation and the signals that help to guide CD4+ Th2 and Tfh cell fate decisions will be expanded on in sections 1.4, 1.5.1, and 1.7.2, respectively.

CD4+ T cells that have differentiated into Th2 cells will then exit the lymph node and travel to peripheral sites of allergic inflammation. Th2 cells produce IL-4, IL-5 and IL-13 cytokines. These cytokines play important roles in mobilizing innate cell populations in response to allergens or helminths and are critical for mediating

clearance of helminths. The regulation and function of these cytokines will be elaborated upon in greater detail in section 1.5.2.

Other activated CD4+ T cells will differentiate into Tfh cells, which migrate to the B cell follicle of lymph nodes and aid germinal center (GC) B cells in their maturation into high-affinity antibody producing cells. Help provided by Tfh cells in the forms of IL-4 and IL-21 cytokines, CD40/CD40L signaling and other cellular signals are essential for the survival, class-switching, and affinity maturation of GC B cells [54-60]. GC B cells that survive the selection process within the GC will migrate into peripheral sites as long-lived antibody producing plasma cells or circulating memory B cells.

High-affinity antibodies produced by plasma cells play an important role during allergic inflammation. IgE antibodies, produced by plasma cells, can be bound by multiple cell types, including basophils and mast cells, on their surfaces via fragment crystallizable-epsilon receptor I (FcεRI) receptors [61,62]. Upon coming into contact with allergens, cross-linking of allergen-specific IgE on cellular Fc receptors will initiate rapid downstream activation of innate cell types that leads to the release of mediators that promote symptoms of allergy and anaphylaxis.

The role of high affinity antibodies during helminth infection is less certain. High affinity antibodies do not appear to be important during primary *N. brasiliensis* infections [63,64]. However, these antibodies may play an important protective role upon secondary infection [63,64]. *N. brasiliensis* infected B-cell deficient or FcεRI

deficient mice expel worms normally after primary infection but have delayed clearance upon secondary infection [64]. B cell deficient mice also have impaired clearance of *H. polygyrus* during secondary infection [63].

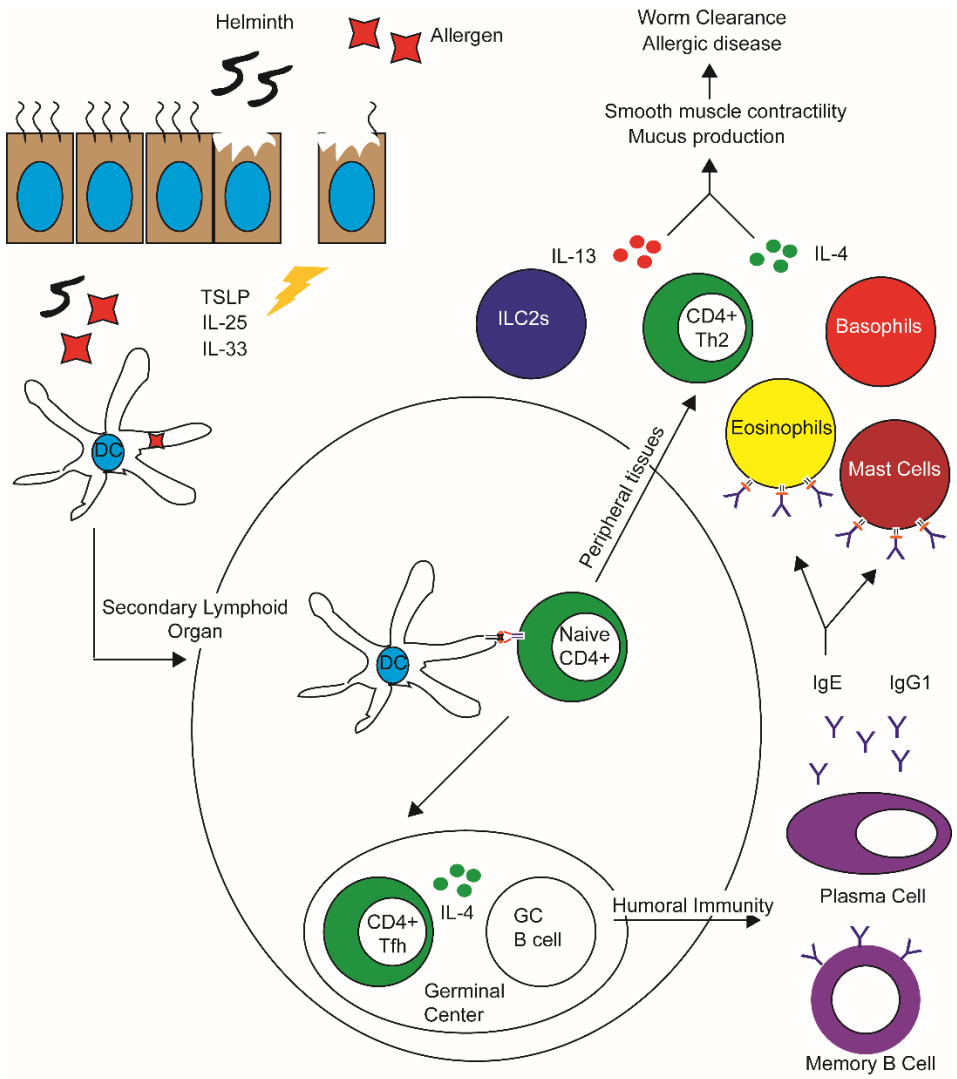

Figure 1: Model of type-2 inflammation

Tissue damage elicited by helminths induces release of TSLP, IL-25, and IL-33 from epithelial cells. These factors will enhance activation and recruitment of numerous

innate cell types. Dendritic cells (DCs) recruited to the site of damage will take up foreign antigen in the form of helminths or allergens. DCs will then travel to secondary lymphoid organs where they may interact with, and present antigen peptide via MHC-II to naïve CD4+ T cell that specifically recognizes the antigen with their TCR. After MHC-II/TCR interactions the naïve CD4+ T cells will differentiate into either a Tfh cell or a Th2 cell. Tfh cells will remain in the lymphoid organ and produce IL-4 to help GC B cell maturation. After their maturation in the GC, GC B cells will migrate to the periphery as memory B cells or antibody producing plasma cells. Antibodies produced by plasma cells will be bound by basophils and mast cells. Conversely, Th2 cells migrate to peripheral inflammatory sites and produce IL-4 and IL-13 which induce smooth muscle contraction and mucus production. The actions of Th2 cells along with basophils, eosinophils, mast cells, and ILC2s perform functions in the periphery that mediate worm clearance and allergic disease.

1.3 Innate immune response in type-2 immunity

In the context of type-2 immunity, several innate cell types play important roles during the immune response to helminths and allergy. These innate cells include: basophils, eosinophils, ILC2s, and mast cells. Upon activation these cells release a number of unique classes of mediators - such as histamines, leukotrienes, proteases, and cytokines. These cells and their mediators are important for parasitic defense, but their actions can also result in unwanted allergic inflammation.

1.3.1 Basophils

Basophils are one of the least abundant cells found in peripheral blood of mice and humans, averaging less than one percent of the total immune cell population [61,65,66]. Despite their relatively low abundance, their impact on allergic inflammation should not be understated. During a type-2 immune response the basophil population expands tremendously in response to type-2 cytokines [42,67]. TSLP produced by

damaged epithelium will induce the development of basophils within the bone marrow [42]. IL-3 also induces the expansion of basophil populations [68]. Basophils produce type-2 cytokines and release high levels of histamines and platelet activating factor (PAF) upon activation. Histamines and PAF enhance vascular permeability and smooth muscle contractility and are important mediators in allergic anaphylaxis as well as allergic responses in the skin and airways [69]. As a result, basophils are major mediators of IgE-dependent [70-72] and -independent chronic allergic inflammation [73,74].

Antigen specific IgE bound on the cell surface can trigger the activation of basophils when contacting specific allergens. IgE is displayed on the outside of basophils by the high-affinity FcεRI, and crosslinking of IgE initiates downstream signaling through FcεRI [61,62]. FcεRI exists as a tetramer on basophils ($\alpha\beta\gamma2$) [61,62]. The α chain is responsible for binding to the Fc portion of IgE. The β chain has four transmembrane domains and one immunoreceptor tyrosine-based activation (ITAM) motif, which is associated with the Lyn kinase. The two identical γ chains are linked via a disulfide bond and each contain one ITAM motif. Upon binding of antigen by IgE, Lyn will phosphorylate tyrosine residues in the ITAM motifs [61]. These phosphorylated ITAM motifs will then recruit the kinase Syk. Syk subsequently activates a multitude of downstream signaling events important for basophil activation and function [61]. In

support for a critical role for Syk kinase during basophil activation, Syk-deficient basophils will not degranulate upon antigen binding to IgE [75].

Basophils can also be activated independently of IgE. Basophil activation can be induced by certain cytokines including: IL-3, IL-18, IL-33, and TSLP [42,68,76]. Basophils cultured *in vitro* with IL-18 or IL-33 in the presence of IL-3 have enhanced production of type-2 cytokines [76]. Bone marrow cells cultured in the presence of IL-3 or TSLP produced enhanced IL-4 mRNA competent basophil populations [42]. Basophils can also be directly activated in an IgE-independent fashion by certain products produced by helminths and house-dust mite. Incubation of basophils with the house-dust mite protease Der p1 enhances basophil production of type-2 cytokines *in vitro* [77]. Proteases secreted by the human hookworm *Necator americanus* similarly enhanced type-2 cytokine expression by human basophils [77]. IPSE-α1, a glycoprotein secreted by *H. polygyrus*, promotes IL-4 production in murine basophils independent of antigen-specific IgE [78]. The mechanisms through which basophils sense and respond to activate proteases remain to be elucidated.

Activation of basophils results in the secretion of cytokines and other factors. Basophils are capable of producing IL-5 and IL-13 [76,77], however the primary cytokine produced by basophils *in vivo* is IL-4 [16]. IL-4 mRNA expression is acquired and maintained during lineage differentiation of basophils in the bone marrow [18,79]. Constitutive expression of IL-4 mRNA allows for rapid protein production in activated

basophils [79]. Basophils are major contributors of IL-4 protein production by five days post *N. brasiliensis* infection [16,80]. However, basophils are not required for the clearance of *N. brasiliensis* or *H. polygyrus* worms after primary infection [81]. Basophil derived-IL-4 may play a critical role in mediating worm clearance during secondary infection [81].

Basophils play critical roles during allergic disease. Consistently, basophil-deficient mice have reduced eosinophil recruitment within various allergic models [74,82]. As supporting evidence, IL-4 derived from basophils increases the expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells [82]. VCAM-1 actively promotes eosinophil recruitment [82]. Furthermore, basophil-derived IL-4 enhanced ILC2 function and promoted lung inflammation during protease-induced airway disease [73].

IL-4 mRNA expressing basophils were also shown to reside in the T cell zone of lymph nodes in papain immunized mice to promote Th2 differentiation [83]. Later work using an IL-4 protein reporter demonstrated that IL-4 production by basophils is restricted to affected tissues; and while basophils are recruited to the lymph node they do not produce IL-4 at this site [80]. Some studies have shown that basophils express MHC-II and may present antigen to CD4+ T cells in lymph nodes to promote Th2 responses [84-86]. However, genetic depletion of basophils or blockade of basophil recruitment to lymph nodes had no effect on Th2 cell generation [80,87]. This finding

was true for both allergic stimuli and helminth infection and suggests that basophils are not essential for Th2 differentiation.

1.3.2 Eosinophils

Eosinophils are a terminally differentiated cell type, derived from myeloid precursors, that are generated in the bone marrow in response to cytokines and are recruited to tissues during type-2 inflammation. They are granule-containing cells that function by releasing mediators from pre-formed granules upon activation. The granules contain several cationic factors including: eosinophil peroxidase, eosinophil cationic protein, eosinophil derived neurotoxin, and major basic protein [88,89]. These factors are mediators of allergic disease and also aid in immune defense against some parasitic worms [88]. Eosinophils also store a large number of pre-formed cytokines within their granules including the type-2 cytokines: IL-4 [90], IL-5 [91], and IL-13 [92]. However, the importance of eosinophilic pre-formed cytokines is still unclear.

Eosinophils are released from the bone marrow into the periphery upon a host response which results in the production of type-2 cytokines. Specifically, IL-5 is the most important cytokine required to induce eosinophil maturation in the bone marrow and subsequent recruitment to inflammatory sites [93]. IL-5 is produced predominately by Th2 cells and ILC2s during type-2 inflammation. Eosinophils are recruited to sites of inflammation and are predominately found in the tissue. Eosinophil migration into inflammatory sites is mediated by the release of chemokines from epithelial cells.

Epithelial cells will release specialized chemokines, known as eotaxins, during type-2 inflammation. IL-4 and IL-13 cytokines induce the release of eotaxins by epithelial cells [94]. Eotaxins interact with CCR3 on the eosinophil cell surface to promote migration to sites of inflammation [95,96]. In support, CCR3 deficient mice and eotaxin deficient mice have major impairments in eosinophil recruitment to airways during OVA-induced allergen challenge [97].

Eosinophilia in the lung can enhance early defense against intestinal parasites. Overexpression of IL-5 during an infection with *N. brasiliensis* leads to enhanced eosinophilia and worm clearance [93,98]. Mice with defective eosinophil trafficking or eosinophil-deficient mice also had impairments in their ability to clear filarial parasites [99-101]. However, some studies have found that IL-5-deficient mice, with impaired eosinophilia, had no defect in their ability to clear the parasites *S. mansoni* [102] or *T. muris* [103]. Therefore, IL-5 and eosinophil contributions during parasite clearance likely depends on the infecting parasite.

Eosinophils are major mediators of allergic disease. During allergen challenge, eosinophils are recruited and contribute to airway hyperreactivity (AHR) through the release of granules containing basic proteins and leukotrienes [89]. High concentrations of eosinophil major basic protein are capable of inducing tissue damage in inflamed airways [104]. Leukotrienes released by eosinophils enhance bronchoconstriction and cellular recruitment by increasing vascular permeability [105]. CCR3 is essential for

eosinophil migration into inflamed tissue [106] and ablation of eosinophils by using an antibody against CCR3 prevents the propagation of allergen induced AHR [107].

1.3.3 Group 2 innate lymphoid cells

The discovery of innate lymphoid cells (ILCs) as a unique cell type began when it was found that IL-25 administration induced the production of Th2 cytokines, IL-5 and IL-13, even in RAG knockout mice (no B or T cells) [12]. In support, another study demonstrated that IL-5 secretion could be induced in a non-B/non-T cell population upon intranasal administration of IL-25 or *N. brasiliensis* infection [108]. Identification of these cells as a unique population was confirmed when studies identified a non-B/non-T population, induced by IL-25, that produced IL4, IL-5, and IL-13 [26]. Importantly, IL-25-deficient animals had delayed Th2 responses and poor expulsion of *N. brasiliensis* [26]. Future studies found that these cells were dispersed in the peritoneal adipose tissue, gut, lung, and mesenteric lymph nodes at rest and expanded rapidly upon infection with *N. brasiliensis* [20-22]. These cells have since formally been termed ILC2s and are described as a lineage (CD3e, TCRβ, TCRδ, CD5, CD19, B220, NK1.1, Ter119, Gr-1, Mac-1, CD11c and FceRIα) negative innate cell population induced by IL-25 and IL-33. Since initially described, studies have found that IL-25 and IL-33 responsive ILC2s represent distinct subsets [109]. ILC2s produce IL-4, IL-5, IL-9, and IL-13 cytokines and have been implicated in defense against helminths as well as mediators of allergic disease [20-22,73,110].

ILC2s express GATA-3 and GATA-3 is essential for the development of ILC2s [111,112]. Deletion of GATA-3 in hematopoietic cells results in a failure to develop ILC2s [111,112]. GATA-3 is also required for the maintenance of ILC2 populations. Temporal ablation of GATA-3 in developed ILC2s results in a significant decrease in ILC2 populations [111,17]. Expression of GATA-3 also appears to be essential for type-2 cytokine production by ILC2s. IL-13 production by ILC2s tracks closely with GATA-3 expression, with IL-13 producers having high expression of GATA-3 [16]. ILC2s with temporal deletion of GATA-3 are incapable of producing IL-5 or IL-13 [17,112].

ILC2s express high levels of the IL-9R, and IL-9 may be critical for their survival [113]. Flow cytometry sorted ILC2s cultured in the presence of IL-9 have enhanced survival marked by increased expression of the pro-survival molecule BCL3. In support, *N. brasiliensis* infected IL-9R knockout mice have reduced numbers of ILC2s compared to wild-type [113]. A caveat of this study is that IL-9R deletion was not confined to the ILC2 population. Therefore, the exact role of IL-9 signaling in ILC2 development and function *in vivo* is still unclear.

ILC2s play an important role in regulating intestinal worm infections. Clearance of *N. brasiliensis* is impaired in RAG-deficient animals [22]. However, impairments in worm clearance in these animals can be rescued by stimulating the expansion and function of ILC2s by administering exogenous IL-25 [22]. In contrast, administration of IL-25 to RAG/common-γ chain-double knockout mice (B, T, and ILC2 deficient) did not

rescue worm clearance [22]. Transfer of ILC2s, followed by IL-25 administration, into RAG/common-γ chain double deficient mice restored worm clearance and eosinophilia in *N. brasiliensis* infected mice [22]. In sum, ILC2s are not sufficient to promote worm clearance unless artificially stimulated with exogenous cytokine.

While playing important roles in worm expulsion, ILC2s have also been identified as mediators of allergic disease. IL-5 produced by ILC2s promoted lung eosinophilia in protease allergen-induced airway inflammation [73]. ILC2 numbers were increased in the skin and associated draining lymph nodes in a mouse model of atopic dermatitis; and inflammation in the skin was dependent on ILC2 function [114,115]. In a model of allergen-induced lung inflammation, RAG-deficient animals still developed significant airway inflammation, marked by eosinophilic infiltrate and mucus production. However, RAG/common-γ chain deficient mice were protected from lung inflammation [116].

1.3.4 Mast cells

Mast cells, similar to basophils, highly express FcεRI on their cell surface and strongly bind allergen-specific IgE [62]. Upon coming into contact with allergen, the IgE will be cross-linked and lead to rapid degranulation of the mast cells with subsequent release of pre-formed granular proteins such as histamine, serine proteases, and proteoglycans from secretory granules [117]. The cytokine TNFα is also pre-formed and rapidly released from the granules upon mast cell activation [117-120]. TNFα released

from mast cells in the periphery can traffic to LNs and induce lymph node enlargement [119,120] Proteins released from secretory granules by mast cells are major mediators of allergic disease and anaphylaxis. These mediators enhance vascular permeability and increase the expression of various adhesion molecules, resulting in enhanced recruitment of other inflammatory cells [121]. Serine proteases released from granules cause serious tissue remodeling and fibrosis in the lung during settings of allergic asthma [62,122]. Histamines released by mast cells enhance smooth muscle contraction and subsequent narrowing of the airways [117,123].

1.4 CD4+ T helper cell activation

The existence of multiple CD4+ Th subsets was originally recognized when it was discovered that two types of CD4+ T cells could be distinguished based on the cytokines they produced and their functional ability to help B cells [124-126]. These first descriptions subset the Th cells as Th1 and Th2. Since this time, several other Th subsets have been discovered and the functions of these subsets have been more fully elucidated. The list of Th subsets now includes: Th1, Th2, Th17, Tregs, and Tfh cells. Tregs are anti-inflammatory and are important for controlling the magnitude and duration of the immune response. Tfh cells are essential for providing B cell help in secondary lymphoid organs to generate the humoral immune response. Th1 cells are essential for activating macrophages and clearance of viruses and intracellular bacteria, while Th17 cells are important for immunity against fungi and extracellular bacteria.

Th1 and Th17 cells do not capably mediate protection against intestinal helminths. Th2 cells are the essential Th subset required for the clearance of intestinal worms and are also the primary adaptive cell type that mediates allergic inflammation in the periphery.

The differentiation of a naïve CD4+ T cell into a specialized Th cell begins in the lymph node when naïve CD4+ T cells become activated. Each CD4+ T cell expresses a unique TCR that binds specifically to a certain peptide bound in the context of a MHC-II complex on an APC. In the periphery, APCs phagocytose and process foreign antigens. In the context of type-2 immunity, these include antigens derived from parasitic helminths as well as, in the case of allergy, innocuous antigens such as house dust mite or certain food antigens. The processed antigens are then loaded into MHC-II molecules to be presented to naïve CD4+ T cells as peptide MHC (pMHC) complexes.

Conventional dendritic cells (cDCs) are the most common APC involved during initial naïve CD4+ T cell activation. cDCs with peptide loaded MHC complexes travel through the lymphatics to lymph nodes. Within the lymph nodes, naïve T cells will search for APCs presenting antigen on MHC-II. Each T cell expresses a unique TCR that recognizes a specific peptide sequence presented in the context of MHC. The frequency of naïve T cells specific for a given antigen is very low with estimates ranging from 1:30,000 to 1: 10,000,000 [127-130]. One study used MHC tetramers for three different antigen epitopes and found that the number of antigen specific T cells varied from 20 to 200 cells per mouse depending on the epitope [127]. Another study using MHC

tetramers specific for the immunodominant *L. major* epitope LACK found approximately 15 cells per lymph node specific for the LACK epitope [128]. Upon interactions of naïve CD4+ T cells with cognate antigen, in the context of pMHC:TCR, the TCR will signal a multitude of downstream signaling cascades that result in the activation and proliferation of the T cell. Signals received from the TCR begin to instruct the naïve CD4+ T cell towards differentiation into a specialized T helper subset.

In addition to TCR signaling, naïve CD4+ T cells require an additional co-stimulatory signal during TCR stimulation to achieve full activation [131,132]. In the absence of a co-stimulatory signal, it was found that TCR-stimulated CD4+ T cells would enter a state of unresponsiveness known as anergy [131-133]. This co-stimulatory signal is provided by CD28. CD28 is expressed on the T cell and resides in the TCR-signaling complex [134,135]. CD28 binds to CD80 and CD86 present on APCs during pMHC:TCR interactions [136]. CD28 binding initiates a multitude of downstream signaling pathways to aid in T cell activation and differentiation. Ligation of CD28 during pMHC:TCR interactions enhances proliferation and IL-2 production by CD4+ T cells [137-139]. Additionally, CD28 signaling enhances the expression of anti-apoptotic factors such as BCL-xL [140,141].

1.5 CD4+ T helper 2 cells

GATA-3 is the Th2 master regulator and the expression of GATA-3 is essential for Th2 differentiation and function [142,143]. The main function of Th2 cells is to

enhance innate cell recruitment to sites of inflammation and to induce mucus secretion in the intestinal epithelium. In the absence of Th2 cells, such as in RAG knockout mice, clearance of intestinal helminths is greatly impaired [20-22]. Additionally, conditional deletion of GATA-3 in activated CD4+ T cells results in major impairments in worm clearance [143]. Th2 cells are also the primary adaptive cell type that mediates allergic inflammation in the periphery and a number of allergic diseases are mediated predominately by Th2 cells.

1.5.1 Th2 cell differentiation

All of the specialized CD4+ T helper subsets are originally derived from naïve CD4+ T cells. The exact mechanisms that drive the fate decisions of naïve T cells into specific functional T helper subsets are not fully understood. As described above, CD4+ pMHC:TCR stimulation is essential to begin the activation and differentiation process of a naïve CD4+ T cell into a specialized Th subset. Signals received from TCRs can vary greatly and a multitude of studies have demonstrated that differences in TCR signaling can have major impacts on fate decisions during Th differentiation. Additionally, the presence or absence of specific signals during TCR activation and during the differentiation process are critical in directing the fate of a naïve CD4+ T cell toward a specialized T helper fate [124,144-146]. The factors important for directing naïve CD4+ T cells into a Th2 fate will be described below.

1.5.1.1 TCR signaling in Th1 vs Th2 differentiation

Signals received from TCRs are heterogeneous and appear to be dictated by a number of factors. These factors include: TCR affinity to pMHC complexes, pMHC:TCR dwell time, and antigen dose. Differences in these TCR characteristics results in varying degrees of TCR signaling strength. As a result, the outcome of pMHC:TCR signaling can have a wide-range of outcomes. In support, the fate decision of a naïve CD4+ T cell to differentiate into a Th1 or Th2 cell has been shown to be dependent on the TCR characteristics delineated above [129,147-151].

Antigen load and dose have been shown to affect TCR signaling and downstream Th cell differentiation [147-149]. Higher doses of antigen increase the number of pMHC molecules displayed by APCs, which is thought to enhance TCR signaling on cognate T cells [129,152]. Studies using T cells expressing transgenic TCRs identified a role for antigen dose in Th differentiation [148,149]. TCR transgenic T cells expressing a TCR specific for moth cytochrome c peptide (pMCC) activated in the presence of APCs and low doses of pMCC promoted Th2 differentiation and cytokine production. In contrast, activation in the presence of APCs and higher doses of pMCC promoted Th1 differentiation and function [149]. In slight contrast to this study, another group showed that activation in the presence of both low and high doses of antigen promoted Th2 differentiation, while intermediate doses of antigen promoted Th1 fate [148].

As described previously, the frequency of T cells specific for a given pMHC complex is very low [127-130]. Importantly, the T cell pool specific for a given pMHC is polyclonal with each T cell expressing a unique TCR [129,145,153]. This suggests that individual T cells may respond to pMHC:TCR ligation differently, depending on their TCR sequence, resulting in unique outcomes. Indeed, differences in TCR binding properties have been observed in polyclonal pools of antigen specific T cells and include TCR affinity [154,155] and dwell time [145,151,156]. TCR affinity has been shown to play a critical role in effector fate determination [150,155]. Alteration of a single amino acid on the D10 TCR clone reduced TCR affinity by approximately 100-fold and altered downstream Th differentiation [150]. Wild-type D10 TCR expressing CD4+ T cells acquired a Th1 phenotype upon antigen stimulation, while CD4+ T cells expressing the altered, lower-affinity D10 TCR acquired a Th2 phenotype [150].

More recent studies using intravital 2-photon microscopy have revealed an important role for pMHC:TCR dwell time in regulating overall TCR signaling strength and downstream Th differentiation [156]. In this study, DCs were treated with either a Th1 or Th2 polarizing adjuvant and interactions with CD4+ T cells were measured *in vivo* with 2-photon microscopy. In brief, the authors found that strength of TCR signaling closely correlated with length of interaction time. Th1 differentiation was induced with longer interaction times and high strength of TCR signaling, while Th2

differentiation occurred with shorter interaction times and lower TCR signaling strength [156].

In sum, the data suggests a model in which Th1 cell differentiation is preferentially induced by greater TCR signaling strength, while Th2 cell differentiation is promoted in settings of lower or weaker TCR signaling (Figure 2). This model is supported by studies that have shown that low doses of antigen, short dwell times, and lower TCR affinities all promote Th2, rather than Th1 differentiation.

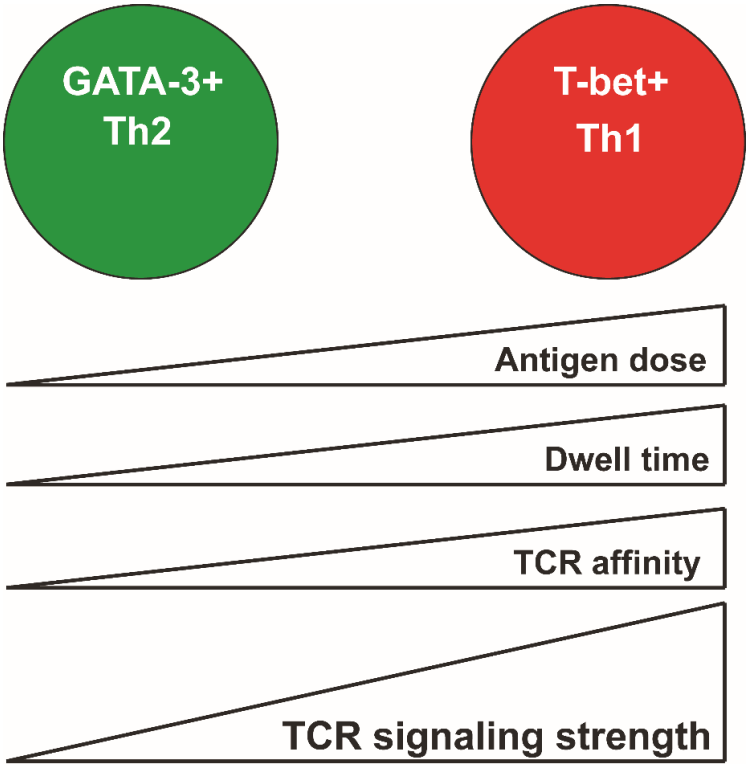

Figure 2: TCR signaling helps to determine Th2 vs Th1 differentiation

Th2 vs Th1 fate decisions are in part dictated by signaling via the TCR. Antigen dose, dwell time of the pMHC:TCR interaction, and overall TCR affinity all contribute to determine overall TCR signaling strength. Low levels of antigen dose, dwell time, and TCR affinity promote Th2 differentiation. High levels of antigen dose, dwell time and

TCR affinity promote Th1 differentiation. Ultimately, lower TCR signaling strength promotes Th2 fate, while higher TCR signaling strength promotes Th1 fate choice.

1.5.1.2 IL-4 signaling and GATA-3 in Th2 differentiation

While TCR signaling is required in the activation and differentiation of naïve CD4+ T cells into specialized T helper cells, the presence or absence of other external signals are also important to help instruct naïve T cells towards a specific T helper phenotype. Therefore, the setting and environment in which naïve T cells are activated is critically important for shaping the downstream immune response. The presence of specific cytokines during activation is important for instructing CD4+ T cell differentiation. In the context of allergic inflammation, the presence of IL-4 plays a major role in promoting Th2 fate polarization. In the absence of IL-4, TCR stimulation leads to only minor expression of GATA-3 [157]. Consistently, expression of the IL-4 receptor (IL-4R) is important for Th2 commitment. IL-4 binding to the IL-4R leads to the phosphorylation of the signal transducer and activator of transcription 6 (STAT6) by Janus kinases. Phosphorylated STAT6 (pSTAT6) monomers will then form homodimers and translocate to the nucleus to serve as a transcriptional activator [158]. In the context of Th2 differentiation, pSTAT6 will enhance GATA-3 expression [159].

Importantly, GATA-3 expression exists in a multi-layered positive feedback loop. First, GATA-3 drives the expression and downstream production of IL-4 protein, which, will lead to enhanced GATA-3 expression through continued IL-4R/STAT6 signaling, as described above [160]. Additionally, the GATA-3 promoter contains a binding site which

when bound by GATA-3 leads to enhanced transcriptional activation [160]. Therefore, Th2 cells are capable of cementing their fate in an autocrine fashion.

Furthermore, GATA-3 promotes Th2 fate while simultaneously inhibiting differentiation into other T helper subsets. GATA-3 inhibits Th1 differentiation by negatively regulating the signaling through the IL-12/STAT4 pathway [161]. Specifically, GATA-3 serves as a negative regulator of both IL-12 and STAT4 expression in Th1 cells [162]. GATA-3 also directly inhibits the production of the important Th1 cytokine, IFN-γ [162-164]. In support, deletion of GATA-3 in cultured Th2 cells results in a reversion from Th2 to Th1 fate [143]. The expression of GATA-3, the production of IL-4, and the targets of downstream IL-4R signaling actively repress differentiation into other T helper subsets. For these reasons, Th2 fate becomes strongly cemented.

While STAT6 and IL-4 are historically believed to be the most important drivers in Th2 fate choice and polarization, several studies have found that the requirement of IL-4/STAT6 pathway during Th2 differentiation may not be absolute [24,165-167]. These studies demonstrated that the presence of IL-4 signaling and active STAT6 expression was required for *in vitro* Th2 differentiation [24]. However, IL-4-deficient and STAT6 knockout mice could still develop Th2 cells in the mesenteric and mediastinal lymph nodes after *N. brasiliensis* infection [24,165]. Importantly, these cells were identified to be true Th2 cells as they expressed comparable levels of IL-4, IL-13, and GATA-3 mRNA compared to CD4+ T cells derived from STAT6-sufficient animals [24]. Despite the

generation of Th2 cells in the lymph node, IL-4 expressing Th2 cell populations were largely absent in the lung [165] and clearance of *N. brasiliensis* from the intestines was impaired [165,168]. These studies are important as they demonstrate that Th2 cells are capable of developing in the absence of IL-4 or STAT6 signaling and that IL-4/STAT6 may be more important in maintaining Th2 fate commitment. It also suggests that other signaling pathways must play a role in driving GATA-3 expression to induce Th2 commitment.

1.5.1.3 Other factors important for Th2 differentiation

Other factors important for regulating Th2 differentiation include STAT5 and cMAF. STAT5 signaling is induced by IL-2 and IL-2 is an important driver of Th2 differentiation [169,170]. Forced expression of STAT5 in naïve T cells induces Th2 differentiation, even in the absence of IL-4 signaling [169]. cMAF promotes Th differentiation towards a Th2 fate and cMAF transgenic mice have enhanced Th2 responses *in vivo* [171]. However, forced expression of cMAF is not sufficient to drive Th2 fate in CD4+ T cells cultured under Th1 conditions [171]. Of relevance, the Notch receptor signaling pathway has also been found to be critical during *in vitro* Th2 differentiation [172,173]. Deletion of Notch receptors or the Notch binding partner RBPJ results in impaired Th2 differentiation *in vitro* [172,173]. The specific role of Notch during Th2 differentiation and function will be elaborated in section 1.6.1.

1.5.2 Cytokine regulation and function

1.5.2.1 Interleukin-4

Th2 cells use the canonical, STAT6- and GATA-3-dependent, pathway to regulate IL-4 production. In support, CD4+ T cells deficient in STAT6 have abrogated production of IL-4 and other Th2 cytokines [158], while Th1 cells overexpressing Stat6 will begin to produce IL-4 [159]. Expression of GATA-3 is critical for maintaining Th2 fate and driving Th2 function, including the production of IL-4. GATA-3 binds to the HSII, CNS-1, and HSVa regions in the Th2 locus to promote IL-4 production. CNS-1 is located in the intergenic space between the IL-13 and IL-4 genes and Th2 cells deficient for CNS-1 have major impairments in their ability to produce IL-4 both *in vitro* and *in vivo* [174]. Deletion of the HSII region results in a significant impairment of IL-4, but not IL-13 production in Th2 cells [175].

HSVa is located downstream of the IL-4 promoter at the 3′ end of the IL-4 gene in an area known as the CNS2 enhancer region. The CNS2 region comprises HSVa as well as HSV. This region is constitutively accessible in Th2, but not Th1 cells [176]. However, Th2 cells deficient for the CNS2 region have only slight impairments in their IL-4 production [175,177,178]. These results were confirmed by assessing CSN2 enhancer activity in various IL-4 producing cell subsets. In brief, Tfh cells had high reporter activity for CNS2, while CNS2 activity was comparatively low in Th2 cells, basophils, and eosinophils [178,179].

Deletion of GATA-3 in fully differentiated Th2 cells does not eliminate IL-4 production, but does result in a significant reduction in the amount of IL-4 produced per cell, as assessed by mean fluorescence intensity (MFI) [143]. This finding demonstrates not only how important GATA-3 is for driving Th2 function, but also suggests that other factors beyond GATA-3 must be important for driving optimal Th2 IL-4 production. Indeed, several other factors are known to be important in initiating and maintaining Th2 IL-4 production.

The transcription factor cMAF is necessary to drive Th2 IL-4 production but is dispensable for the production of other Th2 cytokines, IL-5 and IL-13 [180]. The AP-1 transcription factor, BATF, is also essential for Th2 IL-4 expression [23]. Mechanistically, BATF binds to the RAD50 region and CNS2 enhancer within the IL-4 locus. BATF likely serves as a pioneer factor for other transcription factors to regulate the locus at these sites [23]. BATF-deficient mice did not have permissive epigenetic modifications within the locus. As a result, BATF-deficient mice were incapable of generating IL-4-producing Th2 cells [23]. STAT5 also appears to be capable of driving IL-4 production [169]. Forced expression of STAT5 induces IL-4 production in cultured CD4+ T cells, even when cultured in Th1 conditions [169].

IL-4 is capable of signaling through two unique heterodimeric receptors IL-4rα/IL-2Rγc (type I IL-4R) and IL-4Rα/IL-13Rα1 (type II IL-4R) [181,182]. IL-4 binds directly to the IL-4Rα chain and recruits either IL-2Rγc or IL-13Rα1 to the signaling

complex [183]. Importantly, IL-13 can also signal through the type II IL-4R. Differential expression of the type I and type II IL-4Rs may help explain some of the differential roles of IL-4 and IL-13 in type-2 immunity. The type I IL-4Rs are expressed on many cells of the hematopoietic lineage including CD4+ T cells and B cells [184]. The type II IL-4R is not expressed on the surface of hematopoietic cells and is instead expressed on epithelium [184].

While other cell subsets do produce IL-4 during allergic inflammation, CD4+ Th2 cells are the major producers of this cytokine during a type-2 response [16]. As described above, the main role of IL-4 production during a type-2 immune response is to drive GATA-3 expression and polarize CD4+ T cells to a Th2 fate. Despite the importance of IL-4 in driving Th2 fate, IL-4 deficient animals are still capable of clearing intestinal worm infections, presumably via IL-13 production [28,185]. Although dispensable for the clearance of intestinal helminth infections, recombinant IL-4 administered in high concentrations can aid in the clearance of worms in situations in which chronic worm infections normally persist [25,186].

IL-4 plays a number of other functional roles during an allergic immune response. IL-4 produced by Th2 cells can be bound by other IL-4R-expressing cells, such as eosinophils [187], mast cells [188], and basophils [189]. IL-4R signaling in mast cells and basophils enhances the expression of the high-affinity IgE FcεRI [62,190]. As discussed previously, IgE-dependent mast cell and basophil activation plays a critical

role in mediating the development of allergic asthma. Furthermore, IL-4 signaling through the IL-4R on epithelial and goblet cells can induce mucus secretion [191].

1.5.2.2 Interleukin-5

The major role for IL-5 during an allergic response is to enhance innate cell expansion and recruitment to sites of inflammation. The cells most affected by IL-5 signaling are eosinophils. IL-5 augments eosinophilic maturation in the bone marrow and promotes mobilization of eosinophils from the bone marrow to inflamed tissues [192,193]. Signaling via the IL-5R on eosinophils enhances eosinophil adhesion to plasma-coated glass and bovine serum albumin coated plates *in vitro*. [194,195]. Specifically, IL-5 enhanced β2-integrin mediated adhesion to ICAM-1, which is important for entrance into inflamed sites [195]. Overexpression of IL-5 enhances eosinophil numbers and IL-5 produced by Th2 cells is capable of inducing eosinophil degranulation [196]. Conversely, IL-5-deficient animals have reduced eosinophilic numbers and AHR during allergen-induced models of asthma [197]. IL-5 also promotes the survival of eosinophils, possibly by inhibiting the expression of the pro-apoptotic molecule, BH3 interacting-domain death agonist [198].

1.5.2.3 Interleukin-13

The production of IL-13 by Th2 cells is also dependent on the expression of GATA-3. Signals sent via the IL-4R induces STAT6 which will enhance GATA-3 expression. GATA-3 will then directly upregulate IL-13 expression. In support, GATA-3-

deficient T cells are incapable of producing IL-13 [143,199], and over-expression of GATA-3 in Th1 cells enhances their expression of IL-13 while simultaneously inhibiting IFN-γ production [200,201]. Deletion of GATA-3 in established Th2 cells [143] or cells already producing IL-13 [17] results in a significant loss of IL-13 production and greatly impairs the clearance of *N. brasiliensis* [17,143].

IL-13 signals through the type II, but not the Type I IL-4R complex [158]. Unlike IL-4, however, IL-13 cannot bind directly to IL-4Rα. Instead, IL-13 binds directly to IL-13Rα1, which subsequently recruits the IL-4Rα subunit to the complex to begin downstream signaling events [182]. Additionally, IL-13 can bind directly to IL-13Rα2 and signal through an IL-13Rα1/IL-13Rα2 heterodimer. IL-13Rα2 is expressed almost exclusively on epithelial cells and fibroblasts [184,202].

The production of IL-13 by Th2 cells is essential for mediating peripheral type-2 immune responses. IL-13 produced by Th2 cells at sites of inflammation causes enhanced mucus production, smooth muscle contractility, chemokine production and innate cell recruitment [17,28,185]. Mucus production and smooth muscle contractility are the primary actions in the 'weep and sweep' immune response necessary to eliminate intestinal parasitic helminths. Mice with impaired IL-13 production or signaling have reduced goblet cell hyperplasia and mucus secretion and as a result have significant delays in the clearance of intestinal worm infections [17,28,168,185].

While essential for defense against parasitic helminths, IL-13 produced by Th2 cells is also a major promoting factor in allergic disease. *In vivo* blockade of IL-13 during OVA allergen challenge reduces mucus production by goblet cells and AHR [203,204]. Furthermore, administration of recombinant IL-13 was sufficient to induce goblet cell mucus production and AHR in naïve animals [203,204]. Importantly, IL4Rα deficient and STAT6 deficient animals are protected from IL-13 induced AHR symptoms [204,205]. Reconstitution of STAT6 expression in epithelial cells of STAT6 deficient mice is sufficient to re-establish IL-13 mediated AHR symptoms and epithelial mucus production [205].

1.6 Lymph node architecture and germinal centers

As described previously, lymph nodes are one of the main site in which antigen-specific immune responses begin. Within lymph nodes, T cells interact with APCs to commence their activation and differentiation process into effector T cell subsets. Additionally, T cell-dependent antibody-producing B cells are educated and generated within lymph nodes. Structurally, lymph nodes are divided into two main zones: the paracortex and the B cell follicle. The paracortex lies within the center of the lymph node and is predominately comprised of T cells. The B cell follicle lies closer to the exterior of the lymph node and is comprised of B cells and other cells specialized in aiding the maturation of the B cells. Within the B cell follicle, specialized structures known as germinal centers (GCs) will form.

GCs are the sites where B cells undergo maturation and selection processes that culminate in the generation of high-affinity antibody expressing cells [206]. The GC is a highly organized site that provides the optimal settings for B cell selection and maturation. Within the germinal center B cells undergo two important processes: somatic hypermutation (SHM) and isotype-class switching. Prior to undergoing these processes B cells express low-affinity Ig B cell receptors (BCR), IgM and IgD. Once their selection and maturation process has completed, high-affinity antibody-producing cells will migrate out of the lymph nodes as plasma cells or memory B cells [207]. The antibodies produced by plasma cells play a number of critical roles in the protection against foreign pathogens. In the context of type-2 immunity these antibodies are critical mediators of allergic inflammation and may play protective roles against secondary infections of intestinal helminths [63,64]. Memory B cells play important roles in enhancing secondary responses.

The GC reaction begins when B cells become stimulated by antigen in the B cell follicle. Following antigen stimulation, B cells migrate from the B cell follicle towards the T-B border to a region known as the perifollicular border [208,209]. Interactions between antigen specific B cells and T cells at the perifollicular border can be visualized as early as two days into the primary response [208]. Interacting with T cells at the perifollicular border is essential for B cell survival after antigenic activation [210]. Movement and localization of B cells between the B cell follicle and perifollicular border is mediated by

the chemokine receptors CXCR5 and CCR7 [211]. CCR7 ligands, CCL19 and CCL21, are produced predominately by stromal cells within the T cell zone. In contrast, the CXCR5 ligand CXCL13 is produced primarily by stromal cells, such as follicular dendritic cells (FDCs), in the B cell follicle to promote entry into the follicle [212-214]. Therefore, expression patterns of CXCR5 and CCR7 are largely responsible for determining cellular localization within the lymph node.

Once B cells come into contact with cognate T cells at the perifollicular border, they will begin to undergo rapid proliferation and expansion at the perimeter of the follicle [215]. The B cells will then either differentiate into early plasmablasts at extrafollicular sites and exit the lymph node or will remain in the follicle and serve as founder cells to begin a GC reaction [216,217]. Extrafollicular plasmablasts can undergo Ig class-switching, but not SHM, and then differentiate into plasma cells [218,219]. As a result, they provide a rapid, yet low-affinity antibody response to the current infection [218]. The decision to become an early plasmablast or to seed a GC reaction may be dictated by the length of contacts B cells have with T cells at the perifollicular border [220] as well as initial B cell receptor affinity [221,222].

GCs are seeded by a very small number of initial B cell clones [217,223]. Competition for T cell help at the perifollicular border may be an important selection factor in determining which B cell clones will seed GC reactions. B cells expressing higher affinity BCRs outcompete lower affinity B cells for T cell help at the perifollicular

border and are more likely to attain a GC fate [221]. Antigen presentation by B cells to cognate T cells at the perifollicular border may also be a determining factor in competition for T cell help. B cells with higher affinity BCRs acquire more antigen and express higher concentrations of pMHC [221]. B cells with higher pMHC concentrations on their cell surface outcompete B cells with lower concentrations of pMHC for T cell help [221]. In support, B cells with reduced capacity to present antigen were less likely to seed GCs compared to mice with normal MHC presentation [224]. Overall, competition for T cell help is important for selecting B cell clones to seed GCs, with prolonged interactions with T cells promoting seeding of GCs.

Within the GC, GC B cells undergo SHM and antibody class-switching. These processes, as well as competition for important survival signals results in the selection of a heterogeneous pool of high-affinity antibody expressing B cells [221,222]. Selection and antibody maturation processes occur in two anatomically distinct zones within the germinal center, termed the light and dark zones [206,225]. The light zone is the primary region in which B cells compete for available antigen and other signals for survival. Competition for these signals is the driving factor for selection in the GC. Two specialized cell types reside in the light zone of the germinal center, whose primary function is to guide GC B cells through their maturation and selection processes: follicular dendritic cells (FDCs) and Tfh cells.

FDCs are a stromal cell type that expresses high levels of the complement receptors CD21 and CD35 [226]. These receptors bind non-specifically to antigen-bound immune complexes allowing the FDCs to serve as antigen depots for B cells within the germinal center [227,228]. Competition for this antigen provided by FDCs is one of the primary means of selection within the germinal center. B cells expressing higher affinity BCRs will outcompete B cells with lower affinity BCRs for this antigen with B cells expressing higher-affinity BCRs dominating the B cell pool later in the immune response [206,225,229,230]. The cells that receive these antigenic signals will upregulate the expression of pro-survival and anti-apoptotic molecules. The following pro-survival factors have been shown to be important for regulating GC B cell survival: B cell lymphoma-2 [231,232], BCL-xL [233], and myeloid leukemia cell differentiation protein-1 [234]. Conversely, B cells that do not receive antigenic signals will have enhanced signaling through the death receptor FAS [235,236] and will upregulated expression of pro-apoptotic factors BIM, BAD, BIK, and BAX [237,238] and be cleared from the GC B cell pool.

Tfh cells are the other cell type that resides in the light zones of germinal centers and are critical for aiding GC B cells in their maturation and selection processes. Interactions with Tfh cells are required for the formation and maintenance of germinal centers. Expression of CD40L on Tfh cells is essential to drive germinal center formation [55,56]. Co-ligation of CD40L on Tfh cells with CD40 on B cells prevents the apoptosis of

GC B cells. Mice deficient in CD40 or CD40L do not form GCs and as a result do not have reduced plasma cell differentiation [55,56]. Furthermore, treatment of mice with an antibody against CD40L will dissolve GCs [229]. Humans with mutations resulting in impaired CD40/CD40L signaling develop a severe immunodeficiency known as hyper-IgM syndrome as a result of an inability to generate class-switched antibodies [57].

Expression of signaling lymphocytic activation molecule-associated protein (SAP) on Tfh cells is essential for enhancing long term contacts between Tfh cells and B cells [220]. SAP binds to signaling lymphocytic activation molecule (SLAM) which sends signals that regulate cell adhesion and cytokine secretion [220,239-241]. Lengthier T:B cell interactions occur in the presence of SAP/SLAM signaling and are essential for the generation of an effective humoral immune response [220,242]. Cytokine secretion by Tfh cells is essential for GC B cell affinity maturation and class switching and has been shown to be dependent on positive SAP/SLAM signals [240].

GC B cells expressing higher affinity BCRs are more likely to outcompete B cells expressing lower affinity BCRs for the limitedly available selecting signals within the GC [217]. GC B cells with higher affinity BCRs receive more antigenic signals from FDCs, leading to enhanced activation [243]. Additionally, GC B cells with higher affinity BCRs are more likely to present antigen to Tfh cells and subsequently receive pro-survival and maturation signals from CD40/CD40L ligation and Tfh-derived IL4 and IL-21 cytokines [221]. IL-4 and IL-21 produced by Tfh cells plays important roles in regulating GC B cell

survival, class-switching, and affinity maturation [54,59,60]. The precise roles of Tfh-derived IL-4 and IL-21 will be discussed in section 1.5.5

B cells that successfully receive important survival and selection signals then travel to the dark zone. Within the dark zone, GC B cells undergo SHM and class-switching of their antigen receptors as well as several rounds of replication. Interestingly, it has been demonstrated that GC B cells will cycle between the light and dark zone multiple times [244-246]. This suggests that GC B cells undergo several rounds of antigenic selection in the light zone as well as somatic hypermutation and replication within the dark zone. Multiple selection events allow B cells to fine tune the specificity of their antigen receptors prior to exiting the germinal centers and secondary lymphoid organs as long-lived antibody producing plasma cells or long-term memory B cells. After egress from the lymphoid organs, plasma cells will predominately reside within the bone marrow [218,229], while memory B cells will circulate throughout the lymph scanning for cognate antigen [218,247].

1.7 CD4+ T follicular helper cells

Tfh cells were first described in human tonsillar tissue and were characterized as CD4+ cells with heightened expression of CXCR5 and down regulated expression of CCR7 [214,248]. This chemokine receptor expression profile encourages localization of these cells to the B cell follicle. Expression of co-stimulatory molecules such as CD40L, and production of IL-4 and IL-21 cytokines are essential to establish GCs and aid GC B

cells in their maturation into high-affinity antibody-producing cells [54-56,59,249]. As a result, these cells play crucial roles in long-term protective immunity and are critical in shaping the immune response generated by human vaccines. High-affinity antibodies are also major mediators of some allergic afflictions, such as atopic dermatitis, food allergy, and asthma.

1.7.1 Tfh master regulator BCL6

Tfh cells weren't fully appreciated as a unique T helper subset until the Tfh master regulator, BCL6, was identified [250]. BCL6 is a transcription factor that exerts its effects as a transcriptional repressor. Forced expression of BCL6 in CD4+ T cells promotes Tfh differentiation, while BCL6 deficiency results in an inability to form these cells [250]. BCL6 plays a critical role in Tfh differentiation by inhibiting the expression of BLIMP-1, which is important for naïve T cell differentiation into peripheral effector subsets, including Th1 and Th2. Inhibition of BLIMP-1 is important as it serves as an antagonist of BCL6 expression [250-252]. Consistently, high levels of BLIMP-1 expression inhibit the differentiation of naïve T cells into Tfh cells, while high levels of BCL6 inhibit differentiation into Th1, Th2, and Th17 subsets.

BCL6 binds to and represses a large number of other genes directly involved in regulating the differentiation of other effector T helper subsets [251,253,254]. Over-expression of BCL6 in Th1 cells represses expression of the Th1 master regulator, T-bet [251,254]. BCL6 does not directly inhibit the expression of the Th17 master regulator,

RORγT, but it does inhibit RORγT function [253,254]. However, BCL6 does bind to and inhibit RORα, another ROR family member important for TH17 differentiation [253]. Finally, BCL6 also inhibits differentiation into TH2 cells by inhibiting GATA-3 [253,255]. Although, whether BCL6 directly regulates GATA-3 expression at the transcriptional level is uncertain as there are conflicting results in the current available literature [253-255].

BCL6 has also been implicated in regulating the expression of genes important for determining cellular localization. CD4+ effector T cells exit the lymph node in a sphigoshine-1-phosphate receptor 1 (S1P1) dependent fashion [256]. BCL6 serves as a transcriptional repressor of S1P1, thereby promoting Tfh retention in the lymph node and B cell follicle [253]. KLF2 acts as a positive regulator of S1P1 and CCR7 and a repressor of CXCR5 expression [257]. Therefore, loss of KLF2 expression in CD4+ T cells is essential for Tfh differentiation and follicular localization [257-259]. GC Tfh cells demonstrate high levels of BCL6 binding at the KLF2 promoter [253]. Furthermore, BCL6 can directly bind and inhibit the expression of the T cell zone homing chemokine receptor CCR7 [253,260].

1.7.2 Tfh differentiation

1.7.2.1 TCR signaling in Tfh vs peripheral Th differentiation

The exact mechanisms driving Tfh differentiation are not yet fully understood. Multiple unique models of Tfh differentiation have been proposed. A common feature

of all Tfh differentiation models is that Tfh differentiation begins in the paracortex when antigen-presenting dendritic cells stimulate the TCR on naïve CD4+ T cells [261,262]. In support, differentiation of Tfh cells under physiologic conditions requires antigen presentation by cDCs [261]. Similar to Th1 vs Th2 differentiation described above, the fate decision to differentiate into a Tfh cell or a peripheral effector cell may also be dictated by overall TCR signaling strength. As described in the Th2 differentiation section, the quality of TCR signaling is dictated by a number of factors including: TCR affinity to pMHC complexes, pMHC:TCR dwell time, and antigen load or dose.

Recent studies support a role for TCR in determining fate choice between Tfh and Th1 cells [129,145,155,263]. A polyclonal pool of T cells specific for a given pMHC consistently generated a similar Th differentiation profile in response to *L. monocytogenes* infection. This profile was marked by 50% Th1 and 50% Tfh cells [145]. However, individual clones within the polyclonal pool generated unique profiles of Th differentiation. Some clones differentiating entirely into Tfh cells, others entirely into Th1 cells, and others with a varying mix of both subsets [145]. This finding supports a model in which clonotypic differences, resulting in unique TCR binding properties and signaling strengths, are likely important for regulating Th differentiation.

Similar to Th1 vs Th2 cell differentiation, antigen dose has been shown to play an important role in Th1 vs Tfh differentiation. In an *in vivo* model of *L. monocytogenes* infection, it was found that low and high doses of antigen drove Tfh differentiation,

while intermediate antigen doses were more likely to drive Th1 effector fate decision [145]. In addition to antigen dose, TCR affinity has been shown to play a role in Th1 vs Tfh differentiation. Adoptive transfer of two unique populations of TCR transgenic T cell populations specific for the same pMHC, but with varied affinity, revealed unique differentiation profiles based on TCR affinity after immunization [155]. CD4+ T cells expressing higher affinity TCRs preferentially differentiated into Tfh cells, while T cells with lower affinity TCRs were more likely to adopt a peripheral effector phenotype [155]. Dwell time on pMHC complexes also plays an important role in Tfh vs Th1 differentiation. Similar to antigen doses, short and long periods of dwell time promoted Tfh differentiation while intermediate dwell times enhanced Th1 differentiation [151,155].

Taken together, previous work suggests a model in which both low and high levels of TCR signaling preferentially drives Tfh fate, while intermediate TCR signaling promotes Th1 fate (Figure 3). In support of a model in which high TCR signaling strength is important, Tfh differentiation is impaired in T cells with reduced TCR signaling capacity [263]. Deletion of 6 of the 10 ITAMs found within the TCR signaling complex results in a significant loss of Tfh differentiation after TCR stimulation compared to mice with intact ITAMs on the TCR signaling complex [263].

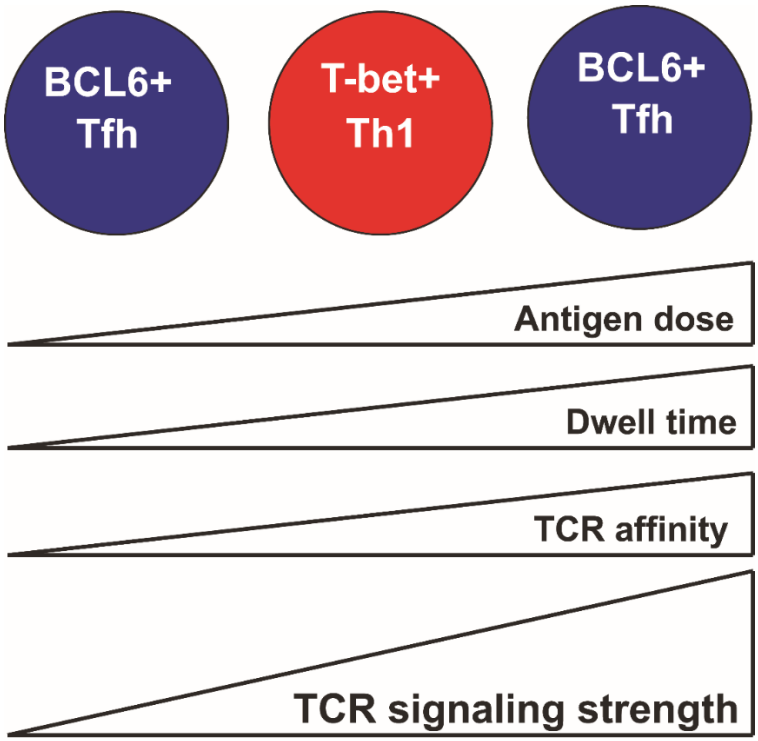

Figure 3: TCR signaling in Tfh vs Th1 differentiation

TCR signaling plays an important role in determining Tfh vs Th1 differentiation. Antigen dose, dwell time, and TCR affinity combine to determine overall TCR signaling strength. Tfh cell differentiation is promoted by both low and high overall TCR signaling strength. Conversely, Th1 differentiation is promoted by intermediate levels of TCR signaling strength.

While a large amount of work supports the TCR signaling model described above, it is important to note that some work has challenged this model. One study demonstrated that naïve transgenic CD4+ T cells were capable of differentiating into Tfh cells in response to low, intermediate, and high affinity ligands [264]. However, Th1 differentiation in this system only occurred in response to high affinity ligands [264]. In support of this finding, another group identified that the expression levels of IRF4 correlate with TCR signaling strength and that the extent of IRF4 signaling is the driving

factor in determining Tfh or peripheral T effector fate choice [265]. This study found that high levels of TCR signaling strength, which drove high levels of IRF4, preferentially promoted Th1 differentiation. In contrast, Tfh differentiation occurred at lower levels of TCR signaling strength and IRF4 expression [265]. Therefore, these two studies instead suggest a model in which high TCR signaling strength promotes Th1 differentiation rather than Tfh differentiation.

While there has been a wealth of studies investigating the role of TCR signaling in Th1 vs Tfh differentiation, there currently have not been any studies to directly investigate how TCR signaling may regulate Tfh vs Th2 differentiation. Therefore, whether TCR signaling strength plays a decisive role in determining IL-4 producing Th2 vs Tfh fate choice is currently unknown. Future studies investigating this dynamic will be important to better determine how TCR signaling helps to shape the type-2 immune response.

1.7.2.2 B cell help during Tfh differentiation

Differentiation of a naïve CD4+ T cell into a Tfh cell fate is a multi-step process that extends beyond TCR signaling [262]. TCR-mediated activation *in vivo* enhances expression of BCL6 and CXCR5, thereby promoting migration of differentiating cells to the perifollicular border [266,267]. Interactions of these CD4+ T cells with B cells at the perifollicular border is an essential second step during the Tfh differentiation process

[59,262,267,268]. The nature of these interactions may be a defining feature in determining Tfh fate choice over peripheral effector fate decisions.

Inducible T cell co-stimulator (ICOS) is a member of the CD28 family [269] and plays an important role in regulating the Tfh population [267,270-272]. ICOSL is expressed on B cells and is important in driving CXCR5 expression in Tfh cells by binding ICOS [273]. Enhanced expression of CXCR5 promotes entry of the CD4+ T cell into the B-cell follicle, thereby promoting Tfh fate. In support, blocking ICOS/ICOSL interactions causes major impairments in Tfh differentiation and GC formation [267,270,272]. Similarly, ICOS deficient mice have an abrogated Tfh population and reduced high-affinity antibody production. ICOS signaling early during Tfh differentiation promotes Tfh fate by inducing BCL6 expression [267]. In contrast, another study found that ICOS is not important during early Tfh differentiation but is essential in maintaining Tfh maintenance by regulating expression of KLF2 [257].

Cognate B cells also serve as APCs at the perifollicular border [274,275]. Although not sufficient to fully induce Tfh differentiation during protein or peptide immunization, antigen presentation by B cells at the perifollicular border can induce a Tfh program in CD4+ T cells during chronic LCMV infection [274]. However, when antigen presentation is restricted solely to B cells Tfh differentiation is impaired, as measured by reduced GC B cell responses. Another study demonstrated that B cells were essential sources of antigen presentation in settings in which antigen was limited

[275]. The requirement for B cells as APCs in limited-antigen conditions was not because they provided unique pMHC:TCR signals but because they quickly became the dominant source of antigen during the immune response and Tfh cells required continued antigen stimulation. B cells were not required to induce Tfh differentiation in settings of high antigen load [275].

1.7.2.3 Other factors contributing to Tfh differentiation

While BCL6 is considered the Tfh master regulator, a number of other transcription factors have been deemed essential for Tfh fate and differentiation. These factors include: BATF, cMAF, IRF4, and ASCL2 (Figure 4). BATF is enhanced in CD4+ T cells upon TCR stimulation in a dose-dependent fashion [276]. Furthermore, IL-6-driven signals received from the IL-6R expressed on the surface of Tfh cells will drive the expression of STAT3, which has been found to be critical for IL-21-induced Tfh differentiation [273,249]. Downstream of the IL-6R, STAT3 will enhance the expression of BATF [277]. BATF can bind to BCL6 gene locus and induce transcription to promote Tfh fate [278]. Mice with global deletions of BATF are unable to form functional Tfh populations after immunization or parasitic infection [23,277].

Similar to BATF, IRF4 expression is enhanced upon TCR-mediated T cell activation[276]. Additionally, IRF4 expression is further enhanced by BATF [277]. IRF4-deficient mice immunized with keyhole limpet hemocyanin (KLH) were incapable of forming Tfh cells [279]. The mechanisms behind how BATF and IRF4 regulate Tfh

differentiation remain unclear. However, studies suggest that BATF binds cooperatively with IRF4 to exert transcriptional regulation at various sites within Tfh cells [277]. Specifically, BATF and IRF4 have been shown to enhance Tfh function by regulating the expression of IL-4 [277].

The transcription factor cMAF is also important for Tfh differentiation. [280,281] cMAF expression is induced early during the Tfh differentiation process by ICOS signaling [281]. T cell-specific deletion of cMAF results in an inability of CD4+ T cells to differentiate into Tfh cells in response to KLH, commensal bacteria, or OVA-NP [280,281]. cMAF expression is induced by BATF in Tfh cells [278]. In support, BATF deficient mice do not express cMAF [278]. As described above, BATF deficient mice do not form Tfh cells. However, Tfh cell generation could be restored in BATF deficient T cells by forced co-expression of cMAF and BCL6 using retroviruses [278]. Interestingly, forced expression of cMAF or BCL6 alone was not sufficient to restore full Tfh development. This finding suggested that cMAF and BCL6 work cooperatively to promote Tfh fate. In support of this, cMAF and BCL6 appear to cooperate to induce expression of PD-1 and ICOS on Tfh cells [260]. Additionally, cMAF may play a role in driving the expression of CXCR5 to promote Tfh localization [260,278].

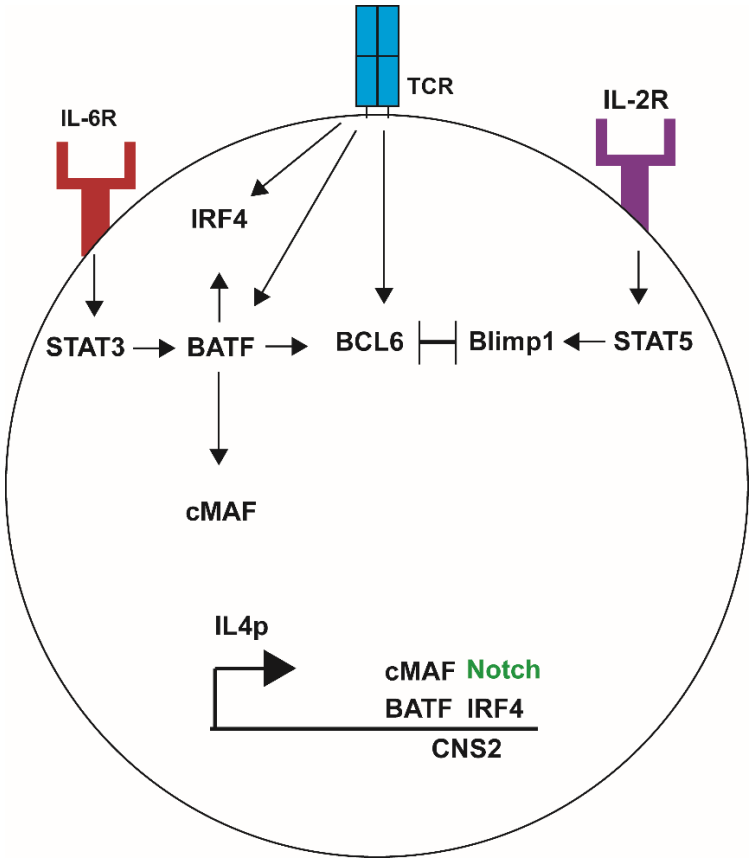

Figure 4: Schematic of the Tfh transcriptional network

The Tfh transcriptional master regulator, BCL6, is induced via IL-6R signaling. Downstream of the IL-6R, STAT3 induces the expression of BATF. BATF capably induces the expression of BCL6 to promote Tfh differentiation. Additionally, BATF promotes the expression of cMAF and IRF4, two factors also deemed essential for Tfh differentiation. BCL6 expression is also induced downstream of TCR signaling. Additionally, TCR signaling also enhances the expression of BATF and IRF4. Downstream of the IL-2R, STAT5 signaling induces BLIMP-1 expression. BLIMP-1 directly inhibits BCL6 expression. Importantly, BCL6 also directly inhibits BLIMP-1 expression. The expression levels of BCL6 vs BLIMP-1 are important to determine peripheral Th vs Tfh differentiation. Functionally, cMAF, Notch, BATF, and IRF4 can bind at the CNS2 locus to induce Tfh IL-4 production.

Another factor deemed essential for Tfh differentiation is achaete-scute homologue 2 (ASCL2). Mice with a global deletion of ASCL2 were unable to generate

Tfh cells; this defect was a result of an inability of CD4+ T cells to upregulate their expression of CXCR5 and downregulate CCR7 upon activation [282]. ASCL2 was found to bind directly to both CXCR5 and CCR7, functioning as a transcriptional activator of CXCR5 and repressor of CCR7 expression. The signals that enhance ASCL2 expression in Tfh cells to promote follicular localization have not been fully elucidated. Use of Wnt signaling agonists promotes ASCL2 and CXCR5, but not BCL6 expression in T cells *in vitro* [282]. Of relevance, Notch signaling has been shown to cooperate with Wnt to exert downstream transcriptional activation in several systems [283-285].

LEF1 and TCF1 are also important factors for regulating Tfh differentiation [286,287]; single deletion of either LEF1 or TCF1 leads to modest impairments in Tfh formation, while dual deletion of these two factors has a more significant effect [287]. Furthermore, enhanced expression of either TCF1 or LEF1 promotes Tfh differentiation over other CD4+ effector subsets [286,287]. TCF1 was capable of repressing the expression of BLIMP-1 while inducing the expression of BCL6, and also functioned by inhibiting IL-2R expression, signaling through which strongly impedes Tfh differentiation [288]. Additionally, LEF1 and TCF1 work cooperatively to upregulate the expression of factors upstream of the IL6R, which promotes Tfh differentiation [287].

STAT5 signaling negatively regulates Tfh differentiation (Figure 4). Constitutive expression of STAT5 in CD4+ T cells selectively blocks Tfh differentiation [289]. Conversely, STAT5 deficient CD4+ T cells upregulated expression of BCL6 and had

enhanced Tfh differentiation *in vivo* [289,290]. STAT5 signaling is induced by IL-2 and deletion of the IL-2Rα subunit enhances Tfh differentiation [289]. Furthermore, treatment of mice with anti-IL-2 neutralizing antibodies prior to LCMV infection enhanced Tfh generation [289]. STAT5 enhances expression of the BCL6 antagonist BLIMP-1 [289,290] and represses expression of cMAF, BCL6, and CXCR5 [290]. Therefore, the IL-2/STAT5 signaling axis likely functions through BLIMP-1 to serve as a general inhibitor of Tfh differentiation.

1.7.3 Cytokine production and function

1.7.3.1 Interleukin-4

Tfh cells produce IL-4 in a non-canonical, STAT6- and GATA-3-independent, fashion [54,291]. Expression of the CNS2 enhancer region in the IL-4 locus is essential for Tfh IL-4 production. BATF, cMAF, and Notch/RBPJ all have putative binding sites within the CNS2 region (Figure 4). Deletion of BATF or cMAF results in a near complete loss of IL-4 production by Tfh cells [23,280].

IL-4 was originally described as an important B cell survival factor [292]. Signaling of IL-4 through STAT6 expressed on B cells results in upregulation of the anti-apoptotic factor B cell lymphoma-extra-large (Bcl-xL) and enhances B cells resistance to ligation of the death receptor Fas [293]. Therefore, germinal center B cells that receive IL-4 signaling are less likely to enter apoptosis. IL-4 signaling by Tfh cells is also essential for the affinity maturation and class-switching of GC B cells to the higher affinity type-2

antibody isotypes, IgE and IgG1 [54,294]. GC B cells bound to Tfh cells producing IL-4 upregulate their expression of activation-induced deoxycytidine deaminase (AID) [54], a molecule essential for antibody affinity maturation and class-switching [295-297]. Consistent with this finding, IL-4, IL-4R, and STAT6 deficient animals all have abrogated class switching to IgE and IgG1 [158,168,17,54]. The small amounts of IgE and IgG1 produced by B cells in IL-4-deficient animals are of a lower affinity than in mice that have sufficient IL-4 production [54].

1.7.3.2 Interleukin-21

IL-21 signals through the heterodimeric IL-21R. The IL-21R is comprised of the IL-21R chain and the common-γ chain [298]. IL-21R is expressed on numerous cell types, including B cells and Tfh cells. Downstream of the IL-21R, IL-21 signals through STAT3 [299]. In support, STAT3 deficient cells are less responsive to IL-21 signals and have reduced expression of downstream IL-21 signaling targets [299,300].

IL-21 produced by Tfh cells is critical for optimal GC formation [60,249,301]. IL-21 deficient animals have significant reductions in GC B cell populations in response to sheep red blood cell immunization (SRBC) [249]. Additionally, IL-21 is important in the differentiation of GC B cells to antibody producing plasma cells and memory B cells. IL-21R knockout and IL-21 deficient mice have significant reductions in plasma cell and memory B cell formation [60]. The reduction observed in plasma and memory B cell populations could be in part due to reduced proliferation. B cell proliferation is

significantly impaired in IL-21 deficient mice after immunization [60]. IL-21 is also important for affinity maturation as memory B cells derived from IL-21R or IL-21 deficient mice expressed lower affinity BCRs [60].

As discussed previously, Tfh differentiation and GC B cell generation are highly reliant on one another. Therefore, the effect on the GC B cell population during IL-21 or IL-21R deficiency could be a T-cell intrinsic effect. Tfh cells express high levels of the IL-21R and IL-21R deficient mice had a significantly impaired Tfh population in response to SRBC immunization [249]. Although, in contrast, another study found no defect in Tfh formation after KLH-NP immunization of IL-21R deficient mice and accurately visualized CD3+ T cells within GCs using histology [60]. Therefore, the exact roles of IL-21 during Tfh differentiation is currently unclear.

1.8 Cytokine-reporter mice

Historically, it has been very difficult to detect type-2 cytokines *in vivo* and many previous studies relied on *in vitro* cultures or *ex vivo* restimulation of cells to assess cytokine production. Unfortunately, *in vitro* studies and restimulations are generally better at showing what cytokines a given cell type is capable of secreting rather than what may truly be produced *in vivo*. The generation of mouse lines that faithfully report type-2 cytokine mRNA expression and production have greatly aided the study of Tfh and Th2 cells (Figure 5). The ability to more easily detect this cytokine expression has greatly enhanced the ability to study the true *in vivo* function of these cells. In these

studies, we have made use of two IL-4 reporter lines: the IL4^{4get} mRNA and IL4^{KN2} protein reporters [165,302].

The IL4^{4get} mouse has an IRES-enhanced green fluorescent protein (GFP) gene knocked-in downstream of the stop codon in the intact IL-4 locus. Once transcription of the *il4* locus has begun, a bicistronic mRNA transcript is generated that allows for both GFP and IL-4 expression. Therefore, IL-4 expressing cells, often termed IL-4-competent, can be easily identified by expression of GFP. The IL4^{KN2} mouse has a human CD2 (huCD2) cassette gene knocked-in in place of the endogenous IL-4 gene. In IL4^{KN2} mice, recent IL-4 protein producing cells are easily identified by huCD2 expressed on the cell surface.

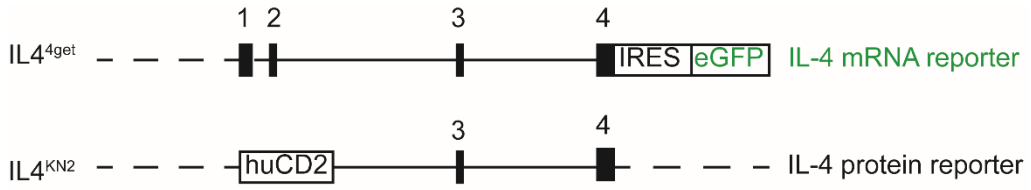

Figure 5: IL4^{4get} and IL4^{KN2} reporter mouse constructs

Black boxes denote exons and numbers above the boxes indicate which exon. The IL4^{4get} is marked by the addition of an IRES-eGFP which was inserted downstream of exon 4 and upstream of 3′ UTR of exon 4 in the *il4* locus. The IL4^{4get} mRNA is bicistronic allowing for endogenous IL-4 production as well as GFP to be produced from the IRES. The IL4^{4get} mouse faithfully reports IL-4 mRNA [165]. The IL4^{KN2} mouse has a human CD2 (huCD2) gene inserted in place of exons 1 and 2 in the *il4* locus. Insertion of the huCD2 disrupts IL-4 production and instead huCD2 is produced and place on the cell surface to report IL-4 protein production [302]. Importantly, all IL-4 regulatory elements within the locus are left intact.

1.9 Notch receptor signaling pathway

The Notch receptor signaling pathway was first discovered in *Drosophila melanogaster*, and consists of four unique receptors, Notch 1-4. Each of these receptors have three common domains: an extracellular ligand binding domain (NECD), transmembrane domain, and an intracellular domain (NICD). There exist five known canonical ligands that bind to each Notch receptor, which stem from two different families. The delta-like family contains three unique ligands (DLL1, DLL3, and DLL4), while the Jagged family has two unique ligands (Jagged 1 and Jagged 2). The receptors and ligands are highly conserved throughout mammalian species. Roles for Notch signaling have been described in a multitude of systems, including neuronal and embryonic development, epithelial differentiation, and the immune system. Within the immune system, Notch signaling has been found to play critical roles in lymphoid progenitor fate choice [303,304], thymic T cell development [305-309], CD4+ and CD8+ effector T cell differentiation and activation [172,310-316], and B cell development [317,318].

1.9.1 Canonical Notch signaling

Canonical Notch signaling is initiated upon ligand binding to the extracellular domain of the Notch receptor (Figure 6). Importantly, initiation of Notch signaling events that occur on the receptor-bearing cell requires the function of an E3-ubiquitin ligase, Mind bomb 1 (MIB1), on the ligand-bearing cell [319]. MIB1 functions by

ubiquitinating and initiating endocytosis of the ligand. Without the expression of MIB1, this process of endocytosis does not occur and downstream signaling on the receptor-bearing cell does not initiate [319].

Once ligand binding has successfully initiated Notch receptor signaling, two proteolytic cleavage steps will begin. The first proteolytic cleavage step is performed by TACE (TNF-α ADAM metalloprotease converting enzyme) and releases the NECD, which remains bound to the ligand and is endocytosed and recycled by the signal-sending, or ligand-bearing cell [320,321]. The second proteolytic cleavage step is performed by the gamma-secretase complex and releases the NICD from the intracellular membrane. Once released from the membrane NICD is free to translocate to the nucleus where it will interact with various co-activators to initiate downstream transcriptional regulation [320]. Canonical Notch signaling requires NICD interacting with the DNA-binding protein CSL/RBPJ. Prior to Notch binding RPB-J functions as a transcriptional repressor. However, once NICD binds to RBPJ the NICD co-activator, mastermind-like protein (MAML), will be recruited to the complex. This NICD/RBPJ/MAML signaling complex will then initiate transcriptional activation of various targets.

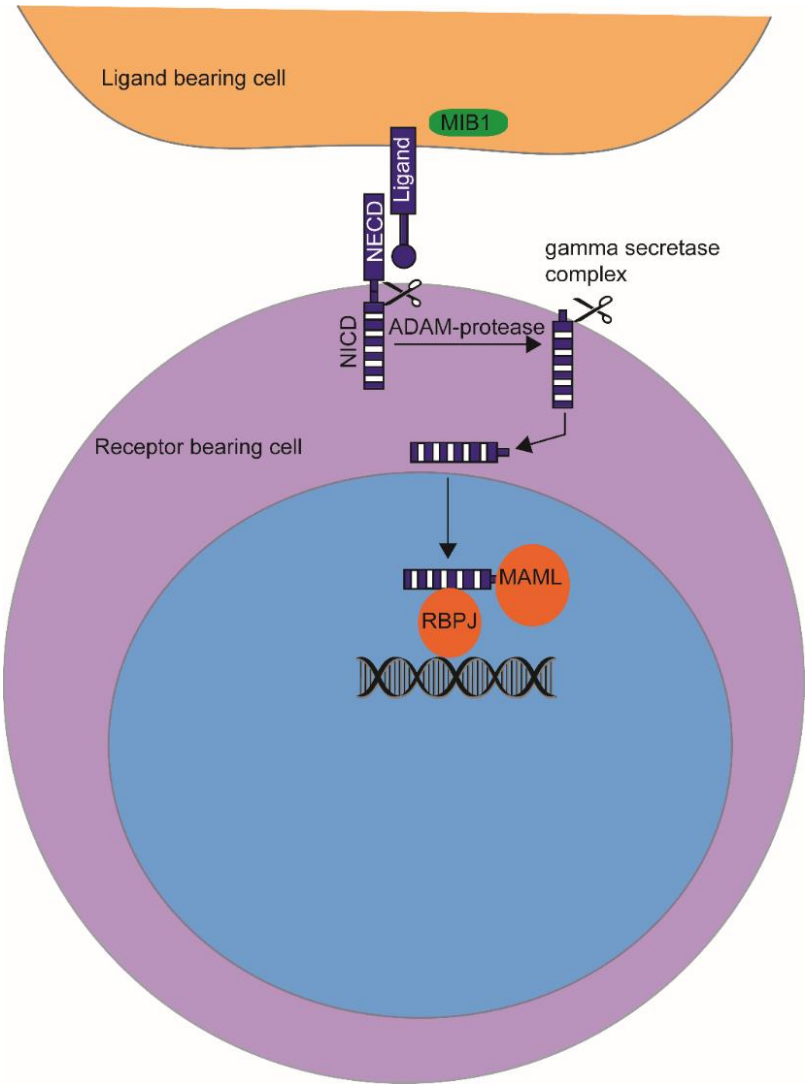

Figure 6: Canonical Notch signaling

Notch ligand is expressed at the surface of the ligand bearing cell. Upon ligation with the NECD of the Notch receptor with a Notch ligand, two proteolytic cleavage steps will be initiated. The first cleavage step results in release of the NECD from cell surface by the ADAM-protease. The NECD remains bound by the ligand and is then internalized by the ligand-bearing cell in a MIB1-dependent fashion. Following release of the NECD and internalization of the NECD-ligand by the ligand-bearing cell, the gamma-secretase complex will release the NICD from the membrane. NICD can then translocate to the nucleus where it will interact with the DNA binding transcription factor RBPJ. The NICD co-activator, MAML, will be recruited to the signaling complex. NICD/RBPJ/MAML will then initiate transcriptional regulation at various sites.

1.9.2 Non-canonical Notch signaling

Non-canonical Notch signaling functions in a ligand and RBPJ/MAML-independent fashion. This non-canonical Notch signaling was first discovered during *in vitro* experiments demonstrating that Notch signaling could inhibit muscle cell differentiation in a fashion that did not require NICD/RBPJ or Notch ligand [322], and this finding was later confirmed *in vivo* using loss of function Notch studies in *Drosophila* [323]. While canonical Notch signaling has been shown to work synergistically with Wnt, the main mechanistic function of non-canonical Notch signaling appears to be to antagonize Wnt/β-catenin signaling [324,325]. Wnt/β-catenin signaling serves several roles in multiple processes, including cellular differentiation, expansion, and development.

Importantly, roles for non-canonical Notch signaling have begun to be elucidated in CD4+ T cells. Studies have found that Notch signaling is important for regulating CD4+ T cell activation and proliferation, even in the absence of RBPJ [326-328]. The ability of Notch to regulate these processes, in the absence of RBPJ, was NF-kb dependent. NF-kb expression is induced upon TCR stimulation and is important for regulating CD4+ T cell proliferation and function [329,330]. Previous studies have identified that Notch1 ICD can bind directly to, interact with, and sustain NF-κB activity [327,331].

1.9.3 Notch signaling in T cell development

Notch signaling plays an essential role in T cell development. Early Notch signals are essential in the bone marrow to direct multi-potent bone marrow progenitor cells to a T cell fate. Constitutive expression of active Notch 1 in the bone marrow restricts progenitor cells to a T cell lineage fate and blocks B cell maturation [303]. In support, re-constitution of irradiated bone marrow with wild-type and Notch1-deficient bone marrow results in a complete block of early T cell development and even leads to the development of a significant B cell population within the thymus [304].

Notch receptors are highly expressed by thymocytes and Notch signaling plays critical roles during thymocyte development [304,305]. After commitment to the T cell lineage, early thymocytes that do not express CD4 or CD8 (double negative; DN) begin rearrangement of their TCR genes. At this point, T cells will transition towards an $\alpha\beta$ or $\gamma\delta$ T cell lineage. Conditional deletion of the Notch1 receptor in DN thymocytes results in a complete block of $\alpha\beta$, but not $\gamma\delta$ T cell development [306,307]. Deletion of Notch1 did not lead to an increase in $\gamma\delta$ T cell development. This suggests that while Notch signals are essential for progression past the DN stage, it may not play an active role in determining $\alpha\beta$ vs $\gamma\delta$ T cell development.

Once thymocytes complete the DN stage, they progress to a stage in which they express both CD4 and CD8 simultaneously (double positive; DP). From this stage, thymocytes will undergo fate choices to become either single positive (SP) CD4 or CD8

cells. Studies suggest that Notch signaling likely plays a critical role in determining CD4+ vs CD8+ T cell development [305,308,309]. Thymocytes over-expressing NICD have a significant reduction in CD4+ T cells and a concomitant increase in CD8+ T cells, suggesting that heightened Notch signaling within the thymus promotes CD8+ T cell development [305,308]. In sum, Notch signaling plays critical roles throughout CD4+ T cell development. This includes critical roles in T cell fate decisions in the bone marrow as well as playing important roles at multiple stages within the thymus.

1.9.4 Notch signaling in Th differentiation

1.9.4.1 Ligand-based and unbiased amplifier models

Notch signaling plays critical roles in regulating peripheral T helper cell differentiation and function. Early studies identified that Notch signaling instructs naïve CD4+ T cells towards a specific T helper fate, depending on which ligand binds the receptor. This has been termed the ligand-based instruction model [321]. Most notably, one study demonstrated that Notch binding with DLLs induces Th1 fate, while binding to Jagged ligands induces a Th2 fate [311]. More recent studies have suggested a different model in which Notch signaling doesn't preferentially induce differentiation into any particular subset, regardless of ligand, and rather Notch serves as an unbiased amplifier during Th differentiation [321,332,333]. This is termed unbiased amplifier model [321,333].

One of the complicating factors in regards to a ligand-based instruction model is that it has been difficult to understand how different Notch ligands are capable of inducing unique signals through the same receptor. One explanation could be based on differences in strength of ligand binding to the receptor itself. Notch signaling appears to be induced to different levels depending on the ligand present and different ligands had varied capacity to bind and activate Notch signaling [334]. In one study, DLL4 was found to have the highest binding potential to Notch receptors, with DLL1 having intermediate binding and Jagged1 having very low binding [334]. These differences in binding were mirrored by unique capacity for each ligand to induce T cell activation, as measured by CD69, and proliferation [334]. Stimulation of CD4+ T cells with anti-CD3/anti-CD28 in the presence of DLL4 induced high levels of CD69 expression and proliferation, while DLL1 induced intermediate levels of both, and Jagged1 induced very low levels of CD69 and proliferation [334].

In support of a ligand-based instruction model, another recent study has further identified ways in which different Notch ligands can induce unique downstream signals. In this study, it was found that DLL4 binding to Notch results in sustained NICD activity, while DLL1 binding results in pulsatile activity of NICD [335]. This difference in Notch activity was accompanied by differences in target genes. Cells stimulated with DLL4 preferentially targeted Hey1, while cells stimulated with DLL1 targeted Hes1 [335]. Functionally, the differences in target genes resulted in opposite cell

fate decisions during myogenesis [335]. These findings are interesting as they suggest that the availability and expression patterns of specific Notch ligands likely plays an important role in determining the outcome of Notch signaling.

The early ligand-based instruction model has been challenged by a model in which the outcome of Notch signaling is not dependent on specific ligands, rather it serves as a general amplifier of T cell differentiation [321,332,333]. Importantly, neither DCs expressing DLLs nor Jagged ligands were capable of inducing Th1 or Th2 independently of exogenous cytokines [332]. Another study supports this 'unbiased amplification' model by elegantly demonstrating that Notch is capable of simultaneously inducing Th1, Th2, and Th17 master transcriptional regulators within the same cell regardless of exogenous cytokines [333]. Polarizing cytokine conditions did alter the magnitude of downstream Notch targets but did not affect the ability of Notch to bind to and regulate any of its gene targets. Together, this model suggests that Notch likely serves a costimulatory role as an unbiased amplifier of T helper cell differentiation and further pushes T helper cells toward specific fates by amplifying signals present during the differentiation process. Indeed, Notch has been implicated as an important molecule for driving Th1, Th2, Th17, Treg, and Tfh differentiation and function.

1.9.4.2 Notch signaling in Th1 differentiation and function

The Th1 master regulator, T-bet, and primary effector cytokine IFN-γ are direct targets of Notch signaling [333,336]. Mice with Notch-deficient T cells displayed

impaired Th1 generation and IFN-γ secretion during *L. major* infection [172,173]. Inhibition of Notch signaling with an inhibitor blocked *in vitro* and *in vivo* generation of Th1 cells by blocking Notch upregulation of T-bet [336]. Furthermore, DLL1 and DLL4 have been shown to promote a Th1 fate [311]. DLL4 expressing APCs promote Th1, rather than Th2 development *in vitro*. DLL4 appears to promote Th1 fate by inhibiting IL-4 production in CD4+ T cells [337].

Contrasting studies found that deletion of MIB1, required for Notch ligand function, had no effect on Th1 differentiation [338]. Furthermore, the ability of Notch to drive Th1 development *in vitro* remained intact in RBPJ-deficient mice [332]. Additionally, Notch ligand expressing APCs could not preferentially instruct *in vitro* Th1 differentiation regardless of which ligand was expressed [332]. Overall, the current literature suggests that DLLs may be more important for Notch-mediated control of Th1 differentiation, but the overall contributions of Notch signaling in Th1 differentiation is likely context dependent.

1.9.4.3 Notch signaling in Th17 differentiation and function

Notch signaling also appears to directly target important factors for Th17 differentiation, including IL-17 and the Th17 master regulator, RORγT [339]. Binding of Notch/RBPJ at these sites was confirmed using chromatin immunoprecipitation (CHiP) [339]. In support, pharmacologic inhibition of Notch resulted in reduced Th17 cell differentiation and cytokine secretion *in vitro* [339,340]. RBPJ has also been shown to

drive the pathogenesis of the autoimmune disorder, experimental autoimmune encephalitis, in mice [341]. In the context of allergic disease, pharmacologic inhibition of Notch signaling alleviated Th17-mediated asthma.

In contrast to the findings described above, mice with T cell-specific deletion of Notch 1 and 2 receptors had enhanced Th17 generation and IL-17 production in the gut at steady state, but strangely these cells exhibited impairments in IL-17 production following immunization [315]. Based on the cumulative data, it appears that Notch plays an important role in both Th17 differentiation and function. However, whether Th17 fate is associated with particular Notch ligands is unknown.

1.9.4.4 Notch signaling in Treg differentiation and function

The Treg master regulator, FoxP3, is positively regulated by RBPJ-mediated Notch signaling in a direct fashion [342]. Both Notch 1 [343] and Notch 3 receptors have been identified as positive regulators of Treg differentiation [344,345]. Enhanced expression of NICD ameliorated symptoms of the experimental diabetes mouse models by enhancing Treg numbers and function [344]. Furthermore, RBPJ-deficient mice had reduced Treg function in a mouse model of acute allograft rejection [346]. The role for Notch in Treg function and differentiation may require Jagged 1 [347].

1.9.4.5 Notch signaling in Th2 differentiation and function

Th2 cells are the most important peripheral T helper subset involved in type-2 inflammation, and Notch signaling in Th2 cells has been studied extensively in this

context. Early studies showed that deficiencies in either RPBJ or Notch receptors resulted in impaired Th2 differentiation *in vitro* [172,173]. In support, overexpression of NICD leads to enhanced Th2 differentiation through direct upregulation of GATA-3 [172,173]. NICD/RBPJ binds directly to the GATA-3-1a promoter to induce transcription and support Th2 differentiation.

In support of an instructive model for Notch signaling in Th2 cells, inhibition of Jagged1 ameliorated airway hyperreactivity in an OVA-induced asthma model while blockade of DLL4 enhanced asthmatic symptoms [348]. In this study, Jagged1 enhanced Th2 responses, while DLL4 was important for Treg differentiation. In support, another study has shown that blockade of DLL4 enhances AHR and mucus production in a model of cockroach allergen [349]. Furthermore, RBPJ-deficient animals were resistant to HDM-driven allergic airway inflammation [321]. However, expression of Jagged1 or 2 on DCs during HDM-driven allergic airway inflammation is dispensable for disease progression [350]. It is conceivable that Jagged ligands could be provided by other cellular sources.

In support of a role for Notch signaling during Th2 responses against intestinal helminths, clearance of the intestinal parasite *T. muris* requires expression of the canonical Notch signaling coactivator MAML [351]. Deletion of MAML in T cells stimulated with *T. muris* antigens led to abrogated IL-4, IL-5, and IL-13 production. As a result, goblet cell mucus secretion was impaired and overall worm burden was

enhanced in mice with T cells deficient in MAML [351]. However, Th2 functionality was restored when *T. muris* infected mice were injected with anti-IFN-γ to promote Th2 differentiation [333]. This suggests that Notch may be more important in enhancing, rather than instructing, Th2 responses.

Studies have also established a role for Notch signaling in driving IL-4 production by Th2 cells. Similar to findings regarding GATA-3, deletion of Notch receptors abrogated *in vitro* generated Th2 IL-4 production, while overexpression enhanced the production of IL-4 [172,173]. Later studies discovered an NICD/RBPJ binding site in the CNS2 enhancer within the IL-4 locus and showed that deletion of this enhancer led to a significant impairment in the generation of IL-4-producing Th2 cells *in vitro* [179,352]. However, this impairment was rescued when recombinant IL-4 was added to the culture system. This finding is important as it suggests that Notch signaling may not be required to drive Th2 differentiation in settings in which IL-4 is not limiting. Deletion of the CNS2 enhancer region also had no impact on peripheral Th2 responses during an OVA-allergen model [352]. This suggests that Notch/RBPJ binding at the CNS2 enhancer may not be necessary for peripheral Th2 function *in vivo*.

Based on the existing body of literature, Notch signaling is clearly important for *in vitro* Th2 differentiation and function. If a ligand-based instruction model is to be believed, then Jagged ligands appear to be more important for regulating Th2 fate. Whether Notch signaling is necessary during *in vivo* type-2 immune responses to drive

Th2 differentiation and function needs to studied further. Deletion of Notch receptors specifically in CD4+ T cells during an *in vivo* model is essential to further elucidate the true role of Notch signaling in Th2 cells. Ultimately, similar to other T helper subsets the role for Notch in Th2 cells may be context dependent.

1.9.4.6 Notch signaling in Tfh differentiation and function

IgE and IgG1 are hallmarks of type-2 inflammation. Previous studies using Notch- or RBPJ-deficient animals found an abrogation in IgE and IgG1 production but labelled these antibody deficiencies as impairments in Th2 cells [172]. We now know, however, that Th2-derived IL-4 plays a limited, if any, role in driving IgE class switching in the lymph node under physiologic conditions. Rather, IL-4 derived from Tfh cells is required to induce GC B cell class switching to IgE and IgG1 [54]. Thus, the antibody production defects were likely due to unappreciated impairments in the Tfh, rather than Th2 population. Therefore, these studies more likely uncovered an unappreciated role for Notch signaling within the Tfh population.

As discussed previously, there have been a considerable number of studies investigating the role of Notch signaling in Th2 cells during type-2 inflammation. However, there are much fewer studies assessing the role of Notch in Tfh cells. The first of these studies generated mice that conditionally deleted Notch receptors in T cells. They found that Tfh differentiation was completely blocked in these Notch-deficient T cells during infections with *Leishmania mexicana and S. mansoni* eggs [314]. Additionally,

mice with Notch-deficient T cells had a major loss in germinal center formation and a nearly complete absence of high affinity IgG1. This study was critical in formally establishing a role for Notch signaling in Tfh differentiation. However, it remains unknown how Notch signaling mediated this outcome at the cellular and molecular level.

A second study confirmed the requirement for Notch signaling in Tfh differentiation and helped to identify which cells are important for providing Notch ligand, as well as identifying which Notch ligand is required for Tfh differentiation [316]. This study used a CCL19^{cre} mouse to conditionally delete specific Notch ligands, DLL1 or DLL4, in lymph node stromal cell populations. Interestingly, deletion of DLL1 had no effect on Tfh differentiation, while deletion of DLL4 in stromal cell populations resulted in a complete block of Tfh differentiation [316]. This finding is intriguing as it identifies that a specific Notch ligand is required during Tfh differentiation and establishes an important role for stromal cells as an essential source of Notch ligands for Tfh cells. While the importance of stromal cell populations in Tfh differentiation is clear, it remains unknown which specific stromal cell populations are required to provide Notch ligands to promote Tfh differentiation. The researchers from this study suggested that FDCs were the most important source of Notch ligand for Tfh cells. However, an FDC-specific requirement could not be formally established because CCL19^{Cre} mediates deletion in all lymph node stromal populations.

Other studies have discovered a potential role for Notch signaling in Tfh function by using mice whose CNS2 region within the IL-4 locus had been deleted. As described previously, the CNS2 region is an enhancer site located downstream at the 3′ end of the IL-4 locus. In these studies, lymph node CD4+ T cells from CNS2-deficient mice had significant impairments in their ability to produce IL-4 [352,178]. While there was no effect of CNS2 deficiency on Tfh differentiation, CNS2 deficiency did result in reduced class switching of B cells to type-2 antibody isotypes. Importantly, while antibody class switching was abrogated, there were only modest impairments in peripheral type-2 immunity. AHR and mucus secretion was intact in CNS2 deficient mice during an OVA-allergen challenge model [178,352]. However, there was a significant reduction in the number of eosinophils in the lung [178]. These findings suggest that the CNS2 enhancer plays an essential role for Tfh-mediated function but it likely plays a limited role for peripheral Th2 immunity. This also establishes further groundwork that IL-4 cytokine production is regulated differently by Tfh and Th2 cells.

While these studies demonstrate that the CNS2 enhancer is critically important for Tfh-derived IL-4 production, they have not yet identified which transcription factors are important for binding to and activating the CNS2 enhancer. Within the CNS2 enhancer lies a consensus NICD/RBPJ binding site, opening the possibility that Notch signaling may play an important role in driving the production of IL-4 by Tfh cells [179,311,352,178]. However, the CNS2 enhancer also contains binding sites for several

other factors that are known to play important roles in Tfh differentiation and function. The bZip transcription factors cMAF and BATF, both of which are critical for Tfh function, are two of the other factors with known binding sites at the CNS2 enhancer [23,277,280]. Therefore, it is still uncertain whether Notch signaling is important for regulating Tfh IL-4 production. Importantly, current literature often discounts an important role for Notch signaling in driving Tfh differentiation or function [353].

The evidence that Notch signaling is required for Tfh differentiation is striking, however, there has been no work done to directly investigate whether Notch signaling is required for Tfh function, and specifically IL-4 production. Also, while it appears that DLL4 is the Notch ligand important for inducing Tfh fate, it remains unclear what are the important cellular sources of DLL4 in the lymph node required for Tfh differentiation. Additionally, it is uncertain whether persistent Notch signaling is important for the maintenance of Tfh fate and function beyond initial Tfh differentiation. Finally, the mechanisms through which Notch exerts its effects to promote Tfh differentiation and function remain to be elucidated.

2. Methods

2.1 Mice

C57BL/6 Notch-1$^{fl/fl}$ mice [354], Notch-2$^{fl/fl}$ [355], CD4^{cre} [356], Rosa26$^{flox\text{-}stop\text{-}YFP}$ [357], CD21^{cre} [358], Rosa26$^{Stop\text{-}flox\text{-}NICD\text{-}GFP}$ [359], ER^{cre} [360], Rosa26$^{flox\text{-}stop\text{-}RFP}$, and C57BL/6 mice were purchased from Jackson laboratories. MIB1$^{fl/fl}$ were generated by Young-Yun Kong (Pohang University of Science and Technology) and rederived at Duke University [319]. CD11c^{cre} mice were provided by Gianna Hammer (Duke University) [361]. IL-4^{4get} and IL-4^{KN2} mice were provided by Richard Locksley (UCSF) [302,165]. OT-II mice were provided by Weiguo Zhang (Duke). All mice were housed in pathogen-free facilities following guidelines set by the Division of Laboratory Animal Resources, Duke University Medical Center, Biological Resource Center at National Jewish Health, and the Institutional Animal Care and Use Committee.

2.2 Infections, worm counts, and immunizations

N. brasiliensis was prepared by culturing feces from an infected rat on a bed of charcoal. Third-stage L3 larvae was collected from the cultured plates and washed with 0.9% saline. Mice were injected subcutaneously in the rear flank with 500 L3 larvae in saline solution. To conduct worm counts, small intestines were removed, opened longitudinally, and incubated in HBSS at 37ºC for 1-2 hours. Adult worms were manually enumerated.

2.3 Notch inhibition studies

Where described, mice were immunized subcutaneously with 100 μg of OVA emulsified in alum in the footpad. On the days indicated, mice were given 10 μmol/kg gamma secretase inhibitor (GSI), Dibenzazepine, (DBZ; Selleckhem) intraperitoneally. GSI was diluted in a solution of 0.5% hypromellose and 0.1% Tween-80. The injection volume was 300 μl. The popliteal lymph node was harvested on the indicated day for subsequent flow cytometric or histologic analysis. In the experiments described, mice were also injected with FTY720 to directly inhibit S1P1. 1 mg/kg FTY720 was administered to appropriate mice on days seven and nine post immunization. The drug was diluted in 0.9% saline and injected I.P. with a total volume of 300 μl.

2.4 Tamoxifen injections

Tamoxifen (T5648; Sigma) was diluted in USP grade corn oil. Two mg of tamoxifen was injected intraperitoneally in 300 μl once every 24 hours for three days prior to infection for the Notch over-expression (ER^{cre}NICD) studies. For the competitive transfer (ER^{cre}Notch1/$2^{fl/fl}$$Rosa^{floxed\text{-}stop\text{-}YFP}$ and $ER^{cre}$$Rosa^{floxed\text{-}stop\text{-}RFP}$ OT-II) studies , tamoxifen was administered on days seven, eight, and nine post OVA/alum immunization.

2.5 Immunofluorescence histology

Lymph nodes were fixed with 4% paraformaldehyde (PFA) for two hours, followed by a wash in PBS for four hours, then incubated in 30% sucrose over-night, and

finally frozen in optimal cutting temperature (O.C.T.) compound. Lungs were inflated with approximately 5 mL of 4% PFA prior to removal from the mouse. To inflate the lung, tissue was removed to expose the trachea. A small incision was made in the trachea and a flexible catheter needle was inserted into the trachea. Surgical string was then used to secure the needle within the trachea and PFA was injected. After injection of the PFA, the needle was removed and the surgical string tightened to retain the PFA within the lung. The lung tissue was then removed and submerged in 20 mL of 4% PFA for 3 hours in a 50 mL conical. The lung was then removed from the PFA and the surgical string was cut to release the PFA from within the lung. Next, the lung was incubated in 20 mL of PBS overnight. Finally, the lung was then incubated in 30% sucrose overnight and then frozen using O.C.T.

Eight-micron sections were cut and epitopes on slides were stained using tyramide amplification. eGFP and YFP were detected using a purified polyclonal rabbit anti-mouse GFP (NB600-308; Novus Biologicals) followed by biotinylated $F(ab')_2$ donkey anti-rabbit (Jackson ImmunoResearch Laboratories). Followed by staining with streptavidin (SA) conjugated to horse radish peroxidase (HRP). Finally, slides were stained with tyramide-FITC. Biotinylated anti-mouse CD4 (RM4-5; Biolegend) and tyramide 555 was used to detect CD4+ T cells. Biotinylated anti-mouse IgD (11-26c; eBiosciences) was used with SA-dylight649 to detect B cells. Biotinylated FDCM2 (212-MK-2FDCM2; ImmunoKontact) was used with tyramide 555 to detect FDC. 4′,6-

Diamidine-2′-phenylindole dihydrochloride (DAPI; 0.5μg/ml; Roche) in PBS was used to counterstain nuclei prior to mounting on coverslips with Vectashield (Vector laboratories). Images were collected with a Zeiss Axio Imager. Analysis of images was performed using FIJI (ImageJ) software.

2.6 Quantification of IL-4 competent CD4+ T cell accumulation in T cell zone

Quantification of GFP within distinct zones was performed. Individual images were taken in the tyramide-FITC (GFP) and SA-dylight649 (IgD) using a 5x objective. Images were then stitched together to compile a single image of the entire lymph node. IgD stains were then used to demarcate the B cell follicle and T cell zones by hand. Outlined regions were saved and then applied to the GFP stain. Thresholding was performed to mark positively stained GFP above background levels. The total GFP pixels were then quantified. The hand-drawn outline of the T cell zone was then applied to the GFP image and GFP+ pixel density was then quantified within the T cell zone. Total GFP+ pixels were then divided by the total pixels within the T cell zone to calculate the percent of GFP+ pixel density residing within the T cell zone.

2.7 Flow cytometry

Lungs were chopped with a razor blade, digested with 250 μg/ml Collagenase XI (C7657; Sigma), 50 μg/ml Liberase (145495; Roche), 1 mg/ml Hyaluronidase (h3506; Sigma), and 200 μg/ml DNase I (DN25; Sigma) in RPMI 1640 for one hour at 37ºC to

prepare single cell suspensions. Single cell suspensions of lymph nodes were prepared by mechanical dissociation. Fc receptors were then blocked to prevent non-specific binding of antibodies using Fcx block (anti CD16/CD32) diluted at 1:100 in 2% FCS in PBS for 10 minutes. Surface stains were performed in 2% FCS in PBS for 30 minutes at 4ºC. All antibodies were diluted 1:200 unless otherwise noted. The following antibodies were used: APC/Cy7 conjugated to anti-mouse CD4 (RM4-5); PerCP-Cy5.5 conjugated to anti-mouse CD11c (N418), anti-mouse CD4 (RM4-5), anti-mouse CD8 (53-6.7), anti-mouse/human CD45R/B220 (RA3-6B2), anti-mouse CD31 (390); PE/Cy7 conjugated to anti-mouse CD279/PD-1 (RMP1-30), CD49b (DX5) and anti-mouse CD21/35 (7E9); Alexa Fluor 647 conjugated to anti-mouse/human GL7 (GL7); APC conjugated to anti-mouse PDPN/GP38 (8.1.1); and PE conjugated to anti-mouse Notch 1 (HMN1-12), anti-mouse Notch2 (16F11), anti-mouse SiglecF (E50-2440), anti-mouse CD131 (JORO50), streptavidin from Biolegend. PE or APC conjugated to anti-human CD2 (S5.5; 1:50) from Invitrogen. APC-eFluor 780 conjugated to anti-human/mouse CD45R/B220 (RA3-6B2) from eBioscience. PE/Cy7 conjugated to anti-mouse CD95 (JO2) and biotinylated anti-rat/mouse CD185/CXCR5 (2G8; 1:100) from BD biosciences. Cells were resuspended in 2% FCS in PBS containing DAPI (0.5 μg/ml). Lymphocyte and singlet gates were performed by size and granularity based on forward and side scatter. Live cells were gated based on DAPI exclusion. Data was collected on a FACSCanto II or

LSR II (BD Biosciences), or Fortessa (BD Biosciences) cytometer and analyzed using FlowJo (TreeStar).

2.8 Transcription factor staining

Surface stains were performed as described above. Next, cells were stained using a Fixable Violet Dead Cell Stain kit (L34964; ThermoFisher) at a 1:1000 dilution in PBS. A Foxp3 transcription factor staining buffer kit (eBioscience) was used to fix and permeabilize cells per kit instructions. Finally, these antibodies were used for transcription factor staining: PE-conjugated to anti-mouse/human GATA-3 (TWAJ; eBioisciences), anti mouse/human cMAF (SymOF1; eBiosciences), anti mouse/human IRF4 (3E4; eBiosciences), anti mouse/human BATF (9B5A13); and AlexaFluor 647 conjugated to anti-mouse BCL6 (K112-91; BD Biosciences).

2.9 FDC isolation

CD21^{cre}Rosa26$^{Stop\text{-}flox\text{-}YFP}$ and Rosa26$^{Stop\text{-}flox\text{-}YFP}$ mice were immunized subcutaneously with ovalbumin (OVA) emulsified in Imject alum (Thermo Fisher) in both rear flanks and upper back. Each mouse received a total of 100 μg of OVA/alum. Mice were sacrificed and axial, brachial, and inguinal nodes were harvested. Lymph nodes were opened using 26G needles and digested at 37ºC for 30 minutes with Colleganase IV (1mg/ml; 17104-019; Gibco); DNase I (40 μg/ml; DN25; Sigma) in RPMI 1640. Pipetting was performed every 10 minutes to assist in digestion of the tissue. After incubation, 5 mM EDTA (03690; Sigma) was added to the digestion to help dissociate

cell aggregates. Cells were filtered through an 80μm mesh. StemCell RapidSpheres (19860A) kit was used to positively select CD45+ cells using biotinylated anti-mouse CD45 (30F11; Biolegend). Flow through, enriched for CD45- cells, was taken and stained for flow cytometry.

To image FDCs by immunofluorescence histology, CD21^{cre}Rosa26$^{Stop\text{-}flox\text{-}YFP}$ and Rosa26$^{Stop\text{-}flox\text{-}YFP}$ mice were immunized subcutaneously in the footpad with 100 μg OVA emulsified in alum. The popliteal lymph node was harvested nine days later and prepared for histologic analysis. Staining for FDCM2 was performed to specifically identify FDCs. FDCM2 signaling was amplified by using tyramide signal amplification (Tyramide-555) as described above. FDCs were identified as FDCM2 positively stained cells co-stained with CD21^{cre}Rosa26$^{Stop\text{-}flox\text{-}YFP}$.

2.10 ELISA

Ninety-six well plates were coated with rat anti-mouse IgE (R35-72; BD Bioscience) and blocked using 5% BSA. Serum samples were added and total IgE detected using biotinylated anti-mouse IgE (R35-118; BD Biosciences) followed by streptavidin-HRP and o-phenylenediamine. An EMax Precision Microplate Reader (Molecular Devices) was used to quantify total IgE concentrations based on purified IgE (MEB-38; Biolegend) standard curves.

2.11 Real-time PCR

CD11c+ MHC-II+ DCs were sorted with a BD FACSAria Fusion or Astrios (Beckman Coulter) and resuspended in 1 mL TRIzol reagent (15596026; Thermo Fisher). Chloroform and ethanol precipitation were used to isolate RNA from the samples. Genomic DNA was eliminated using a DNase I kit (18068015; Thermo Fisher). cDNA was synthesized by reverse transcription (18080051; Thermo Fisher) per kit instructions with oligo (dT) primers followed by RNase H treatment. Real-time PCR was performed on the cDNA using an all-in-one SYBR green qPCR mix (QP001-01; GeneCopoeia) amplified with primers against Mind bomb1 (QT00110453; Qiagen), ASCL2 (Forward: AAGCACACCTTGACTGGTACG; Reverse: AAGTGGACGTTTGCACCTTCA) and β-actin (QT00519526; Qiagen) on an Applied Biosystems StepOnePlus RT PCR system. Expression was calculated relative to β-actin.

2.12 Cell proliferation study

OT-II CD4+ T cells were isolated and stained with 5 μM eFluor670 proliferation dye in HBSS for 30 minutes at 37ºC (65-0840-85; Thermo Fisher). 1.5 x 10^6 OT-II T cells were then transferred I.V. into the mice described. Twenty-four hours post transfer the mice were immunized by subcutaneous injection of 100 μg of OVA emulsified alum in the footpad. Three days post immunization the popliteal lymph nodes were harvested to assess proliferation of the transferred cells by flow cytometry.

2.13 In vitro T cell activation studies

Nintey-six well plates were coated with 2 ug/ml anti-CD28 and the indicated concentrations of anti-CD3 in PBS at 4ºC overnight. The next day, peripheral lymph nodes and spleen was harvested from the indicated mice. CD4+ T cells were then isolated using an EasySep mouse CD4+ T cell isolation kit (StemCell #19851), per kit instructions. $4x10^5$ CD4+ T cells were then cultured in complete RPMI (10% FCS, 1% Pen Strep, L-glutamine, and 50 μM BME) for sixteen hours at 37ºC. Cells were then harvested from the plates and the expression of CD69, CD25, and CD44 was assessed by flow cytometry.

For four-day culture experiments, plates were coated with 2ug/ml anti-CD28 and 1 ug/ml anti-hamster IgG antibodies overnight at 4ºC. The next day, the plate was washed and the indicated concentrations of hamster anti-CD3 were added and incubated for two hours at 37ºC. CD4+ T cells were then isolated from the appropriate mice using the method described above. $1x10^6$ CD4+ T cells were then cultured for four days in complete RPMI in TH0 conditions (10 ng/ml rIL-2, 10 μg/ml anti-IL-4, and 10 μg/ml anti-IFN-γ). The expression of BATF (5 μl/test), IRF4 (1.25 μl/test), and cMAF (5 μl/test) was assessed by flow cytometry using the intranuclear transcription factor staining protocol described above.

2.14 Statistics

Two-tailed paired or unpaired t-tests were performed. P values less than 0.05 and greater than 0.01 are indicated with a single asterisk (*), P values less than 0.01 and greater than 0.001 are indicated with double asterisk (**), and P values smaller than 0.001 are indicated with triple asterisk (***).

3. Notch signaling represents an important checkpoint between Tfh and Th2 cell fate

*Disclosure: portions of the following section were adapted from my published research article: Dell'Aringa M & Reinhardt RL. Notch signaling represents an important checkpoint between follicular T-helper and canonical T-helper 2 cell fate. Mucosal Immunology. Feb. 2018. doi:10.1038/s41385-018-0012-9

3.1 Introduction

As described in the previous chapter, the role of Notch signaling type-2 inflammation remains unclear. Although Notch signaling has been studied in Th2 cells, this work has been performed almost entirely *in vitro* and the *in vivo* role of Notch signaling in Th2 cells has yet to be elucidated. Additionally, what role Notch signaling plays in Tfh cells is only starting to be discovered. Formal evidence that Notch is playing a more general role in Tfh cell-mediated immunity comes from recent work showing that deletion of Notch1 and Notch2 in CD4+ T cells prevents the generation of Tfh cells [314]. The intrinsic nature of Notch signals being required for Tfh cell lineage commitment parallels findings in other T-helper cell subsets where Notch signaling facilitates cell fate choices [362]. Early studies proposed that distinct Notch ligands on dendritic cells could differentially instruct the development of specific T helper cell subsets [311,363]. Although the instruction model has been challenged [364,365], this seminal work laid the foundation to explore the nature of Notch ligands in T helper cell

differentiation and function. As a result, new, non-instruction-based models have emerged. These models advocate that Notch signaling serves as an unbiased amplifier helping to stabilize the cytokine profile already being established in differentiating CD4+ T cells [333,332].

Here, using parasitic helminth infection as a robust model of type-2 immunity, we report that Notch signaling in CD4+ T cells is required for Tfh cell generation, IL-4 production, and IgE class-switching. The defect is specific to Tfh cells as Th2-mediated immunity, worm clearance, and IL-4 expression remained normal in the absence of Notch signaling. Furthermore, an unbiased approach to eliminate all functional Notch ligands using conditional deletion of the E3 ubiquitin ligase Mind bomb1 (Mib1) in dendritic cells, B cells, T cells and FDC shows that the source of Notch ligands driving Tfh cell differentiation is distinct from the cells presenting or harboring cognate antigen during a proto-typical type-2 immune response.

3.1 Deletion of Notch receptors in T cells results in abrogated humoral immunity but normal Th2-mediated immune hallmarks

To address the role of Notch signaling in the development of IL-4-producing Th2 and Tfh cells during a type-2 immune response, we made use of IL-4 reporter mice ($IL4^{4get}$) where Notch receptors are conditionally deleted in all T cells ($CD4^{cre}Notch1/2^{fl/fl}$) [172,165]. The $IL4^{4get}$ reporter background allows for IL-4 cytokine competency to be visualized through the production of green fluorescent protein (GFP). To evaluate type-2 immunity, $IL4^{4get}Notch1/2^{fl/fl}$ (wild-type) and $IL4^{4get}CD4^{cre}Notch1/2^{fl/fl}$ (T cell-

specific, Notch-deficient) mice were infected with the helminth *Nippostrongylus brasiliensis.* Intestinal worm burden, lung eosinophilia, and serum IgE were assessed nine days post infection (Figure 7). In this model, defects in lung eosinophilia and helminth clearance are reflective of impaired Th2-mediated peripheral immunity, while defects in IgE production signify a defect in Tfh-mediated, type-2 humoral responses [17,23]. Similar to wild-type mice, IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ mice mounted a productive type-2 response in the periphery, as worms were cleared from the intestine and significant eosinophilia was observed in the lung (Figure 7a, b). In contrast, mice lacking Notch1 and Notch2 on T cells exhibited significantly decreased IgE levels relative to wild-type animals (Figure 7c).

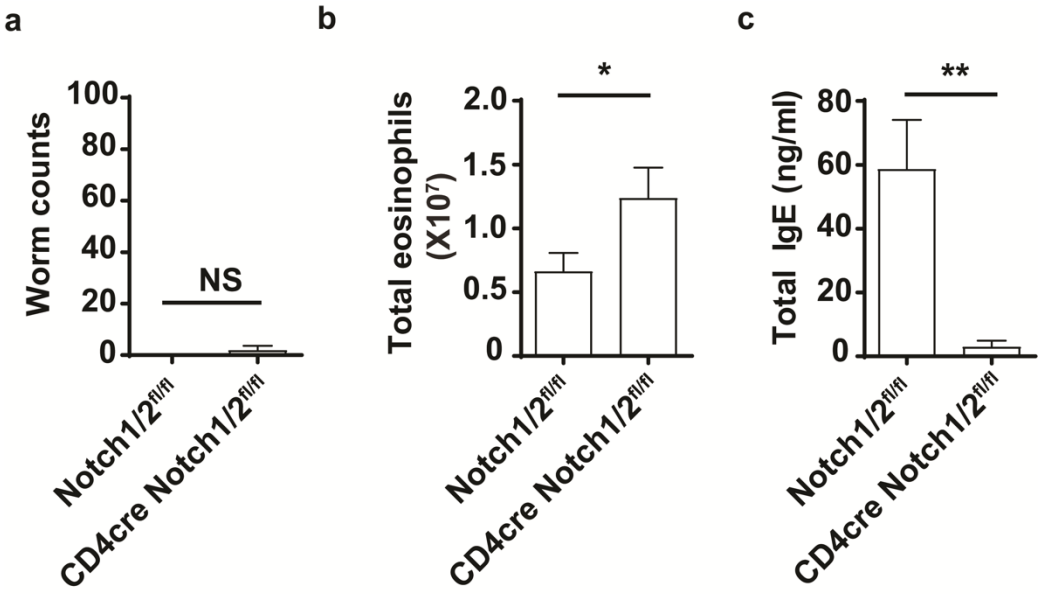

Figure 7: Notch signaling on CD4+ T cells is required for humoral immunity but is dispensable for Th2-mediated type-2 immune hallmarks.

IL4^{4get}Notch1/2$^{fl/fl}$ and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ mice were infected with *N. brasiliensis.* Lung, mediastinal lymph node, and serum were harvested nine days post infection for analysis. **(a)** Intestinal adult worm counts nine days post infection of IL4^{4get}Notch1/2$^{fl/fl}$ (n=13) and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ (n=10). **(b)** Graphs show percentage and number of eosinophils (CD4- B220- CD8- GFP+ CD131+ SiglecF+) of IL4^{4get}Notch1/2$^{fl/fl}$ (n=13) and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ (n=10). **(c)** Total serum IgE of IL4^{4get}Notch1/2$^{fl/fl}$ (n = 13) and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ (n = 10) mice. Error bars represent +/- SEM. Data show is combined from three independent experiments with n = 3-5 mice per group per experiment. **$P < 0.01$, (unpaired two-tailed T-test).

These data indicate that the absence of Notch signaling in T cells has little impact on Th2-mediated immune hallmarks in the periphery but a significant impact on Tfh-mediated immunity. This was not due to a defect in overall CD4+ T cell numbers, as mice lacking Notch receptors on their T cells exhibited an overall increase in CD4+ T cells in both the periphery (Figure 8a) and the draining lymph nodes (Figure 8b) after helminth infection.

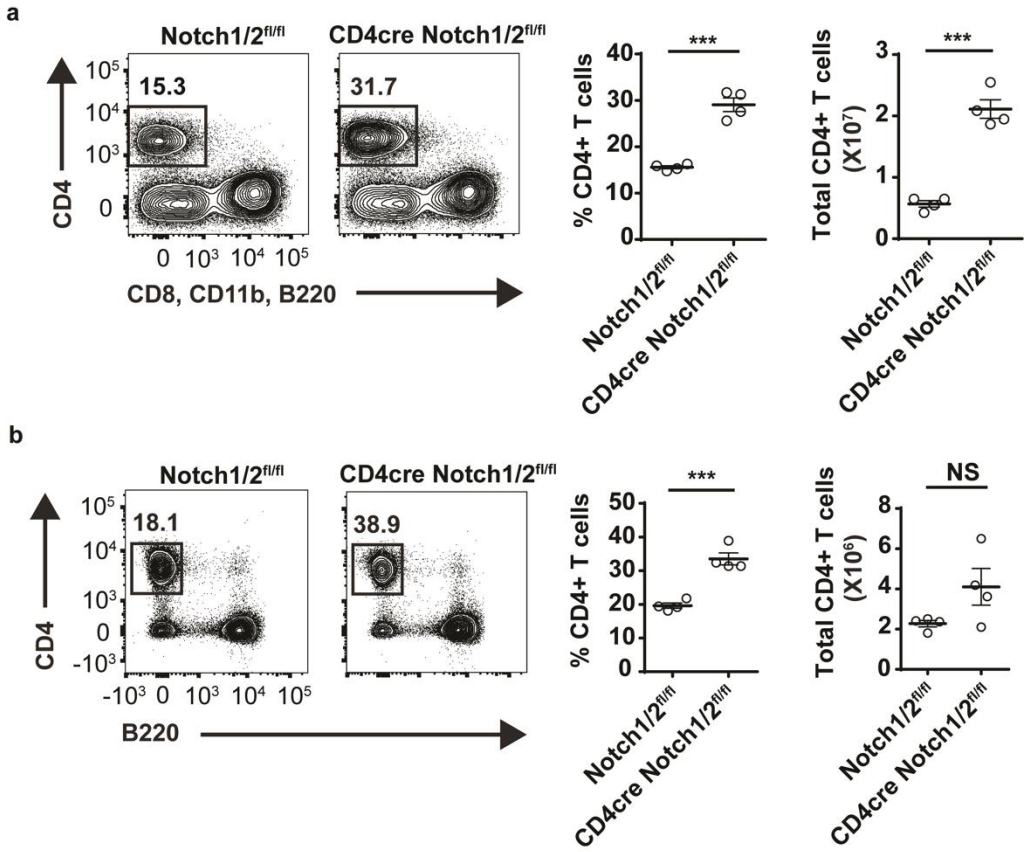

Figure 8: Increased CD4+ T cells in the lung and lymph node in mice with Notch deficient T cells.

IL4^{4get}Notch1/2$^{fl/fl}$ (n = 4) and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ (n = 4) mice were infected with *N. brasiliensis.* Mediastinal lymph nodes and lung were harvested and prepared for flow cytometry nine days post infection. **(a)** Representative contour plots of lung and **(b)** mediastinal lymph nodes CD4+ T cells are shown. Graphs show percentage and total number of CD4+ T cells. Error bars are shown as +/- SEM. Data is representative of four independent experiments with n= 3-5 mice per group. ***P < 0.001 (unpaired two-tailed T-test).

3.2 T cell-specific deletion of Notch receptors leads to a selective reduction in IL-4 competent CD4+ T cells in the lymph nodes compared to the lungs after helminth infection

Given that we observed normal worm clearance and eosinophilia but reduced IgE production in mice lacking Notch in T cells, we hypothesized that type-2 cytokine competency in Notch-deficient Th2 cells would remain intact, while IL-4-competent Tfh cell number or function would be compromised. In support, mice that lacked expression of Notch in T cells showed normal numbers of IL-4-competent cells (GFP reporter positive) in the lung, despite a decreased percentage of IL-4 competency within the total CD4+ T cell pool (Figure 9a). This is consistent with the higher number of CD4+ T cells found in the lungs of these mice after infection (Figure 8a). Despite normal Th2 cell generation, IL-4-expressing CD4+ T cells were significantly reduced in both percentage and number in the lung-draining mediastinal lymph nodes when Notch1 and Notch2 were deleted from T cells (Figure 9b).

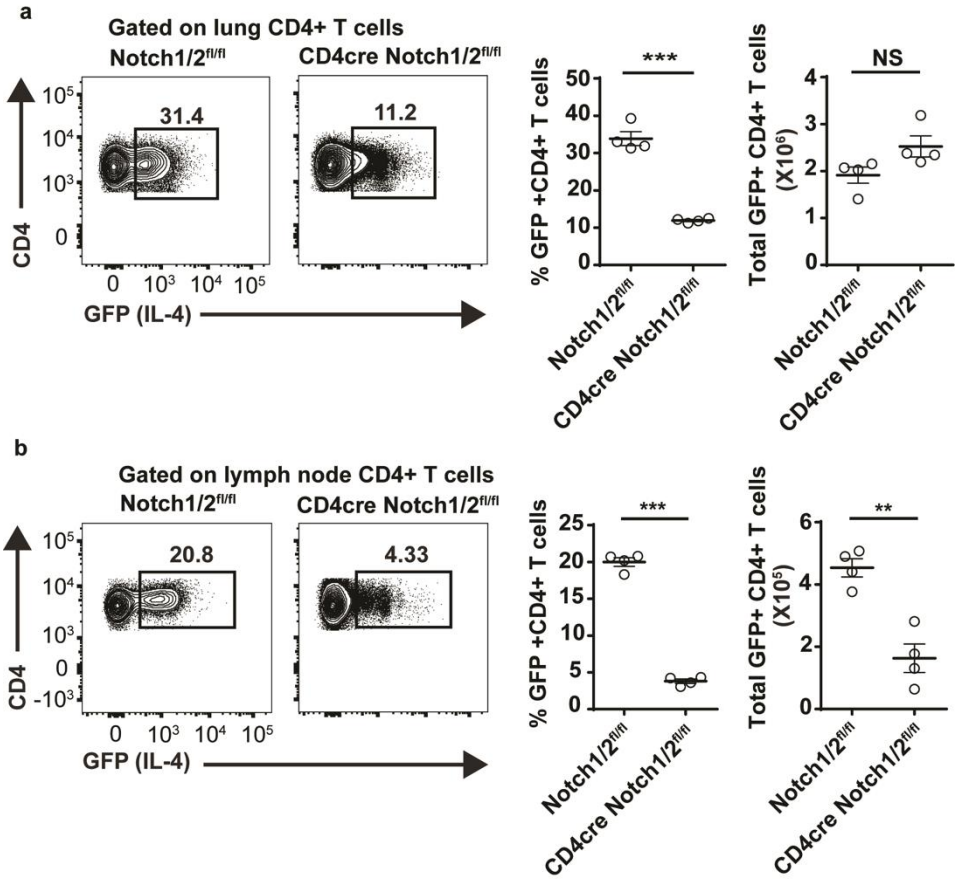

Figure 9: Deletion of Notch receptors on T cells results in reduced IL-4 competency in CD4+ T cells residing in the lymph node but not lung.

IL4^{4get}Notch1/2$^{fl/fl}$ (n = 4) and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ (n = 4) mice were infected with *N. brasiliensis.* Mediastinal lymph nodes and lung were harvested nine days later and prepared for flow cytometry. **(a)** Representative contour plot gated on total CD4+ T cells in the lung and **(b)** lymph node. Gates indicate percent of IL-4 competent cells. Graphs show percent and total number of GFP+ CD4+ T cells. Error bars represent +/- SEM. Data is representative of 4 independent experiments with n = 3-5 per group. **P < 0.01, ***P < 0.001 (unpaired two-tailed T-test).

These findings were further supported by histological analysis of IL-4-expression in the lung and lymph nodes. CD4+ GFP-reporter staining in the lung was similar between both wild-type mice and animals lacking Notch1 and Notch2 in T cells (Figure 10a). The lack of GFP-reporter staining in tissue sections of mediastinal lymph nodes

taken from IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ mice confirms the paucity of IL-4 expressing T cells in both the paracortex (IgD-) and B cell follicles (IgD+) when Notch signaling is absent (Figure 10b). In sum, these data are consistent with a more pronounced role for Notch signaling in establishing type-2 cytokine competency in Tfh cells residing in the lymph nodes compared to lung-resident, canonical Th2 cells.

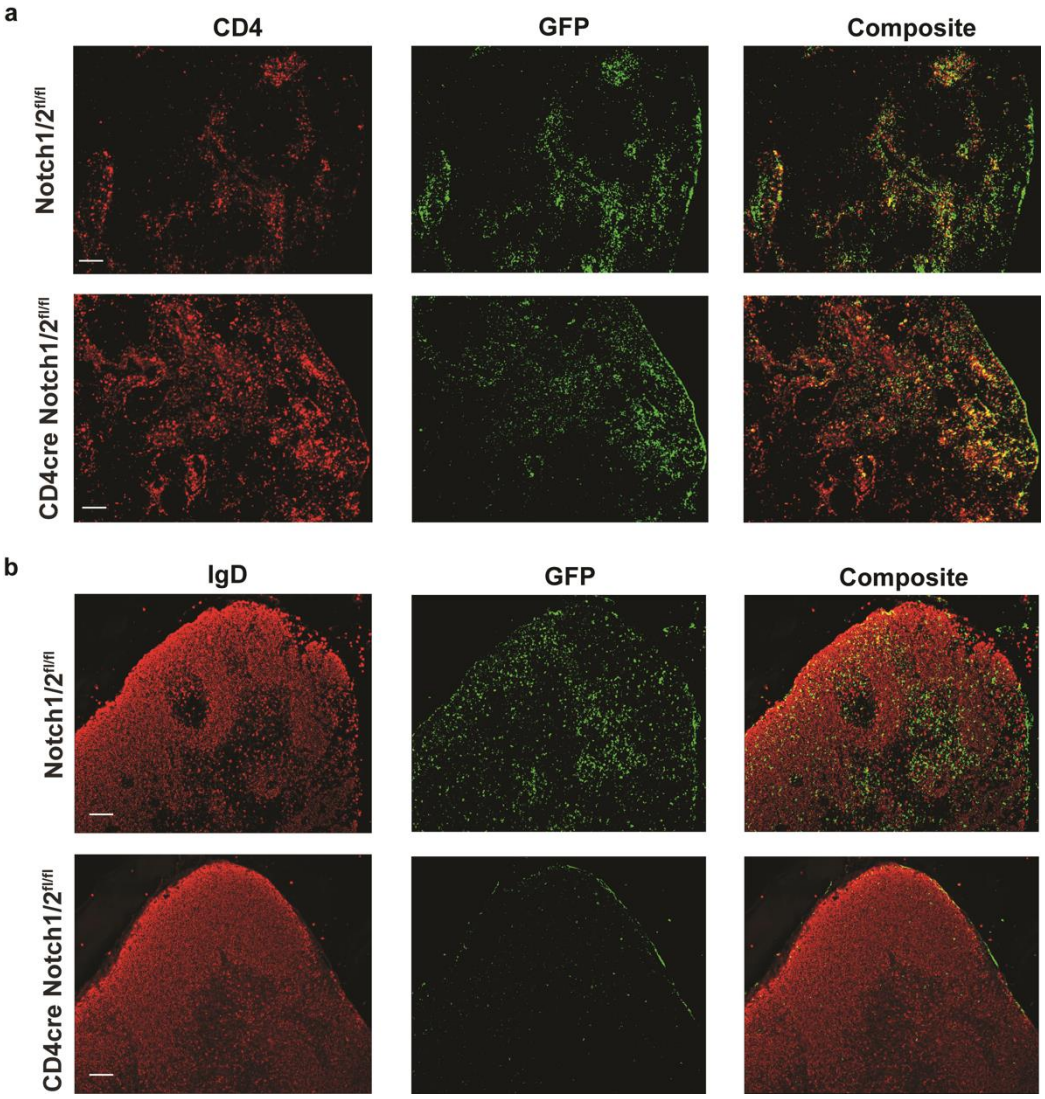

Figure 10: Deletion of Notch1 and Notch2 on T cells results in impaired IL-4 production by LN-resident CD4+ T cells, but not lung resident CD4+ T cells.

IL4^{4get}Notch1/2$^{fl/fl}$ and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ mice were infected with *N. brasiliensis.* Nine days later, lung and mediastinal lymph node were harvested, fixed, and sectioned for subsequent immunofluorescence microscopy. **(a)** Lung sections were stained for CD4 (red) and GFP (IL-4 competency; green). Channels were merged and are shown as a composite image. **(b)**. Lymph node sections were stained for IgD (red) and GFP (IL-4 competency; green). A composite image is shown. Scale bars (bottom left) represent 100 μm. Images shown are representative of three independent experiments with n = 2-3 per group.

Since we observed a significant reduction in the percentage IL-4 expressing CD4+ T cells in the lung we aimed to determine whether there was any change in GATA-3 expression in Notch deficient lung CD4+ T cells. IL4^{4get}Notch1/2$^{fl/fl}$ and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ mice were infected with *N. brasiliensis* and intracellular transcription factor staining was performed on lung CD4+ T cells on day nine. Consistent with the reduced percentage of IL-4 expressing CD4+ T cells, fewer Notch deficient T cells expressed the Th2 master regulator, GATA-3 (Figure 11a). However, similar to what we observed with the IL-4 expressing cells the total number of GATA-3+ CD4+ T cells in the lung was not significantly changed (Figure 11a). Due to the reduced percentage of Th2 cells, we next wanted to investigate if mice with Notch deficient T cells had increases in other Th subsets. Interestingly, we observed enhanced Th1 and Treg populations by percentage and total number in the lungs of mice with Notch deficient T cells based on increased expression of Tbet (Figure 11b) and FoxP3 (Figure 11c), respectively.

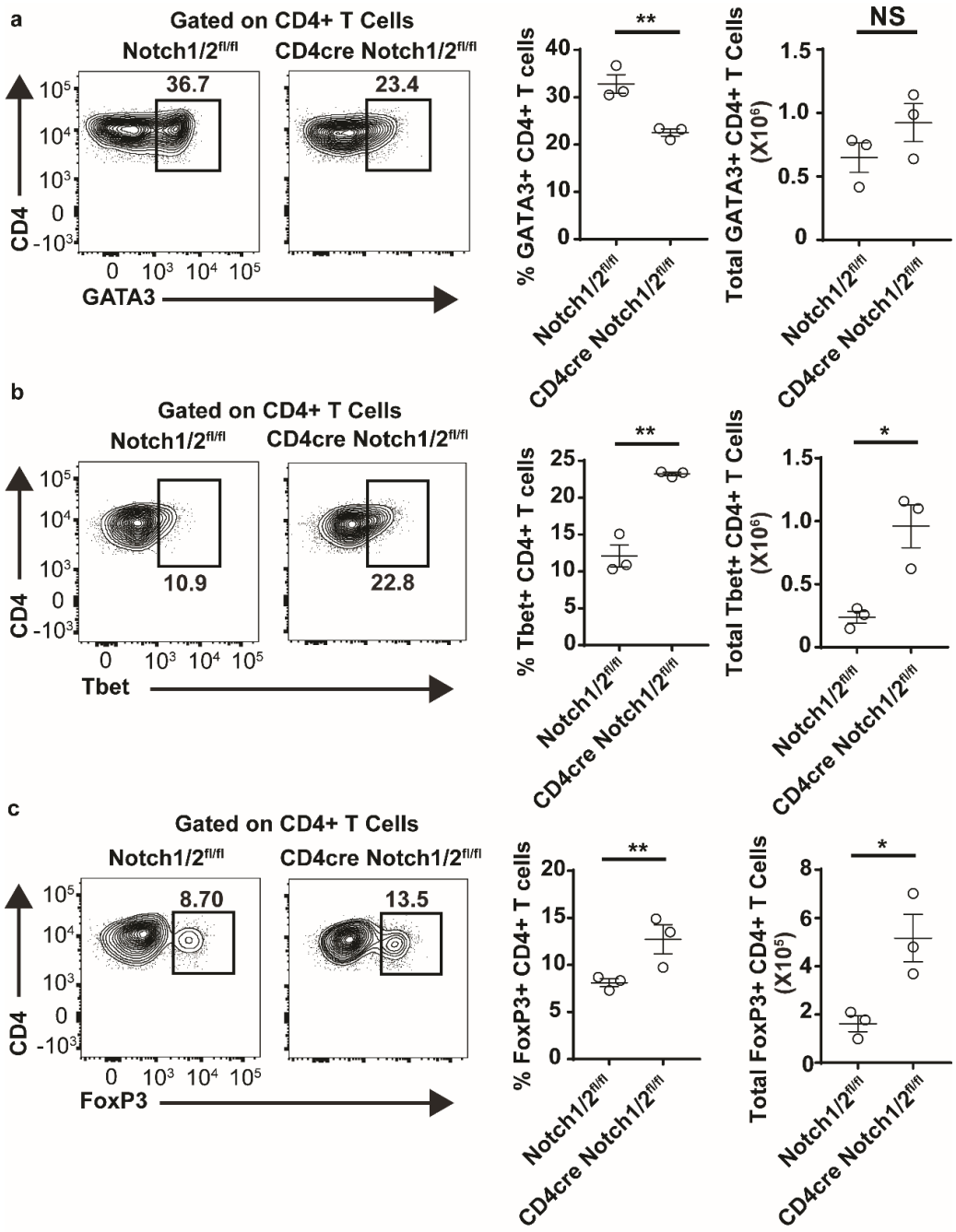

Figure 11: Enhanced Tbet and FoxP3 expressing CD4+ T cell populations in mice with Notch deficient T cells

Lungs of IL4^{4get}Notch1/2$^{fl/fl}$ (n = 3) and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ (n = 3) mice were collected nine days after infection with *N. brasiliensis*. Representative flow plots of CD4+ T cells. Gates represent CD4+ T cell expression of **(a)** GATA-3, **(b)** Tbet, and **(c)** FoxP3. Positive

gates for Tbet expression were set based on positive Tbet staining assessed in the CD8+ T cell population. Graphs show quantification of percentage and total numbers of **(a)** GATA-3+, **(b)** Tbet+, and **(c)** FoxP3+ CD4+ T cells. Error bars represent +/- SEM. Data is representative of two independent experiments with n= 3 mice per group. *P < 0.05, **P < 0.01(unpaired two-tailed T-test).

3.3 Tfh cell generation, IL-4 expression, and germinal center B cell numbers are significantly reduced in mice that lack Notch in T cells

Reduced serum IgE and IL-4 expression in the follicles of the mediastinal lymph nodes suggested that Tfh cell generation and/or function could be impacted by Notch-deficiency in T cells. In support, Tfh cells isolated from helminth-infected mice showed significantly increased Notch1 and Notch2 receptor expression relative to non-Tfh cells (Figure 12a).

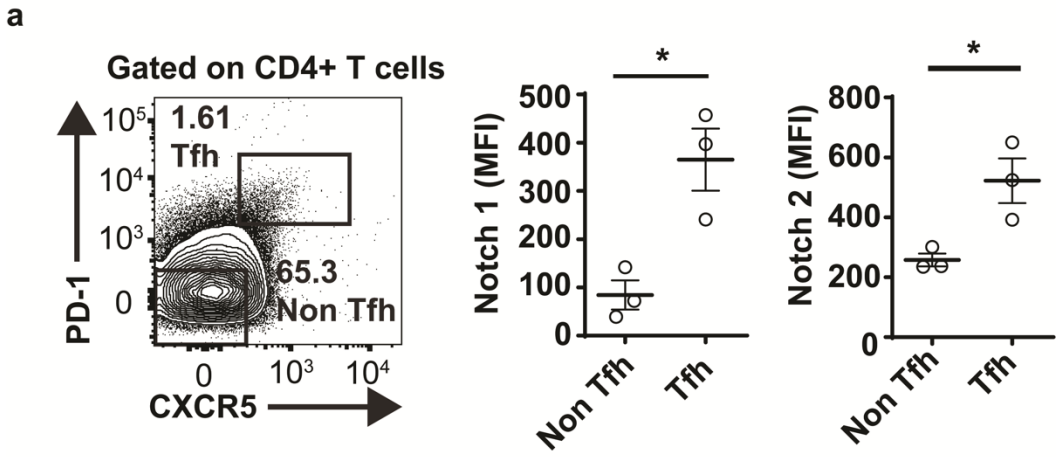

Figure 12: Notch receptors are highly expressed on Tfh cells during helminth infections.

IL-4^{4get} mice were infected with *N. brasiliensis* and the mediastinal lymph nodes were harvested eight days later. **(a)** Representative flow plot of CD4+ T cells. Gates represent non Tfh (PD-1- CXCR5-) and Tfh (PD-1+ CXCR5+) cells. Quantification of mean fluorescence intensity (MFI) of Notch 1 and Notch 2 in Non Tfh and Tfh cells is shown.

*P < 0.05 (unpaired two-tailed T-test). Error bars represent +/- SEM. Data is representative of two independent experiments with n = 3 mice per group.

To directly investigate the necessity of Notch in Tfh cell generation, mediastinal lymph nodes were isolated from IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ and IL4^{4get}Notch1/2$^{fl/fl}$ mice nine days after infection with *N. brasiliensis*, and CD4+ T cells were analyzed for canonical Tfh cell markers CXCR5 and PD-1 (Figure 13a). T cell Notch-deficiency results in a significant impairment in both the percentage and number of Tfh cells within the CD4+ T cell compartment (Figure 13a).

Regarding an effect on IL-4 expression, in the few CXCR5+, PD1+ cells phenotypically resembling Tfh cells generated in infected IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ mice, we observed a 10-fold reduction in GFP (IL-4) reporter expression compared to Notch-sufficient T cells (Figure 13b). This deficiency in Tfh cells and Tfh-derived IL-4 corresponded to impaired germinal center B cell generation, an event largely dependent on Tfh cells in this model (Figure 13c). Together these data identify Notch signaling as a key component in Tfh cell generation and function during type-2 immunity. This result was confirmed as Notch-deficient CD4+ T cells from the mediastinal lymph nodes showed a marked deficit in expression of the Tfh cell lineage-determining factor BCL6 by percent and MFI (Figure 14a). GATA-3, a key lineage-determining factor for Th2 commitment, was not significantly changed in percentage or MFI (Figure 14b).

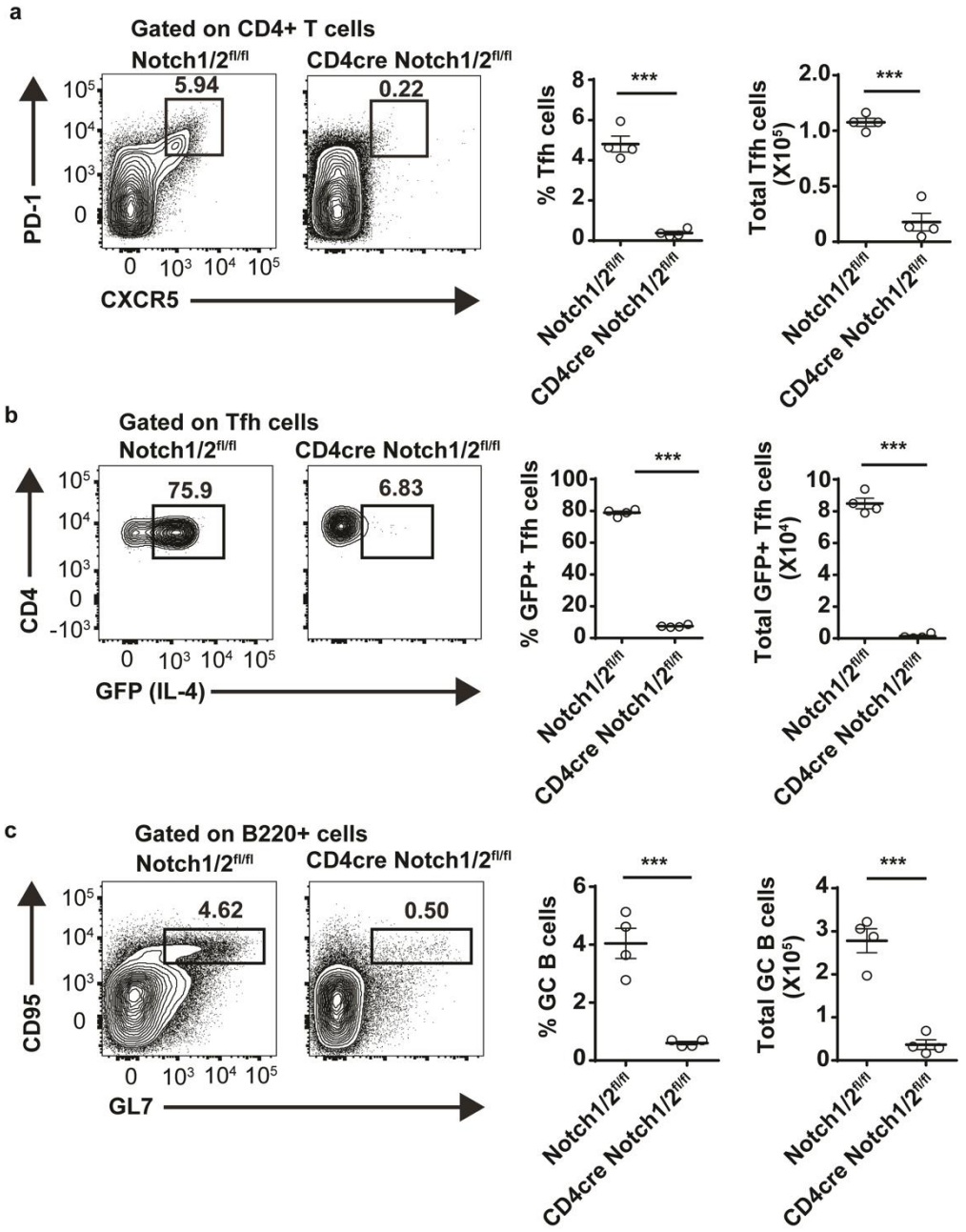

Figure 13: Notch deficiency in T cells results in reduced Tfh cell numbers, Tfh IL-4 production, and germinal center B cells.

Mediastinal lymph nodes of IL4^{4get}Notch1/2$^{fl/fl}$ (n = 4) and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ (n = 4) mice were collected nine days after infection with *N. brasiliensis*. **(a)** Representative contour plots gated on Tfh cells (PD-1+, CXCR5+) of indicated mice are shown. Graphs show quantification of percentage and total number of Tfh cells. **(b)** Representative flow

cytometry plots showing IL-4 competency of Tfh cells shown in panel **(a)**. Graphs show percent and number of IL-4 competent Tfh cells from indicated mice. **(c)** Contour plots pre-gated on B220+ cells. Gate represents germinal center B cells (CD95+ GL7+). Graphs show percent and total number of GC B cells. Error bars represent +/- SEM. Data is representative of four independent experiments with n= 3-5 mice per group. ***$P < 0.001$ (unpaired two-tailed T-test).

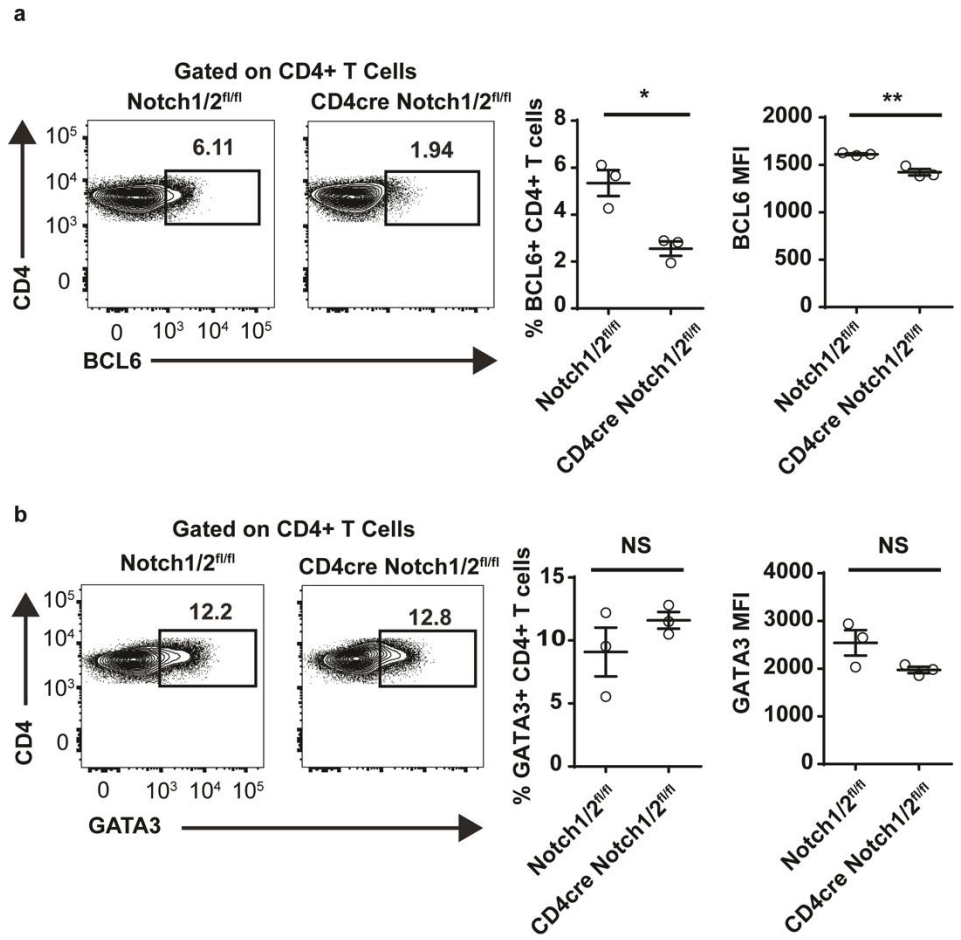

Figure 14: Deletion of Notch receptors on T cells results in a significant reduction in BCL6 expression.

IL4^{4get}Notch1/2$^{fl/fl}$ (n = 3) and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ (n = 3) mice were infected with *N. brasiliensis* and mediastinal lymph nodes were harvested nine days later. (a) Expression of BCL6 or (b) GATA-3 in the total CD4+ population was assessed by intracellular transcription factor staining with percent quantified. Total MFI of the BCL6 and GATA-3 positive populations was determined. Error bars represent +/- SEM. Data

shown is representative of three independent experiments with n = 3-4 mice per group. *P < 0.05, ** P < 0.01 (unpaired two-tailed t-test).

Because the deletion of both Notch1 and 2 resulted in an almost complete loss of Tfh cells, it was difficult to fully assess the role of Notch signaling in IL-4 expression by Tfh cells. To circumvent this issue, we made use of an intermediate phenotype observed in mice that lack Notch1 but retain one allele of Notch2. Deletion of Notch1 alone results in an intermediate loss of Tfh cells compared to Notch-sufficient and Notch1/2-deficient CD4+ T cells (Figure 15a). Importantly, the Tfh cells generated in the absence of Notch1 still exhibited a significant impairment in IL-4 mRNA expression (Figure 15b). In line with these results, elimination of Notch1 alone in T cells caused a moderate reduction in germinal center B cell generation (Figure 15c). Based on these results, expression of both Notch1 and 2 is important to fully drive Tfh commitment and Tfh function.

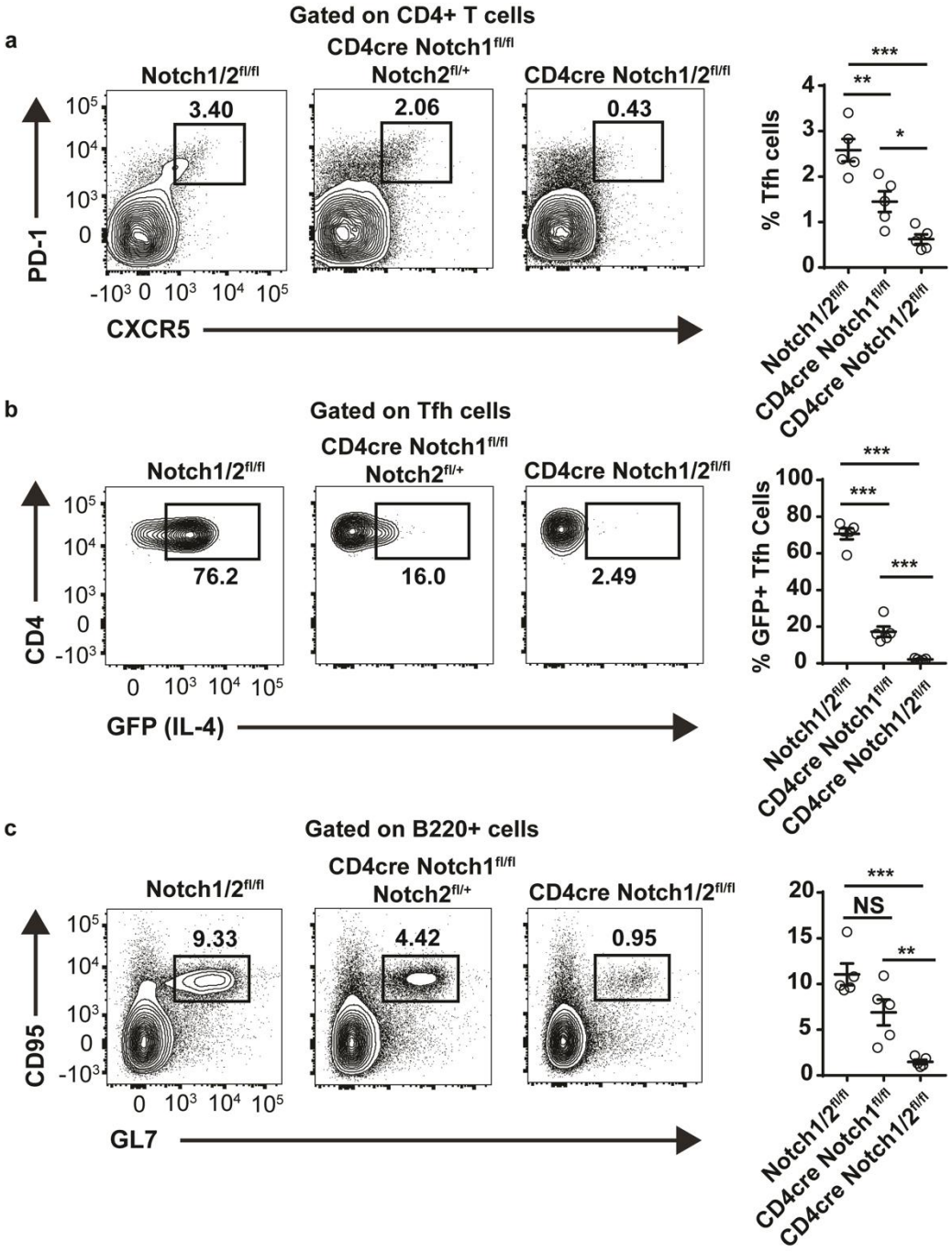

Figure 15: Deletion of Notch1 alone results in an intermediate phenotype compared to T cells deficient in both Notch1 and Notch2.

IL4^{4get}Notch1/2$^{fl/fl}$ (n = 5), IL4^{4get}CD4^{cre}Notch1$^{fl/fl}$Notch2$^{fl/+}$ (n = 5), IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ (n = 5) mice were immunized with OVA emulsified in alum in the footpad. Eight days

after immunization, mice were euthanized and the mediastinal lymph node harvested for analysis by flow cytometry. **(a)** Representative contour plots gated on Tfh cells (PD-1+, CXCR5+) of indicated mice are shown. **(b)** Representative flow cytometry plots showing IL-4 competency of Tfh cells shown in panel **(a)**. **(c)** Contour plots pre-gated on B220+ cells. Gate represents germinal center B cells (CD95+ GL7+). Graphs show percent of Tfh cells **(a)**, IL-4 mRNA competent Tfh cells **(b)**, and GC B cells **(c)**. Error bars represent +/- SEM. Data is representative of two independent experiments with n= 2-3 mice per group. ***$P < 0.001$ (unpaired two-tailed T-test).

3.4 Overexpression of Notch intracellular domain increases IL-4 production by Tfh but not Th2 cells

The data demonstrate that Notch-deficiency in T cells leads to impaired Tfh cell generation and an impaired ability to establish IL-4 competency within those cells. To better uncouple the role of Notch in Tfh generation from its role in Tfh-derived IL-4 production, we investigated whether additional Notch-signaling would lead to increased IL-4 expression in Tfh cells. To do this, IL4^{KN2} reporter mice [302], which report IL-4 production through the cell surface expression of human CD2, were crossed with a tamoxifen-inducible GFP-NICD fusion reporter line [359]. In this IL4^{KN2}ER^{cre}Rosa26$^{Stop\text{-}flox\text{-}NICD\text{-}GFP}$ gain-of-function system, it is possible to directly compare IL-4-production in Tfh cells experiencing endogenous Notch signals relative to those experiencing increased Notch signaling in the same lymph node based on the presence or absence of GFP expression.

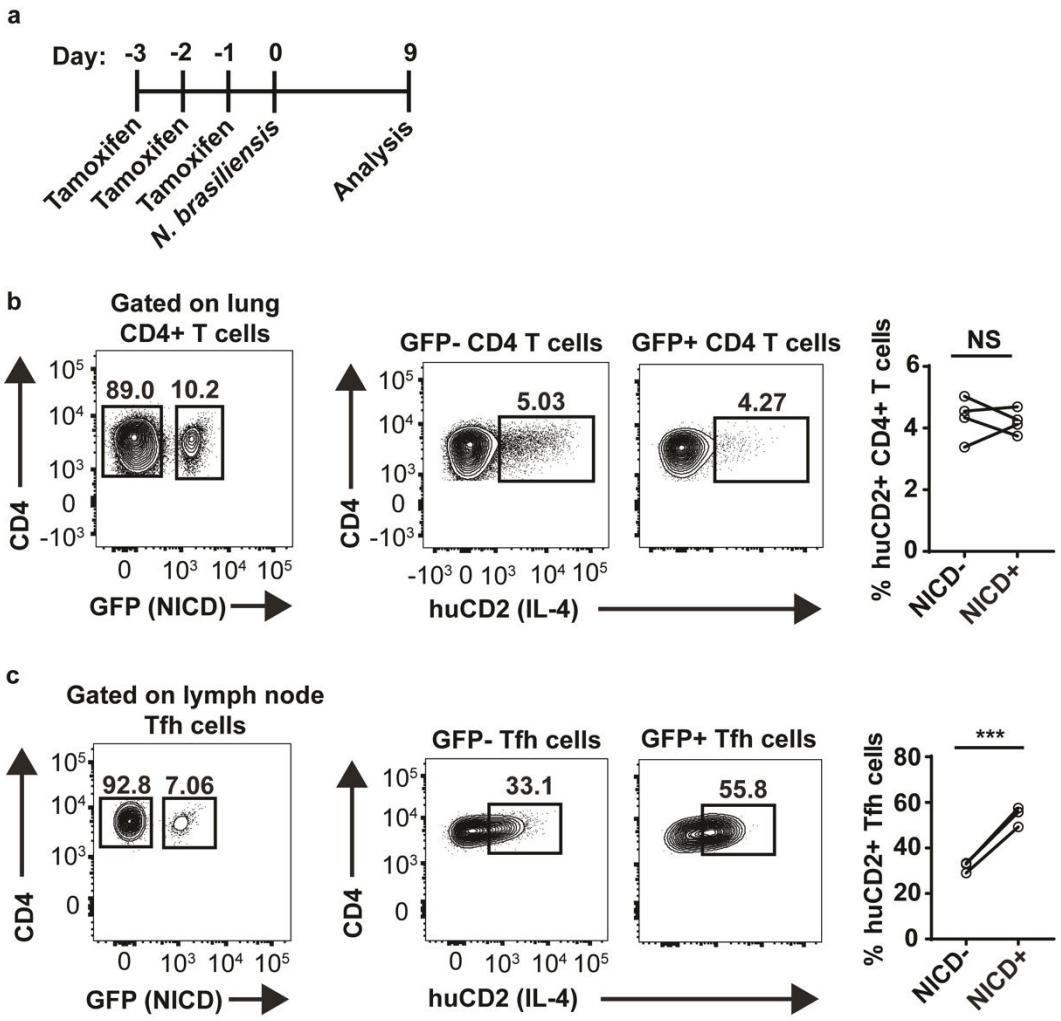

Figure 16: Over-expression of NICD leads to increased IL-4 production by Tfh cells but not lung resident CD4+ T cells.

(a) Diagram of experimental setup: IL4^{KN2}ER^{cre}Rosa26$^{Stop\text{-}flox\text{-}NICD\text{-}GFP}$ (n = 4) mice were injected with tamoxifen once every 24 hours for three days starting on day -3. On day 0 mice were infected with *N. brasiliensis* and mediastinal lymph nodes were harvested on day nine. **(b)** Representative flow plots of CD4+ T cells isolated from the lung. Gates shown represent cells with endogenous Notch signaling (CD4+ GFP-) and cells over-expressing NICD (CD4+ GFP+). IL-4 protein (huCD2) expression was analyzed and quantified as a percent of the gated populations. **(c)** Contour plots of Tfh (PD1+ CXCR5+) cells isolated from mediastinal lymph nodes. IL-4 protein (huCD2) expression was assessed and quantified in Tfh cells with endogenous levels of Notch signaling (CD4+, PD1+, CXCR5+, GFP-) and Tfh cells over-expressing NICD (CD4+, PD1+,

CXCR5+, GFP+). Error bars represent +/- SEM. Data is representative of four independent experiments with n= 3-5 mice per group. ***$P < 0.001$ (paired two-tailed T-test).

IL4^{KN2}ER^{cre}Rosa26$^{Stop\text{-}flox\text{-}NICD\text{-}GFP}$ mice were treated with tamoxifen three days prior to *N. brasiliensis* infection, and mediastinal lymph nodes and lungs from mice were analyzed nine days post infection (Figure 16a). IL-4 protein production, as marked by human CD2, was comparable between CD4+ T cells expressing endogenous NICD (GFP negative) and those over-expressing NICD (GFP positive) in the lung (Figure 16b). In contrast, Tfh cells from the mediastinal lymph nodes of these same animals showed a significant two-fold enhancement of IL-4 production when the GFP-NICD fusion was induced (Figure 16c). Over-expression of NICD also led to an increase in the percentage of non-Tfh cells producing IL-4 in the lymph node (Figure 17a, b).

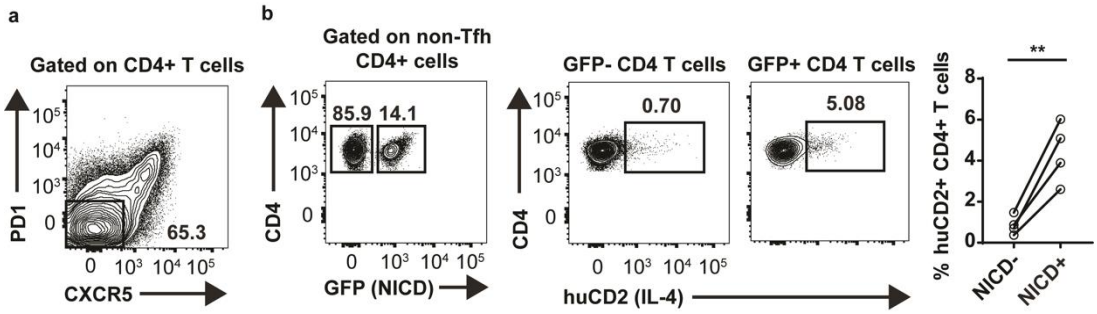

Figure 17: Over-expression of NICD leads to increased IL-4 production in lymph node non-Tfh cells.

IL4^{KN2}ER^{cre}Rosa26$^{Stop\text{-}flox\text{-}NICD\text{-}GFP}$ (n = 4) mice were injected with tamoxifen once every 24 hours for three days starting on day -3. On day 0 mice were infected with *N. brasiliensis* and mediastinal lymph nodes were harvested on day nine. **(a)** Representative contour plot of CD4+ T cells showing a gate for non Tfh (PD1- CXCR5-) cells. **(b)** Contour plot gated through non Tfh cells shown in **(a)** and gated on CD4+ GFP- and CD4+ GFP+ cells. Representative contour plots of GFP- and GFP+ CD4+ T cells are shown. These gates and

the graph show the percent of IL-4 producing CD4+ T cells that are GFP- (NICD-) or GFP+ (NICD+). Data is representative of four independent experiments with n= 3-5 mice per group Error bars represent +/- SEM. $^{**}P < 0.01$, (unpaired two-tailed T-test).

In addition, NICD gain-of-function also led to increased BCL6 and GATA-3 expression in lymph node CD4+ T cells (Figure 18a, b). This finding is consistent with Notch signaling affecting GATA-3 expression, as previously shown during *in vitro Th2* cultures [172],[173], but also highlights a role for Notch signaling in mediating BCL6+ Tfh cell generation or maintenance [17]. BCL6 and GATA-3 in lung CD4+ T cells was not altered in cells over-expressing NICD (Figure 18c, d). Together, these data show that Notch signaling impacts not only Tfh cell development but also IL-4 production by Tfh cells. The observation that over-expression of NICD has a more pronounced effect on IL-4 production and transcriptional regulation in Tfh cells compared to lung Th2 cells further supports a more selective role for Notch signaling in Tfh cells relative to their Th2 cell counterparts.

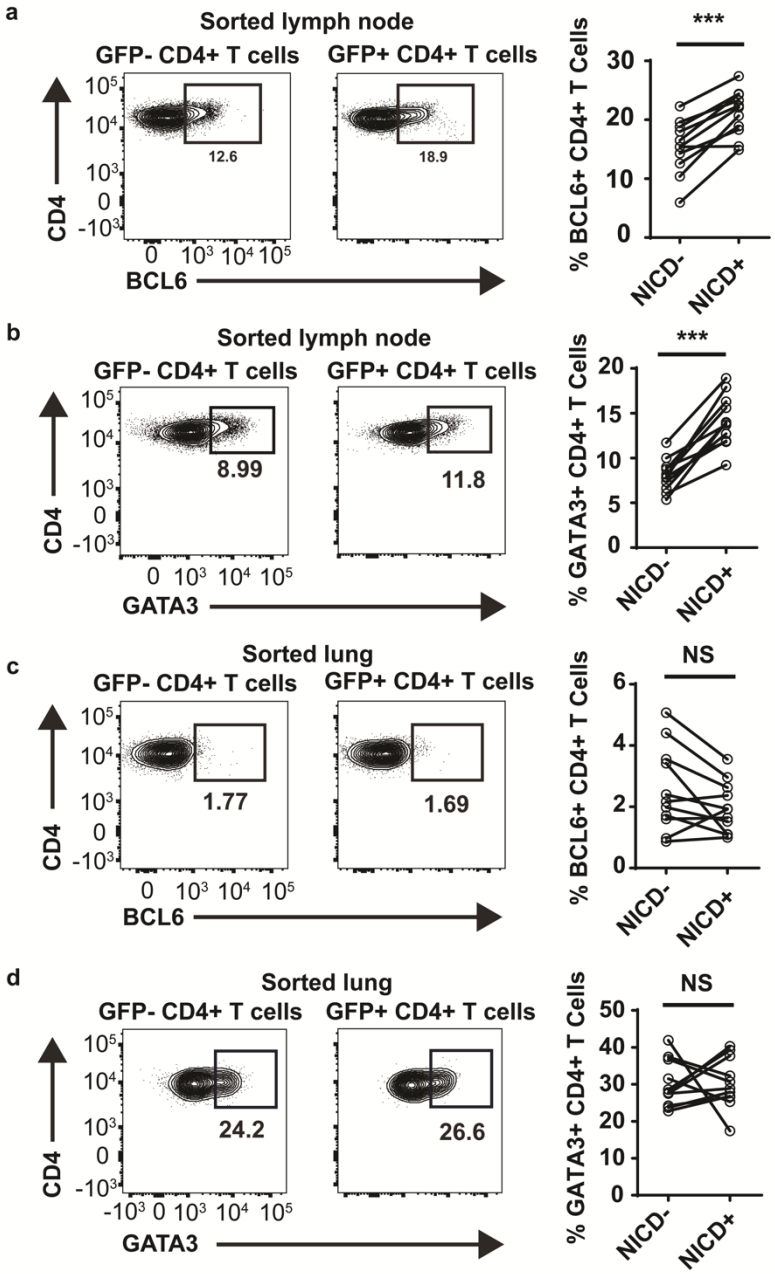

Figure 18: Over-expression of NICD leads to increased expression of BCL6 and GATA-3 in lymph node, but not lung, CD4+ T cells.

IL4^{KN2}ER^{cre}Rosa26$^{Stop\text{-}flox\text{-}NICD\text{-}GFP}$ (n = 11) mice were injected with tamoxifen on days -3, -2, -1 and infected with *N. brasiliensis* on day 0. Mediastinal lymph nodes and lungs were harvested nine days later. CD4+ GFP- (T cells with normal Notch expression) and CD4+ GFP+ (T cells over-expressing NICD) were sorted and prepped for flow cytometry. **(a, b)**

Representative flow cytometry plots of sorted CD4+ GFP- (NICD-) and CD4+ GFP+ (NICD+) lymph node cells. Gates represent percentage of BCL6+ **(a)** or GATA-3+ **(b)** cells. **(c, d)** Contour plots of sorted CD4+ GFP- (NICD-) and CD4+ GFP+ (NICD+) lung cells. Gates depict the percentage of BCL6+ **(c)** or GATA-3+ **(d)** cells. Graphs show quantified percentages of BCL6+ and GATA-3+ CD4+ T cells. Error bars represent +/- SEM. Data is combined from three independent experiments with n = 3-4 mice per group. ***P < 0.0005 (paired two-tailed T-test).

3.5 Functional Notch ligands on conventional dendritic cells can influence Tfh cell fate early during CD4+ T cell differentiation, but are not required for full Tfh commitment.

Although high doses of antigen can drive a Tfh cell fate in the absence of conventional dendritic cells (cDC), in most physiologic settings, the initiation of Tfh cell fate requires antigen presentation by conventional dendritic (cDC) cells [261,274,366]. To assess whether early Tfh cell commitment required functional Notch ligands on cDC subsets, canonical Notch ligand-mediated signaling was prevented by conditionally deleting the E3 ubiquitin ligase molecule, Mind bomb1 (Mib1), in cDC. Notch signaling requires Mib1 for ligand-induced Notch signaling in vertebrates [367]. Deletion in dendritic cells was achieved by CD11c-driven, Cre recombinase-mediated excision of loxP-flanked Mib1 [361,319]. Cre recombinase expression in cDC was confirmed by assessing yellow fluorescent protein (YFP) expression among CD11c+, MHC-II+ cells isolated from the mediastinal lymph nodes of *N. brasiliensis*-infected CD11c^{Cre}Rosa$^{flox\text{-}stopYFP}$ mice (Figure 19a). Mib1 deletion using the CD11c^{Cre}Mib1$^{fl/fl}$ system was confirmed by PCR analysis of dendritic cells isolated and sorted from the mediastinal lymph nodes

five days after *N. brasiliensis* infection (Figure 19b) and bone marrow-derived DC cultures (Figure 19c).

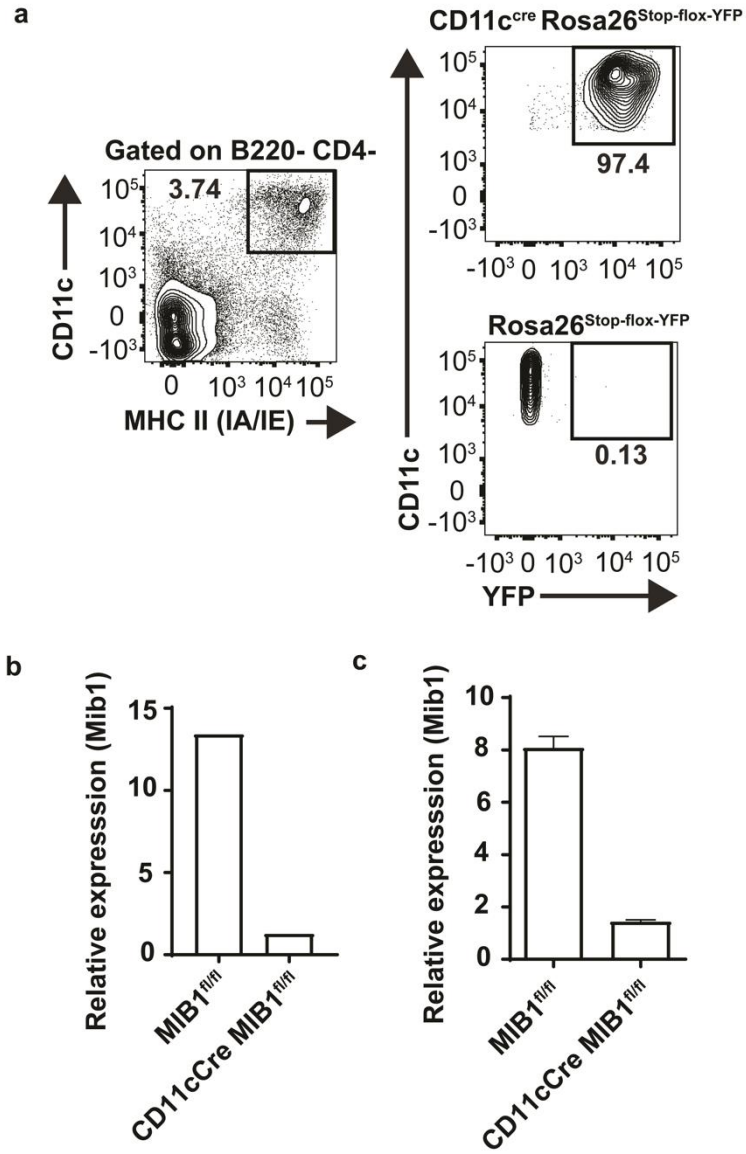

Figure 19: Successful deletion of MIB1 in CD11c+ dendritic cells.

(a) Representative contour plot of CD11c+ MHC-II+ dendritic cells from mediastinal lymph nodes harvested from CD11c^{cre} Rosa26$^{Stop\text{-}flox\text{-}YFP}$ (n = 3) mice five days post infection with *N. brasiliensis*. Percentage of YFP+ cells from CD11c+ MHC-II+ gate is shown for CD11c^{cre} Rosa26$^{Stop\text{-}flox\text{-}YFP}$ (top) and Rosa26$^{Stop\text{-}flox\text{-}YFP}$ (bottom). Data is

representative of two independent experiments. **(b)** IL4^{4get}CD11c^{Cre}Mib1$^{fl/fl}$ (n = 3) and IL4^{4get}Mib1$^{fl/f}$ (n = 3) were infected with *N. brasiliensis* and CD11c+ MHC-II+ cells were sorted from pooled mediastinal lymph nodes five days post infection. Cells were prepared for real-time PCR. The expression of Mind bomb-1 relative to B-actin is shown. **(c)** Bone marrow derived dendritic cells cultured from IL4^{4get}CD11c^{Cre}Mib1$^{fl/fl}$ and IL4^{4get}Mib1$^{fl/f}$ mice were isolated on day nine and sorted and prepared for real-time PCR. Expression of Mind bomb-1 relative to B-actin is shown. Error bars represent +/- SEM.

To assess changes in IL-4 competency and Tfh development in the absence of Mib1 in cDC, we crossed CD11c^{Cre}Mib1$^{fl/fl}$ mice onto the IL4^{4get} reporter system. IL4^{4get}CD11c^{Cre}Mib1$^{fl/fl}$ (Mib1-deficient cDC) and IL4^{4get}Mib1$^{fl/fl}$ (wild-type) mice were infected with *N. brasiliensis.* Mediastinal lymph nodes were isolated on day five and day nine post infection. On day five, mice lacking Mib1 in CD11c expressing cells showed a significant decrease in the percentage and total number of CD4+ T cells expressing the canonical Tfh markers CXCR5+, PD-1+ (Figure 20a). Although the percentage of Tfh cells expressing IL-4 mRNA were similar between mice harboring Mib1-deficient cDC and wild-type cDC, the numbers of IL-4 competent Tfh cells were reduced as would be expected given the decrease in total Tfh cells (Figure 20a, b). However, the dependence on functional Notch-ligands provided by cDC was not absolute as Tfh cell numbers and IL-4 mRNA competency were similar by day nine of infection (Figure 20c).

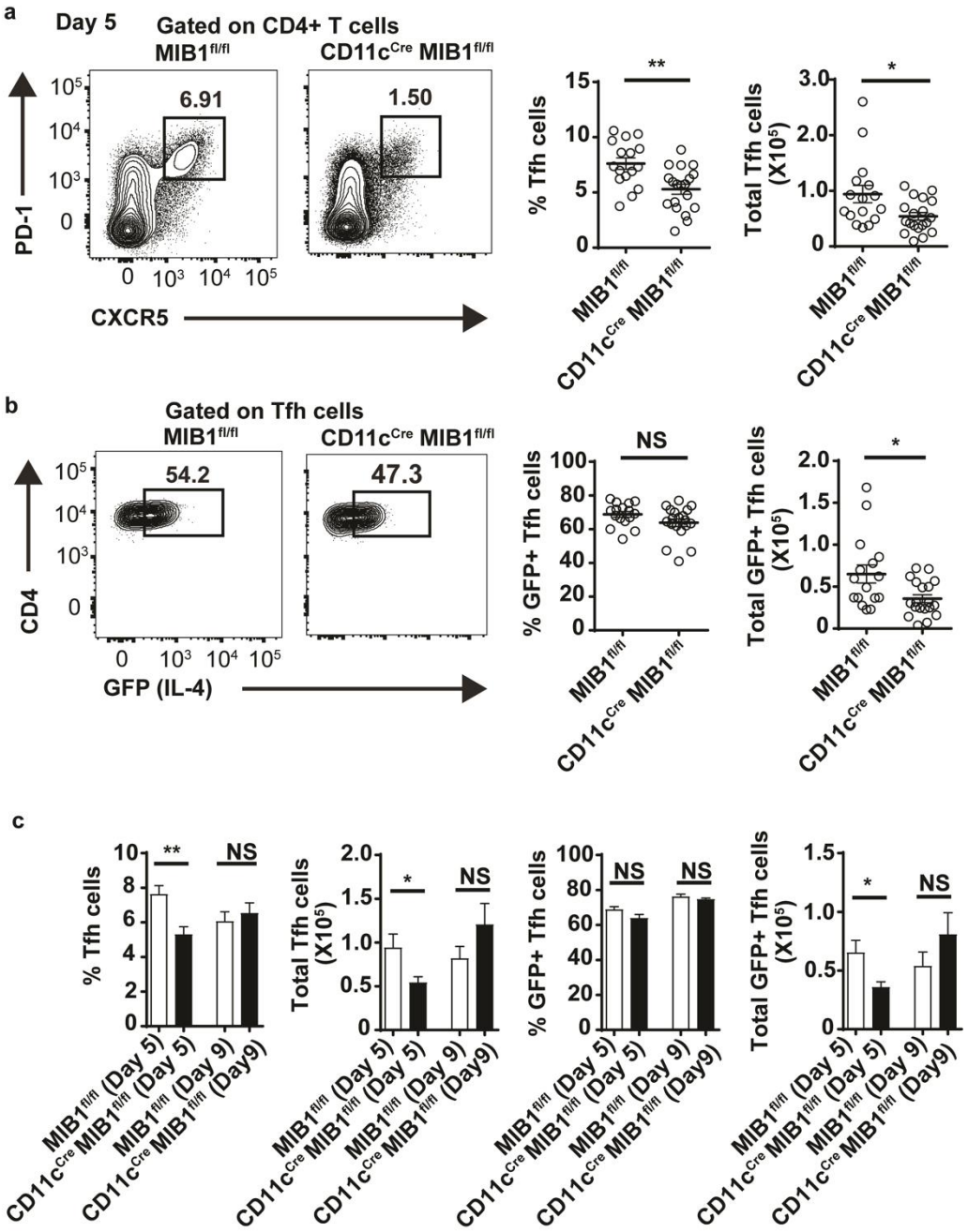

Figure 20: Deletion of MIB1 in conventional dendritic cells is important in early Tfh cell fate decision.

IL4^{4get}CD11c^{Cre}Mib1$^{fl/fl}$ (n = 19) and IL4^{4get}Mib1$^{fl/fl}$ (n = 16) mice were infected with *N. brasiliensis* and mediastinal lymph nodes were harvested five days later for flow cytometry. **(a)** Representative contour plots on Tfh (PD1+ CXCR5+) cells. Graphs show

quantification of percent and total number of Tfh cells. **(b)** Contour plots of Tfh cells. Gates indicate the percent of GFP+ Tfh cells. **(a)**. Graphs show percent and total number of IL-4 expressing Tfh cells. **(c)** Graphs show percentage and total number of Tfh cells and IL-4 expressing Tfh cells from IL4^{4get}CD11c^{Cre}Mib1$^{fl/fl}$ and IL4^{4get}Mib1$^{fl/fl}$ five days (n =19, n = 16) and nine days (n = 11, n = 10) post helminth infection. Error bars represent +/- SEM. Data shown is combined from three (day 9) or four (day 5) independent experiments with n = 3-5 mice per group. *P < 0.05 ***P* < 0.01 (unpaired two-tailed T-test).

The defect observed at day 5 was not due to a general priming defect as IL4^{4get}OT-II CD4+ T cells transferred into CD11c^{cre}MIB1$^{fl/fl}$ mice did not have a significant defect in their proliferation compared to OT-II T cells transferred into control MIB1$^{fl/fl}$ mice (Figure 21a). The transferred cells also had no defect in the expression of early activation markers, CD44 and CD69 (Figure 21b). Consistent with the previous findings in Tfh cells, OT-II T cells transferred into CD11c^{cre}MIB1$^{fl/fl}$ mice exhibited no reduction in the percentage of cells that established IL4 mRNA competency (Figure 21c).

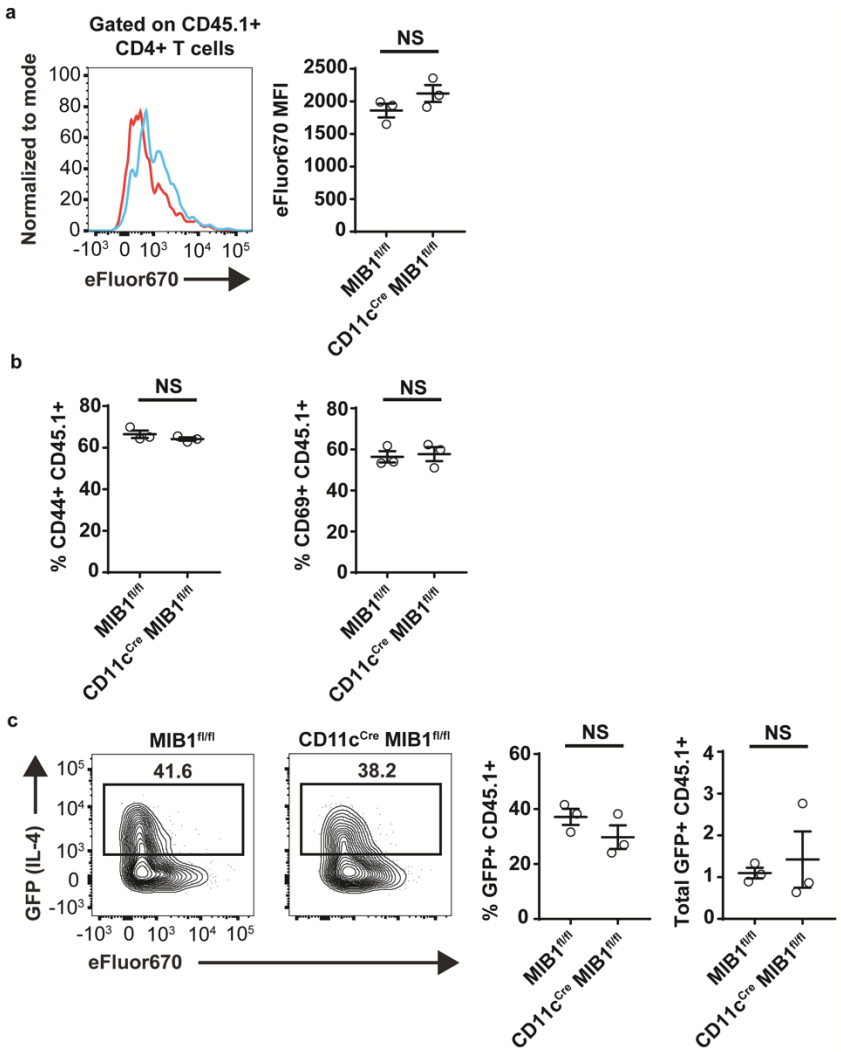

Figure 21: Deletion of MIB1 in conventional dendritic cells does not result in a general proliferation or activation defect in antigen specific T cells.

IL4^{4get} CD45.1 OT-II T cells were isolated and loaded with a proliferation dye (eFluor670). The loaded cells were then transferred into MIB1$^{fl/fl}$ and CD11c^{cre}MIB$^{fl/fl}$ mice. The mice were immunized twenty-four hours later with OVA emulsified in alum and cells then isolated three days post-immunization for analysis. **(a)** Proliferation of OT-II transferred cells from MIB1$^{fl/fl}$ (red) and CD11c^{cre}MIB1$^{fl/fl}$ (blue) mice. eFluor670 MFI of total CD45.1+ CD4+ T cells was quantified. **(b)** Expression of CD44 and CD69 on CD4+ CD45.1+ cells quantified and graphed. **(c)** Representative contour plots of CD45.1+ CD4+ T cells. Gates represent percent of IL-4 mRNA competent cells. Graphs show percent and total number of CD4+ CD45.1+ GFP+ cells. This data represents a single experiment.

3.6 Functional Notch ligands on B cells and follicular dendritic cells are not required for Tfh cell commitment.

Although cDCs often play an important role in the early development of Tfh cells, entry of Tfh cells into the B cell follicles and interaction with B cells themselves appears critical for ultimate Tfh cell commitment [274]. Furthermore, depletion of follicular dendritic cells (FDC) impacts Tfh cell numbers [368]. Given that functional Notch ligands on cDC were not required for Tfh cell commitment after day 5, we pursued the idea that B cells or FDC in the follicles might be the compensatory source of these ligands as the cellular response to *N. brasiliensis* matured. To investigate this mechanism, we conditionally deleted Mib1 in B cells and FDC using a CD21-driven Cre recombinase [369]. CD21 is confined to these two cell types in the mouse immune system [370], and the specificity of CD21-Cre recombinase activity has been confirmed in both B cells and FDC using this system [369,368].

To confirm CD21-Cre activity in FDC and B cells in our hands, we immunized CD21^{cre}Rosa26$^{Stop\text{-}flox\text{-}YFP}$ mice with ovalbumin precipitated in alum at three sites in the back and collected draining axillary, brachial, and inguinal lymph nodes five days post injection. Similar to previous reports, Cre-activity was confirmed in 87.1% of the B cells by YFP expression (Figure 22a). Using flow cytometry, we observed that CD21-cre activity was highly enriched in FDCs compared to other stromal populations based on YFP expression (Figure 22b). Using immunohistochemistry on CD21^{cre}Rosa26$^{Stop\text{-}flox\text{-}YFP}$ mice, we confirmed CD21-cre activity in FDC and B cells by staining for YFP in tissue

sections obtained from isolated lymph nodes (Figure 22c). YFP was co-expressed by both IgD+ (B cells) cells, as well as FDCM2+ (FDC) cells in the IgD- germinal center (Figure 22c).

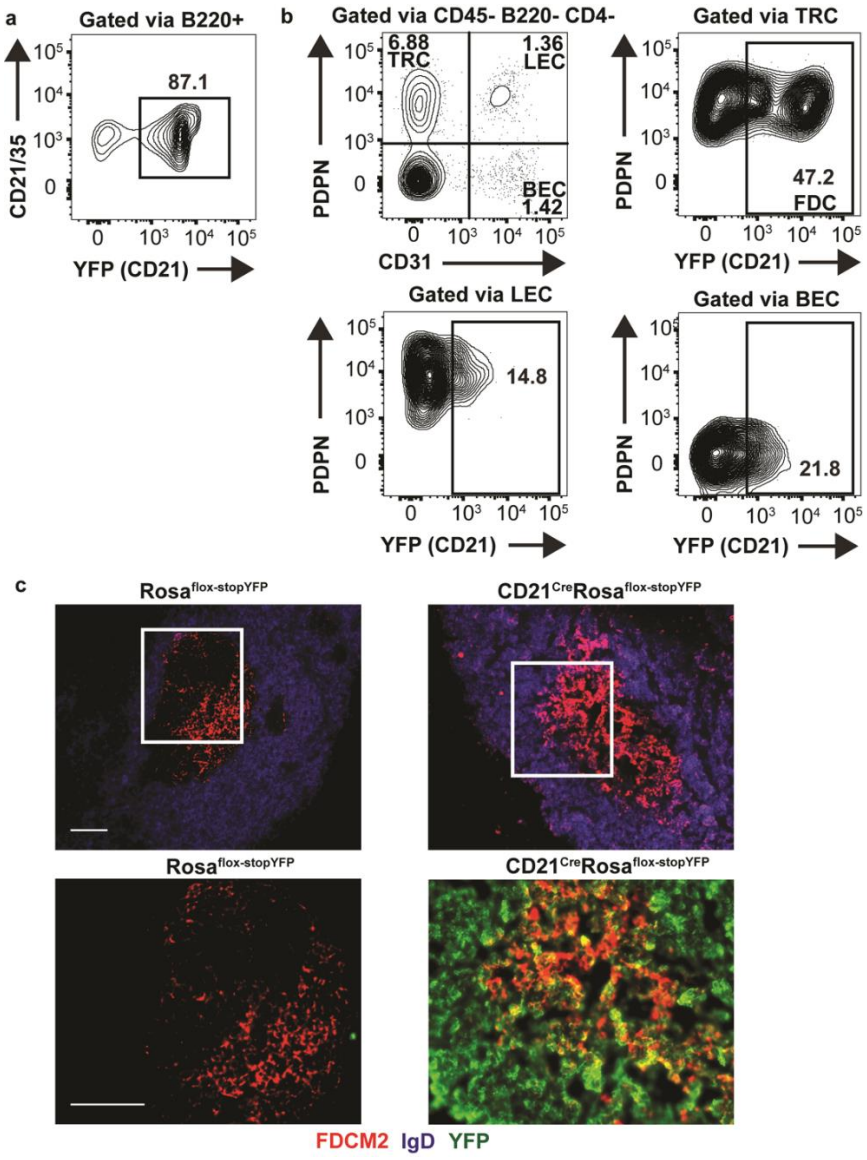

Figure 22: CD21 expression amongst lymph node stromal cell populations.

CD21^{cre}Rosa26$^{Stop\text{-}flox\text{-}YFP}$ and Rosa26$^{Stop\text{-}flox\text{-}YFP}$ mice were immunized with OVA emulsified in alum. Five days later inguinal, axial, and brachial lymph nodes were harvested and

combined. The CD45- population was enriched and prepared for flow cytometry analysis. **(a)** Representative flow plot of the B220+ population. Gate marks YFP+ cells. **(b)** Contour plots from pooled lymph node samples gated on CD45- B220- and CD4-. Gates represent YFP expression on lymph node stromal cell populations: T zone reticular cells (TRC; PDPN+, CD31-, CD21+), lymphatic endothelial cells (LEC; PDPN+ CD31+), and blood endothelial cells (BEC; PDPN- CD31+). FDCs are identified as YFP (CD21) expressing TRCs. Data are representative of three independent experiments with n = 2-3. **(c).** Nine days post subcutaneous footpad immunization with OVA/alum, immunofluorescence microscopy was performed on popliteal lymph nodes harvested from CD21^{cre}Rosa26$^{Stop\text{-}flox\text{-}YFP}$ and Rosa26$^{Stop\text{-}flox\text{-}YFP}$ mice. Sections were stained for IgD (blue), YFP (green) and FDCM2 (red). A composite image of IgD and FDCM2 stains is shown at the top of the panel. A higher magnification of these images (area denoted by a white square) was taken and a composite image of FDCM2 and YFP is shown at the bottom of the panel. Scale bars (bottom left) represent 100 μm.

To assess whether functional Notch ligands on B cells and FDC are required for Tfh commitment, IL4^{4get}CD21^{Cre}Mib1$^{fl/fl}$ (Mib1-deficient B cells and FDC) or IL4^{4get}Mib1$^{fl/fl}$ (wild-type) mice were infected with *N. brasiliensis*, and mediastinal lymph nodes were isolated nine days later. Both CXCR5+, PD1+ Tfh cell percentages and numbers were similar to mice where B cells and FDC remained Mib1 competent (Figure 23a). There was also no significant change in IL-4 mRNA competency among the Tfh cell compartment, as percentage and number of GFP expressing cells remained similar between these two groups (Figure 23b).

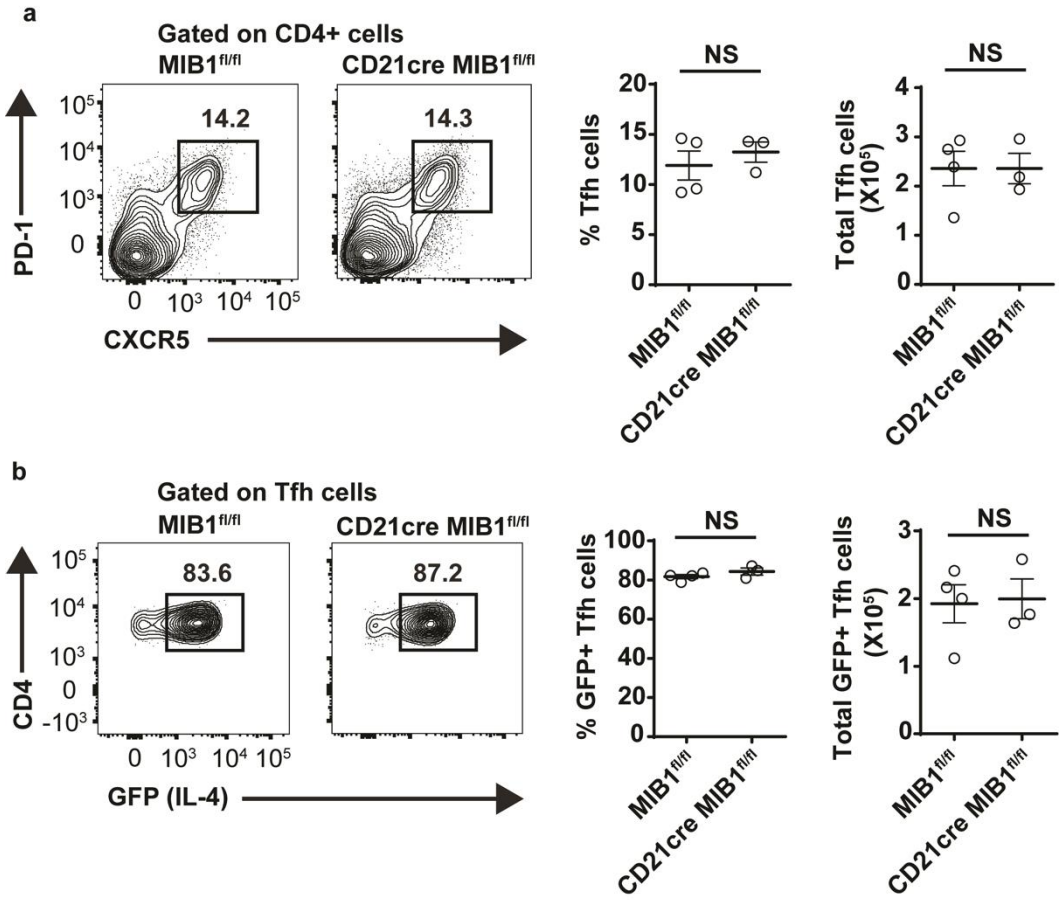

Figure 23: Deletion of MIB1 on FDCs and B cells is dispensable for Tfh differentiation and function.

IL4^{4get}CD21^{Cre}Mib1$^{fl/fl}$ (n = 3) and IL4^{4get}Mib1$^{fl/fl}$ (n = 4) mice were infected with *N. brasiliensis* and their mediastinal lymph nodes were analyzed by flow cytometry nine days post infection. **(a)** Representative contour plots of CD4+ T cells. Gate represents Tfh (PD1+, CXCR5+) cells. Graphs show percent and total number of Tfh cells. **(b)** IL-4 expression was assessed and quantified in Tfh cell populations gated in panel **(a)**. Gate represents GFP+ (IL-4 competent) Tfh cells. Error bars represent +/- SEM. Data shown is representative of two independent experiments with n = 3-4 mice per group.

T cell specific deletion of Mib1 also had no impact on Tfh cell numbers or IL-4 potential (Figure 24a, b). To summarize, functional Notch ligands on conventional

dendritic cells influence early Tfh cell fate and cytokine competency. However, functional ligands on non-hematopoietic cells other than FDC are sufficient in the absence of functional ligands on dendritic cells as the immune response proceeds.

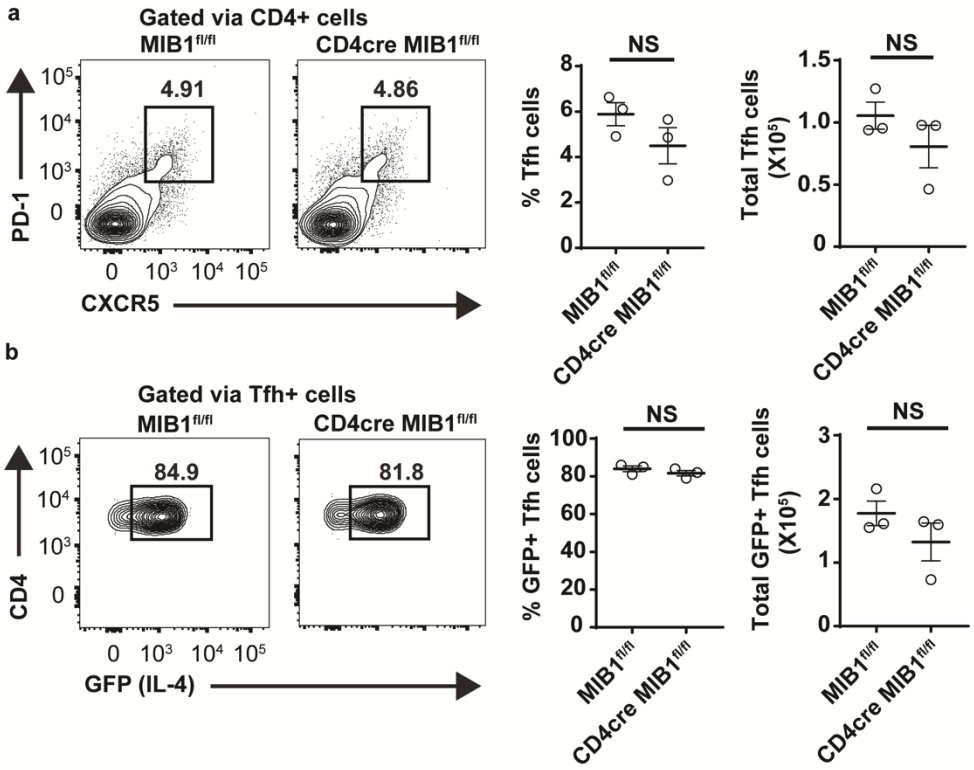

Figure 24: MIB1 deficiency in CD4+ T cells has no effect on Tfh differentiation or Tfh IL-4 production.

IL4^{4get} CD4^{cre} Mib1$^{fl/fl}$ (n = 3) and IL4^{4get} Mib1$^{fl/fl}$ (n = 3) mice were infected with *N. brasiliensis* and mediastinal lymph nodes were harvested nine days later. **(a)** Representative contour plots on Tfh (PD1+ CXCR5+) cells. Graphs show quantification of percent and total number of Tfh cells. **(b)** Contour plots of Tfh cells. Gates indicate the percent of GFP+ Tfh cells. **(a)**. Graphs show percent and total number of IL-4 expressing Tfh cells. Error bars represent +/- SEM. Data is combined from two independent experiments with n = 3-4 mice per group.

3.7 Discussion

In these studies, we establish that Notch signaling in CD4+ T cells represents an important checkpoint in the bifurcation between Tfh cell- and Th2 cell-driven hallmarks of type-2 immunity. Notch receptors 1 and 2 are required for Tfh cell generation, but are largely dispensable for Th2 differentiation in response to parasitic helminth infection. The importance of Notch in this bifurcation is reflected in the biology, as Tfh-mediated type-2 humoral hallmarks, such as IgE production, were impaired, while Th2-orchestrated peripheral immunity, marked by worm clearance in the intestine and innate cell recruitment to the lung, remained intact. Furthermore, these studies reveal that Notch signaling is not only required for development and commitment of Tfh cells, but that Notch signals can enhance type-2 cytokine production in committed Tfh cells. The enhanced cytokine production that results from increased NICD expression also appears specific to Tfh cells as lung-resident CD4+ T cells over-expressing NICD produced similar levels of IL-4 as CD4+ T cells expressing endogenous NICD.

Two competing models for Notch in Th1, Th2, and Th17 cell differentiation have emerged, and are also likely at play in Tfh cell fate determination. The unbiased amplifying or facilitating model implies that Notch signals potentiate an already established cytokine potential in developing subsets [333]. The instructive model suggests that the signals from the engagement of different Notch ligands by Notch receptors can direct differentiation toward a specific cell fate [311]. Importantly, the

results herein support aspects of both amplifying and instructive mechanisms in Notch-driven Tfh cell fate and function. As discussed above, over-expression of NICD showed that Notch-signaling enhances IL-4 production in Tfh, but not Th2 cells. Furthermore, over-expression of NICD had no effect on driving GATA-3 expression in lung CD4+ T cells, while it did lead to enhanced expression of BCL6 in lymph node CD4+ T cells. The selective dependence on Notch ligands in Tfh versus Th2 cell generation also reveals the potential presence of an instructive-based mechanism for Notch in Tfh cell fate choice. In its simplest form, the presence of functional Notch ligands promotes Tfh cell fate, but has a significantly reduced influence on Th2 cell development. This is not to say that Notch does not have a role in GATA-3 expression or in amplifying Th2 cytokine competency, but rather that Th2 cells do not rely on Notch signals *in vivo* to the same extent as Tfh cells to achieve their ultimate effector fate. It is likely that Th2 cell fate is cemented and less influenced by Notch signals once a threshold of GATA-3 expression is achieved in developing Th2 cells. The presence of other IL-4 and IL-13 producers in the lung can reinforce the Th2 phenotype and maintenance of GATA-3 in Th2 cells, potentially making the role of Notch in these settings less critical for driving Th2 responses in the periphery. While we did not see major defects in peripheral Th2 responses in mice with Notch-deficient T cells, we did observe an influx of non-Th2 CD4+ T cells in the lungs of these mice. Given that Notch has been shown to influence

fate and function in Th1, Th2, and Th17 cells, future work investigating the effector fate and transcriptional program of these non-Th2 cells is of great interest.

The near complete absence of Tfh cells and IgE, despite normal Th2 development and peripheral type-2 immunity in mucosal tissues observed in this study is intriguing given conclusions made by prior studies. Several past studies looking at the necessity of Notch in type-2 immune responses have described Notch signaling to be essential for Th2 biology [310]. These prior studies largely based their conclusions on *in vitro* Th2 polarization assays, *ex vivo* restimulation of lymph node resident CD4+ T cells, or used serum cytokines and IgE as surrogates for Th2 function. We now know that these readouts are not particularly good indicators of true Th2 cell biology *in vivo*. For example, IgE is dependent on Tfh-derived IL-4 not type-2 cytokines generated by Th2 cells [54]. Thus, prior studies designed to uncover a role for Notch in Th2 cell biology may have instead identified unappreciated defects in Tfh cells. We believe the experiments shown herein help to reconcile prior results and place them in a more unifying context.

The findings shown herein are of particular interest in light of previous studies investigating the role of Notch during *T. muris* infection, another nematode infection model that requires Th2 cells for parasite expulsion [333,351]. While in the *N. brasiliensis* model we saw normal worm clearance but a complete loss of IgE when T cells lacked Notch1 and Notch2 suggesting normal Th2 function but impaired Tfh function, the *T.*

muris studies showed both a loss of IgE and impaired worm expulsion when T cells expressed a dominant negative form of the Notch co-activator mastermind/MAML (DNMAML) which is considered a pan-Notch inhibitor. This might at first pass suggest that both Tfh and Th2 cells are impaired using the conditional DNMAML system. However, the authors show that administration of anti-CD3 to activate T cells in *T. muris* infected mice elicited no significant change in IL-4, IL-5, and IL-13 in the serum compared to wild-type. This is consistent with Th2 differentiation and type-2 cytokine competency remaining largely intact in this setting. It will be interesting to confirm this by directly assessing Tfh cell and Th2 cell development in this model. Taken together, the data suggests that Notch signaling plays a less critical role in Th2 compared to Tfh differentiation, but supports a role for Notch in amplifying and optimizing Th2 function.

Another interesting area of investigation will be to determine why inhibition of Notch signaling seems to impair IL-4-mediated antibody isotypes more readily than IFN-γ-driven isotype-switching [172,351,312]. One might expect Notch to affect both IL-4-producing and IFN-γ-producing Tfh cells equally as both are found in the germinal centers and affect isotype-switching [54]. In support of Notch being involved in the generation of Tfh cells in both Th2 and Th1 settings, chronic *Leishmania* infection models show that Notch1 and Notch2 deficiency in T cells leads to diminished Tfh cells and Tfh-dependent germinal center formation even in settings of robust type-1 responses. Of note, *L. major* infection of $CD4^{cre}Notch1/2^{fl/fl}$ mice did not affect Th2 cell generation as

Notch1/Notch2-deficiency in T cells promoted lesion development on the normally resistant C57BL/6 background [313]. Lesion development is dependent on the presence of IL-4-producing Th2 cells. However, these findings do suggest that Th1 cell function was impacted in the absence of Notch signaling as their presence would prevent lesion development in this model.

Curiously, blocking of IFN-γ seems to rescue IgE and IgG1 production in *T. muris* infected mice expressing a dominant negative form of MAML in T cells. Whether IFN-γ is impacting Tfh cells directly in this case to promote IgE is not clear. It is likely that IFN-γ from Th1 cells is suppressing systemic levels of type-2 cytokines derived from Th2 cells during chronic helminth infection. Once inhibited by antibody, IFN-γ can no longer suppress Th2 function allowing systemic IL-4 and non-Tfh-derived IL-4 to influence B cells [371,372]. This finding, along with the data discussed above, may also suggest the presence of a non-canonical Notch signaling pathway that differentially involves MAML during Th1, Th2 and Tfh cell generation. Investigating these possibilities will be important for future studies.

The unexpected finding that Th2 cells were less dependent on Notch signals than their IL-4-producing Tfh cell counterparts suggested that these two cell subsets may also require distinct Notch ligands. One such bifurcating-ligand maybe DLL4. Inhibition of DLL4 promotes Th2 cell function in settings of allergic airway inflammation [349,373]. The inhibitory role for DLL4 on Th2 cells is particularly intriguing given that DLL4

serves a promoting role in Tfh cell differentiation [316]. When placed in the context of the data presented here, it is consistent with DLL4 acting as a switch for Tfh cell differentiation. In its absence, responding IL-4 competent cells may default toward a Th2 cell fate. DLL4 acting as a specific trigger for Tfh cells would also be consistent with our Mib1 results, as Mib1 has the highest affinity for DLL4 and is known to play a key role in DLL4-mediated Notch1 signaling during T cell lineage development [374,375].

How and if Notch signals complement TCR signals to direct Tfh cell fate is an interesting area for future investigation. The affinity of the TCR for antigen-MHC complexes and the dwell time spent on APC, are key factors in ultimately determining Tfh cell fate [145,155]. However, additional signals likely influence Tfh cell fate either by increasing dwell time, and/or tuning TCR signals, or by independently promoting a Tfh-specific gene circuit in parallel to TCR signaling [262]. In support of a complementary or parallel role for Notch in TCR-mediated differentiation of Tfh cells, previous studies have shown that TCR signaling increases Notch receptor expression and activation, and that Notch is required for TCR-mediated activation and proliferation of peripheral T cells [327,376]. It is also clear that Notch signals can potentiate or inhibit TCR signals in the thymus and the periphery to regulate T cell fate [377,328].

The advantage of using Mib1 deletion is that it disrupts downstream signaling from all Notch ligands. Thus, it provides an unbiased approach to assess the involvement of Notch ligands in biology. However, as discussed above with regard to

DLL4 in Th2 and Tfh cell generation, this prevents investigation into the individual roles specific Notch ligands might play in type-2 immunity. It is also unable to assess a role for non-canonical Notch ligands as Mib1 may not disrupt non-canonical Notch signaling. Despite these caveats, our findings do show that conventional dendritic cells provide an important early source of Notch ligands. However, functional Notch ligands on cDC are not necessary to establish full Tfh cell effector potential. Similarly, B cells, T cells, and FDC, which interact frequently with Tfh cells in the B cell follicles and germinal centers, are not required sources of Notch ligands for Tfh cell commitment. These findings help to further support a fibroblastic cell source of DLL4 as critical for Tfh cell commitment [316]. Although initially suggested to be FDC [316], our data indicates that other stromal sources are sufficient for Tfh commitment. Notch ligands on cDC, FDC, and B cells may contribute to the Tfh phenotype, but other (likely stromal sources) of Notch ligands can compensate in their absence.

This study places Notch in the company of BCL6 and ASCL2 as factors that selectively regulate Tfh cell development and function relative to Th2 cells. However, unlike BCL6 and ASCL2, which work intracellularly to control Tfh cell fate, Notch and its ligands are present at the cell surface. This suggests that Notch and/or its ligands could represent important targets for immune mediated therapies such as biologics focused on Tfh-mediated disease outcomes, including allergies and other IgE-driven pathology.

4. Continuous Notch signaling is required to maintain Tfh fate and function

*Disclosure: portions of the following section were adapted from my published research article: Dell'Aringa M & Reinhardt RL. Notch signaling represents an important checkpoint between follicular T-helper and canonical T-helper 2 cell fate. Mucosal Immunology. Feb. 2018. doi:10.1038/s41385-018-0012-9

4.1 Introduction

After initial differentiation and expression of GATA-3, Th2 cells become polarized by a GATA-3 driven positive feedback loop [160] and do not require Notch signaling to retain their Th2 fate. While Th2 cells become polarized upon expression of GATA-3, Tfh cells lack a feedback mechanism for fate polarization. In fact, several studies have suggested that Tfh cells exhibit a high degree of plasticity and may have the potential to revert into other T helper subsets when given or deprived of certain signals [378,379]. Currently, which signals are important for driving the maintenance of Tfh fate are unclear. Our studies described in the previous section have already identified Notch signaling as a factor important in driving initial Tfh fate decision. While we know that Notch is required for Tfh differentiation, it is unclear whether Tfh cells require continuous Notch signaling to maintain their fate and function.

Here, we have employed the use of a gamma secretase inhibitor (GSI) in order to study the role of Notch signaling in Tfh maintenance. GSIs specifically inhibit the gamma secretase complex which is required to release the NICD from the membrane.

Without release from the membrane Notch is unable to translocate to the nucleus and perform its downstream roles as a transcription factor. Importantly, the use of a GSI allows us to choose at what point during an immune response we want to inhibit Notch signaling. Since we already know that Notch is required for Tfh differentiation, we can choose to inhibit Notch signaling only after Tfh differentiation has occurred. This allows us to study Notch's role in Tfh maintenance and downstream function without affecting early Tfh differentiation. The studies outlined here demonstrate that continuous Notch signaling is important to maintain Tfh fate and function. Inhibition with GSI after Tfh differentiation reduces the Tfh and IL-4 producing Tfh populations. Additionally, we identify three potential mechanisms through which Notch signaling regulates Tfh differentiation and maintenance.

4.2 Late inhibition of Notch signaling results in a reduction of Tfh fate and function

Notch signaling is indispensable for the differentiation of Tfh cells, but the role of Notch in Tfh maintenance has yet to be investigated [314]. To investigate whether continuous Notch signaling is important to maintain Tfh fate, we employed the use of a gamma secretase inhibitor (GSI) to inhibit Notch signaling in already differentiated Tfh cells. IL4$^{4get/KN2}$ mice immunized with ovalbumin precipitated in alum generate abundant Tfh cells by 7-10 days after subcutaneous immunization [54]. Using this model, ovalbumin immunized mice were given injections of GSI or a mock injection on days seven, eight and nine post immunization. Mice that received the GSI injection had a

reduction in both their percentage and number of Tfh cells (Figure 25a) as identified by the expression of canonical Tfh markers, PD1 and CXCR5.

In the previous chapter we demonstrated that early deletion of Notch receptors results in reduced IL-4 mRNA expression in Tfh cells. However, it is still unclear whether persistent Notch signaling in established Tfh cells is important for maintaining Tfh cytokine competency. Importantly, the percentage and number of IL-4 producing Tfh cells was significantly reduced in mice that received GSI than mice that received a mock injection (Figure 25b). This data suggests that continuous Notch signaling may be important to maintain Tfh fate and function within the B cell follicle beyond Tfh differentiation.

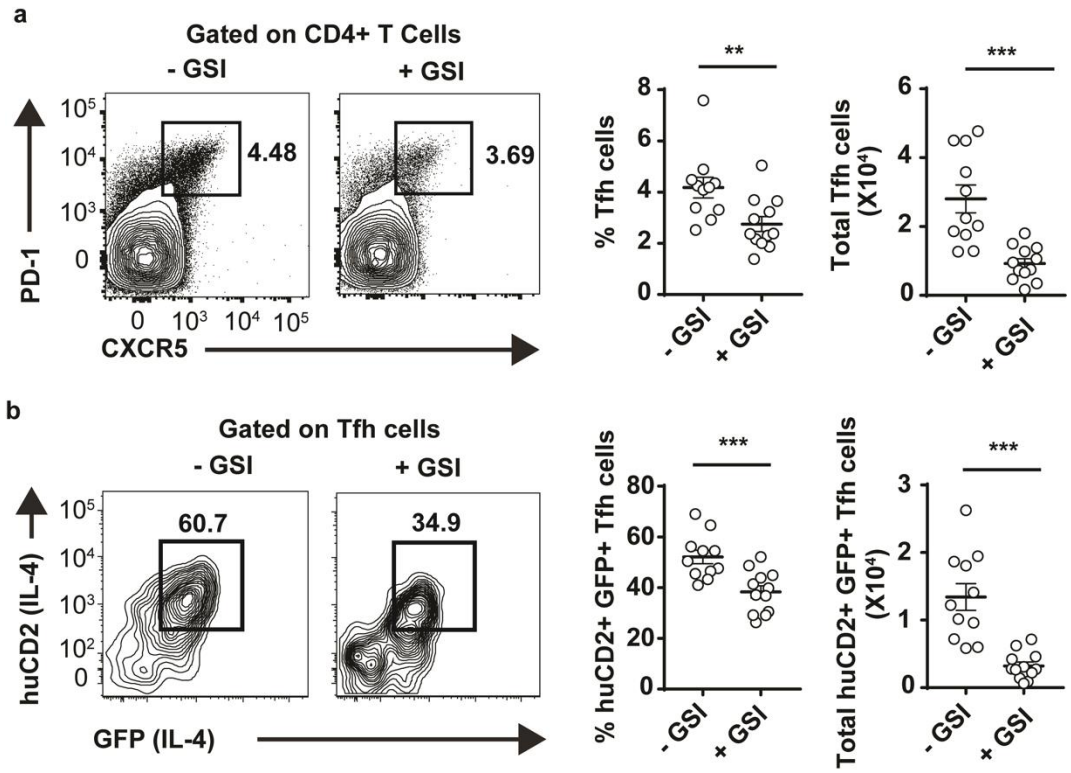

Figure 25: GSI administration after Tfh differentiation leads to reduced Tfh differentiation and IL-4 production.

IL4$^{4get/KN2}$ mice were immunized with OVA emulsified in alum. Seven days post immunization mice were given a control injection (n = 11) or Notch inhibitor (GSI) (n = 12) on days seven, eight, and nine. On day ten, the popliteal lymph node was harvested for flow cytometry. **(a)** Representative contour plots of CD4+ T cells gated on Tfh cells (PD-1+, CXCR5+) of indicated mice are shown. Graphs show quantification of percentage and total number of Tfh cells. **(b)** Representative flow cytometry plots showing IL-4 production of Tfh cells shown in panel **(a)**. Graphs show percent and number of IL-4 producing Tfh cells from indicated mice. Error bars represent +/- SEM. Data is combined from three independent experiments with n= 3-4 mice per group. $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired two-tailed T-test).

Since IL-4 derived from Tfh cells is known to be important in the survival and maturation of GC B cells, we investigated how these reductions in Tfh cells and Tfh IL-4 production affected the GC B cell populations. GC B cell differentiation was not reduced by percentage, but the total number of GC B cells was significantly reduced in mice that received injections of GSI (Figure 26a). These data confirm that continuous Notch signaling is not only important in the maintenance of Tfh fate, but also the downstream function of Tfh cells.

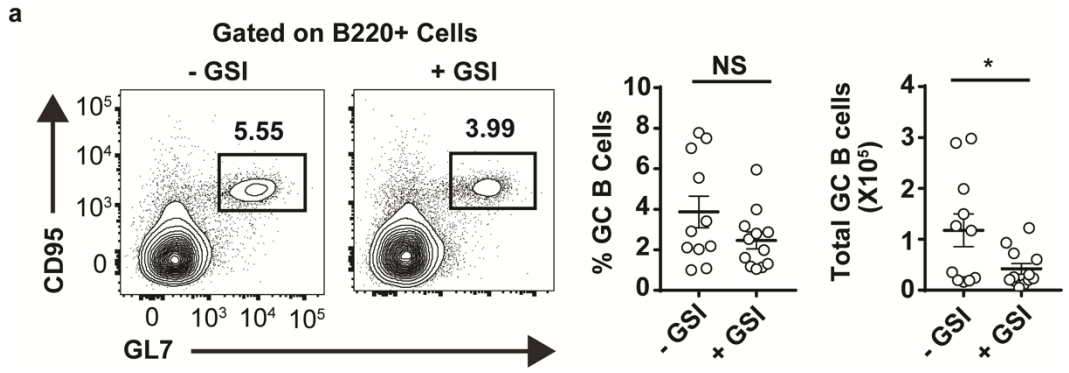

Figure 26: Reduced germinal center B cell population in mice treated with GSI

IL4$^{4get/KN2}$ mice were immunized with OVA emulsified in alum. Seven days post immunization mice were given a control injection (n = 11) or Notch inhibitor (GSI) (n = 12) on days seven, eight, and nine. On day ten, the popliteal lymph node was harvested to assess GC B cell population by flow cytometry. **(a)** Representative contour plots of B220+ cells. Gates indicate germinal center B cells (CD95+ GL7+). Percent and number of GC B cells of the B220+ population was quantified and graphed. Error bars represent +/- SEM. Data is combined from three independent experiments with n= 3-4 mice per group. *$P < 0.05$ (unpaired two-tailed T-test).

4.3 Persistent Notch signaling is required to maintain proper localization of IL-4 competent cells in the lymph node

The localization of T cells within the lymph node is tightly controlled by chemokine receptors, most notably CXCR5 and CCR7. The expression level of these receptors, and the presence of a chemokine gradient, ultimately determine the localization of T cells within the lymph node. CCR7 drives localization towards the cortex of the lymph node, while CXCR5 is important for follicular localization. Upon activation, T cells upregulate CXCR5 and migrate to the perifollicular border. At this B-T border, early interactions with B cells are critically important for Tfh differentiation. Without these interactions, expression of BCL6 will not be maintained, Tfh differentiation will not complete, and germinal center formation will not occur. High expression of CXCR5 retains Tfh cells within the B cell follicle. Currently, the factors controlling the expression of CXCR5 on Tfh cells are not fully elucidated. Furthermore, it is unknown whether Notch signaling plays an important role in regulating the expression of these chemokine receptors.

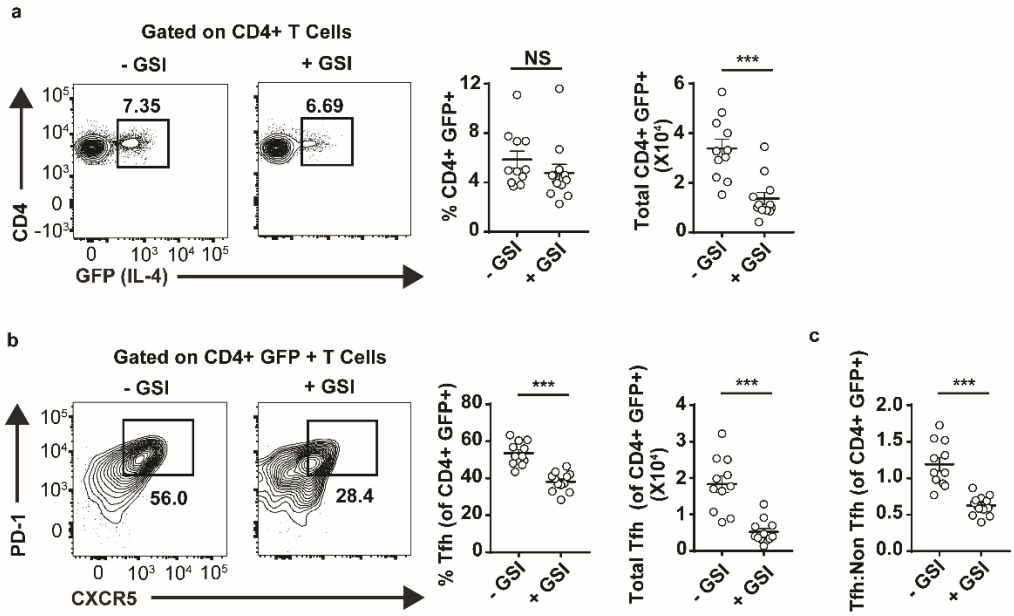

Figure 27: IL-4 expressing cells lose their Tfh phenotype after GSI treatment

IL4$^{4get/KN2}$ mice were immunized with OVA emulsified in alum. Seven days post immunization mice were given a control injection (n = 11) or Notch inhibitor (GSI) (n = 12) on days seven, eight, and nine. On day ten, the popliteal lymph node was harvested for flow cytometry. **(a)** Representative contour plots of CD4+ T cells. Gate represents IL-4 (GFP) expressing CD4+ T cells. Percent and number of IL-4 expressing CD4+ T cells is shown. **(b)** Representative contour plots of IL-4 expressing CD4+ T cells. Gate represents the percent of IL-4 expressing CD4+ T cells that are Tfh (PD1+ CXCR5+) cells. Percent and total number of IL-4 expressing CD4+ T cells that are Tfh cells is quantified. The ratio of total Tfh: Non Tfh IL-4 expressing cells was also quantified. Error bars represent +/- SEM. Data is combined from three independent experiments with n= 3-4 mice per group. ***$P < 0.001$ (unpaired two-tailed T-test).

Previous work has established that IL-4 production in the lymph node is largely restricted to CXCR5^{high} expressing Tfh populations residing in germinal centers [54]. Since local Tfh derived IL-4 production is important for germinal center function, we wanted to assess whether inhibition of Notch signaling affected Tfh marker expression in IL-4 expressing cells. To assess whether Notch signaling had any effect on the

expression of PD1 and CXCR5 on IL-4 competent cells we immunized mice and inhibited Notch signaling with GSI, as described in the previous sections in this chapter. Total number, but not percentage, of IL-4 expressing CD4+ T cells was reduced in mice with inhibited Notch signaling (Figure 27a). As expected, the majority of IL-4 mRNA expressing cells in control injected mice expressed Tfh characteristic markers (Figure 27b). Interestingly, IL-4 mRNA expressing CD4+ T cells in mice treated with GSI exhibited a reduced Tfh phenotype compared to their control injected counterparts, marked by reduced expression of PD-1 and CXCR5 (Figure 27b). The total number of IL-4 expressing CD4+ T cells that were Tfh cells was significantly reduced in GSI-treated mice, as would be expected given the reduction in total IL-4 expressing CD4+ T cells (Figure 27b). Mice treated with GSI had a significantly reduced ratio of Tfh:Non Tfh IL-4 competent cells (Figure 27c). Furthermore, CXCR5 MFI on activated IL-4 expressing (PD1+ GFP+) CD4+ T cells derived from mice that received GSI injections was significantly lower compared to mice that received control injections (Figure 28a).

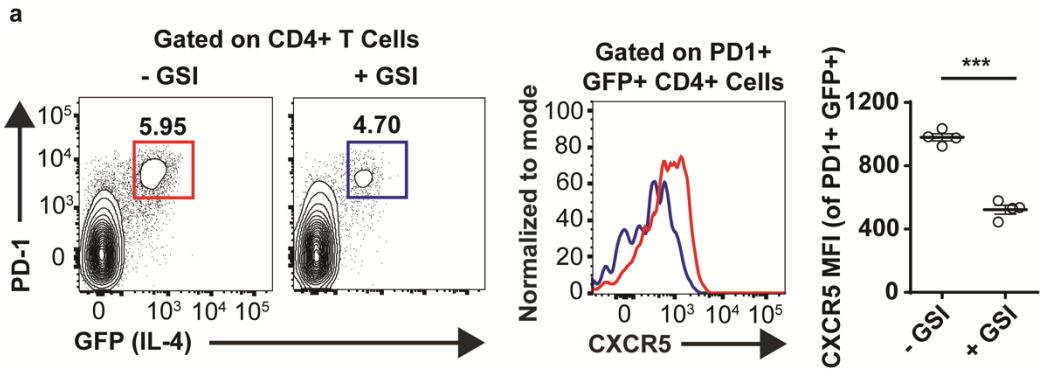

Figure 28: Treatment with GSI leads to reduced expression of CXCR5 by IL-4 expressing CD4+ T cells

IL4$^{4get/KN2}$ mice were immunized with OVA emulsified in alum. Seven days post immunization mice were given a control injection (n = 4) or Notch inhibitor (GSI) (n = 4) on days seven, eight, and nine. On day ten, the popliteal lymph node was harvested for flow cytometry. **(a)** Representative contour plots of CD4+ T cells. Gate represents activated IL-4 expressing cells (PD1+ GFP+). Histogram depicts the expression of CXCR5 in activated IL-4 expressing cells from mice that received mock injections (blue) and mice that received injections of GSI (red). MFI of this population was quantified. Error bars represent +/- SEM. Data is representative of three independent experiments with n = 3-4 mice per group. ***$P < 0.001$ (unpaired two-tailed T-test).

Since Notch inhibition resulted in fewer IL-4 mRNA expressing CD4+ T cells exhibiting a Tfh phenotype, including reduced CXCR5 expression, we hypothesized that we might observe an increased amount of IL-4 mRNA expressing cells in the T cell zone of mice with inhibited Notch signaling. To test our hypothesis, we immunized IL4$^{4get/KN2}$ mice with OVA emulsified in alum and administered GSI injections on days seven, eight, and nine. On day ten, we took the popliteal lymph node, prepared it for histology, and stained it for IgD and GFP (IL-4 mRNA). IL-4 expressing cells were mostly located in the follicle and germinal centers of mice that received mock injections (Figure 29a). Mice administered with GSI exhibited abnormal localization of their IL-4 expressing cells, marked by an accumulation in the cortex, compared to their mock injected counterparts. When quantified, mice that received injections of GSI had twice as many IL-4 mRNA expressing cells residing in the cortex compared to mice receiving mock injections (Figure 29b). This data suggests that Notch signaling may be important in maintaining proper Tfh localization to the B-cell follicle.

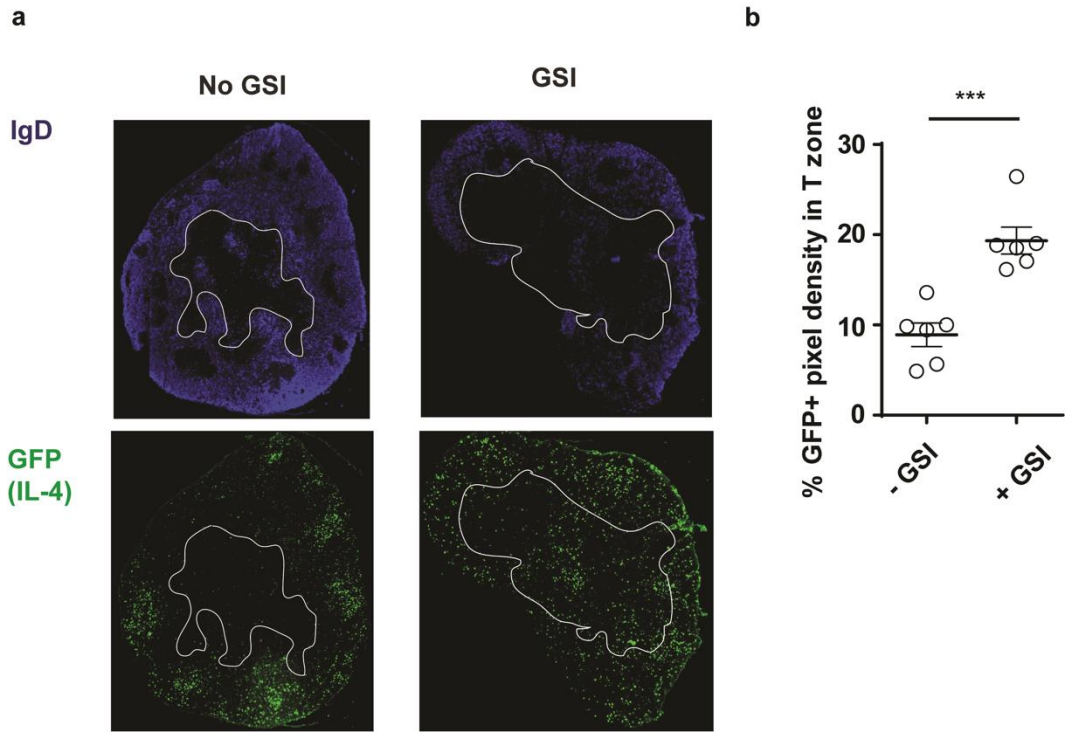

Figure 29: GSI-treatment results in aberrant localization of IL-4 expressing CD4+ T cells.

IL4$^{4get/KN2}$ mice were immunized with OVA emulsified in alum. Mice were given a control injection (n = 6) or Notch inhibitor (GSI) (n = 6) once every twenty-four hours from day seven through day twelve. On day thirteen, the popliteal lymph node was harvested and prepared for histology. **(a)** Fixed popliteal lymph node slices were stained for IgD (blue) and GFP (IL-4; green). White lines were drawn by hand to indicate the T cell zone. **(b)** The percentage of GFP+ pixel density within the T cell zone was calculated for mice that received control injections or injections of GSI. Error bars represent +/- SEM. Data is representative of two independent experiments with n = 3 mice per group. ***$P < 0.001$ (unpaired two-tailed T-test).

Next, we wanted to assess whether the localization phenotype we observed was temporary or if inhibition of Notch for a short period led to sustained increase of IL-4 competent cells in the paracortex. To do this, we again administered GSI to mice from

days seven, eight, and nine post OVA in alum immunization and then gave either GSI or control injections on days ten, eleven, and twelve. On day thirteen, we harvested the popliteal lymph node for histologic analysis of IL-4 mRNA (GFP) expressing cells. Strikingly, GSI again led to an accumulation of IL-4 expressing cells in the T cell zone (Figure 30a, b). However, when Notch inhibition was released on days ten, eleven, and twelve the percent of IL-4 expressing cells residing in the T cell zone returned to normal levels (Figure 30a, b). This further supports a role for the importance of continuous, persistent Notch signaling in controlling Tfh localization.

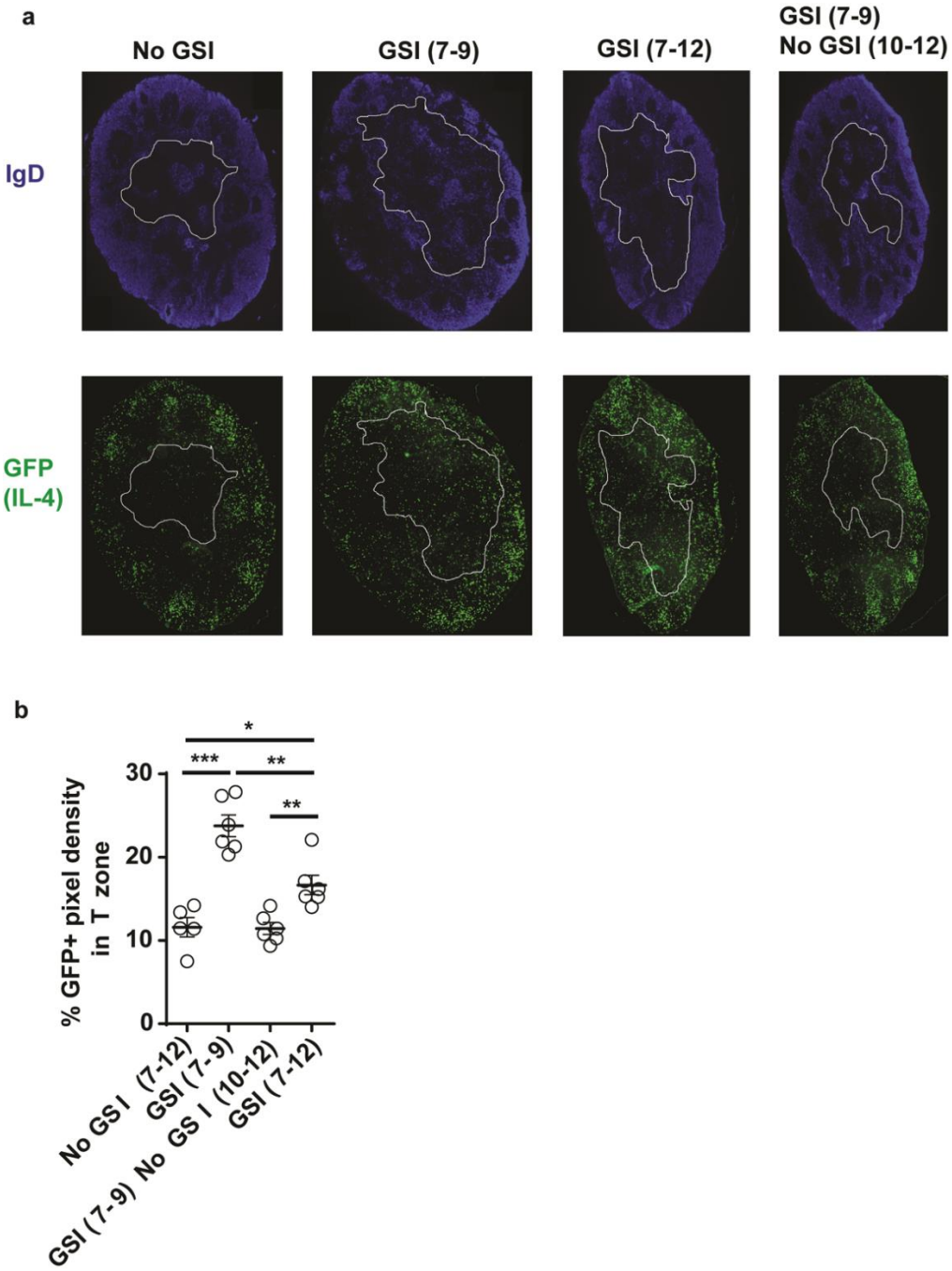

Figure 30: Release of GSI-treatment results in re-localization of IL-4 expressing cells back to the follicle.

IL4$^{4get/KN2}$ mice were immunized with OVA emulsified in alum. Mice were given a control injection (n = 5) or Notch inhibitor (GSI) (n = 6) once every twenty-four hours

from day seven through day twelve. Another group of mice received GSI injections on days seven through nine and then control injections on days ten through twelve (n = 6). On day thirteen, the popliteal lymph node was harvested and prepared for histology. Finally, one group of mice received GSI injections only on days seven through nine and then euthanized on day ten (n = 6). **(a)** Fixed popliteal lymph node slices were stained for IgD (blue) and GFP (IL-4; green). White lines were drawn by hand to indicate the T cell zone. **(b)** The percentage of GFP+ pixel density within the T cell zone was calculated for mice that received control injections or injections of GSI. Error bars represent +/- SEM. Data is representative of two independent experiments with n = 3 mice per group. ***$P < 0.001$ (unpaired two-tailed T-test).

While it appears that Notch signaling could be controlling Tfh localization by regulating CXCR5 expression, it is unclear how Notch signaling might regulate CXCR5. One intriguing transcription factor, ASCL2, is highly expressed by Tfh cells and is important in driving initial Tfh fate by upregulating expression of CXCR5 and down regulating expression of CCR7 [282]. Importantly, Notch has been identified as a driver of ASCL2 expression during epidermal cell development [380]. Taken together, it is possible that Notch signaling may control the expression of CXCR5, and subsequent localization of Tfh cells, by regulating of ASCL2 expression. To begin testing this possibility, we sorted CD4+ GFP+ Non Tfh (PD1- CXCR5-) and CD4+ GFP+ Tfh (PD1+ CXCR5+) cells from control treated and GSI-treated $IL4^{4get/KN2}$ mice. As expected, Tfh cells expressed higher levels of ASCL2 compared to Non Tfh cells (Figure 31a). Intriguingly, Tfh cells derived from mice with inhibited Notch signaling had reduced expression of ASCL2 compared to mice that received control injections (Figure 31b). This data suggests that active Notch signaling may play a role in driving ASCL2 expression,

which simultaneously upregulates CXCR5 and downregulates CCR7 expression, thereby promoting follicular localization and retention in Tfh cells.

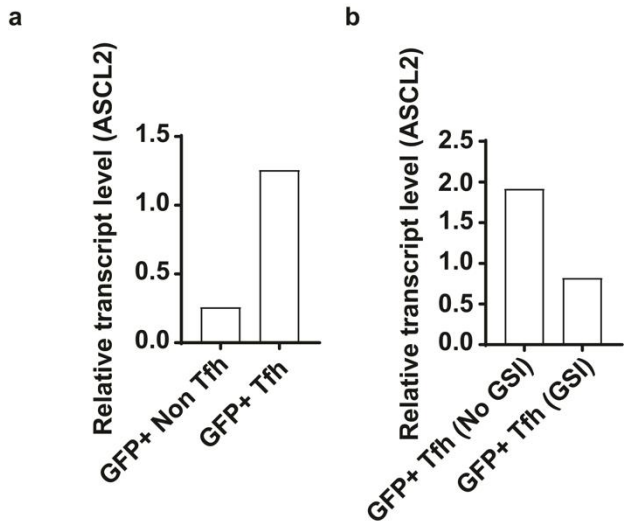

Figure 31: Reduced expression of ASCL2 in Tfh cells from mice treated with GSI.

IL4$^{4get/KN2}$ mice were immunized with OVA emulsified in alum. Seven days post immunization mice were given a control injection (n = 4) or Notch inhibitor (GSI) (n = 4) on days seven, eight, and nine. On day ten, popliteal lymph nodes were harvested and pooled. GFP+ Non Tfh and GFP+ Tfh cells were sorted for subsequent qRT-PCR. **(a)** Relative transcript value of ASCL2 in GFP+ Non Tfh and GFP+ Tfh cells. **(b)** Relative transcript value of ASCL2 in GFP+ Tfh cells there either treated with a control injection or GSI. The relative transcript values were calculated using β-actin as a reference. Data is representative of two independent experiments.

4.4 Accumulation of IL-4 expressing CD4+ T cells in the paracortex of GSI-treated mice requires S1P1.

In the previous section, we observed that lymph node CD4+ IL-4 mRNA expressing T cells with inhibited Notch signaling have aberrant expression of their chemokine receptor CXCR5. As a result, these cells also exhibited abnormal localization in the lymph node compared to control treated animals. Another factor important in the

localization of cells within the lymph node is S1P1. Expression of S1P1 is critical for lymphocyte egress from the lymph nodes to the periphery [256]. Forced expression of S1P1 in activated CD4+ T cells results in abrogated Tfh development by preventing CD4+ T cell homing to the B-cell follicle [259]. Since we have observed a dramatic localization phenotype in the absence of Notch signaling, we wanted to investigate whether S1P1 signaling was playing an important role in driving the aberrant localization of IL-4 expressing cells in animals with inhibited Notch signaling.

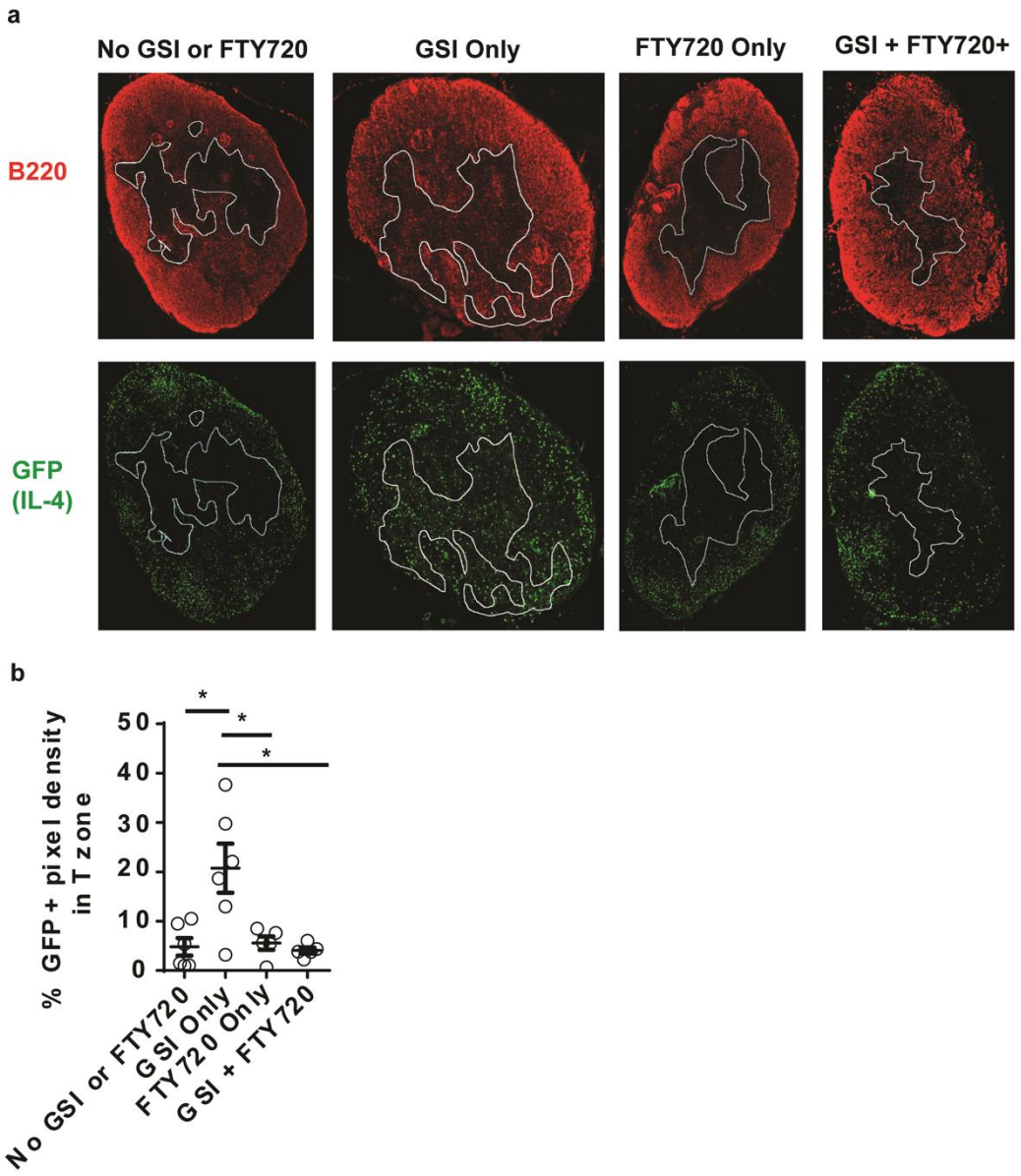

Figure 32: S1P1 inhibition restores normal localization of IL-4 expressing CD4+ T cells observed when administered GSI

IL4$^{4get/KN2}$ mice were immunized with OVA emulsified in alum. Mice were given control injections (No GSI or FTY720; n = 6), GSI injection only (n = 6), FTY720 only (n = 5) or GSI and FTY720 (n = 5). GSI was administered once every twenty-four hours from day seven through day nine. FTY720 injections were given once on days seven and nine. On day ten, the popliteal lymph node was harvested and prepared for histology. **(a)** Tissue sections were stained for B220 (red) and GFP (IL-4; green). **(b)** The percent of GFP+ pixel

density located in the T cell zone was quantified for each group. Error bars represent +/- SEM. Data is combined from two independent experiments with n = 2-3 mice per group. *$P < 0.05$ (unpaired two-tailed T-test).

To answer this question, we employed the use of an S1P1 inhibitor, FTY720. FTY720 has been shown to specifically block signaling from S1P1 and lymphocyte egress from lymphoid tissues [256,381,382]. Concurrent use of FTY720 with our Notch signaling inhibitor, GSI, allowed us to assess whether S1P1 signaling played an important role in the aberrant localization we observed when administering GSI. We immunized IL4$^{4get/KN2}$ mice with OVA emulsified in alum and gave them a control or GSI injection on days seven, eight, and nine. Additionally, we also administered either FTY720 or another control injection on days seven and nine to specified mice to inhibit S1P1 signaling. Consistent with our previous experiments, mice with inhibited Notch signaling again exhibited abnormal localization of their IL-4 mRNA (GFP) expressing cells (Figure 32a). Inhibition of FTY720 alone had no effect on the localization of IL-4 expressing cells compared to control injected mice. Dual administration of GSI and FTY720 resulted in a return of wild-type levels of IL-4 expressing cells (GFP+ pixel density) within the T cell zone (Figure 32a, b). This data suggests that S1P1 signaling is critical for the IL-4 expressing CD4+ T cell re-localization phenotype we have observed when inhibiting Notch signaling.

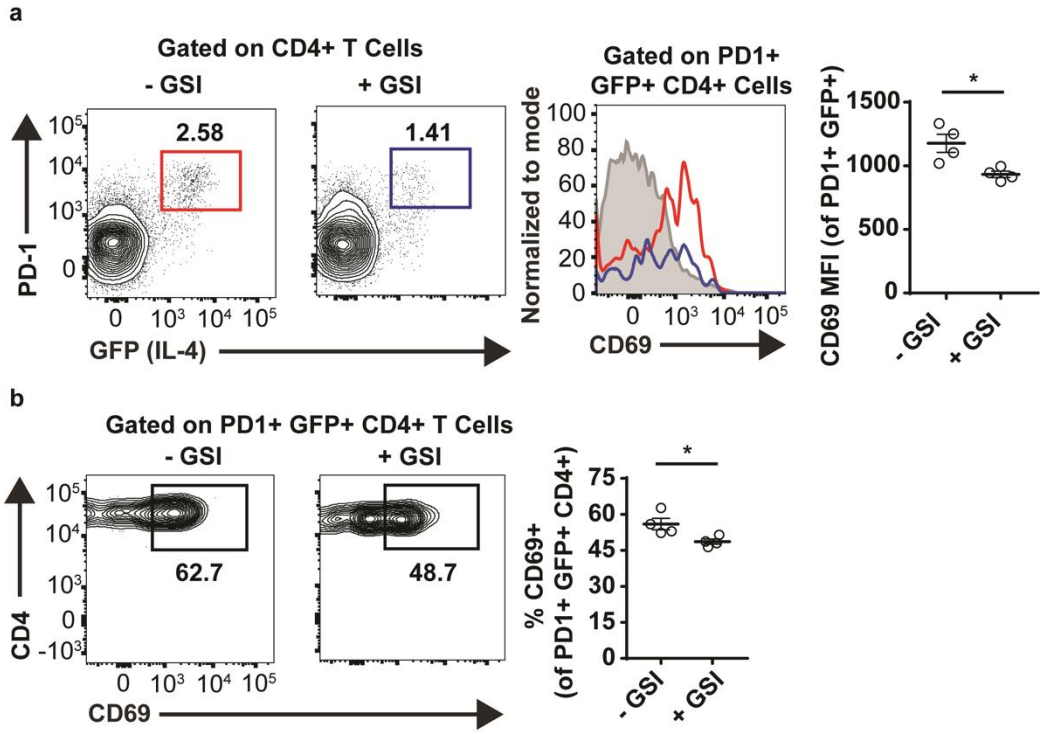

Figure 33: CD69 expression is reduced in IL-4 expressing CD4+ T cells in mice treated with GSI.

IL4$^{4get/KN2}$ mice were immunized with OVA emulsified in alum. Seven days post immunization mice were given a control injection (n = 4) or Notch inhibitor (GSI) (n = 4) on days seven, eight, and nine. On day ten, the popliteal lymph node was harvested for flow cytometry. **(a)** Representative contour plot of CD4+ T cells. Gate represents activated IL-4 expressing cells (PD1+ GFP+). Histograms depict CD69 expression of mice that got control injections (red) and mice that received GSI injections (blue). The filled grey histogram is the CD69 expression of naïve CD4+ T cells. The MFI was quantified and graphed. **(b)** Representative contour plot of activated IL-4 expressing CD4+ T cells (PD1+ GFP+ CD4+). Gate represents the percent of these cells that are CD69+. The percent was quantified and graphed. Error bars represent +/- SEM. Data is representative of two independent experiments with n = 3-4 mice per group. *$P < 0.05$ (unpaired two-tailed T-test).

To further investigate the involvement of the S1P1 pathway, we assessed the expression of CD69 on IL-4 expressing lymph node CD4+ T cells with inhibited Notch signaling. CD69 strongly suppresses S1P1 chemotactic activity and downmodulates

S1P1 expression, thereby enhancing lymphocyte retention in lymph nodes [383]. Since S1P1 signaling appears to be important in our Tfh re-localization phenotype, we hypothesized that Tfh cells with inhibited Notch signaling would have reduced expression of CD69. To test this, we again used GSI to inhibit Notch signaling in IL4$^{4get/KN2}$ mice. Mice were once again immunized with OVA emulsified in alum and given a control or GSI injection on days seven, eight, and nine. On day ten, we harvested the popliteal lymph node and assessed the expression of CD69 on Tfh cells. In support of the previous data, Tfh cells with inhibited Notch signaling had a significantly lower expression of CD69 by percent and MFI (Figure 33a, b). KLF2 directly enhances the expression of S1P1 and loss of KLF2 expression is required for Tfh differentiation [259,257]. Consistent with previous work, KLF2 expression is reduced in IL-4 expressing Tfh (PD1+ CXCR5+) cells compared to IL-4 expressing non-Tfh (PD1- CXCR5-) cells (Figure 34a). In support of a role for S1P1 in our re-localization phenotype, GFP+ Tfh cells sorted from mice with inhibited Notch signaling had significantly higher expression of KLF2 compared to mock injected mice (Figure 34b). These data together confirm that the S1P1 signaling pathway is critical for Tfh re-localization in the absence of Notch signaling.

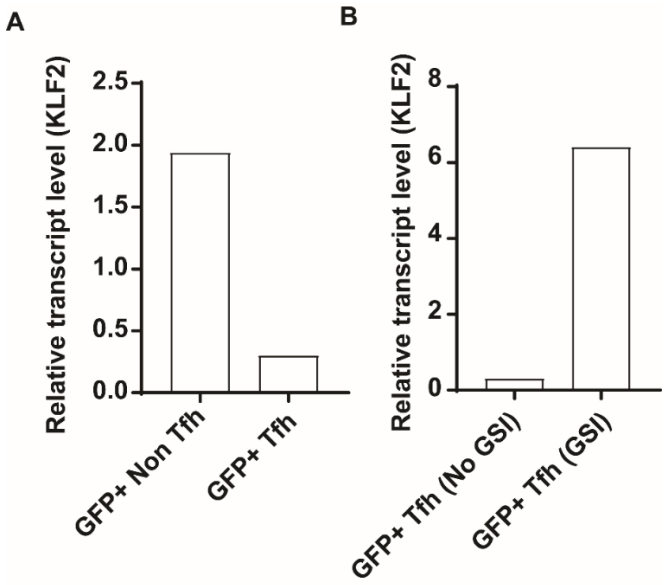

Figure 34: KLF2 expression is enhanced in Tfh cells from mice administered GSI.

IL4$^{4get/4get}$ mice were immunized with 100 ug OVA in alum in the footpad. GSI or a mock injection was administered on days seven, eight, and nine. On day ten, the popliteal lymph node was harvested for cell sorting. IL-4 expressing Non Tfh (CD4+ GFP+ PD1- CXCR5-) and IL-4 expressing Tfh (CD4+ GFP+ PD1+ CXCR5+) cells were sorted. **(A)** Expression of KLF2 in IL-4 expressing Non Tfh and Tfh cells that received mock injections. **(B)** Expression of KLF2 in IL-4 expressing Tfh cells that received either a mock injection (No GSI) or GSI injections. KLF2 expression was calculated relative to β-actin.

4.5 Loss of Notch signaling in established Tfh cells results in a loss of Tfh transcriptional program

While the data to this point suggests Notch may control Tfh fate by regulating the expression of important trafficking molecules, it remains unknown whether Notch may also regulate the expression of important Tfh transcriptional factors. BCL6, BATF, IRF4, and cMaf all play important roles in Tfh cell differentiation [23,384,260,278,280,277]. Many of these factors have also been suggested to play a role in IL-4 expression by Tfh

cells by binding to the CNS2 enhancer in the IL-4 locus [23,277]. Previous studies have shown that these factors work cooperatively to drive Tfh fate and IL-4 production. Currently, there exist no studies that have investigated if, and how, Notch may fit into this important circuit of transcriptional factors in the context of Tfh differentiation and function.

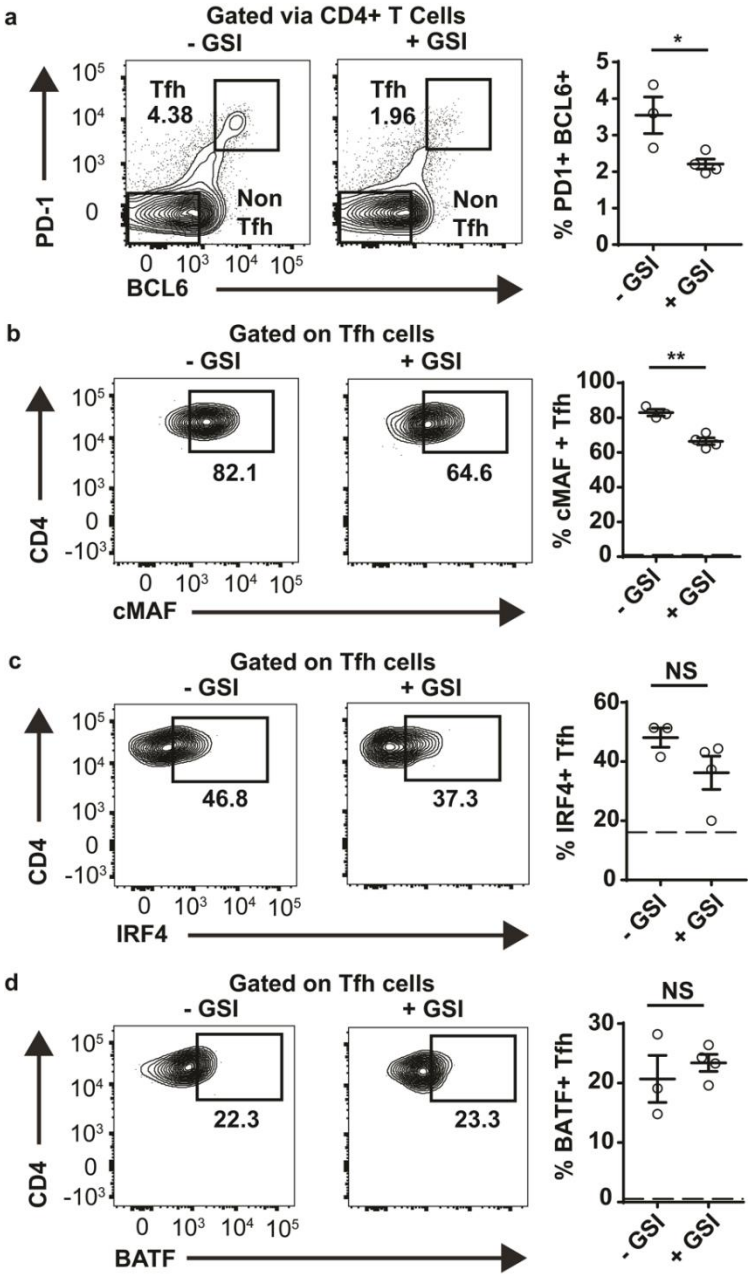

Figure 35: Mice treated with GSI have an altered transcriptional program in their Tfh cells.

IL4$^{4get/KN2}$ mice were immunized with OVA emulsified in alum. Seven days post immunization mice were given a control injection (n = 3) or Notch inhibitor (GSI) (n = 4) on days seven, eight, and nine. On day ten, the popliteal lymph node was harvested for analysis of intracellular transcription factor expression by flow cytometry. **(a)**

Representative flow cytometry plots of CD4+ T cells gated on Tfh cells (PD1+ BCL6+) or Non Tfh (PD1- BCL6-) cells. Graphs show percentage PD1+ BCL6+ CD4+ T cells. **(b, c, d)** Contour plots gated through Tfh cells as shown in **(a)** and showing expression of cMAF **(b)**, IRF4 **(c)**, and BATF **(d).** Graphs show the percent of Tfh cells that are cMAF, IRF4, or BATF positive. Dashed lines indicate the expression of these factors in the Non Tfh population gated in **(a)**. Error bars represent +/- SEM. Data shown in **(a)** and **(b)** is representative of two independent experiments. Data shown in **(c)** and **(d)** represent a single experiment. *P < 0.05, ***P* < 0.01, (unpaired two-tailed T-test).

To assess if Notch signaling influences these Tfh lineage-factors, we again generated Tfh cells using ovalbumin in alum immunization and gave GSI or a control injection on days seven, eight, and nine before performing intracellular transcription factor staining. In support of our previous data, administration of GSI led to decreased Tfh differentiation as assessed by expression of BCL6, the Tfh master regulator, and PD1 on CD4+ T cells (Figure 35a). GSI treatment led to decreased c-Maf expression among BCL6+, PD1^{high} Tfh cells by percent and MFI (Figure 35b; Figure 36b), while the expression of IRF4 and BATF was not significantly changed (Figure 35c, d; Figure 36c, d). C-Maf is known to regulate IL-4 expression in Th2 cells, but its role in Tfh-driven IL-4 expression is unclear [260]. This data further supports a role for Notch signaling as an important regulator of Tfh fate and program and suggests Notch might play a role in regulating the expression of c-Maf in Tfh cells.

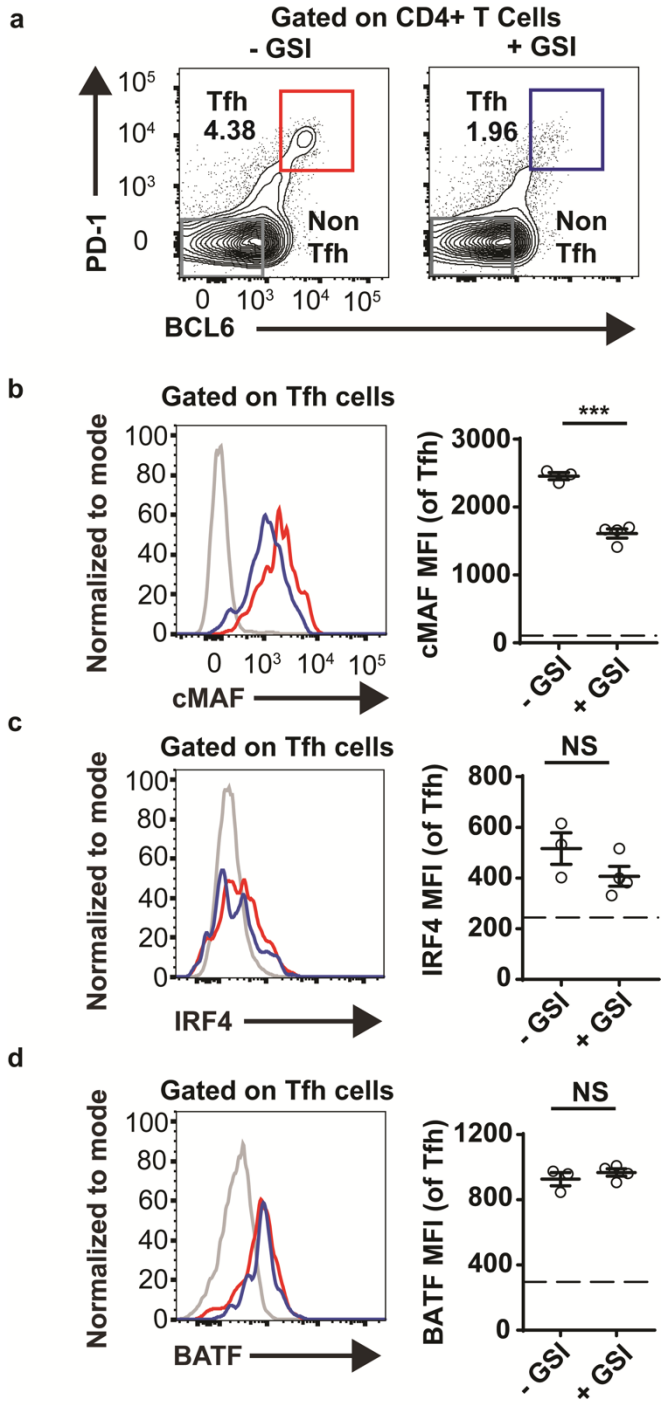

Figure 36: GSI-treatment results in reduced expression of cMAF in Tfh cells.

IL4$^{4get/KN2}$ mice were immunized with OVA emulsified in alum. Seven days post immunization mice were given a control injection (n = 3) or Notch inhibitor (GSI) (n = 4) on days seven, eight, and nine. On day ten, the popliteal lymph node was harvested for

analysis of intracellular transcription factor expression by flow cytometry. **(a)** Representative flow cytometry plots of CD4+ T cells gated on Tfh cells (PD1+ BCL6+) or Non Tfh (PD1- BCL6-) cells. **(b, c, d)** Histograms gated through Non Tfh (gray), Tfh cells from mice given a control injection (red), and Tfh cells from mice given GSI (blue) cells as shown in **(a)** and showing expression of cMAF **(b)**, IRF4 **(c)**, and BATF **(d).** Graphs show quantified MFI of cMAF, IRF4, or BATF in the gated Tfh populations as shown in **(a).** Dashed lines indicate the expression of these factors in the Non Tfh population gated in **(a)**. Error bars represent +/- SEM. Data shown in **(a)** and **(b)** is representative of two independent experiments. Data shown in **(c)** and **(d)** represent a single experiment. ***$P < 0.001$ (unpaired two-tailed T-test).

4.6 Temporal genetic ablation of Notch receptors results in altered profile of IL-4 producing CD4+ T cells in the lymph node

While our work using GSI shows a clear re-localization phenotype of IL-4 expressing CD4+ T cells with inhibited Notch signaling, we cannot currently confirm whether the IL-4 expressing cells accumulating in the T cell zone are Tfh cells that have migrated from the follicle to the T cell zone. Additionally, the use of an inhibitor does not allow for inhibition of Notch signaling specifically in the CD4+ T cell population. To better address some of our remaining questions, we developed a mouse line that allows for specific, temporally controlled deletion of Notch receptors specifically in transferred CD4+ T cells (Figure 37).

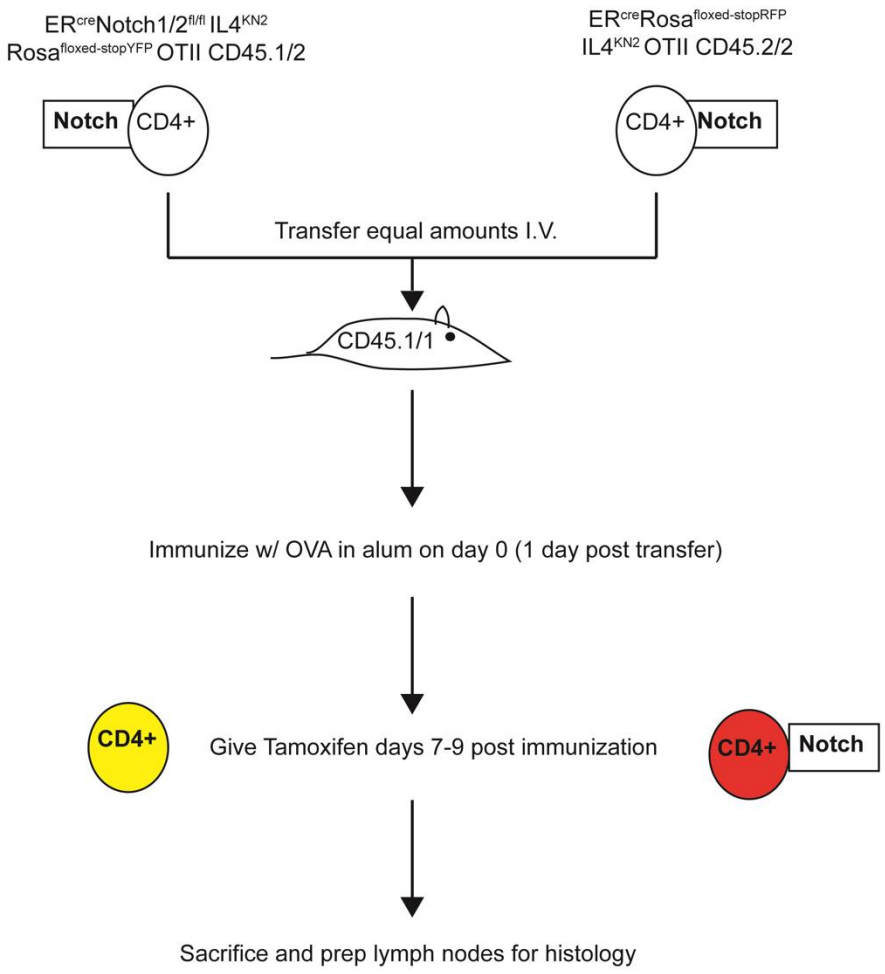

Figure 37: Model for temporal genetic ablation of Notch receptors in transferred OT-II CD4+ T cells

In brief, we crossed our Notch1/2$^{fl/fl}$ mice to ER^{cre} which will genetically delete Notch receptors only when mice are given injections of tamoxifen. Additionally, these mice were crossed onto a Rosa$^{floxed\text{-}StopYFP}$ line so that we could easily identify cells that had successfully undergone deletion of the Notch receptors. Furthermore, these were bred to OT2 mice so the CD4+ T cells expressed a transgenic TCR that was specific for ovalbumin. Finally, these mice were bred to our IL4^{KN2} reporter mouse to track the

production of IL-4 protein. These ER^{cre}Notch1/2$^{fl/fl}$Rosa$^{floxedStop\text{-}YFP}$IL4^{KN2}OT-II mice are congenically marked as CD45.1/2 allowing for easy detection of transferred cells in the recipient mouse. Notch sufficient control cells were obtained from ER^{cre}Rosa$^{floxed\text{-}StopRFP}$IL4^{KN2}OT-II mice which are congenically marked as CD45.2/2.

ER^{cre}Notch1/2$^{fl/fl}$Rosa$^{floxedStop\text{-}YFP}$IL4^{KN2} OT-II and ER^{cre}Rosa$^{floxed\text{-}StopRFP}$IL4^{KN2} OT-II CD4+ T cells were isolated and transferred for analysis in a competitive transfer setting (Figure 37). Briefly, isolated OT-II CD4+ T cells from these mice were transferred intravenously into a congenically marked host. One day post transfer, the host was immunized with OVA emulsified in alum in the footpad. Tamoxifen injections were administered on days seven, eight, and nine post-immunization to induce Notch deletion and YFP or RFP expression. Finally, the popliteal lymph node was harvested on day ten for analysis. Notch deficient CD4+ T cells did not have any defect in overall Tfh differentiation compared to Notch sufficient T cells (Figure 38a), suggesting that deletion of Notch receptors for a short period of time has no effect on the maintenance of the Tfh population.

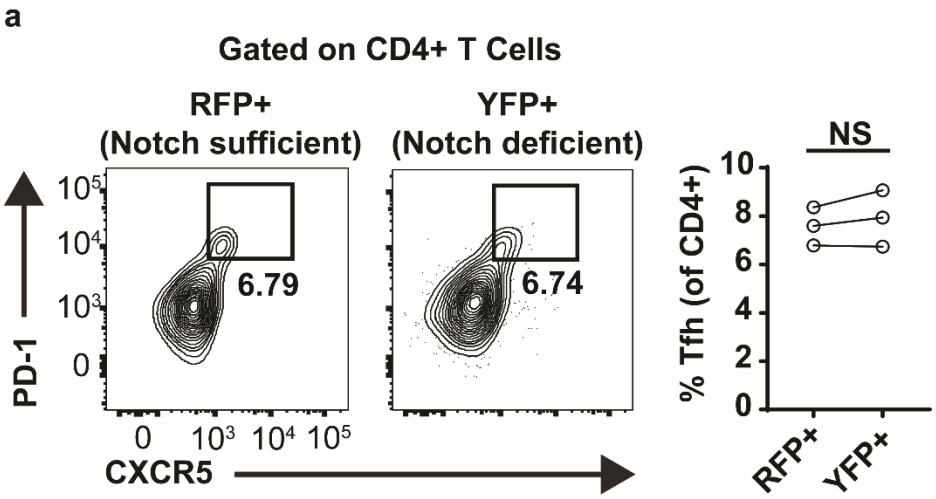

Figure 38: Late temporal deletion of Notch receptors on transferred CD4+ T cells had no effect on Tfh differentiation

CD4+ T cells were isolated from ER^{cre}Notch1/2$^{fl/fl}$Rosa$^{floxedStop\text{-}YFP}$IL4^{KN2} OT-II and ER^{cre}Rosa$^{floxed\text{-}StopRFP}$IL4^{KN2} OT-II mice and transferred in equal numbers into a host mouse. The host mouse was immunized and given injections of tamoxifen as shown in Figure 34. On day ten, the popliteal lymph node was harvested for flow cytometry. **(a)** Representative contour plot of CD4+ T cells that are RFP+ (Notch sufficient) or YFP+ (Notch deficient). Gate represents Tfh (PD1+ CXCR5+) cells. Percentage was quantified and graphed.

Furthermore, total Notch deficient cells had no impairment in their IL-4 production (Figure 39a). However, consistent with our GSI data, IL-4 producing CD4+ T cells with Notch deficiency had significantly reduced expression of canonical Tfh markers, PD-1 and CXCR5, compared to IL-4 producing Notch sufficient cells (Figure 39b). This data suggests that while Notch was not essential to maintain the Tfh population, that Notch does play an important role in maintaining the fate, and possibly localization, of the IL-4 producing CD4+ population.

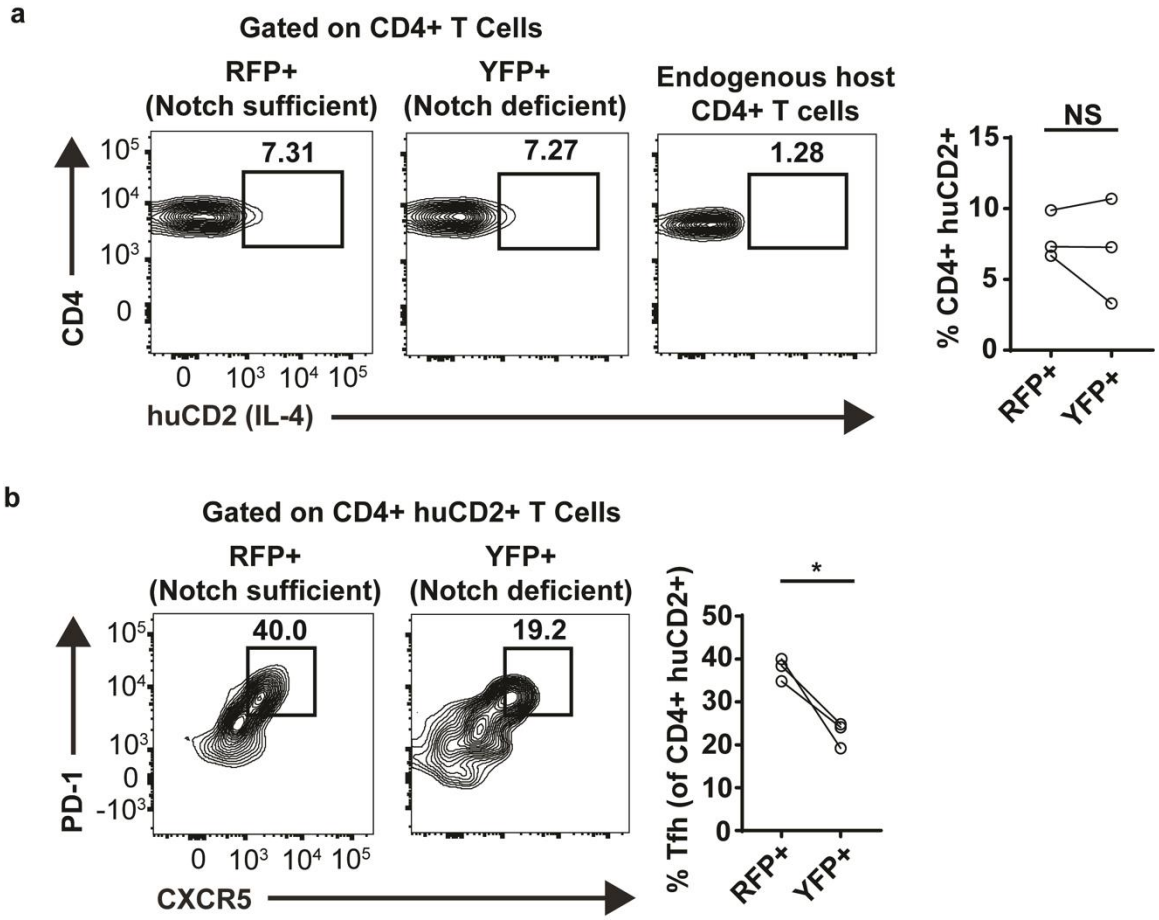

Figure 39: Temporal ablation of Notch signaling results in reduced expression of Tfh characteristic markers by IL-4 producing cells

CD4+ T cells were isolated from ER^{cre}Notch1/2$^{fl/fl}$Rosa$^{floxedStop\text{-}YFP}$IL4^{KN2} OT-II and ER^{cre}Rosa$^{floxed\text{-}StopRFP}$IL4^{KN2} OT-II mice. The cells were transferred into host mice, which were immunized, and given injections of tamoxifen per the experimental outline shown in Figure 37. After ten days post-immunization, the lymph node was harvested for subsequent flow cytometry analysis. **(a)** Representative contour plots of CD4+ T cells that are either Notch sufficient (RFP+), Notch deficient (YFP+), or from the endogenous host (RFP-, YFP-). Gates represent IL-4 producing CD4+ T cells. **(b)** Representative contour plots of IL-4 producing (huCD2+) CD4+ T cells of Notch sufficient and Notch deficient cells. Data is taken from one experiment. Error bars represent +/- SEM. *$P \leq 0.05$ (unpaired two-tailed T-test).

4.7 Genetic ablation of Notch receptors has a minor effect on markers of TCR-mediated activation

Previous studies have investigated the possibility that Notch signaling may play an important role during initial T cell activation and posit that Notch may serve as an important co-stimulatory molecule during TCR-mediated activation of naïve CD4+ T cells [326-328]. Notch signaling activity is heightened in CD4+ T cells which are activated non-specifically with anti-CD28 and anti-CD3, even in the absence of Notch ligand [326,327]. However, the outcome of the Notch signaling on T cell activation and proliferation is unclear. One study demonstrated that inhibition of CD4+ T cells with GSI during non-specific TCR activation leads to reduced activation as measured by CD69 expression [327]. Conversely, the other study discovered that over-expression of NICD in CD4+ T cells activated with anti-CD28 and anti-CD3 had an overall reduced activation phenotype [328].

To investigate the role of Notch signaling in regulating TCR-mediated activation of CD4+ T cells in our hands, we isolated CD4+ T cells from IL4^{4get}Notch1/2$^{fl/fl}$ (Notch sufficient) and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ (Notch deficient) and then activated the cells with varying concentrations of anti-CD3 in the presence or absence of CD28. Activation status of the cells was assessed by measuring the expression of CD69 and CD25 by flow cytometry. Consistent with previous reports, Notch sufficient cells showed enhanced expression of CD69 and CD25 as concentrations of anti-CD3 were increased (Figure 40a). Notch deficient CD4+ T cells didn't show any impairment in their activation by anti-

CD3, as measured by the expression CD69 (Figure 40a) or CD25 (Figure 40b), either in the presence or absence of CD28. These findings suggest that Notch receptors do not play an important role in regulating the early activation status of CD4+ T cells in the absence of ligand.

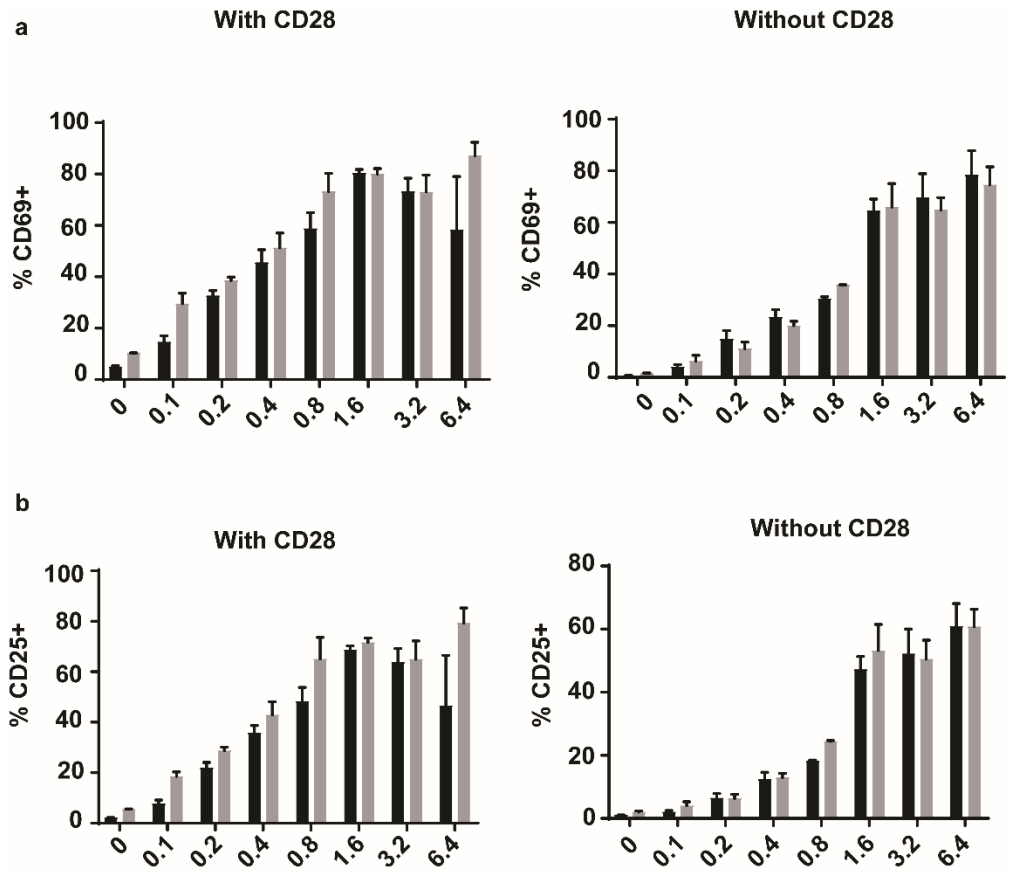

Figure 40: Deletion of Notch receptors has no effect on early TCR-mediated activation

CD4+ T cells were isolated from IL4^{get}Notch1/2$^{fl/fl}$ (Notch sufficient) and IL4^{get}CD4^{cre}Notch1/2$^{fl/fl}$ mice and cultured with varying concentration of anti-CD3 (μg/ml; x-axis) with or without 2 ug/ml anti-CD28. After sixteen hours the cells were isolated for flow cytometry and the expression of **(a)** CD69 and **(b)** CD25 was assessed in Notch sufficient (black bars) and Notch deficient (gray bars) cells. Error bars represent +/- SEM. Data is representative of two independent experiments.

To further investigate a role for Notch signaling during TCR-mediated activation, we cultured isolated Notch sufficient and deficient CD4+ T cells for an increased four-day time period. Previous work has demonstrated that the expression of BATF and IRF4 is enhanced in CD4+ T cells in an anti-CD3 dose-dependent fashion [276]. Consistent with these results, Notch sufficient cells enhanced expression of BATF and IRF4 with increasing anti-CD3 concentrations (Figure 41a, b). Interestingly, Notch deficient CD4+ T cells had reduced expression of BATF+ IRF4+ CD4+ T cells at low concentrations of anti-CD3; however, as anti-CD3 concentrations were increased Notch deficient cells showed comparable levels of BATF and IRF4 expression compared to Notch sufficient cells (Figure 41a, b). These findings suggest that Notch may play an important role at positively regulating CD4+ T cell activation in settings that promote lower levels of TCR activation, while high levels of TCR activation may overcome any need for Notch signaling to fully activate the CD4+ T cell.

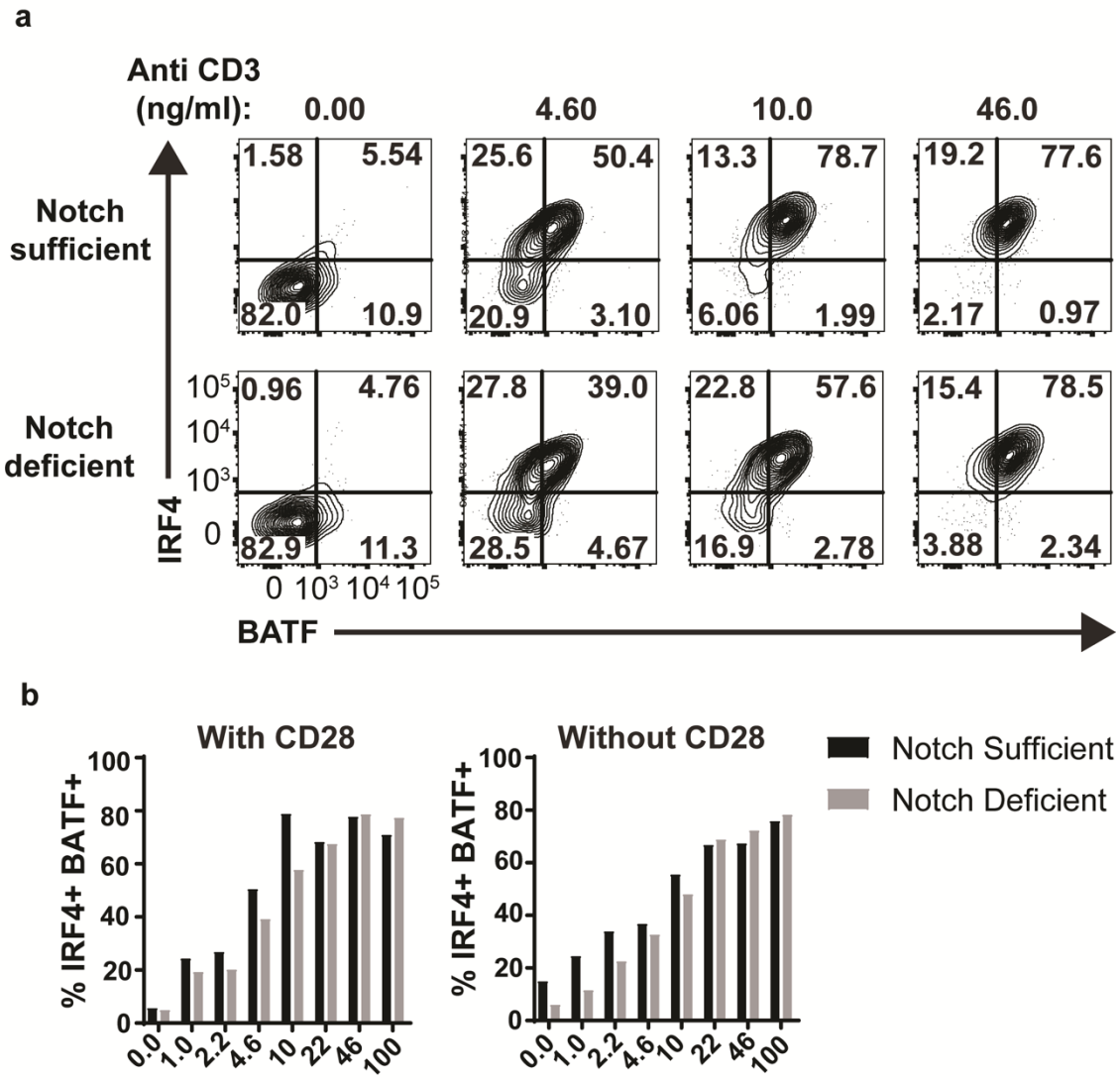

Figure 41: Notch deficient CD4+ T cells have reduced BATF and IRF4 expression upon TCR-mediated activation

CD4+ T cells were isolated from IL4^{4get}Noch1/2$^{fl/fl}$ (Notch sufficient) and IL4^{4get}CD4^{cre}Notch1/2$^{fl/fl}$ (Notch deficient) mice. Cells were than cultured with varying concentrations of anti-CD3 in the presence or absence of anti-CD28 for four days. **(a)** Representative contour plots of CD4+ T cells. Gates represents double negative (BATF- IRF4-), single positive (IRF4+ BATF- or IRF4- BATF+), or double positive (IRF4+ BATF+) cells. **(b)** Quantification of the percent of double positive cells as gated in **(a)**. Graphs show the percent of double positive cells cultured with or without anti-CD28 at varying concentration of anti-CD3 (ng/ml; x-axis).

Discussion

This work establishes, for the first time, a major role for Notch signaling in Tfh cells beyond initial Tfh differentiation. Here, we show that persistent Notch signaling is required in Tfh cells to drive Tfh function and to maintain Tfh fate. The differentiation of Tfh cells, as assessed by expression of Tfh markers PD1 and CXCR5, was reduced in mice whose Notch signaling was inhibited with GSI. In support of this finding, Notch inhibition led to the generation of fewer CD4+ T cells expressing the Tfh master regulator BCL6. In settings of Notch sufficiency, the majority of IL-4 expressing cells in the lymph node are Tfh cells. However, when Notch was inhibited, significantly fewer of these IL-4 expressing cells expressed Tfh fate markers, including major reductions in their expression of CXCR5. This reduction in CXCR5 led to an apparent re-localization of IL-4 expressing Tfh cells from the B-cell follicle to the T-cell zone.

While our work shows a clear localization phenotype of IL-4 expressing CD4+ T cells, we cannot currently confirm whether the IL-4 expressing cells accumulating in the T cell zone are Tfh cells that have migrated from the follicle to the T cell zone. To better answer this question, future studies will use the genetic ablation model described previously. This model will allow us to temporally ablate Notch receptor expression in antigen specific CD4+ T cells *in vivo* in competitive transfer settings. The use of a genetic model will be beneficial for multiple reasons. First, while inhibitor studies are very informative, they come with certain caveats. These caveats include potential off-target

effects as well as an inability to directly target the cells of interest. Another reason our genetic model will be helpful is that it will allow for simultaneous, real-time tracking of Notch deficient and Notch sufficient CD4+ T cells *in vivo.* The use of multi-photon microscopy will allow us to track these cells in real time to better determine the trafficking patterns of Notch deficient compared to Notch sufficient CD4+ T cells.

Our data confirms that Notch signaling is essential for Tfh differentiation and that continuous Notch signaling is important to maintain a Tfh fate. However, it is currently unclear how Notch signaling regulates the differentiating CD4+ T cells to promote and maintain a Tfh fate. Future studies should focus on elucidating the pathways through which Notch signaling exerts its affects to promote the Tfh program.

The studies shown here suggest that Notch signaling may promote Tfh fate by regulating the expression of certain trafficking receptors and subsequent cellular localization. Understanding how Notch signaling may regulate the expression of these trafficking receptors will need to be the focus of future studies. ASCL2 simultaneously upregulates CXCR5 and downregulates CCR7, leading to follicular localization of Tfh cells [282]. As a result, Tfh cells do not develop in the absence of ASCL2. Importantly, previous work has also demonstrated that ASCL2 has an RBPJ/NICD binding site and that canonical Notch signaling can drive the expression of ASCL2 during epidermal cell development [380]. These works taken together suggest a possible role for Notch signaling in regulating Tfh localization through upregulation of ASCL2 and

downstream regulation of CXCR5 and CCR7. In support of this idea, our work demonstrates that established Tfh cells with impaired Notch signaling have reduced expression of ASCL2. Investigations into the molecular interactions of NICD and its co-activators with ASCL2 in Tfh cells will be an important focus of future studies.

Another trafficking receptor that requires future investigation is S1P1. The re-localization of IL-4 expressing cells observed when Notch signaling is inhibited appears to require S1P1 signaling, as dual inhibition of S1P1 and Notch signaling led to normalized localization of IL-4 expressing CD4+ T cells. As further support of the involvement of S1P1 signaling pathway in our system, we observed a significant decrease in the expression of CD69, a negative regulator of S1P1 signaling, in IL-4 expressing cells whose Notch signaling was inhibited with GSI. Furthermore, an important positive regulator of S1P1, KLF2 [258,259], was enhanced in Tfh cells with inhibited Notch signaling. With this data taken together, our studies suggest that S1P1 may play an important role in regulating Tfh localization within the lymph node.

Many factors including BCL6, BATF, ASCL2, ICOS, and c-Maf have been deemed essential for Tfh cell development [23,262,277]. These factors are part of a developmental circuit which helps to determine Tfh cell fate [262]. However, Notch has largely been left out of this conversation despite its requirement in Tfh development [314]. Here, we show further evidence supporting a role for Notch in the Tfh developmental circuit, as inhibition of Notch signaling leads to decreased expression of

c-Maf in Tfh cells. Of note, BATF, c-Maf, and RBPJ have all been reported to bind at the CNS2 enhancer of the IL-4 locus [179,23,277,280]. Given the importance of the CNS2 region in the expression of IL-4 by Tfh cells [352,178], a likely mechanism as to how Notch signaling modulates IL-4 in Tfh cells is via NICD binding to RBPJ to modulate IL-4 expression. Whether and how RBPJ and NICD interact with c-Maf and BATF at this site is not known. As such, further work investigating how Notch selectively mediates cell fate and influences type-2 cytokine potential in the context of these other factors will be an important next step in understanding Tfh cell generation and function.

Notch signaling has been suggested to serve a co-stimulatory role during TCR-mediated activation of naïve CD4+ T cells [326-328]. Based on the current body of literature, it remains unclear whether Notch signaling positively or negatively regulates CD4+ T cell activation and proliferation. Our studies suggest that Notch signaling plays a positive role, as Notch deficient CD4+ T cells had reduced expression of BATF and IRF4 after non-specific activation. Elucidating how Notch signaling might serve in a co-stimulatory role during TCR-mediated activation is another avenue for future studies.

Other T helper subsets, including Th2 cells, become strongly polarized and their fate largely becomes cemented [160]. Tfh cells do not become strongly polarized and must require some exogenous signals in order to maintain their fate. While other factors have been previously shown to play critical roles in initial Tfh cell differentiation, little is known about the factors that are important in maintaining a Tfh fate. The work

presented here demonstrates an important role of Notch signaling not only in Tfh differentiation, but also in the maintenance of Tfh fate. In the absence of Notch signaling, IL-4 expressing cells down-regulated their expression of CXCR5 and appear to re-localize from the B-cell follicle to the T cell zone. Whether this is truly Tfh re-localization needs to be confirmed with future studies. Additionally, Tfh cells with inhibited Notch signaling have an altered transcriptional program compared to mice with active Notch signaling.

Notch receptors represent an intriguing target for future pharmacologic studies aiming to target Tfh cells and reduce their function. BCL6, ASCL2, cMAF, BATF, and IRF4 have all been shown to be required in Tfh differentiation [23,250,260,277,280,384]. However, BATF [23], cMAF [171], and IRF4 [385,386] also play important roles in regulating the differentiation and function of other T helper cell subsets. BCL6, ASLC2, and Notch appear to be selectively important for Tfh cells and are therefore the best targets for specifically manipulating Tfh cell populations. The problem with targeting BCL6 or ASCL2 is they exist in their entirety as intranuclear transcription factors. Historically, developing pharmacologic tools to target these factors has been very difficult. Thus, Notch presents a more promising target as it is expressed first as an extracellular receptor before being cleaved and translocating to the nucleus to activate downstream transcriptional targets. Notch ligands are also expressed on the cellular

surface and could represent interesting pharmacologic targets in addition to the Notch receptors.

5. Final conclusions and future directions

5.1 Concluding remarks: Notch in type-2 Immunity

The studies shown herein identify that Notch signaling represents an important step in the bifurcation of Th2 and Tfh differentiation. *N. brasiliensis* infection of mice with Notch deficient T cells resulted in a near complete loss of Tfh differentiation, while peripheral Th2 numbers were unaffected. As a result, peripheral Th2-mediated clearance of intestinal worms was intact, while IgE production, which requires Tfh cells, was abrogated. This finding suggested that Notch signaling is likely more important for regulating the Tfh, than the Th2 population. In support, over-expression of NICD on lung CD4+ T cells had no effect on their IL-4 production. Conversely, over-expression of NICD within the Tfh population resulted in significantly enhanced IL-4 production.

Which cells serve as important sources of Notch ligand for Tfh cells are unknown. Previous studies have identified that cDCs are required for the differentiation for Tfh cells under physiologic conditions. Elimination of functional Notch ligands on cDCs by deletion of MIB1 led to impaired Tfh differentiation early during infection but these cells proved to be ultimately dispensable as sources of ligand as the infection proceeded. Previous work identified stromal cells as important sources of Notch ligand during Tfh differentiation. Specifically, DLL4 but not DLL1 was essential for Tfh differentiation. Exactly which stromal cell type was important to provide DLL4 was still unclear. FDCs, like Tfh cells, reside in the light zone of GCs and were a strong candidate

as required sources of Notch ligand for Tfh cells. However, deletion of MIB1 on FDCs had no effect on Tfh differentiation or function. Thus, our studies indicate that non-FDC stromal sources can provide Notch ligand to Tfh cells to direct Tfh differentiation.

While previous studies, as well as our work shown here, demonstrates that Notch is important for Tfh differentiation it wasn't certain if Notch signaling played any role after Tfh differentiation. We present here, for the first time, evidence that continuous Notch signaling is essential to maintain Tfh fate and function beyond initial Tfh differentiation. Inhibition of Notch signaling with GSI after Tfh differentiation led to reductions in the Tfh population and Tfh IL-4 production. We also demonstrated that IL-4 expressing T cells with reduced Notch signaling have a reduced Tfh phenotype and abnormal localization.

Taken together, our data suggests a model in which Notch signaling represents an important bifurcation between Th2 and Tfh mediated immunity. Here we suggest a model in which the presence of ample Notch signaling during Th differentiation promotes Tfh fate, while a dearth of Notch signaling encourages Th2 fate in the context of type-2 immunity (Figure 42). Furthermore, we demonstrate the importance of continued Notch signaling for the maintenance of Tfh fate and function beyond initial Tfh differentiation, whereby, loss of Notch signaling after Tfh differentiation results in a loss of Tfh fate (Figure 42).

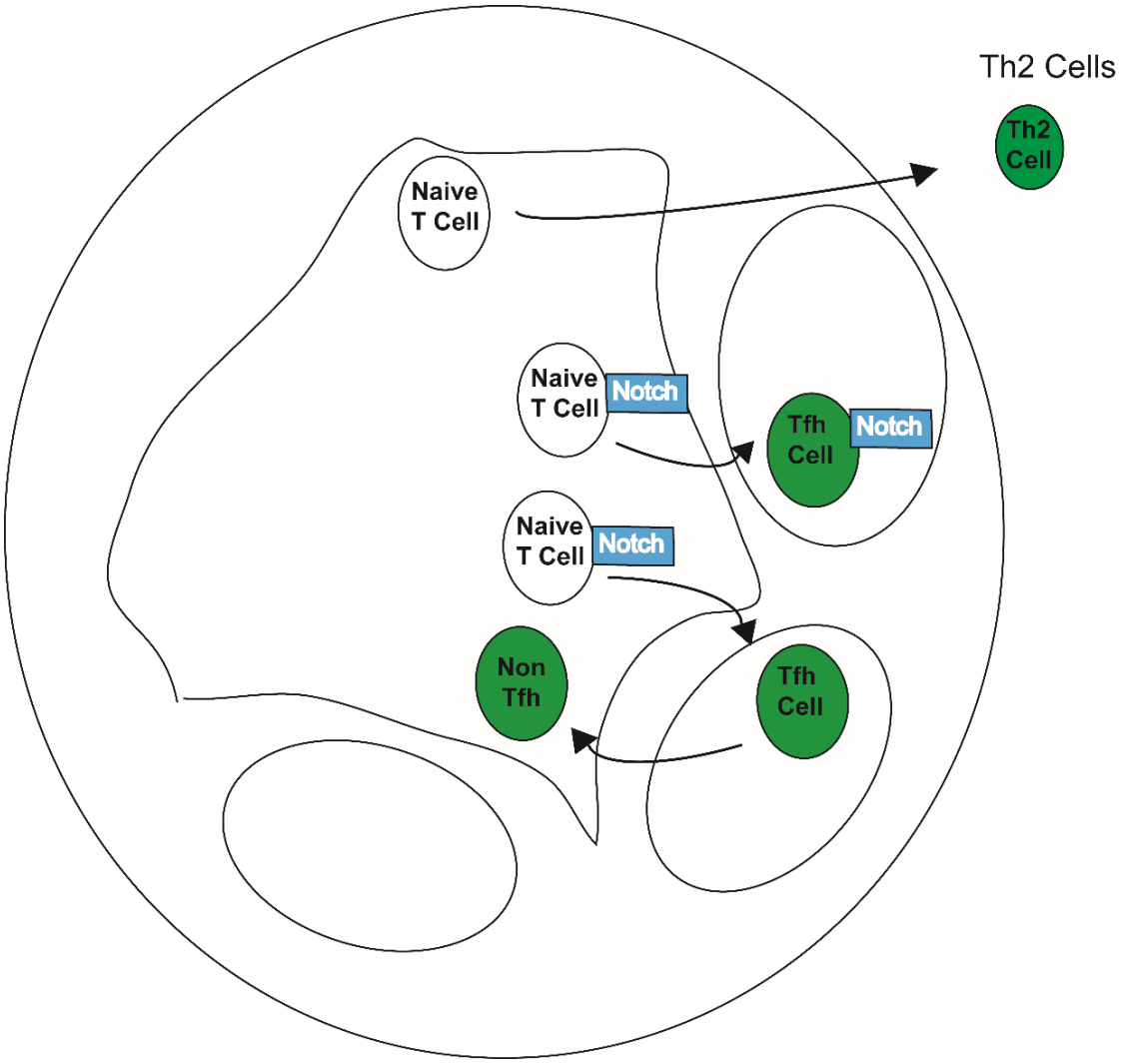

Figure 42: Updated model for the role of Notch signaling in type-2 immunity and T helper cell differentiation

Notch signaling plays an essential role during Th differentiation. Our previous work shows that Notch signaling is essential for IL-4 expressing (green) Tfh differentiation, but is dispensable for IL-4 expressing Th2 cell differentiation. Furthermore, continuous Notch signaling is essential to maintain a Tfh fate. When Notch signaling is lost, IL-4 expressing cells in their lymph node lose their Tfh phenotype and may re-localize from GC back to T cell zone. Future studies are required to formally establish that Tfh cells are truly re-localizing upon loss of Notch signaling.

5.2 Future directions: How does Notch signaling promote Tfh fate and function

The mechanism through which Notch exerts its effects on Tfh cells is not known, but we have identified several pathways Notch may modulate to promote Tfh fate and function. Currently, our studies point to three potential pathways that Notch may regulate during Tfh differentiation, maintenance, and function but we cannot conclusively identify the pathway or pathways Notch modulates. The three areas of focus for future studies should be to: assess the role of Notch in regulating Tfh cell localization, determining whether Notch signaling is essential to modulate the Tfh transcriptional program, and to identify if Notch receptors play a significant role in modulating TCR signaling. One of the major caveats of the data shown here is that some of it relied on the use of a pharmacologic inhibitor to reduce Notch signaling. While inhibitors are useful tools, they can have off target effects and do not allow for cell specific inhibition. Therefore, future studies investigating the mechanistic role of Notch signaling in Tfh fate and function should use genetic models, rather than pharmacologic inhibition, to specifically modulate Notch signaling in T cells.

5.2.1 Determine if Notch signaling regulates cellular localization of Tfh cells

The most promising mechanism through which Notch signaling may promote Tfh fate is by regulating the expression and activity of important trafficking receptors. In support, activated IL-4 expressing CD4+ T cells had reduced expression of the important

follicular homing chemokine receptor, CXCR5. Consistent with this reduced expression of CXCR5, we observed abnormal localization of IL-4 expressing T cells within the lymph node. Specifically, mice with inhibited Notch signaling had a greater accumulation of IL-4 expressing CD4+ T cells within the T cell zone compared to mice with intact Notch signaling. This finding suggested, but could not formally prove, that Tfh cells with impaired Notch signaling may re-localize back to the paracortex.

To better determine whether Notch signaling is essential to maintain appropriate Tfh localization, we will use mice that we have generated that allow for temporal deletion of Notch receptors in transferred OT-II T cells (ER^{cre}Notch1/2$^{fl/fl}$Rosa$^{floxedStop-YFP}$IL4^{KN2} OT-II and ER^{cre}Rosa$^{floxedStop-RFP}$IL4^{KN2} OT-II). Our genetic system allows for temporal deletion of Notch receptors and subsequent tracking of Notch deficient (YFP+) and Notch sufficient (RFP+) antigen specific cells in a competitive transfer system. Briefly, CD4+ T cells are isolated from these mice followed by OVA/alum immunization the next day. After seven days, tamoxifen injections are administered to induce deletion of Notch receptors and/or expression of YFP (Notch deficient) or RFP (Notch sufficient). Early studies with these mice have demonstrated that deletion of Notch receptors after Tfh differentiation had no effect on the percentage of OT-II T cells differentiating into Tfh cells. Furthermore, there was no reduction in the percentage of IL-4 producing cells. However, deletion of Notch receptors led to a significant reduction in the percentage of IL-4 producing cells that exhibited a Tfh phenotype. This finding suggests that Notch

signaling may play a significant role in regulating the localization of IL-4 producing cells within the lymph node.

Continued studies with these mice will be informative to formally determine the role of Notch in regulating the localization of differentiating cells. First, histology should be performed to visualize whether Notch deficient (YFP+) cells are more likely to localize to the paracortex compared to Notch sufficient (RFP+) cells. Our current flow cytometry data suggests that deletion of Notch receptors appears to most affect the IL-4 producing population. Therefore, we will need to set up an experiment that allows us to specifically assess the localization of IL-4 producing cells. We may need to perform these experiments in non-competitive settings as staining for huCD2 (IL-4 producing cells) must be done in fresh-frozen, non-fixed lymph node sections. In contrast, RFP and YFP expression can only be visualized in fixed lymph node sections. Importantly, Notch sufficient and deficient cells both contain at least one allele of CD45.2. Therefore, we can use fresh-frozen sections to stain for huCD2 and also stain sections for CD45.2 to determine localization of transferred cells. Since both Notch sufficient and deficient cells have an allele of CD45.2, this experimental setup requires a non-competitive transfer setting.

To definitively prove that Notch signaling plays an essential role in regulating T cell localization, we need to visualize T cell trafficking *in vivo.* To perform this, we will use floxed-photo-activatable-GFP (PA-GFP) mice. These mice allow for expression of a

special GFP molecule in cre-recombinase expressing cells that only fluoresces once activated at a certain wavelength with 2-photon microscopy. We will cross our PA-GFP$^{fl/+}$ mice to ER^{cre}Notch1/2$^{fl/fl}$ OT-II mice so that we can temporally delete Notch receptors in transferred T cells followed by photoactivation. Control (Notch sufficient) cells will be isolated from ER^{cre}PA-GFP$^{fl/+}$Rosa$^{floxed\text{-}stop\text{-}RFP}$ OT-II mice. Cells will be transferred into a wild-type host and immunized one day post transfer. On day seven, eight and nine mice will be given injections of tamoxifen. On day ten, we will perform intravital 2-photon microscopy to photoactivate T cells. Use of 2-photon microscopy to photoactivate will allow us to specifically photoactivate cells within the B-cell follicle or GC in an intact lymph node. After photoactivation, minor surgery will be performed on the host animal and it will be returned to the animal facility. Three days post photo-activation, we will isolate the lymph node and perform histology to determine the localization of GFP+ (photoactivated Notch deficient T cells) and GFP/RFP double positive cells (photoactivated Notch sufficient T cells).

The transcription factor ASCL2 has been shown to positively regulate CXCR5 and negatively regulate CCR7 expression to promote follicular localization of Tfh cells [282]. Importantly, Notch signaling has been demonstrated to directly enhance ASCL2 expression in mouse epithelial cells [380]. Taken together, we hypothesized that Notch signaling may regulate ASCL2 in Tfh cells. In support, mice with inhibited Notch signaling have reduced expression of ASCL2. Therefore, Notch signaling may promote

follicular localization of Tfh cells by positively regulating ASCL2. To further investigate this possibility, we will use our competitive transfer system to assess the expression of ASCL2, CXCR5, and CCR7. Additionally, we will perform *in vitro* transwell studies with Notch deficient and sufficient T cells using CCL19/CCL21 and CXCL13; which bind CCR7 and CXCR5, respectively. If our hypothesis is accurate, we should see reduced expression of ASCL2 and CXCR5 and enhanced expression of CCR7 in Notch deficient T cells. This chemokine receptor expression profile should result in preferential migration towards CCL19/21, rather than CXCL13.

A previous study has demonstrated NICD/RBPJ binding at the ASCL2 locus in epithelial cells by using chromatin immunoprecipitation (CHiP) followed by sequencing at the ASCL2 locus [380]. Use of this technique in Tfh cells could definitively define whether NICD/RBPJ is binding at the ASCL2 locus during Tfh differentiation. A positive result would suggest that Notch signaling promotes follicular localization by positively regulating ASCL2 expression. It would be interesting to assess binding of RBPJ at this locus both during the early phase of Tfh differentiation and within fully differentiated Tfh cells. By assessing early and late time points, we would be able to better identify whether Notch signaling is important not only during Tfh differentiation but also if persistent Notch signaling is important to maintain ASCL2 expression and follicular localization.

Mice that allow for selective and temporal enhancement of Notch signaling in CD4+ T cells will be generated to functionally assess a cooperative role of NICD and ASCL2. Here, I propose the generation of IL4^{KN2}ER^{cre}Rosa$^{floxed\text{-}stop\text{-}NICD\text{-}GFP}$ OT-II and IL4^{KN2}ER^{cre}Rosa$^{floxed\text{-}stop\text{-}RFP}$ OT-II mice. With these mice, we can assess the specific effect of NICD over-expression at early/steady state (no immunization), middle (during Tfh differentiation), and late (post Tfh differentiation) time points in a competitive transfer setting. If our hypothesis is correct, cells over-expressing NICD should have enhanced expression of CXCR5 and will preferentially localize to the B cell follicle. To assess the involvement of ASCL2 in this system we will use this over-expression system with ASCL2 deficient T cells (IL4^{KN2}ER^{cre}Rosa$^{floxed\text{-}stop\text{-}NICD\text{-}GFP}$ ASCL2$^{-/-}$ OT-II). If we see the reversal of a phenotype, this would suggest that NICD is functioning through ASCL2 to drive CXCR5 and follicular localization. By investigating NICD/ASCL2 cooperation at early and late time points we can assess whether NICD is capable of inducing a Tfh like phenotype in the absence of a TCR stimulus and if NICD continues to regulate the Tfh phenotype beyond initial differentiation. The IL-4 producing populations in the lymph node appear to be the cells most affected by alteration in Notch signaling so addition of the IL4^{KN2} reporter may be necessary to fully identify the phenotype.

S1P1 signaling is essential for T cell egress from lymph nodes out to the periphery and the expression of S1P1 is reduced in Tfh cells. Here, we show that simultaneous inhibition of S1P1 and Notch signaling resulted in a return to normal

localization of IL-4 expressing CD4+ T cells in the lymph node compared to mice with inhibition of Notch signaling alone. Consistent with this finding, we observed enhanced expression of S1P1 in Tfh cells with inhibited Notch signaling. Furthermore, activated IL-4 expressing cells had reduced expression of CD69, which negatively regulates the signaling potential S1P1. KLF2 positively regulates S1P1 function and loss of KLF2 is essential for Tfh differentiation [257-259]. In addition, previous work has identified that KLF2 is capable of directly repressing Notch1 expression in human cell lines [387]. Here, we show enhanced expression of KLF2 in Tfh cells whose Notch signaling has been inhibited. Studies analyzing whether Notch signaling directly regulates KLF2 are of great interest. The expression of KLF2 and Notch may exist on an axis during Th differentiation. If this is true, KLF2 and Notch signaling may work similarly to BCL6 and PRDM1, which function as antagonists of each other to direct differentiating cells towards Tfh or peripheral Th fate, respectively. It would be interesting to use CHiP-seq analysis to determine whether Notch/RBPJ binds and modulates expression at the KLF2 locus in mouse and human Tfh cells.

Based on our data, S1P1 signaling is important for the re-localization of IL-4 expressing cells when Notch signaling is inhibited. It would be informative to repeat our S1P1 inhibition experiment using the Notch1/2 temporal genetic deletion model that we have generated (ER^{cre}Notch1/2$^{fl/fl}$Rosa$^{floxedStop\text{-}YFP}$IL4^{KN2} OT-II and ER^{cre}Rosa$^{floxedStop\text{-}RFP}$IL4^{KN2} OT-II). If we observe abnormal localization of transferred Notch deficient cells (YFP), it

would be interesting to determine if that localization defect is restored if S1P1 signaling is also impaired. We will administer FTY720, to inhibit S1P1, at the same time tamoxifen is given to delete Notch receptors. A reversal in phenotype would suggest that S1P1 is essential for the re-localization of IL-4 producing cells.

Taken together, our data suggests a model in which Notch directs Th cell differentiation by regulating the expression and activity of chemokine trafficking receptors (Figure 43). Specifically, Notch regulates Tfh localization by manipulating S1P1 signaling potential through modulation of KLF2 and CD69 expression. Additionally, our data suggests Notch signaling may drive ASCL2 expression to subsequently enhance CXCR5 and reduce CCR7 expression. These combined actions generate a trafficking receptor profile that promotes follicular localization. Further work investigating how Notch signaling regulates these factors is critical for future studies.

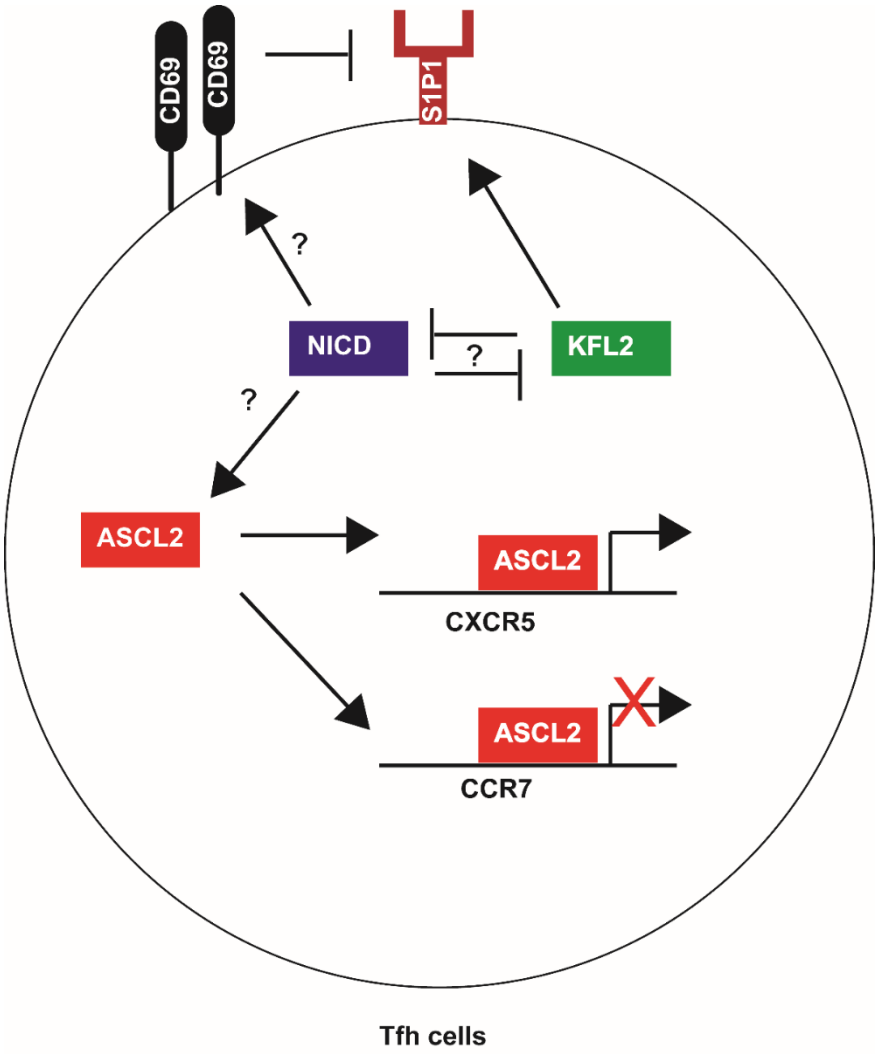

Figure 43: Model for Notch regulation of follicular localization

ASCL2 is essential in Tfh cells to drive CXCR5 expression and repress expression of CCR7. Notch signaling (NICD) may drive ASCL2 expression to promote Tfh fate. S1P1 is promotes T cell egress from follicles and lymph nodes. KLF2, which blocks Tfh differentiation, enhances S1P1 expression and CD69 inhibits S1P1 activity. Mice with impaired Notch signaling have reduced CD69 expression and enhanced expression of KFL2. In sum, our data suggests that Notch signaling may modulate the expression of certain trafficking receptors to promote Tfh fate.

5.2.2 Identify whether Notch signaling is important to initiate the Tfh transcriptional program

Another mechanism through which Notch might promote Tfh maintenance is by regulating the expression of transcription factors important for Tfh fate and function. BATF, IRF4, and cMAF have been previously identified as factors essential for Tfh differentiation and Tfh IL-4 production [23,277,279,280,384,386]. Notch signaling has largely been left out of the larger conversation in terms of factors important in regulating Tfh differentiation and function. Here, we show additional evidence that Notch signaling fits into the transcriptional circuit of Tfh cells (Figure 44). Tfh cells with inhibited Notch signaling had normal expression of BATF and IRF4, but had substantially reduced expression of cMAF. Determining how Notch signaling regulates cMAF in Tfh cells is of interest for future investigation.

Previous work has shown that RBPJ and cMAF proteins do not interact directly but whether NICD/RBPJ can bind at the cMAF locus has yet to be investigated [341]. ChIP of RBPJ would confirm whether Notch is capable of directly regulating cMAF. In the event that RBPJ does not bind at the cMAF locus, Notch signaling could still regulate cMAF in a non-canonical or indirect fashion. Our data shows that over-expression of NICD enhances the expression of BCL6 in lymph node CD4+ T cells. Previous work has demonstrated that cMAF and BCL6 work cooperatively early during the differentiation process to promote Tfh fate [280]. Additionally, cMAF expression is essential for Tfh differentiation [278,280]. By crossing our $ER^{cre}Rosa^{floxed\text{-}stop\text{-}NICD\text{-}GFP}$ mice with $cMAF^{fl/fl}$ OT-

II mice, we can determine whether NICD promotion of BCL6 expression requires cMAF. Additionally, we can determine whether NICD over-expression is sufficient to drive Tfh differentiation despite deficiencies in cMAF expression.

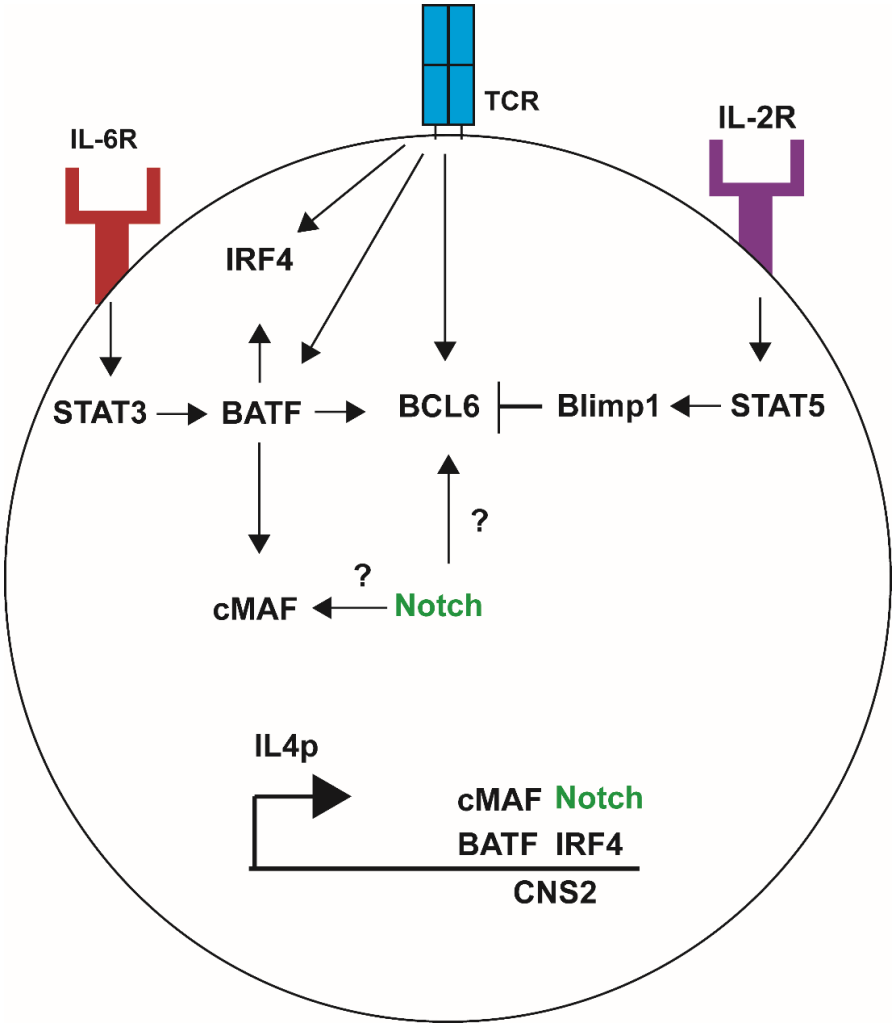

Figure 44: Updated model of Tfh transcriptional network

This schematic is an updated model of that shown in Figure 4 and shows how Notch fits into the Tfh transcriptional network. cMAF is essential for Tfh differentiation and Notch signaling may be important for enhancing cMAF expression during Tfh differentiation. Furthermore, Notch signaling may play a role in regulating BCL6 expression. Deletion of Notch signaling results in reduced BCL6 expression, while enhanced Notch signaling promotes BCL6 expression. How Notch signaling might regulated cMAF and BCL6 has yet to be determined.

5.2.3 Assess if Notch signaling regulates TCR-mediated activation

The final mechanism through which we proposed that Notch signaling might promote Tfh differentiation is by regulating TCR related signals. Previous work has identified that Notch receptor expression and signaling is enhanced upon TCR engagement [326-328]. Two groups have investigated the role Notch signaling plays during TCR activation; the first determined that inhibition of Notch resulted in reduced T cell activation and proliferation [326,327], while the second observed that co-ligation of Notch signaling during TCR activation led to reduced peripheral T cell activation and proliferation [328]. These results are in direct contradiction, but could in part be explained by the methods by which these groups chose to manipulate Notch signaling. To assess the involvement of Notch signaling during TCR activation in our hands, we cultured Notch1/2-sufficient and -deficient T cells in the presence of anti-CD3 and anti-CD28. In our studies, we found no difference in the early activation status of Notch1/2-deficient T cells as measured by CD25 and CD69 expression. However, we did observe a defect in the expression of BATF and IRF4; which are typically enhanced in T cells upon TCR stimulation in a dose dependent fashion. This finding suggests that Notch signaling may indeed play a role in modulating downstream TCR activity.

To directly assess the role of Notch signaling in regulating TCR signaling strength, I recommend the generation of CD4^{cre}Notch1/2$^{fl/fl}$ OT2 Nur77^{GFP} (Notch deficient) and Notch1/2$^{fl/fl}$ OT-IINur77^{GFP} (Notch sufficient) mice. Nur77^{GFP} mice

faithfully report TCR signaling strength, with high levels of Nur77^{GFP} expression reporting high levels of TCR signaling strength [388]. With this model, we could determine in competitive transfer settings whether Notch sufficient and Notch deficient cells have differences in TCR signaling strength. This model is ideal as the T cells will be on the OT-II background, therefore Notch sufficient and deficient T cells will express identical TCRs and we could directly assess the role of Notch signaling in regulating TCR signaling strength.

Dwell time is one of the characteristics thought to be important in regulating overall TCR signaling strength. Importantly, we have generated tools that will allow us to determine whether Notch signaling can regulate pMHC:TCR dwell time. We have generated CD4^{cre}Notch1/2$^{fl/fl}$ OT-II mice and Notch1/2$^{fl/fl}$ OT-II mice. I propose isolation of CD4+ T cells from these mice followed by labelling these cells with fluorescent dyes and adoptive transfer into the same host. Next, we will isolate DCs incubate them with OVA peptide and transfer them into the host. By differentially labelling the CD4+ cells we will be able to visualize the interactions of Notch sufficient and Notch deficient T cells with peptide-loaded DCs in a competitive system by using 2-photon microscopy.

Another method to assess whether Notch signaling helps to regulate dwell-time would be to use APCs that do not express functional Notch ligands. To this end, we will use CD11c^{cre}MIB1$^{fl/fl}$ (Non-functional Notch ligands) and MIB1$^{fl/fl}$ (Functional Notch ligands) mice in a competitive transfer experiment. Briefly, OT-II CD4+ T cells will be

isolated, dye labelled, and transferred into a host mouse. The next day, DCs will be isolated from CD11c^{cre}MIB1$^{fl/fl}$ and MIB1$^{fl/fl}$ mice and labelled differentially with fluorescent dyes. Following labelling, the DCs will be incubated with OVA peptide and transferred in equal numbers into the host mouse. The following day, the popliteal lymph node will be harvested and 2-photon microscopy will be performed to analyze contacts between OT-II T cells with CD11c^{cre}MIB1$^{fl/fl}$ and MIB1$^{fl/fl}$ DCs.

As described previously, TCR signaling has been shown to play an influential role in Th cell differentiation and Notch signaling. Mice expressing different transgenic TCRs (OT-II, TEa, or SM1) produce different patterns of Th effector cell differentiation when infected with *L. monocytogenes* expressing peptide specific for their TCRs [145]. OT-II and SM1 mice generated an effector pool that had significantly larger numbers of Tfh cells compared to Th1 cells, while TEa mice had an effector pool that was Th1 dominant [145]. Since it appears that the outcome of Th differentiation does rely in some part on TCR signaling strength and Notch signaling might regulate TCR signaling strength, it would be interesting to see whether manipulations in Notch signaling strength could alter the outcome of Th differentiation in these TCR transgenic T cells. For example, I propose the generation ER^{cre}Rosa$^{floxed\text{-}stop\text{-}NICD\text{-}GFP}$ TEa TCR transgenic mice. Upon infection, TEa mice generate an effector population that has greater numbers of Th1 than Tfh cells; it would be interesting to see if over-expression of NICD leads to an effector pool that is now more dominated by Tfh than Th1 cells.

5.3 Tfh cells and Notch signaling in humans and disease

Similar to mice, human Tfh cells play critical roles in regulating antibody responses by instructing maturing GC B cells. Human and mouse Tfh cells express many of the same markers including: PD-1, ICOS, CXCR5, and BCL6. However, humans, unlike mice, have a unique population of CXCR5+ Tfh cells found circulating in the vascular system. These cells do not express BCL6 but are characterized and split into various subsets by their differential expression of PD-1, ICOS, CXCR3, and CCR6 [389]. Functionally, these cells are able to induce antibody secretion when cultured with B cells *in vitro* [389]. However, the functional role of circulating Tfh cells *in vivo* is currently unknown. Whether these cells can re-enter lymph nodes to provide help to maturing B cells is uncertain. Discovering when/if these cells re-enter lymph nodes and which signals dictate their migration could help to identify their functional relevance *in vivo.*

Due to the importance of chemokine receptors in regulating cellular localization, it would be interesting to determine if circulating Tfh cells have reduced expression of ASCL2; leading to comparatively lower expression of CXCR5 and heightened expression of CCR7 or other chemokine receptors. Since we believe Notch signaling may play an important role in regulating Tfh localization by enhancing ASCL2 expression, it would be very interesting to determine whether circulating Tfh cells have reduced Notch expression and signaling compared to Tfh cells residing in B cell follicles. KLF2 and S1P1 are important for initiating T cell egress into the periphery [256,258,259,383]and it is

therefore plausible that these factors could be expressed more greatly on circulating Tfh cells compared to traditional Tfh cells residing in B cell follicles.

Tfh cells are believed to play an important role in a multitude of human diseases including allergy [390,391], autoimmune disease [392-394], and some post-germinal center lymphomas [395,396]. Due to the importance of Notch signaling in regulating mouse Tfh cell function and development it will be interesting to investigate whether Notch signaling is similarly important for human Tfh cell development and function. Importantly, Notch signaling has also been implicated as a driver of human autoimmune disease and some cancers. Whether Notch function and Tfh cells are linked during these diseases is not currently known. Importantly, GSIs are now being investigated in clinical trials to target Notch signaling in a number of cancers and autoimmune disorders [397].

Many autoimmune diseases including systemic lupus erythematosus (SLE), vasculitis, rheumatoid arthritis (RA) and others are mediated by antibodies that react to self-antigen. Due to the importance of Tfh cells in the development of high-affinity antibodies, they have been implicated as drivers in various antibody-mediated autoimmune diseases [392-394]. Support for a role of Tfh cells in autoimmunity comes from studies using *Sanroque* mice [398,399]. *Sanroque* mice have a point mutation in their roquin-1 gene that results in dysregulated roquin-1 function. In the context of Tfh cells, this roquin-1 mutation results in enhanced ICOS expression and signaling [398]. This

increase in ICOS signaling results in significantly higher Tfh and GC B cell populations [398]. Furthermore, these mice generated higher populations of autoreactive T cells and autoantibody producing B cells. As a result, *Sanroque* mice generate SLE-like symptoms marked by enhanced production of autoantibodies to dsDNA, glomerulonephritis, anemia, and hepatitis [398]. Dysregulation of GCs appears to be the causative effect as deletion of one allele of BCL6 reduced the GC phenotype and SLE-like symptoms [400].

IL-21 production by Tfh cells has also been implicated as a mediator of some autoimmune disorders. As described previously, IL-21 secretion by Tfh cells supports GC B cell maturation into high-affinity antibody producing cells [60]. Non-obese diabetic (NOD) mice generate spontaneous diabetes, marked by destruction of pancreatic islets [401]. Interestingly, IL-21R deficient NOD mice are resistant to diabetes generation [402]. Furthermore, over-expression of IL-21 in normally diabetic resistant mice was sufficient to induce diabetes [403].

IL-21 has also been identified as a potential driver of RA [404,405]. IL-21R deficiency is protective in a mouse model of rheumatoid arthritis [405]. Furthermore, patients with active RA had increased serum levels of IL-21, which correlated closely with numbers of Tfh-like cells found in the periphery, compared to healthy controls (HC) [404]. Additionally, B cells isolated from RA patients showed greater proliferation and antibody production when stimulated with IL-21, compared to HC [404].

The Notch signaling pathway has been implicated in autoimmune pathogenesis [406,407]. Notch receptor expression and downstream signaling events are enhanced in T cells of patients with active RA, compared to HC and patients with inactive disease [406]. Of note, inhibition of Notch signaling with GSI during in an animal model of RA resulted in reduced RA severity [407]. Although, this inhibition of Notch signaling was not confined to Tfh cells and it cannot be concluded that Tfh-derived Notch signals were important drivers of disease in this model. Ultimately, whether increased Notch signaling and Tfh activity are linked during RA and other autoimmune disorders has yet to be investigated.

Tfh cells have also been linked to numerous forms of malignant T cell and B cell lymphomas. Angioimmunoblastic T cell lymphomas contain neoplastic Tfh cells that express PD-1 and BCL6 and produce CXCL13, the CXCR5 ligand [395,396]. Non-cancerous Tfh cells have also been suggested to support tumor microenvironments through the production of various cytokines including IL-4, IL-21, and IFN-γ. IL-4 promotes B cell survival and may enhance the survival of follicular tumors as enhanced levels of IL-4 are correlated with poor prognosis in patients with follicular lymphomas [408,409]. Furthermore, IL-4 and IL-21 production has been shown to enhance leukemic cell proliferation [410]. Circulating Tfh cells are enriched in some chronic leukemias and their expression of CD40L, IL-21, and IL-4 is thought to enhance tumor growth and survival [410].

Notch signaling is also dysregulated in various lymphomas. Notch signaling and NF-κB signals have been shown to work cooperatively to enhance tumor survival and proliferation [411]. Importantly, gain-of-function mutations of Notch1 are associated with up to 50% of all T-cell acute lymphoblastic leukemias (T-ALL) [397,412]. Inhibition of Notch with GSI led to reduced survival of human lymphoma cell lines [411,413]. Due to the potential role for Notch signaling in promoting tumor growth and survival, GSIs are currently being used in a multitude of clinical trials as a potential cancer treatment [397].

To conclude, Notch signaling and Tfh cell biology represent important areas of focus for future studies in mouse and humans. Our data formally identifies that Notch signaling plays an essential role in Tfh differentiation, function, and maintenance. Deletion of Notch receptors results in a nearly complete block in Tfh differentiation and impairs Tfh IL-4 production. Importantly, peripheral effector Th2 function remained intact when Notch receptors were abolished. Furthermore, Notch signaling appears to play a continuous role in Tfh cells. Reduced Notch signaling beyond initial Tfh differentiation results in impaired Tfh IL-4 production and fate maintenance. Future studies investigated how Notch signaling exerts its effects on Tfh cell biology is critical. Additionally, determining if and how Notch signaling regulates Tfh function in humans will be of great interest.

Based on the current body of literature, as well as the data presented here, Notch receptors and ligands are strong candidates for pharmaceutical targeting for two reasons: (1) First, our data suggests that Notch signaling can be targeted in diseases and malignancies propagated by Tfh cells without negatively affecting peripheral T helper cell function. (2) Notch receptors and ligands exist extracellularly at the cell surface before exerting downstream affects intracellularly. Therefore, pharmaceutical targeting of Notch receptors and ligands will be easier than targeting other Tfh-specific factors like BCL6 and ASCL2, which exist entirely intracellularly.

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Biography

Mark Dell'Aringa was born in Arlington Heights, Illinois on August 17th 1988. He received his high school diploma from Barrington High School in 2007 and graduated from the University of Illinois Urbana-Champaign in 2011 with a Bachelor of Science in molecular and cellular biology, with honors. Mark joined the Duke immunology department in 2011 and he began working in Dr. Lee Reinhardt's lab in March 2012.

Mark is an author on two peer-reviewed articles. These include: 1) Dell'Aringa M, Reinhardt RL (2018) Notch signaling represents an important checkpoint between follicular T-helper and canonical T-helper 2 cell fate. Mucosal Immunol. doi:10.1038/s41385-018-0012-9 and 2) O'Brien TF, Bao K, Dell'Aringa M, Ang WX, Abraham S, Reinhardt RL (2016) Cytokine expression by invariant natural killer T cells is tightly regulated throughout development and settings of type-2 inflammation. Mucosal Immunol 9 (3):597-609. doi:10.1038/mi.2015.78. Mark also wrote two chapters for an upcoming book entitled 'Type-2 Immunity: Methods and Protocols'. The chapters are entitled: 1) 'Using cytokine reporter mice to visualize type-2 immunity *in vivo*' and 2) 'Live imaging of T follicular helper cells in explanted lymph nodes'.