TRANSCRIPTION FACTOR REGULATION OF T HELPER SUBSET FUNCTION

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The immune system protects the body from foreign organisms. T cells and B cells are integral components of the ability of the immune system to generate focused immune responses. The development of specialized subsets of T helper cells is governed by transcription factors. Previous work demonstrated a requirement for the transcription factor PU.1 in the development of IL-9-secreting Th9 cells. Work in this dissertation demonstrates that the Th9 subset is not stable in vitro, and that PU.1 expression decreases during long-term culture. To examine a role for PU.1 in Th9-independent immunity we examined a model of multiple sclerosis termed experimental autoimmune encephalomyelitis (EAE). Mice that lack PU.1 expression in T cells (*Sfpi1*^{lck}-/- mice) demonstrated more severe disease with attenuated recovery compared to control mice, and this was accompanied by an increase of T cells in the central nervous system. We also observed that following multiple routes of immunization *Sfpi1*^{lck}-/- mice had increased numbers of T follicular helper (Tfh) cells and increased germinal center responses. This correlated with increased expression of the cytokine IL-21 and the surface protein CD40L in T cells that lacked PU.1 expression and resulted in increased numbers of germinal center B cells and antigen-specific antibody titers compared to control mice. The increased germinal center B cells and antibody titers were attenuated with blocking CD40L antibody but not with neutralizing IL-21 antibody. These results suggest that PU.1 limits the expression of CD40L on

Tfh cells to regulate the humoral immune response. Together, the data in this dissertation demonstrate Th9-independent functions of PU.1. Moreover, this work shows that transcription factors promoting the development of one subset of T helper cells can simultaneously have negative effects on distinct T cell lineages.

Mark H. Kaplan, Ph.D.-Chair

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC	Antigen presenting cells
BAL	Bronchoalveolar lavage
BAFF	B-cell Activating Factor
BCL	B cell lymphoma
BCR	B cell receptor
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CNS	Central Nervous System
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Ets	E-twenty six
FasL	Fas ligand
FBS	Fetal bovine serum
FACS	Fluorescence-activated cell sorting
Foxp3	Forkhead box protein 3
GATA3	GATA binding protein 3

GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
IBD	Inflammatory bowel disease
ICOS	Inducible co-stimulator
ICS	Intracellular staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IN	intranasal
IP	intraperitoneal
IRF	Interferon regulatory factor
iTreg	inducible T regulatory
IV	Intravenous
JAK	Janus family tyrosine kinase
LPS	Lipopolysaccharide
LT-α	Lymphotoxin alpha
Maf	Musculoaponeurotic fibrosarcoma
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
MS	Multiple Sclerosis
NK	Natural killer

nTreg	Natural T regulatory
OVA	Ovalbumin
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristake 13-acetate
Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
PU.1	SFFV proviral integration 1
qRT-PCR	quantitative real-time polymerase chain reaction
RA	Rheumatoid arthritis
ROR	Retinoid-acid-related orphan receptor
Runx	Runt-related transcription factor
SAP	Slam-associated protein
SC	Subcutaneous
SCID	Severe Combined Immunodeficiency
Sfpi1	Spleen focus forming virus proviral integration site-1
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
Tbx21	T-box transcription factor 21
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
Tfh	T follicular helper

TLR	Toll-like receptor
TLSP	Thymic stromal lymphopoietin
TNF	Tumor necrosis factor
Treg	T regulatory
WT	Wild type

INTRODUCTION

The Immune System

The human body is a complex system and protection against potentially harmful entities is crucial for survival. Over time the body has evolved in such a way that requires the penetration of multiple physical and functional barriers in order for an outside organism to initiate an internal infection. The skin is the body's first layer of protection from the outside world. Intact skin is a natural deterrent for potentially invasive organisms and enzymes produced by the skin can eliminate foreign organisms before they can penetrate the skin (1, 2). Mucosal layers that line various areas of the body including the lungs and digestive tract provide another layer of protection. Through the production of mucous and chemical mediators in the lung (3) and digestive tract (4), outside organisms are prohibited from establishing infection and disease. However, when an organism is able to penetrate the skin or mucosal layer it is the responsibility of the immune system to eliminate the invading organism. The immune system is a systemic network of cells and molecules that work together to initially identify and eventually remove the invading organism.

The immune system has two components, the innate and adaptive immune systems. The cells of the innate immune system are important initiators of the immune response. Dendritic cells are innate immune cells that are strategically located throughout the body (5). Dendritic cells constantly sample antigens present within their local environment. Through the expression of pattern recognition receptors (PRR), dendritic cells are able to identify foreign organisms.

When PRRs are triggered dendritic cells become activated and mature into cells that are able to migrate to local lymph nodes and stimulate cells of the adaptive immune system called T- and B-lymphocytes. The activation of T-lymphocytes by dendritic cells requires close contact between the two cell types. When dendritic cells encounter a foreign organism they present processed antigens bound to major histocompatibility class II (MHC II) molecules on their cell surface which can be recognized by $CD4^+$ T-cells that express T-cell receptors (TCR) specific for the peptide:MHC II complex. Mature dendritic cells also express surface molecules such as CD80, CD86, and CD40, which interact with T-cell surface molecules such as CD28 and CD40L promoting the survival and further activation of T-cells. Further, cytokines produced by dendritic cells bind to receptors on the surface of activated $CD4^+$ T-cells, directing the differentiation of activated T-cells into helper cells with distinct qualities.

The indispensible nature of the immune system is demonstrated in individuals that lack different factors that are important for the innate and adaptive immune response. Chronic granulomatous disease (CGD) can develop in individuals that have genetic mutations in genes that are important for the killing of invading organisms after they have been phagocytosed (6). Therefore, individuals with CGD are susceptible to a wide range of infectious agents. Severe combined immunodeficiency (SCID) is a category of disorders in which the development of T and B-lymphocytes is impaired. Mutations in genes for CD40L, JAK3, IL-7 and other genes have been shown to cause SCID (7). Alternatively, uncontrolled

activity of the immune system can also be detrimental. The incidence of allergic disease is increasing (8). Allergic disease occurs in individuals that are genetically predisposed to enhanced immune activity in response to certain exogenous antigens. Typically, individuals who suffer from allergic disease have increased expression of type 2 cytokines and increased circulating serum IgE upon allergen exposure. Autoimmune disease is another example of improper activity of the immune system. There are multiple mechanisms in place to prevent the immune system from attacking the host (9). However, disruption of self-tolerance can occur when there is a breakdown in one or more of these mechanisms. Together, these diseases demonstrate the necessity of proper functioning of the innate and adaptive immune system.

T-helper cells play a crucial role in promoting the elimination of pathogens and promoting the development of memory responses. Through the production of cytokines T-helper cells can further activate cells of the innate immune system to directly kill invading pathogens. T-helper cells can also recruit other cells of the immune system to sites of infections. One of the most important jobs of T-helper cells is to help B-cells produce antibodies that are highly specific for invading pathogens. The importance of T-helper cells in different parts of the immune response and the variety of pathogens encountered by T-cells, requires the development of distinct subsets of T-helper cells. Th1 and Th2 cells were the first two subsets of T-helper cells described in the late 1980's(10) and since then multiple other subsets have been discovered (11) (Figure 1).

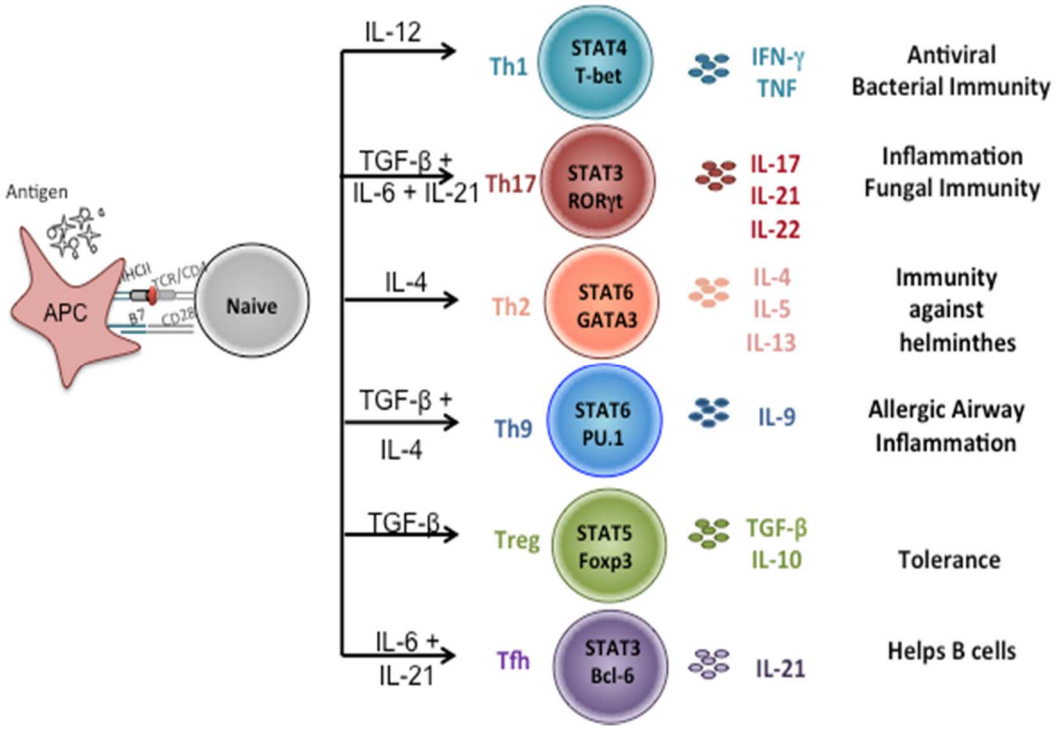


Figure 1. Diagram of T-helper Cell Differentiation

Appropriate contained responses to foreign organisms are key to removal of the invading organism without causing harm to the host. However, unrestrained or exacerbated production of cytokines by T-helper cells and other cells of the immune system often in combination with a break down in self tolerance mechanisms can lead to acute or chronic inflammatory disease. During certain helminth infections that occupy the portal vein system the Th2 cytokine, interleukin 13 (IL-13), is an important component of the antiparasitic response. However overtime persistent production of IL-13 can lead to fibrosis and significant pathology within the liver increasing the morbidity and mortality rates of infected individuals. Other Th2 cytokines such as IL-4 can also be beneficial in

that IL-4 can abrogate the development of autoimmunity by inhibiting the differentiation of Th1 and Th17 cells, two principal cell types in the development of autoimmune disease. For a long time, Th1 and Th2 cells were the only known T-helper subsets identified and the different manifestations of autoimmune disease were either due to the activity of Th1 or Th2 cells. Through the production of IFN-γ and TNF-α, Th1 cells mediated macrophage activation and the production of chemical mediators that promoted damage of joints in the case of rheumatoid arthritis or central nervous tissue in multiple sclerosis. Th2 cells were the promoters of B-cell antibody production. The discovery of the Th17 subset was a pivotal occurrence because it explained the conflicting evidence that surrounded the importance of Th1 cells in autoimmunity and it paved the way for a clearer understanding of the mechanisms involved in promoting autoimmunity. IL-17, the principal cytokine of the Th17 subset can promote tissue destruction in a variety of ways. IL-17 can lead to the production of chemokines and the resulting recruitment of inflammatory cells and increase the production of matrix metalloproteinases, which can directly destroy the extracellular matrix and surrounding tissue (12). The advances in understanding the significance of the different cell types and cytokines involved in autoimmune disease have largely been a product of disease studies in animal models. One mouse model that has been used extensively to study the inflammatory nature of the different T-helper subsets is the model of multiple sclerosis called experimental autoimmune encephalomyelitis (EAE). Although, EAE does not recapitulate every aspect of

multiple sclerosis, it has been a useful tool to better understand the involvement of the different T-helper subsets in the context of autoimmune disease.

EAE: A model for understanding T-helper cell biology

EAE was first developed in the early twentieth century and it was the first model of aseptic central nervous system inflammation and autoimmunity in general (13). As EAE is a disease characterized by demyelination within the CNS, induction of EAE in mice occurs through immunization with myelin-associated proteins such as myelin oligodendroctye (MOG) protein or MOG peptides, myelin basic protein or peptide, and proteolipid protein. An adjuvant such as complete Freund's adjuvant (CFA), a mixture of *Mycobacterium tuberculosis* and oil, is often administered in an emulsion containing the myelin-associated protein. CFA is thought to bolster the immune response and help in breaking down the blood brain barrier facilitating the entry of immune cells (13). The histological findings of EAE mirror those observed in patients who suffer from demyelinating diseases of the central nervous system (CNS) such as multiple sclerosis (MS) (13). Thus, EAE is a tool for exploration into the development of diseases such as MS and possibly the development of treatment modalities. Although, only a small number of drugs developed from EAE studies have advanced to clinical use to date (13), there is an abundance of knowledge that we have today concerning how the immune system works that can be attributed to evidence gathered using the EAE model.

In particular, the discovery of the Th17 subset stemmed from investigations looking to determine the prevailing cytokines of EAE and autoimmunity. Before the discovery of Th17 cells Th1 cells, through the production of IFN-γ, were thought to be the impetus of autoimmunity. However, there is much stronger evidence that the IL-12 family member IL-23, an important cytokine in the differentiation of Th17 cells, has a much more important role in promoting the inflammatory response observed in EAE. Th9 cells have also been shown to be pathogenic in EAE. Adoptive transfer of in vitro derived Th9 cells can induce EAE. However, the contribution of IL-9 to the pathology of EAE is still unclear. Further, whether the transcription factor PU.1, which regulates IL-9 production from Th9 cells, is required in $CD4^+$ T-cells for EAE development is also unclear. To begin to clarify the role of IL-9 and PU.1 in EAE, we induced EAE in $Sfpi1^{lck}$-/- mice, which specifically lack the expression of PU.1 in $CD4^+$ T-cells. $CD4^+$ T-cells from *Sfpi1*^{lck}-/- mice produce very small amounts of IL-9 when differentiated under Th9 conditions. In a model of allergic inflammation of the lung, *Sfpi1*^{lck}-/- mice were resistant to disease because of their deficiency in IL-9 production. Thus, *Sfpi1*^{lck}-/- mice will be a great tool for better understanding the involvement of IL-9 and PU.1 in the pathogenesis of EAE and potentially MS.

T-helper Cells and Their Transcription factors

The transcription factor network within the different T-helper subsets regulates the characteristics of the T-helper subsets. Cytokine production, cellular

trafficking and other properties of T-helper cells are often controlled by multiple transcription factors. However, there are a few transcription factors that have a more dominant effect on T-helper cell polarization. These transcription factors are referred to as master transcription factors. GATA3 is required for Th2 cell differentiation and GATA3 was the first master transcription factor described (14). GATA3 expression in Th2 cells regulates the expression of Th2-associated cytokines and the absence of GATA3 expression causes T-cells to become more Th1 like as indicated by increases in IFN-γ (14). A couple years after the discovery of GATA3, T-bet was described as the lineage-defining transcription factor for Th1 cells (15). Similar to GATA3's control of IL-4, T-bet expression regulates the expression of the hallmark Th1 cytokine, IFN-γ (15). Over the past decade the newest T-helper subsets and their master regulators have been described. Th17 cells require RORγt (16), T-regulatory cells require Foxp3 (17-19), Tfh cells require Bcl6 (20), and Th9 cells require PU.1 (21). The T-helper cells and their associated transcription factors and cytokines have been shown to play important roles in promoting disease in mouse models and in humans. However, there are still many questions about the function of the T-helper subsets that have not been addressed.

In the following paragraphs, I touch on some of the main characteristics of the different T-helper subsets and their involvement in immunity against foreign pathogens. I also highlight what is currently understood about the newest T-

helper subset, Th9 cells, and importance of PU.1 in T-helper cell immunity beyond regulation of IL-9 expression by Th9 cells.

Th1 cells

The differentiation of Th1 cells from naïve $CD4^+$ cells requires upregulation of the IL-12Rβ2 through TCR signaling and IL-12 produced by antigen presenting cells (APC) within the local environment (11, 22). IL-12R signaling leads to the phosphorylation and dimerization of the signal transducer and activator of transcription-4 (STAT4). Once, in the nucleus STAT4 can induce the expression of IFN-Y. IFN-γ can then bind to the IFN-γ receptor on the surface of Th1 cells and induce more IFN-γ production through the activity of STAT1. IFN-γ can also upregulate IL-12 production by phagocytes further potentiating Th1 differentiation (22). Both STAT1 and STAT4 signaling can induce T-bet expression (23). T-bet works to stabilize the Th1 lineage by promoting Th1-cell specific genes but T-bet also limits the expression of Th2 associated genes (24). The Th1 polarizing ability of T-bet is demonstrated as overexpression of T-bet in Th2 cells decreases Th2 cytokine production and increases the production of IFN-γ (15).

Th1 cells are responsible for promoting immunity against intracellular pathogens. Through the production of IFN-γ, Th1 cells can activate phagocytes to kill cells that have been infected by viruses and other intracellular pathogens. Th1 cells also produce IL-2 and lymphotoxin, and TNF-α (22). The importance of IFN-γ

production by Th1 cells is demonstrated in the increased susceptibility of infection in mice that lack IFN-γ and the interferon gamma receptor 1 (IFNGR1) (25). IFN-$\gamma^{-/-}$ and IFNGR1$^{-/-}$ mice are unable to clear a variety pathogens including *Leishmania major*, *Listeria monocytogenes*, and other obligate intracellular pathogens. Patients that lack functional IFN-γ receptors also show increased susceptibility to infection and often die in childhood (25). Th1 cells are also important for tumor immunity. Through the production of IFN-γ, Th1 cells can activate macrophages to release reactive oxygen species which can lead to tumor cell death (26). IFN-γ can also cause tumor cells to upregulate MHC II expression increasing the possibility of detection (26). IL-2 production by Th1 cells can increase the expansion of cytotoxic lymphocytes, which can directly kill tumor cells (26). The importance of Th1 cell development during certain types of infection and in promoting antitumor immunity is clear. However, Th1 cells have also been shown to contribute to various disease pathologies. Transfer of Th1 cells into naïve mice has been shown to induce experimental autoimmune encephalomyelitis (EAE) (27, 28), the mouse model of the human disease multiple sclerosis. Th1 cells are also harmful in diseases such as inflammatory bowel disease (29) and other autoimmune disorders (30).

Th2 cells

The signature cytokines of Th2 cells are IL-4, IL-5, and IL-13. Th2 cells play a critical part in immunity against extracellular pathogens especially helminthes.

The polarization of Th2 cells can be influenced by a variety of factors but IL-4 is one of the most important. Signaling through the IL-4R leads to phosphorylation and homodimerization of STAT6, which induces the expression of GATA3, the master transcription factor of the Th2 subset (14, 31). The initial source of IL-4 is currently unclear but a variety of cells including TCR stimulated naïve $CD4^+$ T-cells and basophils are potential candidates. STAT5 activity is also required for complete Th2 differentiation (32). IL-2 signaling induces STAT5 and in combination with GATA3, STAT5 can induce IL-4 expression. The importance of IL-2 and STAT5 signaling in Th2 differentiation is highlighted in studies were IL-2 signaling is absent and Th2 differentiation is attenuated (33). Cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 have also been shown to promote the Th2 phenotype (31). Epithelial cells can express TSLP, IL-25, and IL-33 and the ability of these cytokines to enhance the Th2 phenotype is linked to their contribution to type 2 immune responses that occur during the pathology of allergic disease. Notch and OX40 signaling can also promote the Th2 phenotype especially in the absence of IL-12 (31).

The involvement of Th2 associated cytokines in the development of allergic disease and asthma has been understood for many years. The ability of cytokines such as IL-4 to promote B-cell class switching to IgE, IL-5 to promote the migration of eosinophils, and IL-13 to promote airway remodeling highlights the significance of Th2 cytokines in the pathology of allergic disease and asthma (31). Also, Th2 cytokines have been observed in patients with asthma and

allergic disease further supporting the importance of Th2 cells in the pathology of these diseases (8). Th2 cells are also vital for resistance to helminthic infections. The $CD4^+$ T-cell response to helminth infections can vary based on the infectious agent. However, clearance of the infectious agent often requires the development of Th2 cells. IL-4 is important for B-cell antibody class switching to IgE, which can directly bind to the invading parasite. IL-5 produced by Th2 cells is vital for the development of eosinophilia and the release of anti-parasitic chemical mediators upon interaction of the IgE constant region with its receptor on the surface of eosinophils and mast cells. IL-4 and IL-13 are both important for promoting smooth muscle contraction within the intestine, which helps to expel parasites from the host. However, persistent IL-13 can also be harmful during a parasitic infection because IL-13 can promote fibrosis and this is especially important when the liver is involved (31).

Th17 cells

The discovery of the Th17 lineage began with in vivo studies of autoimmunity. Th1 cells were thought to be the major driver of inflammation in many autoimmune mouse models. However, the dominant Th1 effector cytokine, IFN-γ, was found to be dispensable for the induction of EAE and disease was found to be exacerbated in the absence of IFN-γ (34). This finding prompted further investigation into other cytokines involved in autoimmune pathology. It was eventually found that IL-23, a cytokine vital for the commitment of the Th17

lineage, and subsequently IL-17 are important for the development of autoimmunity using the EAE model (35, 36). In vitro differentiation of Th17 cells stemmed from studies looking at cytokines that could inhibit the development of the Foxp3^{+} regulatory T-cell (T-regulatory cells, Treg) lineage. It was found that IL-6 was a potent suppressor of Treg development and that instead of expressing high levels of Foxp3, cells cultured with TGF-β and IL-6 or IL-21 expressed IL-17 (34, 37-39). It was later demonstrated that TGF-β and IL-6 induced the expression of RORγt a transcription factor that when overexpressed in naïve T-cells can induce IL-17 production (34). The expression of RORγt is partly influenced by the amount of TGF-β, as lower levels of TGF-β in combination with IL-6 favor the expression of RORγt (34). Interestingly, although RORγt expression on its own can induce IL-17 production, the absence of RORγt does not completely abolish the development of IL-17 secreting cells in vivo. Another transcription factor that belongs to the same family as RORγt, RORα has been shown to have a somewhat similar impact on Th17 development (34). Other transcription factors such as IRF4 have also been shown to be important for Th17 development (34).

The association of Th17 cells with autoimmunity has grown stronger as transgenic mice lacking cytokines, receptors, and transcription factors important for Th17 induction, are found to be show a range of resistance to the development of different mouse models of autoimmune disease (34). These mice have also been used to highlight the importance of Th17 cells in promoting

immunity against some extracellular pathogens (34). Importantly, Th17 associated cytokines have been shown to be involved in a variety of human disease including rheumatoid arthritis, and asthma. Consequently, treatments targeting Th17 associated cytokines are being pursued (34).

Tfh cells

T follicular helper (Tfh) cells help B-cells produce high affinity antibodies, which target invading pathogens for destruction. During Tfh cell development, Tfh cells upregulate the expression of the chemokine receptor CXCR5 enabling Tfh cells to migrate into germinal centers, which are specialized regions in which antigen specific germinal center B-cells undergo selection (40). Tfh cells provide survival signals to B-cells that express B-cell receptors (BCR) that are specific for the pathogen being targeted. Tfh development requires the expression of Bcl6 (40). The absence of Bcl6 expression in vivo impairs the development of Tfh cells (40). Bcl6 is a transcriptional repressor that either represses the expression or the activity of the master transcription factors of the Th1, Th2, and Th17 subsets (40). However, Bcl6 alone cannot induce all of the Tfh features necessary for B-cell help. Therefore, transcription factors such as c-MAF, IRF4, STAT3, and others have also been demonstrated to be important for Tfh development (40). Further, cognate interactions between Tfh surface molecules such as the Slam associated protein (SAP), CD28, ICOS, and CD40L and their interaction partners expressed on the surface of germinal center B-cells have been shown to be

required for Tfh development (40). Tfh cells also express cytokines. IL-21 is produced by Tfh cells and through binding of the IL-21R, IL-21 increases the proliferation and antibody class switching of germinal center B-cells (40). At the moment, the ability of IL-21 to impact differentiated Tfh cells is still unclear, however IL-21 has been shown to contribute to the differentiation of Tfh-like cells in vitro (40). Other cytokines such as IL-4, IL-9 (41) and IFN-γ are important for antibody class switching (40). However, if Tfh cells can produce IL-9 is unclear and whether the IL-9 promoting transcription factor, PU.1, has a role in the Tfh phenotype has not been studied.

Regulation of Tfh cell development is essential to prevent the possible development of disease. The Tfh surface molecule PD-1 negatively regulates the proliferation of Tfh cells (42). Also, transcription factors such as Klf2 (43, 44), STAT5 (40), and PU.1 as identified by our work are important negative regulators of Tfh development. Dysregulation of Tfh cell development and function could have significant consequences on human health. Increased numbers of circulating Tfh-like cells have been identified in individuals with various autoimmune diseases including systemic lupus erythematosus (SLE) (45). Similarly, increased numbers of Tfh cells have been demonstrated in mouse models of SLE. Alternatively, mutations impacting STAT3, SAP, CD40L, and ICOS have shown to negatively effect the development of humoral immunity in both humans and mice (45).

Among the molecules that mediate Tfh function, IL-21 and CD40L are two of the most important. CD40L is required for germinal center development and blocking the CD40-CD40L interaction early or late in T-cell dependent B-cell responses attenuates germinal center development (46). CD40L provides germinal center B-cells with survival and maturation signals. The transcriptional regulation of CD40L expression in Tfh cells is not well understood but it is clear that CD40L expression is essential for the maintenance of germinal centers. IL-21 production by Tfh cells is important however, it appears to play a lesser role in germinal center maintenance compared to CD40L. Germinal centers can develop in the absence of IL-21 expression. However, fewer germinal center B-cells are present and less IgG class switching is observed (47). There has been some advancement in understanding the regulation of IL-21 production by Tfh cells but there are still many questions that need to be addressed including what transcription factors regulate the production of IL-21 by Tfh cells.

T-regulatory cells

T-regulatory cells (Tregs) play a vital role in controlling inflammation and protecting against autoimmunity. There are two types of Tregs that can develop. Thymus derived Tregs develop in parallel with other $CD4^+$ cells in the thymus and iTregs develop in the periphery. nTregs and iTregs require the master transcription factor Foxp3 for development and function (48). Humans and mice that lack Foxp3 expression develop a variety of diseases and disorders including

thyroiditis, dermatitis, and uncontrolled lymphoproliferation (48). The effector-mediated mechanisms of tolerance used by Tregs to maintain tolerance include cytokine production, and induction of cytolysis through the production of granzymes. Cytokines such as IL-10, TGF-β and IL-35 have been shown to mediate the suppressive activity of Tregs (48). In addition, human and mouse Tregs have been shown to produce granzyme A and granzyme B respectively, inducing the killing of target cells (48). Although, Tregs are important for limiting the immune response in order to prevent harm to the host, the immunosuppressive activity of Tregs negatively impacts the anti-tumor effector cell responses. Through the production of cytokines and granzyme molecules Tregs can eliminate T-effector cells, NK cells, and cytotoxic T-cells from the local tumor environment thereby allowing for continued tumor growth and expansion (49). Treatments that target Tregs in cancer patients are currently being pursued but the exact role of Tregs in different tumor environments is still not clear (49).

Th9 cells

Th9 cells are a subset of T-helper cells that primarily secrete IL-9. IL-9 was first described as a T-cell growth factor but has since been shown to target a variety of cell types including mast cells, astrocytes, keratinocytes, and lung epithelial cells (27, 50-53). The cytokines IL-4 and TGF-β induce a network of transcription factors within naïve $CD4^+$ T-cells necessary for IL-9 production in both mice and humans (54). In addition to the requirement of TGF-β and IL-4, other co-

stimulatory molecules and cytokines have been shown to augment or inhibit IL-9 production by Th9 cells (Figure 2). Both murine and human Th9 cells increase IL-9 production in response to IL-1β, nitric oxide and thymic stromal lymphopoietin (TSLP) (55-57) and down regulate IL-9 production in response to interferon IFN-γ and IL-27 (58-60). Cultured human Th9 cells also increase IL-9 production when exposed to IL-6, IL-10, IFN-α, IFN-β, and IL-21 (60); however, the relative importance of these cytokines in regulating Th9 cellular activity during the onset and progression of different disease states is still in question. In addition to the cytokines that can induce IL-9 production there are a number of transcription factors that contribute to the Th9 phenotype. Importantly, the expression of the transcription factor PU.1 has been shown to be vital for the in vitro development of human and mouse Th9 cells. The absence of PU.1 expression in mice attenuates the development of allergic inflammation of the lung in an IL-9 dependent manner. Other transcription factors that have been shown to be important for Th9 cell development include IRF4, BATF, STAT5, and STAT6. Studies from our lab looking at the regulation of genes that are highly expressed within the Th9 population when compared to Th2 and Tregs, helped to identify genes distinctive to the Th9 subset (21). Genes for the B-cell activating factor (BAFF) and IL-1 receptor antagonist (IL-1RA) were among the category of genes identified that are typically associated with the innate immune system. Also present were genes for chemokine receptors such as CCR4. At the moment, it is unclear of how BAFF and the other genes identified contribute to the Th9

phenotype however; these genes along with others have provided a framework for understanding the characteristics that makeup the Th9 subset.

Th9 cells act in a pro-inflammatory or an anti-inflammatory manner depending on the experimental disease model being tested. The inflammatory nature of Th9 cells is often directly related to the ability of IL-9 to act on various cell types within the lung, skin, brain, and other non-immune privileged organs. IL-9 can also enhance the effector function of other T-helper cell subsets. For example, the binding of IL-9 in combination with TGF-β and IL-6 to human $CD4^+$ T-cells leads to higher levels of $CD4^+IL\text{-}17A^+$ cells compared to cultures without IL-9 (61). A similar observation was made in mice but IL-6 was not required (62). Conversely, IL-9 has been associated with promoting the activity of T-regulatory cells, which are a subset of T-helper cells that through the expression of anti-inflammatory cytokines IL-10 and TGF-β dampen the immune response (62, 63). The impact of IL-9 on human T-regulatory cells has not been closely examined and will require further study to determine if observations made using murine cells can be expanded to humans. The proclivity of Th9 cells to lead to enhanced or reduced inflammation may depend on the dominant cell type within the immediate environment.

A signature feature of the T-helper subsets is their ability to persist over long periods of time in vivo or after multiple rounds of in vitro culturing. The stability of

Th9 cells, being the most recently described T-helper cell subset, is still in question. There are a few reports that show transient IL-9 production after adoptive transfer of Th9 cells in a model of autoimmunity (64) and during helminth infections (65). Currently, there are no reports about the in vitro stability of Th9 cells. The development of tools that will allow monitoring of IL-9 production will facilitate studies looking into the stability of Th9 cells. One group has generated an IL-9 reporter mouse (65) and we are currently working on generating our own. With these mice we will be able to track IL-9 producing cells in vivo, analyze their cytokine secreting abilities ex vivo and conduct in vitro culturing of Th9 cells over multiple rounds. The stability of the Th9 subset will be important to understand if treatments targeting IL-9 production by Th9 cells will be developed in the future.

The importance of Th9 cells during various inflammatory processes in the human body is still not well defined. There are some disease pathologies in humans where IL-9 expression has correlated with disease severity and in analogous experimental mouse models where IL-9 was found to be a prominent factor linked to the observed pathology. For example, in mouse models such as EAE, Th9 cells were shown to promote a distinct pattern of inflammation suggesting that Th9 cells may play a unique role in the pathology of multiple sclerosis. Many of the investigations involving Th9 cells have focused on the impact of IL-9, however the importance of PU.1 expression by T-cells during these disease processes has not been studied.

The role of IL-9 in Central Nervous System Inflammation

The opposing functions of T-regulatory cells and the pro-inflammatory Th1, Th2, and Th17 subsets regulate the balance between tolerance and inflammation. Th9 cells likely impact this balance as well. When the balance between tolerance and inflammation is tipped to favor pro-inflammatory effector cells, autoimmune diseases can arise. Experimental autoimmune encephalomyelitis (EAE), a mouse model of the human disease multiple sclerosis characterized by demyelination within the central nervous system (CNS), has been used to demonstrate the impact of the T-helper subsets on the initiation and progression of autoimmune conditions. Currently, there are conflicting views on the contribution of Th9 cells during EAE because it is thought that IL-9 can promote either a pro-inflammatory or anti-inflammatory state depending on the microenvironment when IL-9 is expressed and the time during disease progression where IL-9 is produced. Adoptive transfer experiments demonstrated that naïve T-cells from mice expressing a myelin oligodendrocyte glycoprotein (MOG)-specific-TCR skewed under Th9 conditions can induce EAE (27).

The pathogenic capability of Th9 cells contrasts the reported ability of IL-9 to augment the activity of T-regulatory cells (62). In one study, mice deficient in the expression of the *Il9r* showed earlier onset of EAE and increased disease severity compared to WT mice. When Il9r-/- or Il9r+/+ $CD4^+CD25^+$ Tregs were adoptively transferred into WT mice after induction of EAE, IL-9R-deficient Tregs had a reduced capacity to suppress T-cell proliferation suggesting that the

absence of IL-9 signaling during EAE diminishes Treg activity and exacerbates CNS disease (62). The protective role of IL-9 in this study differs from the pathogenic role of IL-9 presented in other studies. One study links IL-9 production in the CNS to the production of CCL20 by astrocytes expressing the IL-9R and suggests that the CCL20 produced can lead to the migration of Th17 cells (66). Other studies that utilized IL-9 blocking antibodies, *Il9*-/- mice, and *Il9r*-/- mice observed delayed onset of disease and decreased disease severity (67-69). The proposed mechanisms employed by IL-9 in promoting EAE pathology in these different models include activation of STAT1 and STAT3 pathways, increasing mast cell numbers in the CNS, and enabling activation of antigen specific T-cells. The diverging observations concerning the significance of IL-9 in the pathology of EAE will require further investigation. However, a study conducted by Elyman et al., provides a possible explanation for the conflicting results (69). Stimulation of Notch receptors through Jagged2- expressing A20 B-cells induced the production of IL-9. Pretreating WT mice with Jagged2 before the induction of EAE led to less severe disease and simultaneous treatment with Jagged2 and induction of EAE led to greater disease severity. The authors suggest that the disparity in outcomes is due to changes in the Treg/Th17 ratio brought on by differing cell targets for IL-9. When mice are pretreated with Jagged2, the Treg/Th17 ratio increased suggesting that in this relatively non-inflammatory microenvironment where Tregs may predominate, IL-9 is able to promote Treg activity and dampen subsequent CNS inflammation. However, the presence of IL-9 as the immune response is being initiated leads to even greater

inflammation, possibly by expanding pro-inflammatory cells such as Th17 cells. Currently, Th17 and Th1 cells are the primary T-helper subsets found to be associated with the human disease multiple sclerosis. However, increasing amounts of evidence in mouse models suggest that further study into the possible contribution of Th9 cells during multiple sclerosis would be revealing. Moreover, a role for PU.1 in EAE has not been investigated to date. The requirement of PU.1 for the production of IL-9 by T-helper cells suggests that PU.1 may also be an important factor in CNS inflammation.

PU.1

PU.1 is a transcription factor that regulates the development and function of various cell types within the immune system. The necessity of PU.1 expression during the early stages of myeloid and lymphoid progenitor cell development has been well characterized although some questions still require further investigation. PU.1 is essential for the development and maintenance of macrophages and dendritic cells. In dendritic cells, PU.1 regulates the gene expression of many of the crucial factors of macrophage and dendritic cell development and function. PU.1 regulates the expression of the macrophage colony stimulating factor receptor (M-CSFR) (70), granulocyte-macrophage colony stimulating factor receptor (GM-CSFR) (71) and the Flt3 receptor (72), all of which are important for either dendritic cell or macrophage development. PU.1 also regulates major histocompatibility class II (MHC II) (73) expression on the

surface of dendritic cells. In contrast, expression of PU.1 within the lymphoid lineages is only required during the early stages of T and B-cell development (74). However, lack of PU.1 expression in mature T and B-cells does have significant effects on the phenotype of T and B-cells. PU.1 regulates the expression of large variety of genes within germinal center B-cells and mature B-cells including light and heavy chain antibody genes (74, 75).

Interactions between PU.1 and members of the interferon regulatory family of proteins have also been shown to regulate gene transcription in mature B-cells. PU.1 in combination with IRF4 increases the expression of the Igκ gene by binding to the 3' enhancer region (76). Further, it was recently shown that PU.1 in combination with IRF8 is required to limit plasma cell differentiation, with the absence of PU.1 and IRF8 expression leading to increased differentiation of antibody secreting cells and antibody production. Similarly, the absence of PU.1 in $CD4^+$ T-cells has a major impact on the differentiation of naïve T-cells. We have shown that T-cells that lack PU.1 expression have increased TCR expression (77) and a higher expression of Th2 associated cytokines (77, 78). As mentioned in the Th9 section of this introduction, PU.1 is also essential for the differentiation of Th9 cells. PU.1 expression has also been detected in other T-helper subsets (21). However, it is not clear whether PU.1 has any functional significance in the other T-helper subsets. In this dissertation we provide evidence for a broader role of PU.1 in T-cells through the regulation of the Tfh phenotype and increased T-cell infiltration into the central nervous system (CNS)

after EAE induction in *Sfpi1*^{lck}-/- mice. Further, we observe enhanced germinal center activity in *Sfpi1*^{lck}-/-, through increased expression of CD40L and IL-21, using two different models.

The range of PU.1 expression by cells of the immune system suggests that PU.1 could have a major impact in human health. As PU.1 is a master transcription factor in cells of the myeloid lineage it is no surprising that many of the diseases associated with PU.1 are related to the inactivation of PU.1 in myeloid cells. Mutations in the PU.1 binding sites in the gene encoding gp91^{phox}, a component of the NADPH oxidase complex, results in CGD (79). In the context of cancer, lack of sufficient expression of PU.1 early in myelopoiesis leads to leukemia in mice (80) and mutations in PU.1 have been observed in patients with acute myeloid leukemia. (81). Disruption of PU.1 expression alone is not thought to completely transform cells but in-line with most other cancer causing mutations, mutations in PU.1 can provide the initial hit needed to begin the cancerous transformation(81). The finding that PU.1 regulates IL-9 expression has expanded the inquiry of the impact of PU.1 in disease. We have shown that the absence of PU.1 expression in T-cells abrogates the development of allergic disease in mice. Another group has shown that intestinal tissues from patients with ulcerative colitis are positive for IL-9 and PU.1 (82), supporting a report that showed transfer of IL-9^{+} cells can exacerbate colitis in mice (83).

The studies in this dissertation are focused on gaining a better understanding of Th9 biology and the PU.1 function in the immune system.

Research goals

PU.1 is a master transcription factor of cells of the myeloid lineage and initially it was thought that the role of PU.1 in mature lymphocytes was limited to the B-cell lineage. However, recent work by our lab showing that PU.1 is essential for selective IL-9 production by Th9 cells has uncovered a new role for PU.1 in regulating the polarization of T-helper cells. The goal of this research is to investigate the potential role of PU.1 in regulating other aspects of T-helper cell immunity. Our first aim is to use retroviral overexpression of PU.1 in $CD4^+$ T-cells to determine what genes are regulated by PU.1 under neutral conditions. Our second aim is to look at the importance of PU.1 expression in T-helper cells in the context of inflammation of the central nervous system through the use of the mouse EAE model of multiple sclerosis. The last aim of this research will be to determine the role of PU.1 in Tfh cells and whether it has any impact on germinal center activity.

MATERIALS AND METHODS

Mice

C57BL/6 mice (WT) were purchased from Harlan Bioscience. PU.1 conditional mutant mice were generated by crossing *Sfpi1* $^{fl/fl}$ mice on the C57BL/6 background with mice containing the Cre recombinase transgene under the control of the *Lck* promoter (21, 84). Mice were kept in a specific pathogen-free environment and all studies were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine.

T helper Cell Differentiation

Naïve $CD4^+CD62L^+$ T cells were isolated from spleen and lymph nodes by magnetic separation (Miltenyi Biotech). Naïve cells were cultured in complete RPMI-1640 medium (supplemented with 10% (vol/vol) FBS (Atlanta Biologicals), 1mM glutamine (BioWhittaker), 100 U/mL penicillin (BioWhittaker), 100 μg/mL of streptomycin (BioWhittaker), 10mM HEPES, pH 7.3 (BioWhittaker), 1 mM sodium pyruvate (BioWhittaker) and 50 μM 2-mercaptoethanol) on α-CD3 (2μg/mL; 145-2C11; BioXcell) coated plates in the presence of soluble α-CD28 (1-2μg/mL) under Th1 (5ng/mL IL-12; 50 U/mL IL-2 and μg/mL anti-IL-4, 11B11), Th2 (10ng/mL IL-4; and 10μg/mL anti-IFN-γ, XMG), Th9 (10ng/mL IL-4; 2ng/mL TGF-β; and 10μg/mL anti-IFN-γ, XMG) , Th17 (100ng/mL IL-6; 10ng/mL IL-1β; 2ng/mL TGF-β; 10μg/mL anti-IFN-γ, XMG; 10μg/mL and anti-IL-4, 11B11)and T regulatory cell conditions (2ng/mL TGF-β;10μg/mL anti-IFN-γ, XMG; 10μg/mL and anti-IL-4, 11B11). Cells were expanded after three days with fresh media

and cytokines for Th1 (media only), Th2 (media only), Th17 (50ng/mL IL-6; 5ng/mL IL-1β; and 20U/mL of IL-2) Th9 (10ng/mL IL-4; 2ng/mL TGF-β; and 50U/mL IL-2), and T-regulatory cells (50U/mL IL-21). After 5 days, cells were restimulated on α-CD3 coated plates for 24 hours and supernatants were collected for ELISA. For CD40L staining, naïve $CD4^+$ T cells were stimulated with PMA (50ng/mL) and Ionomycin (500ng/mL) for 2 hours. Cells were either stained for surface CD4+(RM4-5) and CD40L expression or permeabalized for intracellular CD40L staining.

ELISA Detection of IL-1RA and BAFF

T-helper subsets were differentiated under Th1, Th2, Th9, Th17 and Treg conditions for 5 days. On Day 5 cells (5.0×10^5 to 1.0×10^6) were restimulated on α-CD3 coated plates for 24 hours. The supernatant from restimulated cells was collected and stored at -20 degrees Celsius for later use. 96-well plates were coated with affinity purified IL-1RA polyclonal antibody (R&D) dissolved in 0.1 M $NaHC0_3$ buffer (pH 9) at 4^oC overnight. Wells were washed three times with ELISA wash buffer (0.1% Tween-20 in PBS) and blocked with ELISA buffer (2% BSA, 0.01% NaN_3 in PBS) for 2 h at RT. Diluted IL-1RA standard (R&D) and cell-free supernatants were added to plates and incubated at 4^oC overnight. Wells were washed three times with ELISA wash buffer, biotinylated antibodies (1 µg/ml; BD Biosciences) dissolved in ELISA buffer were added and plates were incubated at RT for 2 h. Wells were washed three times with ELISA wash buffer and incubated with streptavidin alkaline phosphatase (1:2000; Sigma) dissolved

in ELISA buffer at RT for 1 h. Wells were washed three times with ELISA wash buffer and phosphatase substrate (5 mg/ml; Sigma 104) dissolved in ELISA substrate buffer (10% diethanolamine, 0.05 mM $MgCl_2$, 0.02% NaN_3; pH 9.8) was added. The absorbance was read at 405 nm (BioRad microplate reader model 680). BAFF ELISAs were conducted using protocol provided by BAFF ELISA kit (R&D).

Chemotaxis Assay

Th2 and Th9 cells were cultured as described in T-helper differentiation section. Th2 and Th9 cells were washed in pre-warmed complete RPMI and resuspened at 1 $x10^6$ cells/ 100µL. Recombinant CCL22 at concentrations from 1- 1000 ng/mL was diluted in serum free- RPMI and 600 µL of diluted chemokine was added to the bottom chamber of the transwell plate. Add 1 $x10^6$ cells of Th2 or Th9 cells to the top chamber of the transwell plate where appropriate. Covered plate was placed at 37 degree Celsius for 4 hours. After 4 hours, top chamber of transwell plate was carefully removed and the media from the bottom chamber was collected. Cells were counted with a hemacytometer.

Gene expression analysis (quantitative RT-PCR)

Total RNA was isolated from unstimulated cells (for genes encoding transcription factors and surface proteins) or anti-CD3 (2 µg/ml) stimulated cells (for cytokine

and cytotoxic genes) using Trizol reagent (Ambion Life Technologies) and reverse transcribed to make cDNA according to the manufacturer's instructions (Invitrogen). Quantitative PCR reactions were set up by adding cDNA, primers (Applied Biosystems), TaqMan Fast Universal Master Mix (Applied Biosystems) and DEPC water to a final volume of 10 µl in MicroAmo Fast Optical 96-well plates (Applied Biosystems). Quantitative PCR was performed on duplicate samples using the 7500 Fast Real-Time PCR system. Samples were normalized to the expression of β_2-microglobulin mRNA and relative expression was calculated using the change-in-threshold ($-\Delta\Delta C_T$) method.

BAFF Retroviral Transduction

The coding region for murine BAFF was cloned into a MIEG-EGFP vector. The Plate-E packing cell line were transfected with 15µg of MIEG-GFP or MIEG-BAFF plasmid by calcium phosphate precipitation. 24 hours later DMEM media was replaced with fresh DMEM media and the cells were allowed to continue to grow for another 24 hours. After 1 and 2 days the supernatant containing virus was collected and stored at -80 degrees Celsius. Naïve $CD4^+$ $CD62L^+$ cells were isolated from OT-II mice and cultured under Th9 conditions. On day 2 of culturing, cells were transduced with 2mL of retroviral supernatant containing 8µg/mL of polybrene by centrifugation at 2000rpm at RT for 1 hour. Viral supernatant was removed and replaced with initial culturing media supplemented

with 50U IL-2. Cells were placed at 37 degrees Celsius and continued to grow for an additional three days, with expansion occurring on Day 3.

Adoptive Transfer of OT-II Th9 cells expressing BAFF control virus and induction of allergic airway disease (AAD)

OT-II Th9 cells infected with MIEG-BAFF or MIEG-GFP virus were harvested after 5 days of culturing. Cells were placed in PBS at a concentration of 1.0x 10^7 cells/mL and sorted based on GFP expression. 1.5 x 10^6 GFP^+ cells were transferred by i.v., injection into 8-10 week old naïve C57Bl/6 mice. Mice were challenged with 100 µg of OVA i.n. for 5 days and 18 hours after the last challenge mice were sacrificed with ketamine. The trachea was cannulated and the lungs were lavaged 3 times with 1 ml PBS. The cells recovered in the BAL fluid were counted with a hemacytometer. Lungs were collected for histology.

Induction of experimental autoimmune encephalomyelitis (EAE)

Induction of EAE disease has been previously described (86). In brief, 8-10 week old female mice were immunized subcutaneously (s.c.) on days 0 and 7 with myelin oligodendrocyte glycoprotein (MOGp35-55) antigen peptide (100 µg; Genemed Synthesis) emulsified in complete Freund's adjuvant (150 µl; Sigma). Mice were injected i.p. with pertussis toxin (100 ng; Sigma) on days 0 and 2.

Mice were sacrificed 19 days after induction of disease and spleen and brain were harvested.

Brain mononuclear cell isolation and staining

Mononuclear cells were isolated from the brain of EAE mice 12 and 25 days after immunization with MOG_{35-55} peptide. Mice were perfused with PBS intracardially and brain tissue was isolated after separation of cranial bones. Brain tissue was either digested in a solution of collagenase D (5mg/mL) and DNASE I (1mg/mL) at 37 degrees for 45 minutes to 1 hour or manually dissociated. Mononuclear cells were then isolated from brain tissue using a 30%/70% Percoll gradient. Isolated cells were stimulated with PMA (50ng/mL; Sigma Aldrich) and Ionomycin (500ng/mL; Sigma Aldrich) for 4 hours. Surface staining of cells was conducted using CD4 Alexa Flour 700 (Biolegend; Clone,). Intracellular staining was done with IFN-γ (Pe-Cy7; BD pharmingen), IL-17a Alexa Fluor 647 (eBioscience), IL-9-PE (Biolegend; Clone), and Foxp3- FITC (eBioscience).

Treg Suppression Assay

The Treg suppression assay has been described previously(87). Splenocytes and peripheral lymph nodes from wild type and *Sfpi1*^{lck}-/- mice were collected and a portion of cells were set aside and nTregs were isolated with a $CD4^+CD25^+$ bead sorting kit (MIltenyi Biotec). $CD4^+$ $T_{responder}$ cells were collected from the flow through after pre-enrichment for $CD4^+$ cells followed by positive selection of

$CD4^+CD25^+$ T-cells. T-cells from the remaining splenocytes were depleted using CD90.2 microbeads (Miltenyi Biotec). After depletion of T-cells, the remaining APCs were treated with mitomycin C to a final concentration of 50µg/m for 20 minutes. Excess complete RPMI was added to mitomycin C reaction and cells were centrifuged at 1500rpm for 5 minutes. Cells were washed an additional 2x in complete RPMI. nTregs were labeled with CFSE and $T_{responder}$ cells were labeled with Cell Trace Violet. nTregs were resuspended at 1.0 x 10^6 cells /mL and the $T_{responder}$ cells at 5 x 10^5 cells/mL. Soluble α-CD3 (2C11) was added to the $T_{responder}$ cells at a concentration of .5µg/mL. 50µL of culture complete RPMI and 5.0 x 10^4 T-cell deplete splenocytes was added to each reaction well in 96-well plate and 2.5 x 10^4 $T_{responder}$ cells were added to appropriate wells followed by serially diluted nTregs. 96-well plate was placed at 37 degrees Celsius for 72 hours. $T_{responder}$ proliferation was determined by CellTrace Violet dilution as measured by flow cytometry.

Surface and intracellular staining

Splenocytes were treated with Fc-block for 5 minutes at RT and stained with Tfh markers CXCR5 (SPRCL5, Biolegend), CD4(RM4-5, Biolegend), PD-1 (J43, Biolegend), and ICOS (C398.4A, eBioscience). CXCR5 staining was carried out at RT for 45 minutes and washed. Antibodies for CD4, PD-1, and ICOS were subsequently added. GCB cells were stained with Fas at 4^0 for 45 minutes, washed, and stained for B220 and GL-7. Cells were stimulated for 2 or 4 hours in the presence of PMA and Ionomycin for CD40L and IL-21 staining, respectively.

After 1 hour and 2 hours, for CD40L and IL-21 staining, respectively, cells were treated with 3μM monensin. After stimulation cells were surface stained for Tfh markers, and stained for IL-21. IL-21 staining was conducted using the IL-21 chimera antibody (R&D systems) as described previously (88) and intracellular CD40L was conducted using the MR-1 antibody clone.

Retroviral transduction

Bicistronic retroviral expression vectors expressing either eGFP (MIEG), or eGFP in combination with the mouse gene for PU.1, *Sfpi1* (MIEG-*Sfpi1*), were described previously (78). T- cells cultured in Th17 conditions were transduced with retroviral supernatant, MIEG or MIEG-*Sfpi1*, 2 days after culturing in the presence of 8 μg/mL polybrene. Cells were also given IL-2 and expanded 3 days after culturing. After 5 days, cells were sorted based on GFP expression and stimulated on α-CD3 coated plates for 24 hours. Supernatants were collected for ELISA.

MOG_{35-55} peptide and SRBC immunizations

Mice were immunized with 100-150 μg of MOG_{35-55} peptide (Genemed Synthesis) subcutaneously (s.c.) in an emulsion of complete Freud's Adjuvant (CFA) containing 1mg/mL of heat killed H37RA strain of *Mycobacterium tuberculosis* (Sigma-Aldrich) in the hind leg region. Pertussis toxin (List Biological Laboratories, Inc) in PBS was injected intraperitoneally (i.p.) at a dose of 100-250

μg on the day of immunization and again 2 days after. sRBC (VWR Intl.) immunizations were done with 1 x 10^9 sRBC injected i.p. After 7 days, mice were sacrificed and splenocytes stained with Tfh and GC B cell markers.

Tfh gene expression

Wild-type and *Sfpi1*^{lck}-/- mice were given one injection of 1 x 10^9 SRBCs i.p. Seven days after immunization mice were sacrificed and splenocytes were stained with CXCR5, CD4, and PD-1 antibodies. $CD4^+CXCR5^{High}PD\text{-}1^{High}$ (Tfh) and $CD4^+CXCR5^-PD\text{-}1^-$ (non-Tfh) cells were sorted by flow cytometry. RNA from sorted cells was isolated with Trizol to generate cDNA. Quantitative PCR was conducted to measure gene expression.

CD40L blocking experiments

Wild type and *Sfpi1*^{lck}-/- mice were given one injection of 1 x 10^9 sRBCs i.p. CD40L blocking antibody (MR1, BioXcell) or control antibody (hamster IgG, BioXcell) were given i.p. on days 5 and 6 at concentration of 125-250μg/mL in PBS on each day. Mice were sacrificed on day 7, serum was collected to determine antibody titer and splenocytes were stained for Tfh and GCB cell markers.

RESULTS

Part I Characterization of Th9 cells

Genes Enriched in Th9 cells

When Th9 cells were first described, their ability to secrete high levels of IL-9 in the absence of other lineage-associated cytokines was highlighted, and suggested that Th9 cells could be a separate T-helper subset. The different subsets of T-helper cells share various characteristics however there are many idiosyncrasies that when taken together distinguishes each T-helper subset as a unique cell type. The cytokines IL-4 and TGF-β help to shape the differentiation of naïve cells into Th9 cells. IL-4 also is vital for Th2 development and TGF-β for T-regulatory cell development. However, IL-4 and TGF-β alone are not effective promoters of IL-9 production. Therefore, the combination of IL-4 and TGF-β appears to direct T-helper differentiation in a distinct manner. To gain a more complete understanding of how Th9, Th2, and T-regulatory cells differ our lab conducted a microarray experiment (85). There were 629 genes enriched in the Th9 population and from these 629 genes we selected 17 to investigate further. Three of the genes selected were the genes for IL-1R antagonist (IL-1RA), CCR4 and B-cell activating factor (BAFF). To confirm our microarray findings and assay the function of these genes we conducted in vitro culturing of Th9 cells and looked for the expression of these genes by qPCR, ICS, and ELISA.

Th9 cells express more IL-1RA than other T-helper subsets

The IL-1RA is a molecule that can bind IL-1 receptors but cannot induce intracellular signaling. The activity of the IL-1RA can dampen the immune response that would normally be enhanced by the binding of IL-1 cytokines to their receptors. Production of IL-1RA has been demonstrated in different tissues of the body and different cell types including monocytes, macrophages and neutrophils (89). Secretion of the IL-1RA has not been examined in T-helper cells. However, the gene for the IL-1RA was enriched in our array of the Th9 population. To confirm that Th9 cells express the IL-1RA we conducted qPCR to look for the expression of the Il1rn gene by Th9 cells and the other T-helper subsets. We found that the IL-1RA was indeed expressed by Th9 cells and we saw minimal IL-1RA expression by any of the other T-helper subsets (85). To determine if IL-1RA was also secreted by these T helper subsets, we tested supernatants from stimulated Th subsets using ELISA. We saw similar results with Th9 cells being the primary producers of IL-1RA among the different T-helper subsets (Figure 2A).

Increased CCR4 expression by Th9 cells promotes Th9 cell migration

We next looked at CCR4 expression. CCR4 is a chemokine receptor that binds the chemokines CCL22 and CCL17. Th2 cells in mice and Th17 cells in humans have been shown to express CCR4 (90-94). We compared CCR4 expression amongst the different T-helper subsets. We confirmed by qPCR (85) that Th9

cells express more CCR4 mRNA compared to the other T-helper subsets. Specifically, comparing CCR4 expression by Th2 and Th9 cells by flow cytometry, a higher percentage of Th9 cells are positive for CCR4 (Figure 2B) compared to Th2 cells. To determine if the increased expression of CCR4 by Th9 cells also reflected a higher propensity of Th9 cells to migrate in response to chemokine we conducted a migration assay comparing the ability of Th2 and Th9 cells to migrate in response to a CCR4 ligand. We cultured Th2 and Th9 cells for 5 days and washed cells to remove any residual culturing media. We then setup the migration assay with the Th2 or Th9 cells in the top porous portion of the transwell plate and the bottom wells of the plate contained increasing concentrations of CCL22. The amounts of CCL22 tested were within the range demonstrated to be produced by different immune cell types cultured in vitro (95). In the absence of chemokine we observed minimal migration (Figure 2C). However, when CCL22 was present we observed a greater number of Th9 cells compared to Th2 cells, migrate in response to CCL22 at all concentrations tested (Figure 2C). This data suggests that the higher expression of CCR4 by Th9 cells promotes increased migration of Th9 cells in response to chemokine when compared to Th2 cells.

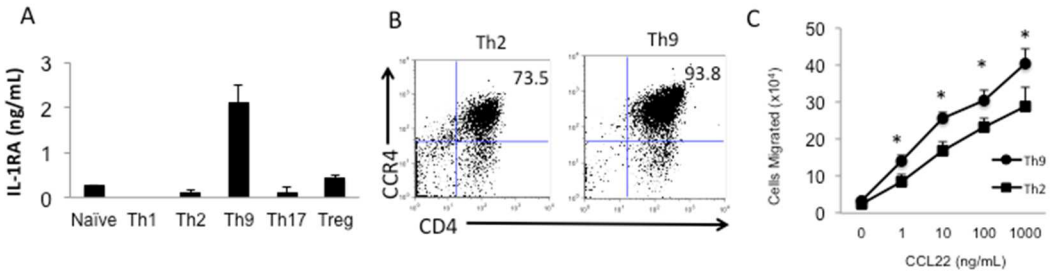


Figure 2. IL-1RA and CCR4 expression in Th9 cells. We identified the genes for the IL-1RA and CCR4 as genes enriched in the Th9 population. For confirmation of our microarray studies we cultured the T-helper subsets and measured IL-1RA(A) expression by ELISA. We cultured Th2 and Th9 cells stained cells for CD4 and CCR4 surface expression. A migration assay was conducted using Th2 and Th9 cells and measured cellular migration (B) in response to increasing concentrations of CCL22.* p<.05.

B-cell Activating Factor (BAFF) is highly expressed by Th9 cells

The gene for BAFF, Tnfsf13b, was also enriched in the Th9 population. BAFF is a cytokine that is a member of the TNF family and BAFF can be produced by a variety of cell types. Under specific conditions dendritic cells, monocytes, neutrophils and activated B-cells and T-cells among other cell types have been shown to produce BAFF (96). BAFF exists in a membrane bound form and a soluble form. We looked for the expression of BAFF in T-helper subsets by qPCR, ELISA, and intracellular staining. Th9 cells expressed the highest levels of BAFF mRNA followed by Th2 cells and little to no BAFF was expressed by the remaining T-helper subsets (Figure 3A). When we looked at protein secretion by

ELISA we detected a large amount of BAFF secretion by Th9 cells, a small amount in Th2 cells (Figure 3B) and no secreted BAFF was detected in supernatants from the remaining T-helper subsets. We observed similar results by ICS for BAFF in Th2 and Th9 cells (Figure 3C). The MFI data for BAFF ICS support the increased BAFF protein expression in Th9 cells compared to Th2 cells (Figure 3D). Our data suggests that Th9 cells express the highest levels of BAFF both at the mRNA and protein levels compared to the other T-helper subsets and Th9 cells are the primary subset that actively secretes soluble BAFF.

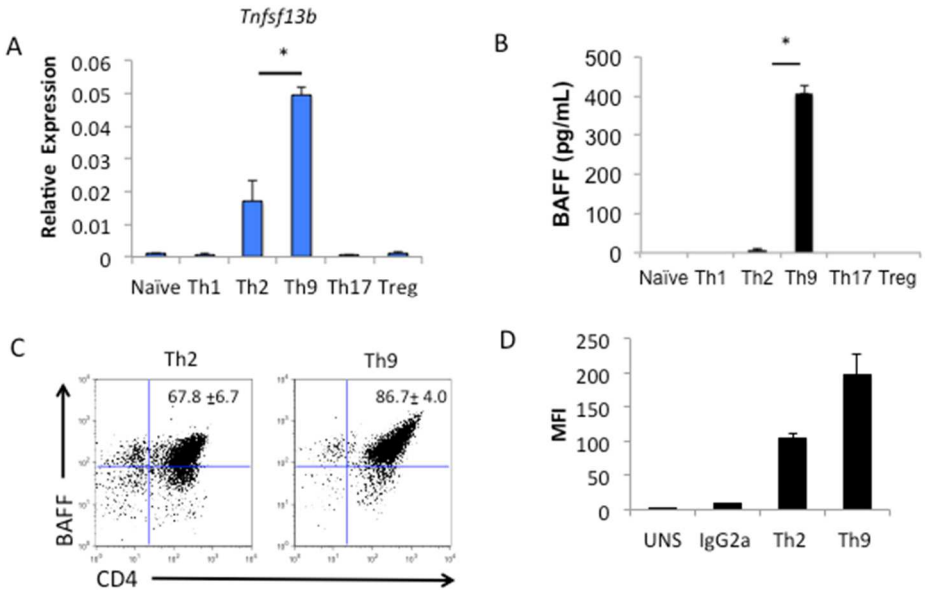


Figure 3. BAFF expression by the T-helper subsets. T-helper cell subsets were cultured under appropriate condition. BAFF mRNA expression (A) and protein secretion (B) was assayed in the different T-helper subsets. Intracellular staining for BAFF in Th2 and Th9 cells (C) and MFI (D) of BAFF from ICS.

We also looked at the mRNA expression of APRIL, *Tnfsf13*, a protein that has some functional similarity to BAFF. Naïve cells showed the highest mRNA expression of APRIL and a similar lower level of APRIL expression was observed in the T-helper subsets (Figure 4A). Further, when we compared the expression of APRIL to BAFF, APRIL expression was consistently much lower in all T-cell subsets assayed compared to BAFF (Figure 4B). We next looked at the expression of the BAFF receptors. BAFF can bind to three different receptors. The B-cells maturation antigen (BCMA), transmembrane activator and calcium modulator and cyclophilin ligand interacter (TACI), and the BAFF receptor are expressed at varying levels at different stages of B-cell maturation (96). BAFF has also been shown to induce T-cell activation and Th17 cell differentiation and expansion (97-99). Currently, It is not clear which BAFF receptor(s) are expressed on T-helper cells. To determine which of the BAFF receptors are expressed by naïve $CD4^+$ T-cells and T-helper subsets we looked at the expression of the BAFF receptors by qPCR. We observed that both the BAFF-R and TACI (Figure 3C, D) are highly expressed by naïve cells and a lower level of expression by the other T-helper subsets was observed. BCMA mRNA expression could not be detected (data not shown) in any of the T-helper subsets.

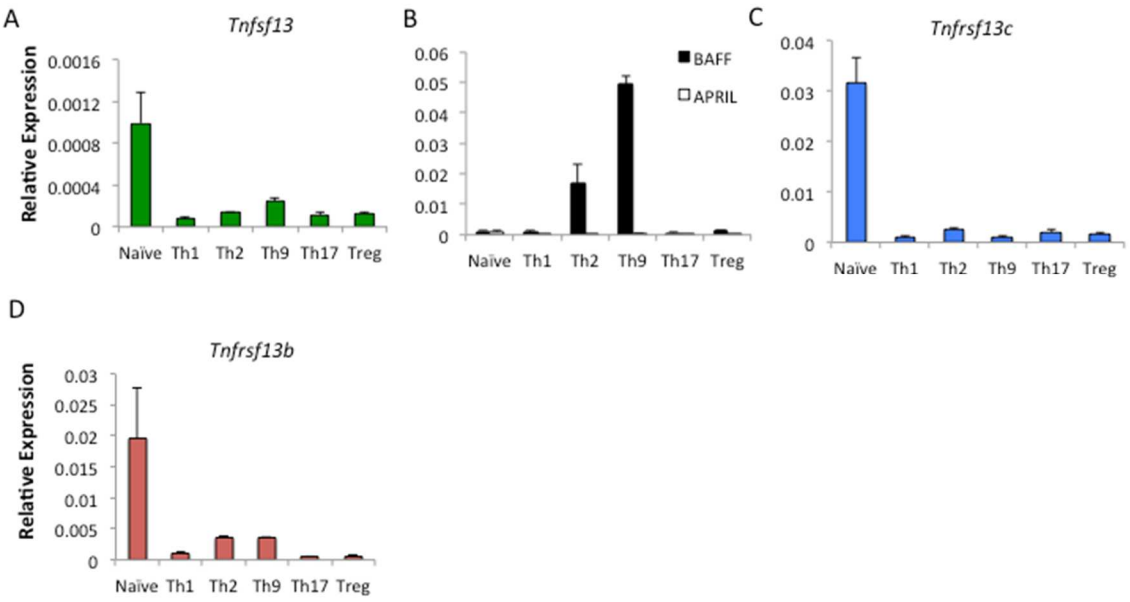
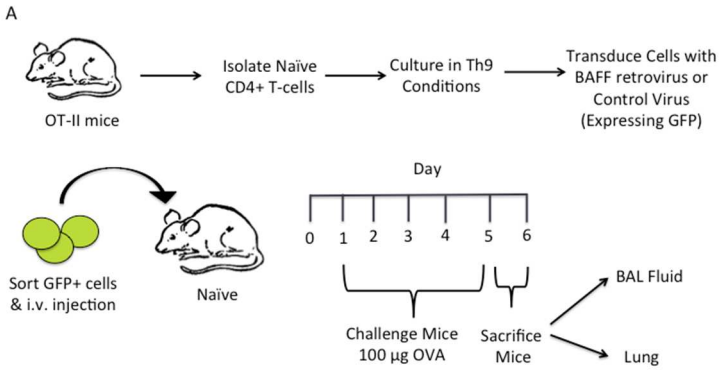


Figure 4. mRNA Expression of APRIL and BAFF receptors in T-helper subsets. T-helper subsets were differentiated from naïve cells over a period of five days. On day 5, cells were stored in Trizol for later RNA extraction and cDNA synthesis. The level of expression of APRIL (A), BAFF-R (C), and TACI (D) by the different T-helper subsets in represented in bar graph form. BAFF and APRIL (B) expression by the T-helper subsets is compared. The data are representative of 2 experiments with n=2.

After, looking at the expression of BAFF and its receptors we wanted to determine if BAFF expression by Th9 cells had any functional applications. It has been reported that mice that overexpress BAFF systemically are resistant to the development of allergic inflammation of the lung (100). To investigate if BAFF expression by Th9 cells is important for limiting IL-9 induced allergic inflammation

of the lung we retrovirally transduced OT-II Th9 cells with BAFF or control virus, sorted the cells based on GFP expression and transferred the GFP^+ cells into naïve mice (Figure 5A). We then challenged the mice with OVA for 5 days. Mice that received control virus transduced Th9 cells had significantly higher number of cells within the bronchial lavage fluid (BAL) (Figure 5B) compared to mice that received cells overexpressing BAFF and mice that received PBS. More specifically, mice that received cells transduced with control virus had significantly more $CD4^+$ T-cells, granulocytes, and eosinophils (Figure 5C-E) compared to mice with cells overexpressing BAFF and mice that received PBS. Histology of the lungs also showed the mice that received control virus transduced Th9 cells had more cellular infiltration and mucus production than mice that received the BAFF-transduced Th9 cells (Figure 6). However, these data could not be experimentally confirmed.



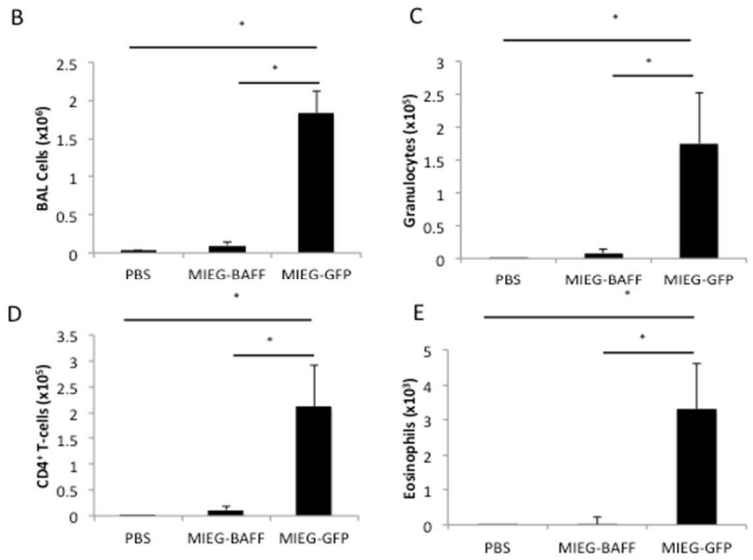


Figure 5. Induction of Allergic Pulmonary Inflammation with OTII-Th9 cells infected with BAFF virus . (A) Schematic diagram of experimental setup. Naïve $CD4^+$ T-cells from OT-II mice were isolated and cultured under Th9 conditions. On day 2 of culture cells were infected with MIEG-BAFF retrovirus , MIEG-GFP (control) retrovirus. Cells were then sorted based on GFP expression and transferred into naïve mice. Mice were then challenged for 5-6 days and sacrificed 18 hours after the last challenge. Cells from the BAL Fluid were collected and analyzed for surface marker expression and the lungs were collected for histology. The total number of cells (B) in the BAL were counted and the number of (C) granulocytes, (D) $CD4^+$ T-cells, and (E) eosinophils was determined. Statistical significance was determined with a two-tailed t test. *, $p<0.05$.

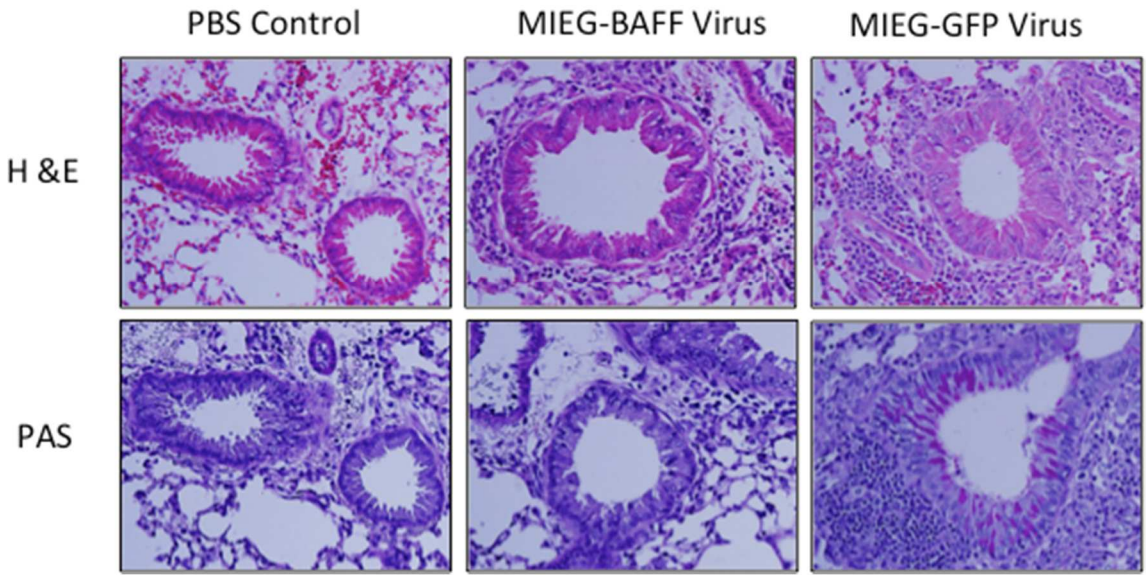


Figure 6. Lung Histology from mice receiving Th9 cells infected with BAFF virus and control virus. Mice that received OT-II Th9 cells infected with BAFF virus, control virus, or PBS were challenged for 5-6 days with OVA and sacrificed 18 hours after the final challenge. Lung tissue was collected from each mouse, sectioned, and stained with hematoxylin and eosin (H&E, upper panels) and Periodic- acid-Schiff (PAS) staining (lower panels).

Stability and Plasticity of Th9 cells

An effective immune response requires coordination and tailoring of cell types to provide optimal conditions for elimination of foreign organisms. Dendritic cells play an important role in directing the differentiation of $CD4^+$ T-cells to form distinct subsets with unique characteristics that facilitate the removal of foreign organisms.

For a period of time, it was thought that once a $CD4^+$ T-cell acquires features of a particular T-helper subset, the phenotype of that cell did not change. Early on when T-helper cells were first discovered and Th1 and Th2 cell lines were the dominant tools used for studying T-helper cell differentiation, IFN-γ produced by Th1 cells and IL-4 along with IL-13 produced by Th2 cells, were the major cytokines observed (10). The selective cytokine expression and absence of any evidence of overlap of IFN-γ and IL-4 secretion by Th1 and Th2 cells over long periods of time suggested that the Th1 and Th2 subsets were stable and committed cell types. The discovery of Th17 cells which primarily produce IL-17 but are capable of producing IFN-γ both in vitro and in vivo (101, 102) under certain conditions, provided evidence that overlapping cytokine production by the different T-helper subsets was possible and suggested that there was some flexibility in the phenotype of T-helper cells. There is also evidence that suggests that the master transcription factors assigned to the different T-helper subsets can actually be expressed in other T-helper subsets under certain conditions which further support a the idea of more flexibility in T-helper cell differentiation (103).

The plasticity of Th2, Th17, and T-regulatory cells has been shown in a number of different studies (103). However, questions still remain about the more recently discovered Th9 cells. One of the primary questions being asked about Th9 cells is, whether Th9 cells are a stable distinct T-helper cell subset or are they Th2 cells that have the ability to produce IL-9. Also, if Th9 cells are a separate subset,

do they show a similar flexibility in cytokine production both in vivo and in vitro like Th17 cells?

Generation of IL-9 reporter mice

To begin to investigate the stability of Th9 cells in vivo we generated constructs for two reporter mouse strains. The construct of the first mouse has the gene for Thy1.1 expressed in tandem with the IL-9 gene (Figure 7A). Thus, the mice have been given the name NITE mice or Nine Induced Thy1.1 Expression. These mice will facilitate the identification of cells that are expressing IL-9 without the use of intracellular staining which would require cell fixation, thereby killing the cell. The second mouse has a similar construct to the NITE mice however in place of the Thy1.1 gene is the gene for Cre recombinase and these mice are called NICE mice. The NICE mice will be crossed with mice that express the Rosa-YFP allele, which is flanked, on its 5' end by a stop codon surround by loxP sites. The NICE/Rosa-YFP mice will allow us to track cells that are currently or previously expressed IL-9 (Figure 7B,C).

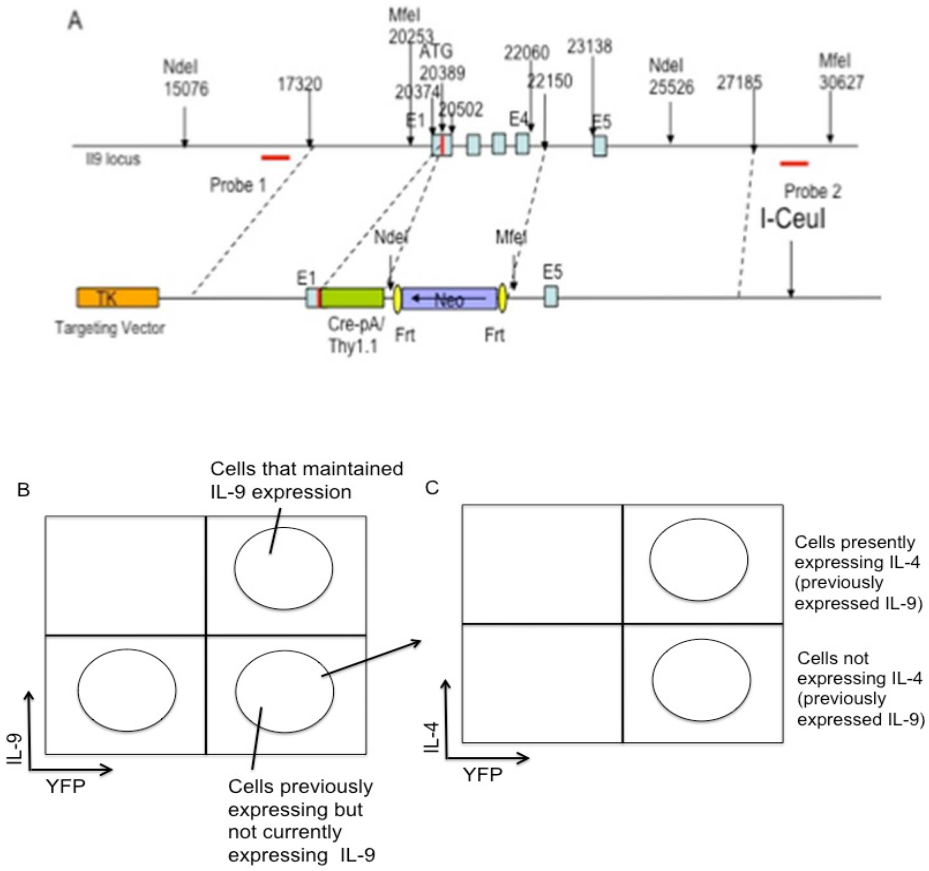


Figure 7. IL-9 Reporter Mice (A) Detailed map of the *Il9* locus and targeting vector to knock Thy1.1/Cre into exon 1 of *Il9*. **(**B**)** Schematic of Rosa-EYFP system where cells with no expression are on the bottom left and cells that are Cre+ or were previously Cre+ are on the right. Cells that are currently expressing IL-9 will also be EYFP+ (upper right) and cells that were previously IL9+ but are presently IL-9- will be EYFP+ (bottom right) (C) Schematic of predicted results for FACs analysis of NICE reporter mice. Cells gated from bottom right quadrant of (B) will be further assessed for EYFP and IL-4 (as shown) or other cytokine secretion.

In vitro Stability of Th9 cells and Plasticity of Th1, Th2, and Th17 cells

IL-9 secretion has been attributed to different T-helper subsets but Th9 cells are believed to produce the highest levels of IL-9. However, if Th9 cells are an actual committed T-helper cell lineage is not clear. To investigate the stability of Th9 cells we cultured naïve $CD4^+CD62L^+$ cells under Th9 and Th2 conditions for 5 days and looked at IL-9 expression by intracellular cytokine staining, qPCR, and ELISA. ICS showed that Th9 cells expressed a higher percentage of IL-9 compared to Th2 cells (Figure 8A) and more IL-4 was detected in Th2 cells. ELISA and qPCR looking at IL-9 production by Th9 cells supported our ICS findings (Figure 8B-C). We took a portion of the Th9 cells from the first round of culture and restimulated the cells with plate bound α-CD3, soluble α-CD28, TGF-β and IL-4. We expanded the cells after three days and analyzed the cells on day 5. After two rounds of in vitro culturing, the level of IL-9 detected by intracellular staining decreased by more than sixty percent (Figure 8D). ELISA and qPCR data also showed a similar decrease in IL-9 production (Figure 8E, F). We also looked at the gene expression of PU.1 after multiple rounds of culture. The expression of PU.1 (Figure 8G) decreased by more than 50 percent after the 2^{nd} round of culture. To determine if Th9 cells begin to secrete other cytokines after 2 rounds of culture we did intracellular staining for IL-4, IL-17a, and IFN-γ. We did not detect any IFN-γ after the 2^{nd} round of culture however a small amount of IL-4 and a higher percentage of $IL\text{-}17a^+$ cells could be detected (data not shown). From these studies we were able to conclude that the combination of TGF-β and IL-4 were not sufficient for maintaining IL-9 production by Th9 cells.

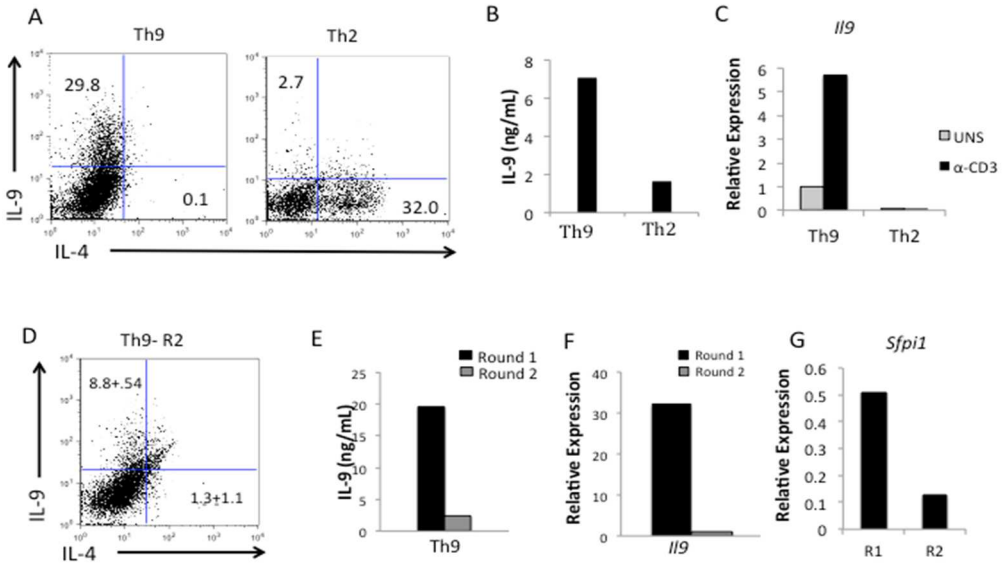


Figure 8. IL-9 Secretion decreases after multiple rounds of culturing. Th9 and Th2 cells (A) were stained for IL-9 and IL-4 expression by ICS. Five day cultured Th9 and Th2 cells were restimulated for 24 hours on α-CD3 coated plates and supernatants were collected and IL-9 secretion (B) was measured by ELISA. IL-9 mRNA (C) expression after 0 hours (no stimulation) or 6 hours of restimulation of Th9 and Th2 cells on α-CD3 coated plates. On day 5 a portion of the 5-day culturedTh9 cells were then re-plated and cultured for an additional five days under standard Th9 culturing conditions and stained for IL-9 and IL-4 (E) on day 10. After Round 1 and Round 2 of cell culturing cells were collected for analysis of IL-9 protein secretion (E) and IL-9 (F)and PU.1 (G) gene expression after restimulatiion.

Since the discovery of Th9 cells, various transcription factors and cytokines have been shown to be important contributors to Th9 differentiation. IL-2 was identified

as a cytokine that is required for Th9 differentiation (104). Other cytokines such as IL-1β (105-107), IL-25 (108), and IL-21 (60) were also suggested to promote IL-9 secretion. To investigate if changing Th9 culturing conditions to include IL-1β, IL-25, and IL-21 would promote maintenance of IL-9 production by Th9 cells we cultured Th9 cells under varying conditions. We looked at what impact the addition of IL-1β, IL-25, IL-21 or combinations of these cytokines from the first round of culture would have on IL-9 secretion after two rounds of culture. Adding IL-1β to standard Th9 conditions increased IL-9 production after one round of culture as detected by ELISA and qPCR (Figure 9). However, the addition of IL-21 to standard Th9 skewing cytokines drastically decreased IL-9 secretion after one round of culture. Adding IL-1β in cultures with IL-21 helped to attenuate the decrease in IL-9 observed with just IL-21 alone. Although, IL-1β increased IL-9 production after one round of culture, IL-9 secretion and mRNA expression by Th9 cells was still very low after 2 rounds of culture in culturing conditions where IL-1β was present (Figure 9A,B). Consistent with the negative impact IL-21 had on IL-9 production by Th9 cells after 1 round of culture, IL-9 production after 2 rounds with IL-21 was the lowest observed (Figure 9A,B).

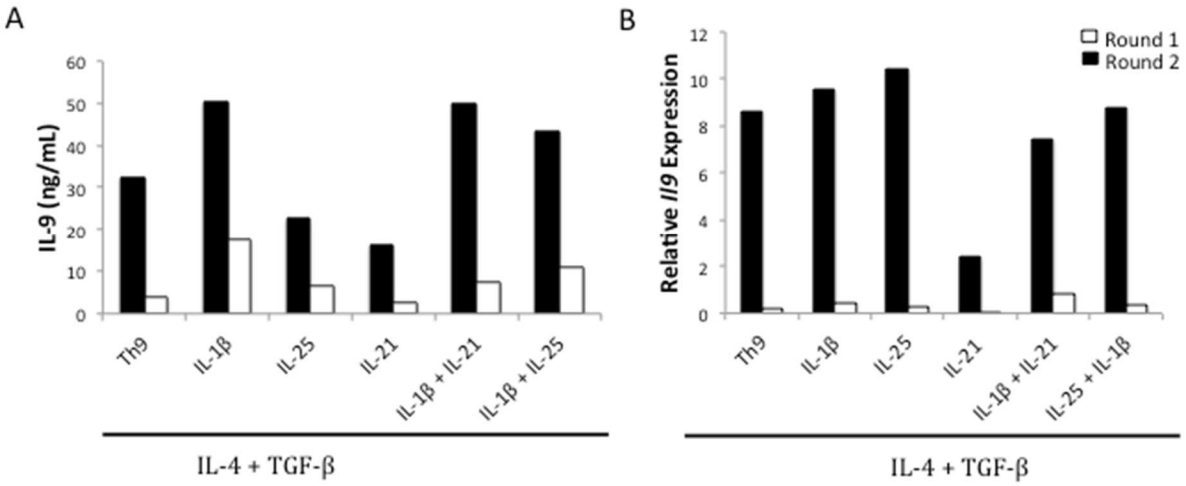


Figure 9.The addition of IL-1β, IL-21, or IL-25 has moderate effects on IL-9 secretion by Th9 cells after multiple rounds of culturing. Naïve $CD4^+CD62L^+$ cells were cultured in the presence of IL-4,TGF-β +/- IL-1β, IL-21, and IL-25 for 5 days. On day 5 cells were restimulated on α-CD3 coated plates and supernatants were collected and IL-9 secretion (A, black bars) was measured by ELISA. A portion of cells were re-plated and cultured under the same conditions from Round 1. On day 10 cells were restimulated on α-CD3 coated plates and supernatants were collected and IL-9 secretion (A, white bars) was measured by ELISA. On day 5 and day 10 a portion of cells were restimulated on α-CD3 coated plates for 6 hours. After, RNA isolation and conversion to cDNA IL-9 mRNA was measured from Round 1 (B, black bars) and Round 2 (B, white bars) of culturing.

IFN-γ has been shown to inhibit Th9 development (58). The transcription factors T-bet and STAT4 are downstream of IFN-γ signaling. We used cells from T-bet

and STAT4 double knockout (DKO) mice to test if diminished IFN-γ production would increase IL-9 production after multiple rounds of culture. We did not see any difference in IL-9 production after 1 or 2 rounds of culture when cells from DKO mice were cultured when compared to our cultures with cells from wild type mice (Figure 10A, 8A-B). We saw a similar decrease in IL-9 production by ICS when cells were cultured under varying conditions for multiple rounds (Figure 10A-C). These results suggest that IFN-γ is not the cytokine negatively impacting IL-9 production by Th9 cells under the conditions we tested.

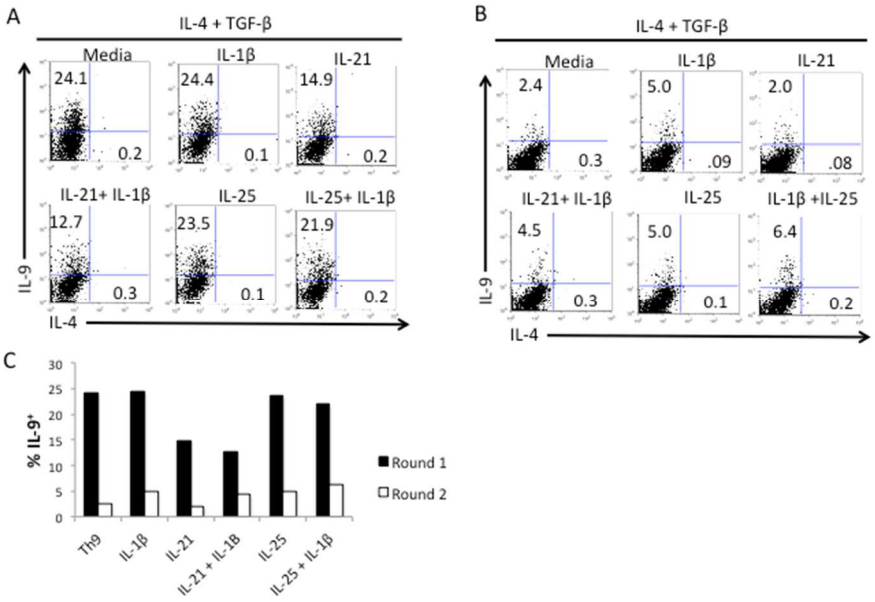


Figure 10. *Stat4*-/-*Tbx21*-/- double knockout Th9 cells decrease IL-9 production after multiple rounds of culture. Naïve T-cells from STAT4-/-, Tbet -/- mice were cultured in presence of IL-4, TGF-β +/- IL-1β, IL-21 and IL-25 for five days. On day 5, cells were collected for ICS for IL-9 and IL-4(A). Cells were cultured under the same conditions from round 1 for an additional round.

ICS for IL-9 and IL-4 (B) was conducted on day 10. Bar graph of IL-9$^+$ cells (C) after 1 and 2 rounds of culture.

Th1, Th2, and Th17 cells produce IL-9 when cultured under Th9 conditions

T-helper cell subsets are associated with the production of specific cytokines. The expression of these cytokines helps to define the phenotype of the different T-helper subsets and continued expression of these cytokines help to affirm the "committedness" of a specific lineage. Traditionally, Th1 cells express IFN-γ, Th2 cells express IL-4, IL-5, and IL-13, and Th17 cells express IL-17A, IL-17F and IL-21. However, this paradigm is shifting as under altered conditions the cytokines produced by a specific subset can change. To determine if Th1, Th2, and Th17 cells have the capacity to produce IL-9 after multiple rounds of culture, we cultured 5-day differentiated Th1, Th2, and Th17 cells (Figure 11A) in the presence of TGF-β and IL-4. Round 2 Th1 cells continued to express a high percentage of IFN-γ, although there was a fifty percent decrease in IFN-γ$^+$ cells (Figure 11B). A small percentage of IL-9$^+$ cells was detected by ICS in Th1 cultures after the 2nd round of culture (Figure 11B). Th2 cells cultured under Th9 conditions for the 2nd round expressed slightly lower amounts of IL-4 and a higher percentage of IL-9$^+$ cells was detected by ICS (Figure 11A-B). We also detected a five-fold increase in IL-9 production when measured by ELISA (Figure 11C) and a more than two fold decrease in IL-4 secretion by Th2 cells (Figure 11D) cultured under Th9 conditions. Th17 cells have been reported to produce IL-9. After one round of culturing of Th17 cells we did not detect any IL-9 (Figure 11A).

However, some IFN-γ$^+$ and IL-17A$^+$IFN-γ$^+$ cells were detected after one round of Th17 culturing (data not shown). When Th17 cells were cultured in the presence of IL-4 and TGF-β the percent of IL-9$^+$ cells increased from 0 percent to 8% IL-9 single positive cells and while IL-17A$^+$ cells was reduced to 7 % (Figure 11B). When we looked at IL-9 secretion by Th17 cells using ELISA we saw more than a five fold increase in IL-9 secretion by Th17 cells after switching to Th9 culturing conditions (Figure 11E), a finding demonstrated previously (109). Further, Th17 cells increased their expression of IL-17A by about three fold (Figure 11F). Our findings suggest that cells from Th1, Th2, and Th17 cultures have the capacity to secrete IL-9.

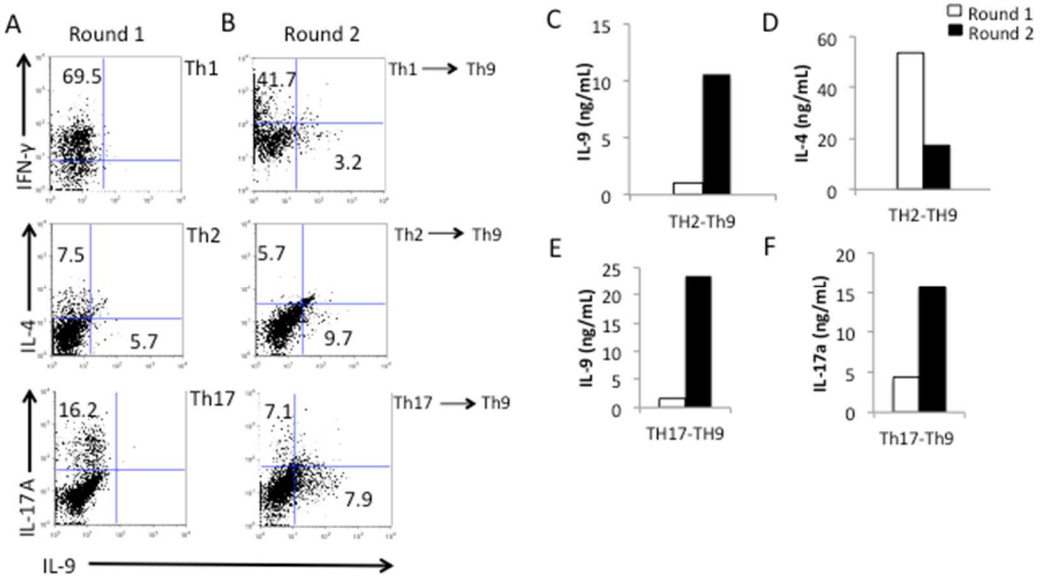


Figure 11. IL-9 can be detected in Th1, Th2, and Th17 cultures after restimulation under Th9 conditions. Naïve T-cells were cultured under Th1, Th2, and Th17 conditions for 5 days. On day 5, cells were restimulated for 6 hours and ICS staining for IL-9, and signature cytokines (A) for each T-helper

subset was conducted. A portion of Th1, Th2, and Th17 cells were re-plated and cultured under Th9 skewing conditions (IL-4 and TGF-β) for 5 days. On day 10, cells were restimulated for 6 hours and ICS for IL-9 and other cytokines (B) was conducted. On day 5 and day 10 Th2 and Th17 cells were restimulated for 24 hours and supernatants were collected for IL-9 (C.E), IL-4(D), and IL-17a (F) ELISAs.

The role of PU.1 expression in CD4$^+$ T-cells in CNS Inflammation

Sfpi1^{lck}-/- mice have exacerbated EAE

Data from our previous work showed that *Sfpi1*^{lck}-/- mice produce limited amounts of IL-9 when differentiated under Th9 conditions (21). *Sfpi1*^{lck}-/- mice were also used to demonstrate that IL-9 is necessary for the development of allergic inflammation of the lung. To further investigate the role of IL-9 during the induction of EAE we immunized *Sfpi1*^{lck}-/- and wild type mice with an emulsion of CFA and $MOG_{35-55.}$ peptide. We monitored disease progression daily for thirty days and by day 15 *Sfpi1*^{lck}-/- mice had significantly higher disease scores compared to wild type mice (Figure 12A). *Sfpi1*^{lck}-/- mice continued to show attenuation of disease recovery up until day 30 when compared to wild type mice (Figure 12A). Analysis of cellular infiltration of brain tissue in *Sfpi1*^{lck}-/- and wild type mice twelve days post immunization (p.i) showed brain tissue from *Sfpi1*^{lck}-/- mice had significantly more total cells (Figure 12C) and CD4$^+$ cells (Figure 12D) compared to wild type mice. Histological staining of spinal cord sections with hematoxylin and eosin 12 days p.i. supported our finding of increased cellular

infiltration into the CNS of *Sfpi1*^{lck}-/- EAE mice (Figure 12B). We found similar results when looking at cellular infiltration 25 days p.i. *Sfpi1*^{lck}-/- mice had significantly more CD4^{+} cells infiltrating the brain compared to wild type mice (Figure 12E). However a similar number of total cells were found in brain tissue from *Sfpi1*^{lck}-/- mice and wild type mice (Figure 12F).

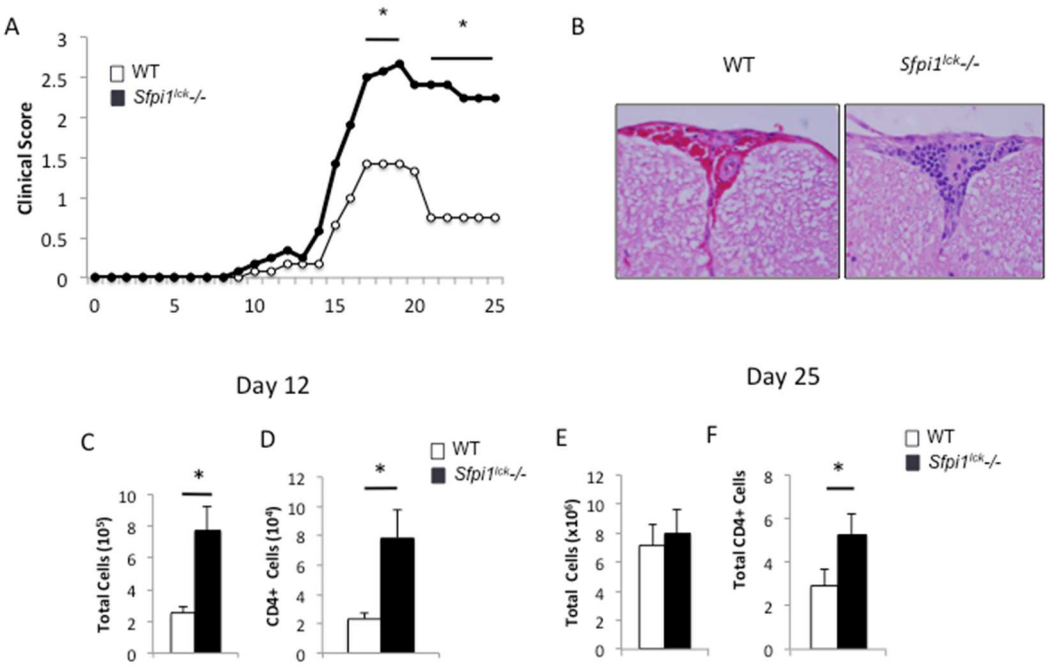


Figure 12. *Sfpi1*^{lck}-/- mice have exacerbated EAE. Clinical disease scores of MOG$_{35-55}$ immunized *Sfpi1*^{lck}-/- and wild type mice were collected daily over a period of thirty days. (A) Graph displays the overall trend in clinical disease until day 25. (B) H & E staining of spinal cord sections from EAE mice. Mononuclear cells from brain tissue of immunized mice were collected. The number of total cells and CD4^{+} cells were counted on day 12 (C,D) and 25 (E,F). Statistical significance was determined with a two-tailed t test. *, $p<0.05$.

Our lung studies with *Sfpi1*^{lck}-/- mice demonstrated that in the context of allergic inflammation of the lung PU.1 is required for IL-9 production and disease initiation. To determine if a similar reduction in IL-9 was present during CNS inflammation we analyzed cytokine expression by cells isolated from brain tissue 12 and 25 days p.i. We stimulated cells for 4 hours and examined IFN-γ, IL-17A, IL-9, and IL-4 production by intracellular staining. Twelve days p.i. we did not observe any difference in cytokine expression when comparing *Sfpi1*^{lck}-/- mice and wild type mice (Figure 13A). However, when we looked 25 p.i. there were significantly more CD4^{+} cells that were positive for IFN-γ, IL-17A, and IL-9 expression in *Sfpi1*^{lck}-/- mice compared to wild type mice (Figure 13B). There were also significantly more IL-17A^{+}IFN-γ^{+} cells in *Sfpi1*^{lck}-/- mice compared to wild type mice (Figure 13C,D). We did not observe any difference in Foxp3^{+} cells in *Sfpi1*^{lck}-/- and wild type mice (Figure 13A,B). The increase in the pathogenic IFN-γ^{+} and IL-17A^{+} cells correlated with the increased disease score observed in *Sfpi1*^{lck}-/- mice 25 days p.i. However, the increase in IL-9 positive cells was an interesting finding and suggested that the expression of IL-9 during inflammation in the CNS may be regulated in a PU.1-independent manner.

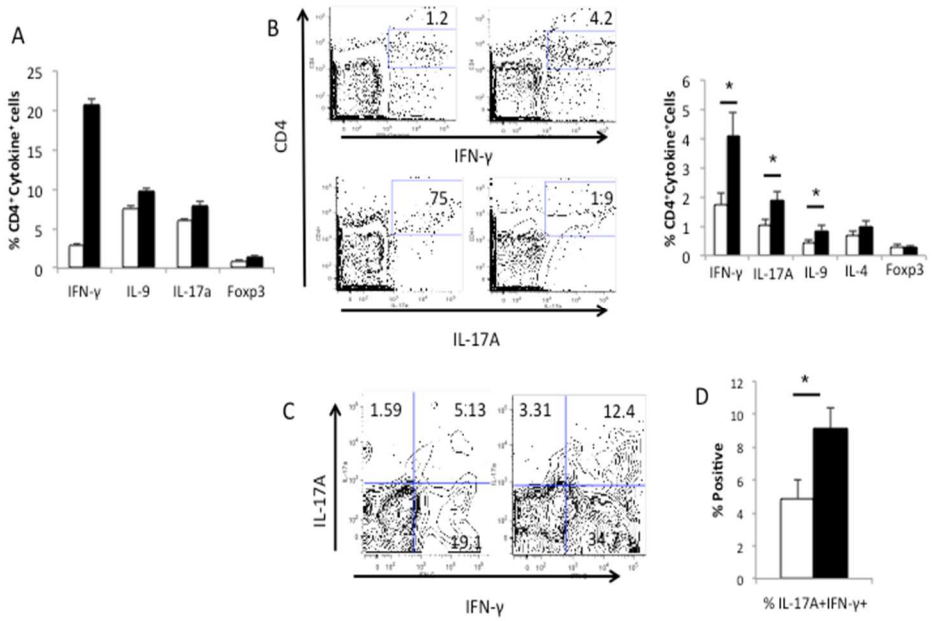


Figure 13. Mononuclear cells from *Sfpi1*^{lck}-/- EAE express more IFN-γ, IL-17A, and IL-9. Mononuclear cells from Sfpi1^{lck}-/- and wild type EAE mice were isolated from the brain tissue at designated times post immunization. Cells were restimulated and stained for proinflammatory cytokine production followed by flow cytometry. The production of IFN-γ, IL-9 and IL-17a was analyzed 12(A) and 25 (B,C,D) days post immunization. Statistical significance was determined with a two-tailed t test. *, $p<0.05$.

Suppressive Function of Sfpi1^{lck}-/- Tregs is not impaired

Tregs are important in the resolution phase of EAE (110, 111) and there is evidence in humans that suggests Tregs may be important for alleviating inflammation within the CNS(112, 113). In humans, it has been reported that the frequency of Tregs in the blood and periphery is similar(114, 115) between patients with MS and healthy patients. However, some studies find that the

function of Tregs in patients with MS is impaired compared to healthy controls(114-117). In our investigation we observed a similar frequency of Foxp3^{+} cells infiltrating the CNS (Figure 13A, 13B). To determine if nTregs from *Sfpi1*^{lck}-/- mice have diminished suppressive abilities we isolated nTregs from *Sfpi1*^{lck}-/- and wild type mice by bead sorting. In a Treg suppressor assay we compared the ability of *Sfpi1*^{lck}-/- and wild type Tregs to suppress the α-CD3 induced expansion of T-responder cells from WT and *Sfpi1*^{lck}-/- mice. We did not observe any difference in the suppressive abilities between *Sfpi1*^{lck}-/- and wild type mice (Figure 14A,B). Tregs from both groups were able to inhibit the expansion of T-responders from *Sfpi1*^{lck}-/- and wild type mice in a similar manner. Our results suggest that the in vitro suppressive function of nTregs from *Sfpi1*^{lck}-/- mice is not impaired, although the in vivo function of these cells needs to be investigated further.

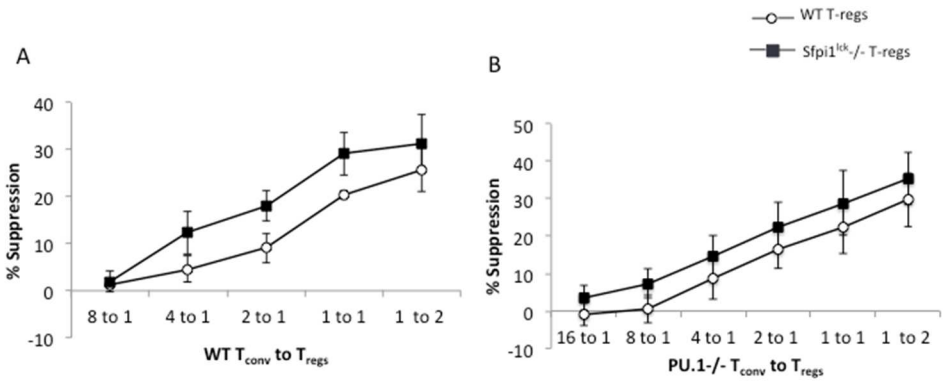


Figure 14. nTregs from *Sfpi1*^{lck}-/- and wild type mice have similar suppressive abilities. nTregs from Sfpi1^{lck}-/- and wild type mice were tested for their ability to inhibit proliferation of T-responder cells. Suppression of wild type

(A) or *Sfpi1*^{lck}-/- (B) T-responder cells by nTregs from *Sfpi1*^{lck}-/- and wild type mice was measured and compared to T-responders cultured only in the presence of soluble anti-CD3 and APCs.

Part II PU.1 Regulates T-cell dependent Germinal Center Responses

Results

PU.1 regulates the expression of CD40L and IL-21

Although PU.1 induces IL-9 production in Th9 cells, whether it has functions in other Th subsets is not known. Results from a study comparing T cells with ectopically expressed PU.1 to control cells demonstrate that PU.1 can activate and repress gene expression in T cells (Figure 15A). Consistent with our previous reports (21, 77, 78) we observed repression of Th2 cytokine genes and induction of chemokines associated with type 2 inflammation (Figure 15A). Among other PU.1-repressed genes we observed genes encoding CD40L and IL-21. To investigate whether endogenous PU.1 regulates the expression of CD40L in CD4+T cells, CD4+ T cells were isolated from wild type and mice with a conditional allele of *Sfpi1* that encodes PU.1 crossed to an Lck-Cre transgenic strain (termed *Sfpi1*^{lck}-/- mice) and stimulated with PMA and Ionomycin for 2 hours. After 2 hours of stimulation, surface and intracellular CD40L expression was determined by flow cytometry. An increase in the percentage of $CD4^+$ cells that stained positive for surface and intracellular CD40L was observed when stimulated CD4+ cells from *Sfpi1*^{lck}-/- mice were compared to cells from wild type mice (Figure 15B,C). The CD40L mean fluorescence intensity was also

increased in stimulated CD4+ cells from *Sfpi1*^{lck}-/- mice (Figure 15D). These data demonstrate that PU.1 limits the expression of CD40L in CD4+ T cells.

Many of the Th subsets express IL-21 (88, 118-120). We differentiated Th1, Th2, Th9, Th17 and T-regulatory cells in vitro for 5 days, restimulated equal numbers of cells on α-CD3 coated plates for an additional 24 hours, and collected supernatants to measure IL-21 using ELISA. Th17 cells, which are believed to secrete the highest levels of IL-21 amongst the Th subsets, derived from *Sfpi1*^{lck}-/- mice produced significantly more IL-21 compared to wild type mice (Figure 15E). However, IL-17A production was similar between both groups (Figure 15F). To demonstrate that replacement of PU.1 in Th17 cells normalizes the increased IL-21 expression, we ectopically expressed PU.1 in naïve *Sfpi1*^{lck}-/- cells cultured under Th17 conditions. Retroviral expression of PU.1 in *Sfpi1*^{lck}-/- cells decreased IL-21 expression (Figure 15G). Thus, PU.1 regulates the expression of CD40L and IL-21.

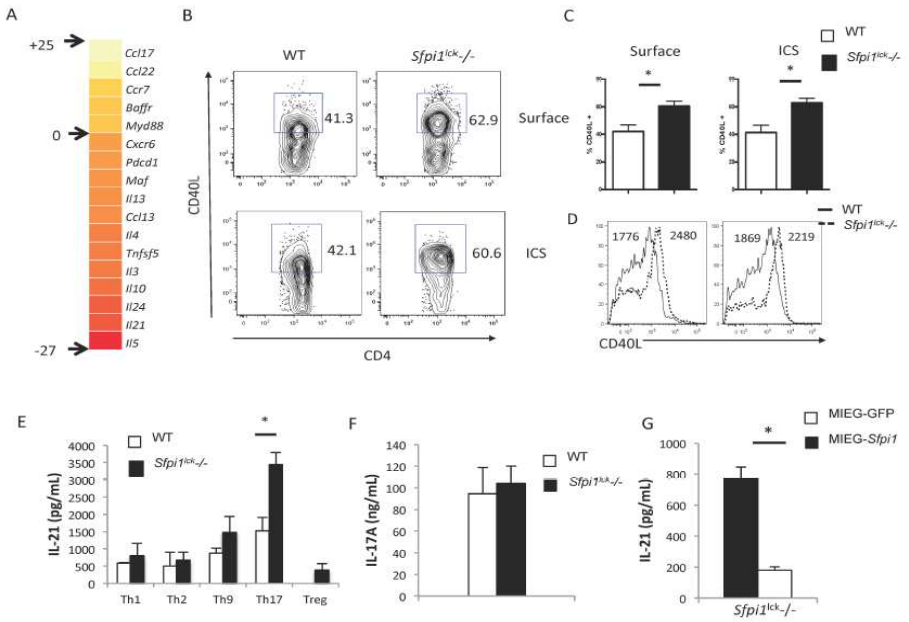


Figure 15. PU.1 regulates CD40L and IL-21 expression in vitro. (A) Heat map showing changes in expression of genes from overexpression of PU.1 in $CD4^+$ T cells. Numbers indicate fold change in expression compared to control transduced cells. (B-C) Total $CD4^+$ T cells from *Sfpi1*^{lck}-/- and WT mice were stimulated with PMA + Ionomycin for 2 hours. Surface and intracellular staining for CD40L was conducted and flow cytometry plots are shown (B). Bar graphs indicate the percent of $CD4^+CD40L^+$ cells (C) and mean fluorescence intensity for CD40L staining that is shown by histogram (D). (E-F) Naïve $CD4^+CD62L^+$ T cells were isolated from WT and *Sfpi1*^{lck}-/- mice and cultured under Th1, Th2, Th9, Th17, and T-regulatory conditions. IL-21 production by each Th subset (E) and IL-17A production by Th17 cells (F) was measured by ELISA. (G) Naïve cells from *Sfpi1*^{lck}-/- mice were cultured under Th17 conditions and subsequently transduced with retrovirus expressing GFP, MIEG-GFP, or a vector expressing the PU.1 gene, MIEG-*Sfpi1*. After 5 days of culture, cells were sorted by GFP expression and restimulated with anti-CD3 coated plates. Supernatants were collected and IL-21 secretion was measured by ELISA. Data are representative of two independent experiments with a total of 3-5 samples of each genotype per experiment. Statistical significance was determined with a two-tailed t test. *, $p<0.05$.

MOG_{35-55} immunized Sfpi1^{lck}-/- mice show decreased resolution of germinal center activity

CD40L and IL-21 promote germinal center activity and germinal center B-cell differentiation (46, 47, 121-123). The cognate interaction between CD40-expressing GCB cells and CD40L-expressing Tfh cells is important for germinal center B-cell survival, proliferation, and maturation. Lack of CD40L expression in humans and mice impairs proper germinal center formation and function (46, 124-128). IL-21 also contributes, though is not essential for the differentiation and expansion of GCB cells (121). The findings that PU.1 can regulate CD40L and IL-21 expression led us to hypothesize that PU.1 may be important in Tfh and germinal center activity. To investigate if PU.1 has any impact on Tfh development, wild type and *Sfpi1*^{lck}-/- mice were immunized with an emulsion containing myelin oligodendricyte glycoprotein (MOG) peptide in CFA. At several time points after immunization the spleens from wild type and *Sfpi1*^{lck}-/- mice were analyzed for expression of *Il21*, *Tnfsf5* (encoding CD40L), and *Bcl6* by qPCR. *Il21* expression in splenocytes from immunized *Sfpi1*^{lck}-/- mice was significantly increased at 12 and 25 days after immunization, compared to splenocytes from wild type mice, but expression of *Bcl6* was not significantly altered (Figure 16A-B). *Tnfsf5* expression was also higher compared to wild type mice, though only significantly at the later time point (Figure 16 A-B). We then examined splenic Tfh cells using flow cytometry. *Sfpi1*^{lck}-/- mice showed an increased percentage of Tfh cells ($CD4^+CXCR5^+PD\text{-}1^+$) 12 days after immunization compared to wild type mice (Figure 16C). The increase in the percentage of Tfh cells in *Sfpi1*^{lck}-/- mice persisted on Days 25 and 28 (Figure

2C). Interestingly, by day 28, the Tfh response had entirely waned in wild type mice, contrasting *Sfpi1*^{lck}-/- mice that maintained a smaller but significant percentage of Tfh cells. The mean fluorescence intensity (MFI) of PD-1 and ICOS expressed on the surface of Tfh cells was greater in *Sfpi1*^{lck}-/- compared to wild type mice (Figure 16D). Increased PD-1 expression is consistent with an ability of PU.1 to repress *Pdcd1* expression (Fig. 15A).

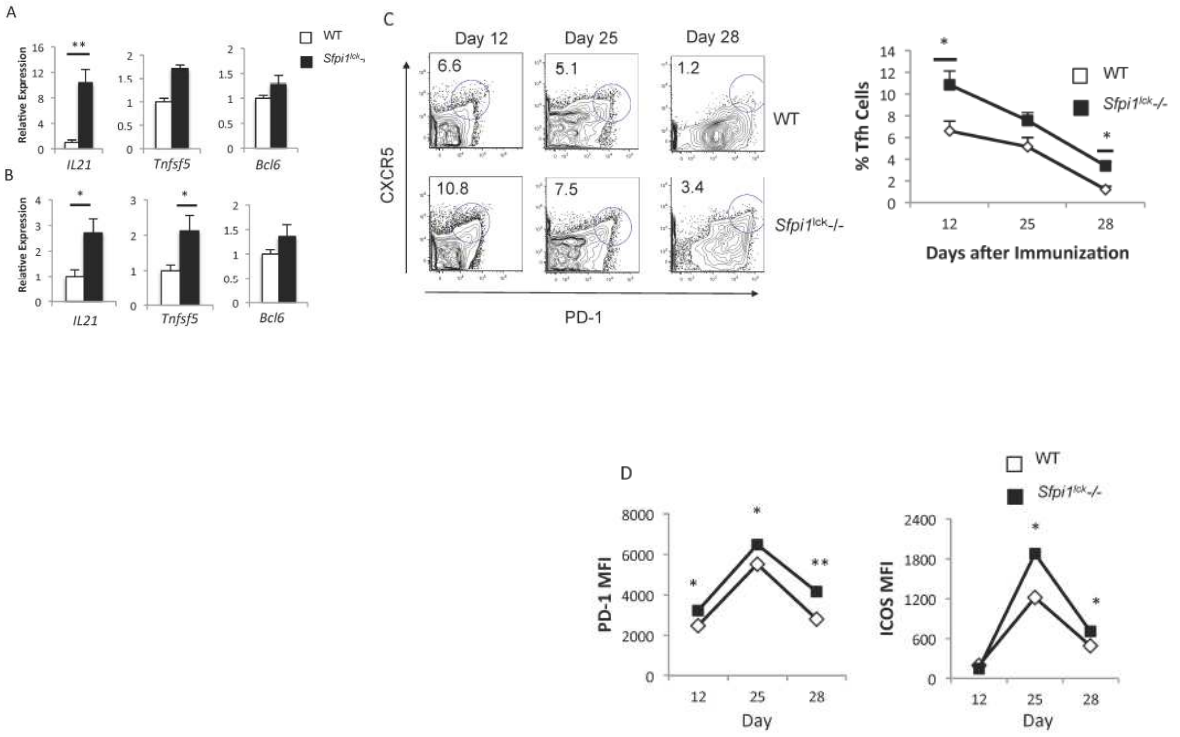


Figure 16. *Sfpi1*^{lck}-/- mice have increased Tfh cells, and IL-21 and CD40L expression after immunization with MOG$_{35-55}$. WT and *Sfpi1*^{lck}-/- mice were immunized with MOG$_{35-55}$ and sacrificed 12, 25, and 28 days after initial immunization. The spleens from immunized mice were harvested and splenocytes from wild type and *Sfpi1*^{lck}-/- mice were collected for mRNA analysis.

mRNA levels of *Il21*, *Tnfsf5* (CD40L), and *Bcl6* 12 (A) and 25 (B) days after immunization were determined by qPCR. Splenocytes from immunized mice were stained for Tfh markers and analyzed by flow cytometry. (C) Percentage of Tfh in wild type and *Sfpi1*^{lck}-/- mice on days 12, 25 and 28 were determined from dot plots that are gated on CD4+ cells. (D) PD-1 and ICOS expression by WT and *Sfpi1*^{lck}-/- Tfh cells were also measured by flow cytometry.. Data are representative of 2-3 experiments with 3-6 mice per group (A-D). Statistical significance was determined with a two-tailed t test. *, $p<0.05$; **, $p<0.005$.

To determine if increased Tfh numbers and function resulted in increased germinal center B (GCB) cells, we examined GCB populations using flow cytometry and antigen-specific antibody production in the serum. We observed increased GCB cells in *Sfpi1*^{lck}-/- compared to wild type mice beginning on day 12 (Figure 17A). The percent of GCB in *Sfpi1*^{lck}-/- mice increased over the 28 day period examined, and was significantly greater than observed in wild type mice at all time points (Figure 17A). In agreement with the increase in GCB cells, we also observed increased IgG titers at day 12 (Figure 17B) and increased MOG_{35-55}-specific IgG and $IgG2_c$ titers in *Sfpi1*^{lck}-/- mice compared to wild type mice on day 25 (Figure 17C). The enhanced germinal center activity in *Sfpi1*^{lck}-/- mice suggests that PU.1 is a negative regulator of Tfh cells and indirectly regulates GCB cell formation and without PU.1 expression in Tfh cells, the resolution of germinal center activity is attenuated.

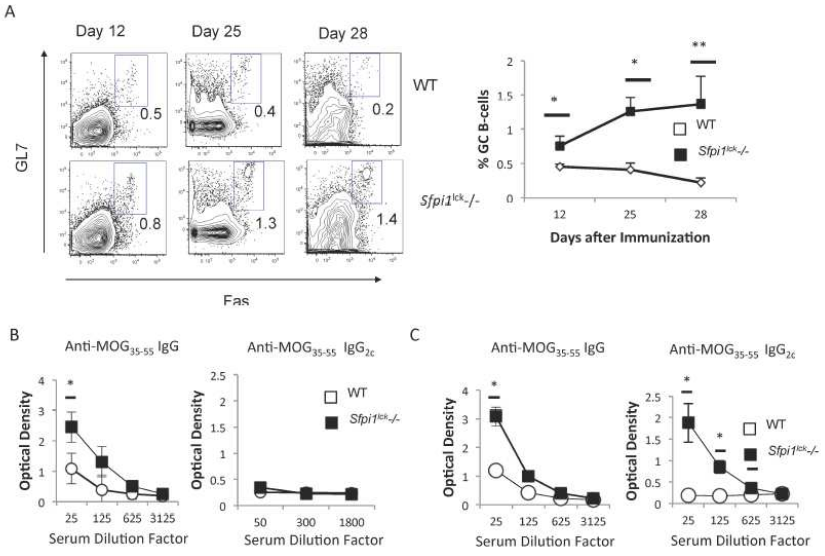


Figure 17. *Sfpi1*^{lck}-/- mice have enhanced germinal center B-cell development after MOG$_{35-55}$ immunization. WT and *Sfpi1*^{lck}-/- mice were immunized with MOG$_{35-55}$ and after 12, 25, and 28 days splenocytes were stained with B220, Fas and GL7 antibodies to identify GCB cells and analyzed using flow cytometry. Data shown are gated on B220+ cells (A). Antigen-specific serum antibody titers for IgG and IgG2a were measured on days 12 (B) and 25 (C). Data are representative of 2-3 experiments with 3-6 mice per group. Statistical significance was determined with a two-tailed t test. *, $p<0.05$; **, $p<0.005$.

Increased germinal center formation in the absence of PU.1 following SRBC immunization

We then wanted to examine PU.1 expression in the Tfh population. To test this, we immunized wild type and *Sfpi1*^{lck}-/- mice with sheep red blood cells (SRBCs). SRBCs are highly immunogenic, resulting in a robust GC activity, allowing for the study of greater numbers of Tfh cells. We sorted the CD4+CXCR5+PD-1+ Tfh

population from wild type mice and analyzed PU.1 expression by qPCR. PU.1 expression was higher in Tfh cells compared to non-Tfh cells (CD4+CXCR5-PD-1-) (Figure 18A).

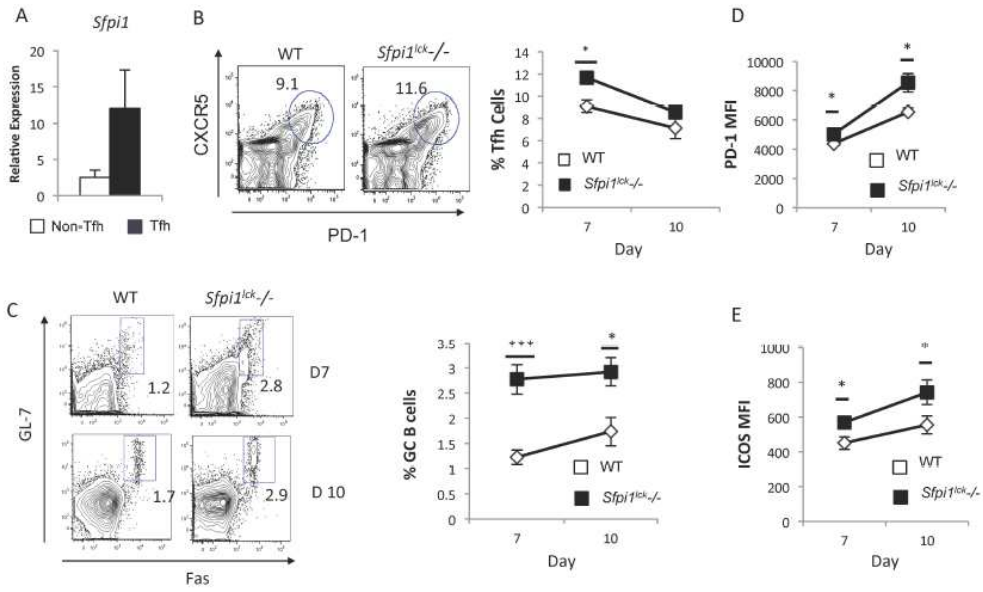


Figure 18. Tfh and GC B cell analysis in WT and *Sfpi1*^{lck}-/- mice after SRBC immunization. WT and *Sfpi1*^{lck}-/- mice were immunized with sheep red blood cells (SRBC) for analysis of germinal center activity. (A) Splenocytes from WT mice were sorted into CD4$^+$CXCR5$^+$PD-1$^+$ Tfh cells (black bar) and CD4$^+$CXCR5$^-$ PD-1$^-$ Non-Tfh cells (white bar) and PU.1 expression was determined by qPCR. (B) Splenocytes from SRBC immunized mice were stained for Tfh cells at day 7 (B) or GC B cells at day 7 and 10 (C) and analyzed by flow cytometry. (D-E) The mean fluorescence intensity of PD-1 and ICOS on Tfh cells 7 and 10 days after immunization were determined by flow cytometry. Data are representative of 2 experiments with 6 mice per group (A) and 2 experiments with 8 mice per group

(B-E). Statistical significance was determined with a two-tailed t test, is indicated as follows: *, p<0.05; ***, p<.0001.

The SRBC system also allowed us to test our conclusions from the adjuvant-dependent system using an adjuvant-independent approach. We examined Tfh and GCB cells seven and ten days after SRBC immunization. *Sfpi1*^{lck}-/- mice showed a higher percentage of Tfh and GCB cells compared to wild type mice seven days after immunization (Figure 18B,C). On day 10, the percentage of Tfh cells in *Sfpi1*^{lck}-/- mice and wild type mice was similar, however, the percentage of GCB cells in *Sfpi1*^{lck}-/- mice was significantly higher than in wild type mice (Figure 18B, C). Tfh cells from *Sfpi1*^{lck}-/- mice also showed significantly higher levels of PD-1 and ICOS on both day 7 and day 10 (Figure 18D,E). To further understand what factors may be contributing to the increase in GCB cells in *Sfpi1*^{lck}-/- mice, we looked at the expression of CD40L and IL-21 in Tfh cells from *Sfpi1*^{lck}-/- and wild type mice. Tfh cells from *Sfpi1*^{lck}-/- mice had increased expression of CD40L compared to wild type mice (Figure 19A,B). *Sfpi1*^{lck}-/- Tfh cells also had a higher percentage of IL-21-positive Tfh cells with a higher MFI compared to wild type mice (Figure 19C, D and data not shown). The number of IL-21-positive cells within the spleen of *Sfpi1*^{lck}-/- mice was two-fold larger than the number seen in wild type mice (Figure 19E). Apart from the increased CD40L and IL-21 expression by *Sfpi1*^{lck}-/- Tfh cells the increase in GCB cells in immunized *Sfpi1*^{lck}-/- could also be due to decreased follicular regulatory cells. However, the percentage of Tfr cells within the spleens of *Sfpi1*^{lck}-/- and wild type

mice were similar (data not shown). SRBC protein specific IgG1 and $IgG2_c$ antibody titers were also significantly elevated in *Sfpi1*^{lck}-/- mice compared to wild type mice (Figure 19F,G).

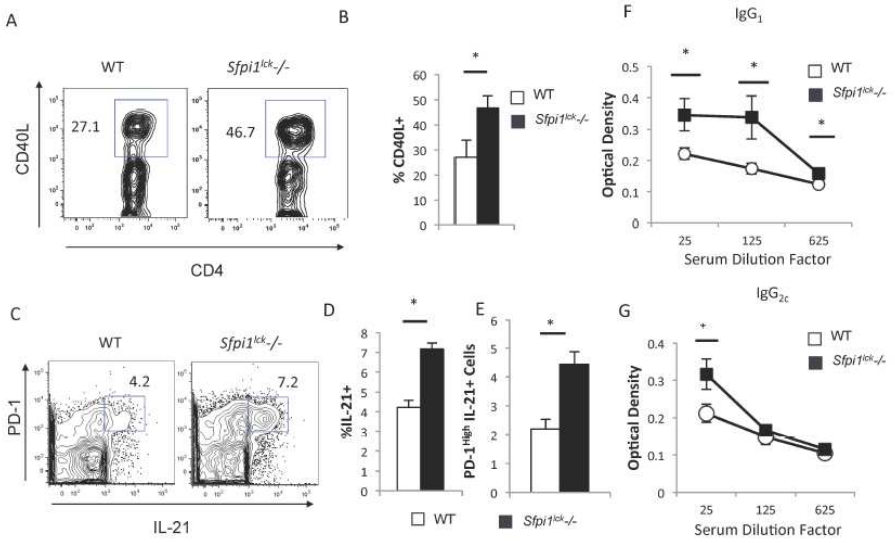


Figure 19. CD40L and IL-21 expression and Ig titers from WT and *Sfpi1*^{lck}-/- mice after SRBC immunization. WT and *Sfpi1*^{lck}-/- mice were immunized with SRBC and stained for CD40L and IL-21 expression in Tfh cells. CD40L (A,B) and IL-21 (C,D) expression by WT and *Sfpi1*^{lck}-/- Tfh cells was determined by flow cytometry. (E) The percentage of $CD4^+CXCR5^+$ $PD\text{-}1^{High}$ cells that are $IL\text{-}21^+$ was determined by flow cytometry. Serum from SRBC immunized WT and *Sfpi1*^{lck}-/- mice were collected and IgG_1 (F) and IgG_{2c} (G) titers for SRBC protein specific antibodies were determined by ELISA. Data are representative of 2 experiments with 5-6 mice per group (A-D) and 2 experiments with 6 mice per group (C-G). Statistical significance was determined with a two-tailed t test. *, $p<0.05$.

We next determined if there were alterations in the expression of other Tfh-associated genes caused by the absence of PU.1, we sorted Tfh cells ($CD4^+CXCR5^+PD\text{-}1^+$) from *Sfpi1*^{lck}-/- and wild type mice 7 days after sRBC immunization. *Sfpi1*^{lck}-/- Tfh cells showed a significant increase in *Tbx21* mRNA expression (Figure 20), consistent with previous reports of increased T-bet expression in IL-21-secreting Tfh cells (129) . We also observed that *Sfpi1*^{lck}-/- Tfh cells showed a significant increase in *Bcl6*, and *Maf* expression (Figure 20). *Sfpi1*^{lck}-/- Tfh cells also showed a decrease in *Irf4* mRNA expression (Figure 20). We did not observe any difference in *Gata3, Ifng, or Il4* expression by wild type and *Sfpi1*^{lck}-/- Tfh cells (Figure 20). Together, these data indicate an important requirement for PU.1 in limiting Tfh development and regulating multiple genes within the Tfh population.

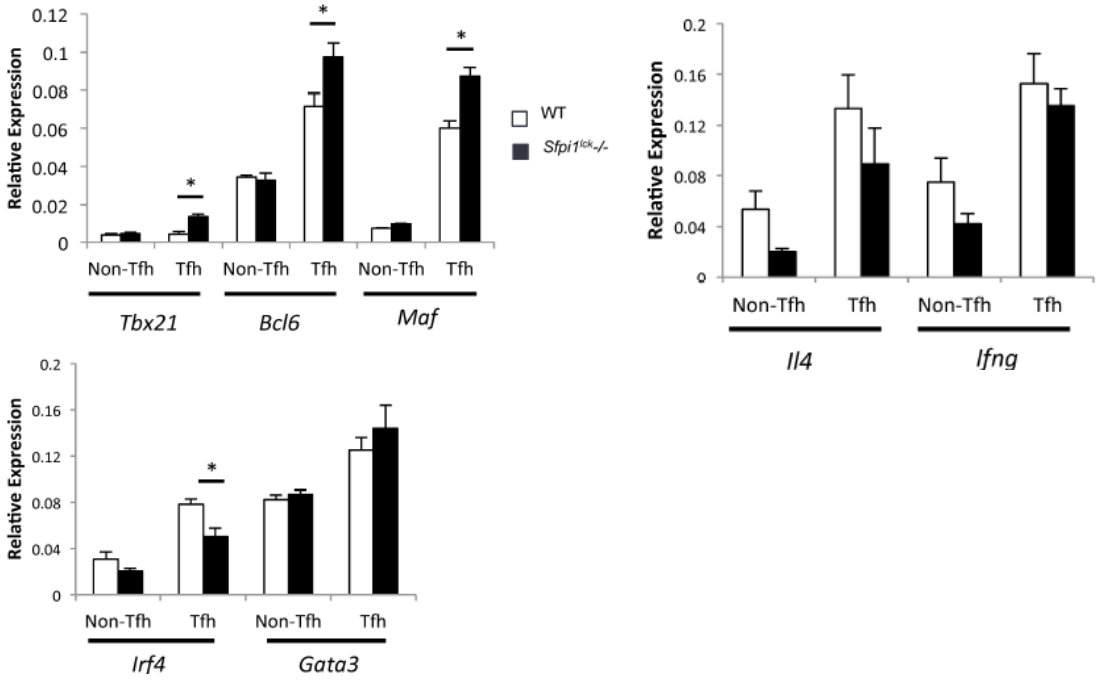


Figure 20. Gene expression analysis of sorted Tfh cells from SRBC immunized WT and *Sfpi1*^{lck}-/- mice. Tfh and Non-Tfh cells were sorted from SRBC immunized WT and *Sfpi1*^{lck}-/- mice and expression of the indicated genes was determined by qPCR. Data are representative of 2 experiments with 6 mice per group. Statistical significance was determined with a two-tailed t test. *, $p<0.05$.

Blocking CD40L in Sfpi1^{lck}*-/- mice decreases Germinal Center B cells and restores normal Immunoglobulin levels*

CD40L and IL-21 both play an important role in the expansion of GCB cells. However, the absence of CD40L expression in humans and mice appears to have a more dramatic impact on GC development and subsequently B-cell function (46, 118, 122). We used blocking antibodies against IL-21 or CD40L to determine if either factor was contributing to the enhanced development of GCB cells seen in *Sfpi1*^{lck}-/- mice. We immunized *Sfpi1*^{lck}-/- mice with SRBC and injected mice with IL-21 blocking antibodies on days 2, 4, and 6. On day 7 mice were sacrificed and GC Tfh and B cells were assessed. We found that blocking IL-21 did not change the number of GCB cells in wild type or *Sfpi1*^{lck}-/- mice (data not shown). We next blocked CD40L signaling after SRBC immunization by treating *Sfpi1*^{lck}-/- and wild type mice with control antibody or antibodies against CD40L. Blocking CD40L significantly decreased the number of GCB cells in *Sfpi1*^{lck}-/ (Figure 21A). Despite the reduction in GCB cells in *Sfpi1*^{lck}-/- mice that received CD40L blocking antibody, there were still elevated GCB cell numbers in

Sfpi1^{lck}-/- mice compared to wild type mice that received control antibody. We observed a slight but not statistically significant decrease in Tfh cells in *Sfpi1*^{lck}-/- mice after CD40L antibody treatment (Figure 21B). To determine if the anti-CD40L-mediated reduction in GCB cells was reflected in function, we analyzed the titers of IgG_{2c} in mice that received control and CD40L blocking antibodies. Anti-CD40L treatment attenuated the increase in IgG_{2c} compared to *Sfpi1*^{lck}-/- mice that received control antibody (Figure 21C). The titers of IgG_{2c} observed in *Sfpi1*^{lck}-/- mice that received anti-CD40L antibody was indistinguishable from titers observed in wild type mice (Figure 21C). Thus, PU.1 negatively regulates Tfh cell development and controls GCB cell numbers by a mechanism that is at least partly dependent upon CD40L.

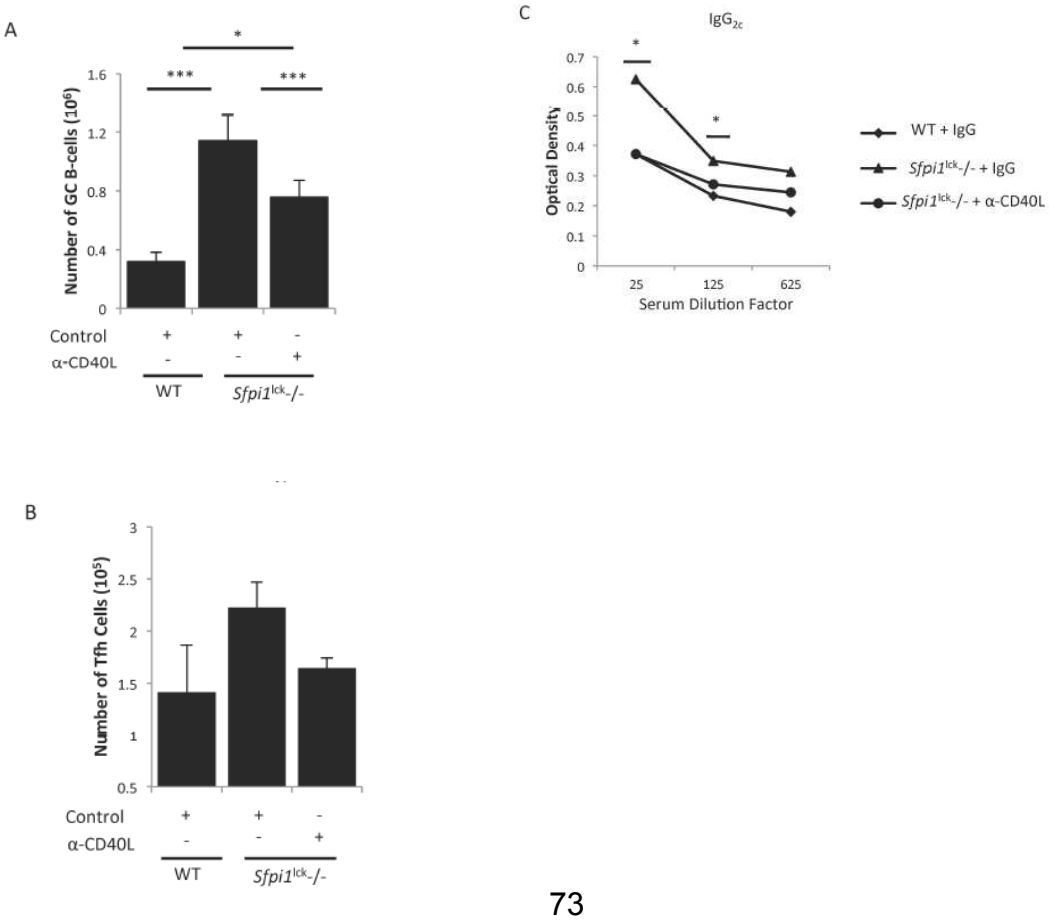


Figure 21. Blocking of CD40L abrogates germinal center B-cell increases in *Sfpi1*^{lck}-/- mice. WT and *Sfpi1*^{lck}-/- mice were immunized with SRBC and treated with control antibody or CD40L blocking antibody on days 5 and 6 post-immunization. On day 7 mice were sacrificed and splenocytes analyzed for Tfh cells and GCB cells by flow cytometry. The total number of GCB cells (A) and Tfh cells (B) are indicated. (C) SRBC protein specific IgG2c titers were analyzed by ELISA. Data are representative of 2 experiments with 4-8 mice per group (A-C). Statistical significance was determined with one way ANOVA (A,B) and two-tailed t test (C), is indicated as follows: *p<0.05; ***p<.0001.

Discussion

Characterization of Th9 cells

The purpose of T-helper cell polarization is to promote the production of appropriate cytokines and other factors necessary for elimination of the infectious agent. T-helper cells are often identified by the cytokines they express and initially Th9 cells were identified by their selective production of IL-9. Work in our lab has focused on expanding the understanding of what other characteristics can be attributed to the Th9 population. We identified Th9 cells as the major T-helper subset expressing CCR4, IL-1RA and BAFF. We conducted functional studies looking at the effect of CCR4 expression on the migration of Th9 cells in response to chemokine and observed that more Th9 cells migrate in response to chemokine when compared to Th2 cells. We also looked at the effect of BAFF expression by Th9 cells on the development of allergic inflammation of the lung.

Preliminary data suggested that overexpression of BAFF by Th9 cells attenuates the development of allergen induced pulmonary inflammation.

The in vivo significance of increased expression of CCR4, IL-1RA and BAFF by Th9 cells has not been investigated. Although, we have demonstrated a possible functional role for increased CCR4 expression by Th9 cells it is not clear if Th9 cells actually express CCR4 in vivo. Further, the importance of IL-1RA expression by Th9 cells will also require further investigation. The ability of BAFF to limit allergic inflammation of the lung has been demonstrated previously by another group (100), however BAFF has also been shown to affect other aspects of immunity and whether BAFF derived Th9 cells can contribute in these areas will be an important question to address. BAFF is an important regulator of B-cell activity (96). Depending on the receptor present, BAFF can promote B-cell survival or apoptosis through BAFF-R and TACI signaling, respectively. Th9 derived BAFF may be important for controlling the local B-cell numbers during an immune response. IL-1RA produced by Th9 cells may have a similar effect in limiting the immune response. IL-1β can promote Th17 cell development (130) and the production of IL-1RA by Th9 cells may work to curtail the development of Th17 cells within the microenvironment of an immune response. The expression of BAFF and IL-1RA by Th9 cells and the potential anti-inflammatory function of these molecules suggest that Th9 cells have the ability to dampen the immune response. Further, the ability of IL-9, the signature cytokine of Th9 cells, to promote the function of Treg in the context of certain disease models further

supports the idea that Th9 cells can be anti-inflammatory (62, 63). Moreover, CCR4 expression by Th2 cells leads to the recruitment of Th2 cells during allergen induced lung inflammation (131). We have shown that IL-9 is required for the induction of allergen induced lung inflammation. The expression of CCR4 may be important for recruiting Th9 cells to the lung when allergen is present. Although, the connection of CCR4 expression by Th2 cells and their presence in the lung has been questioned due to studies where blocking CCR4 had little impact on abrogating the detection of $CCR4^+$ Th2 cells in the lung (132), it is possible that blocking CCR4 may have a more significant impact on the recruitment of Th9 cells in a different disease setting.

Having a better understanding of the unique set of genes expressed by Th9 cells will be key for future studies into the mechanisms utilized by Th9 cells to cause disease. Targeting IL-9 or PU.1 during disease pathogenesis will require a better understanding of the stability of the Th9 subset and the requirement of PU.1 for maintenance of IL-9 production. Our in vitro studies looking at conditions necessary for maintenance of IL-9 secretion by Th9 cells suggested that the standard conditions for polarizing Th9 cells, IL-4 + TGF-β, were insufficient for maintaining IL-9 production after multiple rounds of culture. The addition of IL-1β, IL-25, and IL-21, cytokines, which enhance IL-9 production from Th9 cells after one round of culture, did not rescue the dramatic decrease in IL-9 production after multiple rounds of culture. In vivo studies in mice where Th9 cells were transferred into mice also suggests that Th9 cells lose their ability to produce IL-9

after a period of time, but still retain IL-9-dependent functions (27, 64). There are still many questions about the stability of Th9 cells and it will be important to thoroughly understand if Th9 cells can persist in inflammatory conditions in order to develop a timetable for treatments targeting Th9 cells. Variation in IL-9 and PU.1 expression has been observed in patients with ulcerative colitis (UC) (82). Patients with active disease showed significant production of IL-9 and PU.1 when compared to healthy patients or patients with inactive ulcerative colitis. The variation in IL-9 production by UC patients may be evidence of the instability in IL-9 production during a disease process.

The role of IL-9 and PU.1 in CNS Inflammation

Our studies looking at a greater role for PU.1 in T-helper cell immunity lead us to investigate if the deletion of PU.1 from T-cells would impact the development of EAE. We observed that the absence of PU.1 in T-cells leads to increased mononuclear cells in the CNS of *Sfpi1*^{lck}-/- mice and the CD4^{+}cells in *Sfpi1*^{lck}-/- mice express higher levels of proinflammatory cytokines. As wild type mice began to recover from disease, the recovery of *Sfpi1*^{lck}-/- mice was attenuated. There was also greater cellular infiltration in the spinal cords of *Sfpi1*^{lck}-/- mice compared to wild type. Although, we were not able to address our initial hypothesis concerning the role of IL-9 in EAE, we did observe changes in the expression of other cytokines that may explain the phenotype of *Sfpi1*^{lck}-/- EAE mice.

There was a significant increase in the expression of IL-21 in the spleens of *Sfpi1*^{lck}-/- mice 12 and 25 days p.i. We have demonstrated that PU.1 can regulate the expression of IL-21 by Th17 cells and Tfh cells. Therefore, the increased IL-21 expression in the spleens of *Sfpi1*^{lck}-/- EAE mice could be from increased production of IL-21 by Th17 cells and Tfh cells. Initially there was conflicting evidence concerning the requirement of IL-21 for induction of EAE but there is strong evidence suggesting that IL-21 is not required for initiation of EAE (133, 134). One report looked at the impact of treating EAE mice with IL-21 before or after MOG_{35-55} immunization. Treating EAE mice with IL-21 before immunization resulted in worse disease and treating mice with IL-21 after immunization did not alter the disease course. The mechanism of the IL-21 induced exacerbation of disease course was found to be dependent on NK cells, which produced higher levels of IFN-γ when treated with IL-21 (135). In our EAE experiments we observed a higher percentage of IFN-γ^+ cells infiltrating the CNS. The higher levels of IL-21 in the spleens of *Sfpi1*^{lck}-/- EAE mice may have triggered more NK cell activation leading to increased production of IFN-γ within the spleen causing increased differentiation of MOG-specific Th1 cells. In contrast to the proinflammatory role of IL-21 in EAE, induction of EAE in IL-21 -/- and IL-21R -/- mice lead to exacerbated disease (133) suggesting that IL-21 may be important for attenuating the EAE disease severity. Similarly, another group has shown that blocking IL-21 during EAE leads to exacerbated disease (136) and we observed similar results in studies where we blocked IL-21 in *Sfpi1*^{lck}-/- and WT mice. Thus, IL-21 appears to have different effects in the pathogenesis

of EAE and it is not clear if the elevated levels of IL-21 in *Sfpi1*^{lck}-/- EAE mice contributed to the disease course observed or if it was a result of other processes occurring in the spleen.

Sfpi1^{lck}-/- EAE mice also showed increased levels of CD40L expression in the spleen. CD40L is a co-stimulatory molecule expressed by cell types including activated T-cells and Tfh cells. The absence of CD40L expression completely abrogates the development of EAE (137). Our studies show that PU.1 can regulate the expression of CD40L by activated T-cells. The ability of PU.1 to regulate the expression of CD40L leads us to speculate that the increase in CNS cellular infiltration and disease severity seen in *Sfpi1*^{lck}-/- mice could be due to increased CD40L expression on T-cells. It has been demonstrated that cytokines such as IL-2 can induce the expression of CD40L (138) and IL-2 is also important for T-cell proliferation. Our previous work showed that activating cells from *Sfpi1*^{lck}-/- mice with increasing amounts of α-CD3 leads to significantly more production of IL-2 (77). Twelve days p.i. our *Sfpi1*^{lck}-/-EAE mice show a two fold increase in the number of $CD4^+$ cells present in brain tissue compared to WT mice and we see a similar increase 25 days p.i. The increase in $CD4^+$ cells infiltrating the CNS of *Sfpi1*^{lck}-/-EAE mice could be a result of increased IL-2 expression in *Sfpi1*^{lck}-/- mice inducing the expansion of MOG- specific T-cells. The IL-2 could also lead to increased CD40L expression by *Sfpi1*^{lck}-/- encephalitogenic T-cells in the CNS, allowing for continuous activation of APCs thereby attenuating the resolution of disease.

The role of PU.1 in Tfh and Germinal Center Activity

Mounting a lasting immune response against invading organisms is a key component of the adaptive immune response. Tfh cells and GCB cells are vital factors in the production of high affinity antibodies during an initial infection and later during subsequent infections. A balance of initiation and resolution of the germinal center response is required for prevention of recurrent or persistent infections or on the other side of the spectrum, autoimmunity. Therefore, a comprehensive understanding of the factors that positively or negatively regulate germinal center activity is vital. Tfh cells express the surface proteins that facilitate cognate B cell interactions within the germinal center. Among the proteins that facilitate these interactions, CD40L and IL-21 are principal orchestrators within germinal centers that enable Tfh cells to provide help to B cells. Yet, how CD40L and IL-21 are regulated in Tfh cells is not completely understood.

We present evidence that shows PU.1 is important for regulating CD40L and IL-21 expression by Tfh cells. These results point to PU.1 as a negative regulator of CD40L and IL-21 and consequently germinal center B-cell development and antigen specific antibody production. IL-21 expression by Tfh cells has been shown to be downstream of ICOS and c-maf (139). The array data shows that ectopic expression of PU.1 in $CD4^+$ T cells leads to decreased *Il21* and *Maf* mRNA, but not ICOS. Our in vivo studies show increased expression of IL-21

protein and *Maf* mRNA in *Sfpi1*^{lck}-/- Tfh cells, compared to controls. PD-1 expression was also decreased in the array and Tfh cells in *Sfpi1*^{lck}-/- mice express greater amounts of PD-1. Whether the regulation of PD-1 on T cells is a direct effect of PU.1, or whether it might result from altered interactions with GCB cells is not yet clear.

T-cell receptor (TCR) signaling is necessary for multiple stages of Tfh and germinal center development. A combination of cytokines such as IL-6 (140), surface receptor interactions (141, 142), and TCR signaling (143, 144) contribute to initial Tfh differentiation. Strong TCR signaling may also induce dendritic cells to produce higher levels of IL-6 in a manner that is dependent upon the induction of CD40L on the T cell (137). We have previously shown that the absence of PU.1 in CD4$^+$ T cells leads to increased TCR expression by splenocytes and increased T cell activation (77). Thus it is possible that the increase in CD40L expression by *Sfpi1*^{lck}-/- naïve CD4$^+$ T cells we report here could be linked to an increase in TCR expression leading to an increase in IL-6 production by DCs and increased Tfh differentiation. TCR signaling is important for initial Tfh development outside of the germinal center and within the germinal center. The interaction between TCR expressed by germinal center Tfh cells and p:MHC II complexes expressed by GCB cells facilitates the selection of B cells expressing antibodies of highest affinity. Overexpression of TCR could affect the selection process by providing survival signals to B cells that would have normally

undergone apoptosis. As such, the altered threshold of activation in *Sfpi1*^{lck}-/- T cells might impact increased Tfh and GCB cell numbers described in this report.

Our findings also suggest that changes in the expression of PU.1 in T cells might play a role in autoimmunity. Gene association studies have linked increased PU.1 expression in CD4+ T cells to systemic lupus erythematosus (145) and the ability of PU.1 to alter Tfh functions might be a potential mechanism for increased autoantibody production. Moreover, the ETS DNA recognition motif is enriched in sequences near enhancer regions associated with human Tfh cell gene regulation (146). These data would suggest that altered PU.1 expression might contribute to autoantibody production in patient populations.

Our studies have provided evidence for an expanded role for PU.1 in T-helper cell biology. The ability of PU.1 to regulate gene expression within different cell types is largely dependent upon the level of PU.1 expression. In myeloid cells where PU.1 serves as a master regulator of gene expression, PU.1 expression levels are very high, in contrast to B-cells where PU.1 is present but is not required to induce the phenotype of mature B-cells. A similar framework of PU.1 expression appears to exist in T-helper cells. On one hand you have Th9 cells, where expression of PU.1 is high and PU.1 is required for polarization of Th9 cells and IL-9 production. On the other hand, low levels of PU.1 expression in Th2 cells has some impact on the Th2 phenotype but PU.1 is not required for the

development of Th2 cells. Where Tfh cells fit in on the spectrum of PU.1 expression is not clear. We did show that PU.1 expression is higher in Tfh cells compared to non-Tfh cells but we have not compared PU.1 expression from in vivo derived Th9 and Th2 cells specifically. Further, our in vitro and in vivo studies indicate that dysregulation of PU.1 expression in Th9 and Tfh cells would have the greatest impact on the balance between health and disease. In support of a role for PU.1 in promoting human disease, we have shown that Th9 cells differentiated using naïve cells from atopic patients have increased expression of PU.1 and IL-9 in comparison to Th9 cells differentiated using naïve cells from non-atopic patients (147). Our mouse in vivo studies also demonstrates that PU.1 plays a significant role in the development allergic pulmonary inflammation (21). Moreover, data presented in this dissertation using in vivo mouse models of disease provides evidence that PU.1 is required to regulate Tfh-cell dependent germinal center B-cell development and antibody production, and CNS inflammation. However, whether PU.1 is important for regulating Tfh mediated germinal center B-cell responses and CNS inflammation in humans is not clear. In the future it will be important to investigate PU.1 expression in humans in the context of Tfh responses and CNS inflammation, as PU.1 may be a new molecule for drug development.

Future Directions

Characterizing Th9 Cells

The recent discovery of Th9 cells identifies a primary source of the cytokine IL-9. Whether Th9 cells are a distinct T-helper lineage on their own was initially questioned but studies both in humans and in mice provide support for Th9 cells being a unique subset of cells that produce IL-9 without co-expression of other cytokines (53, 54). Many of the studies looking at the involvement of Th9 cells during various pathologies have been preclinical, utilizing mouse models of atopic disease, autoimmune disease, and tumor immunity to elucidate the contribution of Th9 cells during disease pathology. The focus of these studies has been on IL-9 production by Th9 cells. In the future, It will be important to begin to look at other aspects of the Th9 phenotype such as the expression of BAFF, CCR4 and IL-1RA in these mouse models to determine how these factors contribute to the development of Th9 mediated pathology. It is possible that BAFF and IL-1RA may act to dampen Th9 induced inflammation, which suggests like IL-1RA, BAFF could be used therapeutically to inhibit inflammation. Moreover, in our studies, BAFF secretion was detected in Th2 and Th9 cultures but not in the remaining T-helper subsets. Cytokines produced by Th2 and Th9 cells have different effects on the development of allergic inflammation in the lung and BAFF expression by these two subsets may have a different contribution to disease. To determine if BAFF has similar effects during a Th2 or Th9 induced allergic lung responses, transferring OT-II Th2 or Th9 cells that over express BAFF into naïve mice and challenging with OVA as we have done

previously, could provide more insight into the importance of BAFF during allergen induced lung inflammation. Further, the possible contribution of Th9 cells in collagen-induced arthritis, a mouse model for rheumatoid arthritis, has not been studied. If Th9 cells are shown to be involved it would be interesting to look at the impact of IL-1RA expression by Th9 cells on disease. Experiments where IL-1RA is removed or overexpressed in Th9 cells could demonstrate a role for Th9 derived- IL-1RA in limiting joint inflammation.

Looking ahead, some questions that have not been addressed concerning Th9 stability is whether PU.1 is required for persistence of IL-9 production by Th9 cells and what signals are required to maintain high levels of PU.1 over a sustained period of time. Previously, we've demonstrated that PU.1 is downstream of TGF-β (148), however when we culture Th9 cells for a second round in the presence of IL-4 and TGF-β, there is a significant decrease in PU.1 expression. We have not looked at the expression of the TGF-β receptors after multiple rounds of culture. It is possible that the TGF-β receptors or IL-4R are downregulated after one round of culturing. Smad2, Smad3, and Smad4 are also downstream of TGF-β and are required for IL-9 production (149, 150). Investigating the expression of the Smad proteins after multiple rounds of culturing could also be informative. Further, due to poor reagents and reduced cell viability it is difficult to assess PU.1 protein expression during multiple rounds of Th9 culturing. Work in our lab has demonstrated that PU.1 increases access to the IL-9 locus by recruiting histone acetlyases (151). In the future if better

antibodies for the detection of PU.1 are developed it will be important to assess the level of PU.1 expression, which may be impact the accessibility of the IL-9 locus.

The role of IL-9 and PU.1 in CNS Inflammation

In our studies we did not see the decrease in IL-9 production in *Sfpi1*^{lck}-/- EAE mice that we expected to see. As it has been reported, there are a few other pathways that induce IL-9 expression. For example, Jagged2 signaling through the notch receptors can induce IL-9 in combination with Smad3 signaling. Jagged2 is expressed by T-cells and APCs during EAE (69). To determine if the increased expression of IL-9 in *Sfpi1*^{lck}-/- EAE mice is due to increased Jagged 2 signaling we could block Jagged2 signaling during EAE development in *Sfpi1*^{lck}-/- mice and determine if IL-9 levels are reduced. We could take a similar approach to block the OX40-OX40L (152) and TL1A/DR3 (153) signaling pathways as both pathways have been shown to induce IL-9 production in a PU.1 independent manner. Although, these experiments would address the increased IL-9 production, they may not provide an answer to why *Sfpi1*^{lck}-/- EAE mice have worse disease. Passive induction of EAE through adoptive transfer of MOG_{35-55} primed splenocytes from wild type and *Sfpi1*^{lck}-/- mice into naïve mice may provide further insight into the attenuated disease recovery of *Sfpi1*^{lck}-/- EAE mice. Our studies looking at the function of tTregs from wild type and *Sfpi1*^{lck}-/- mice show that tTregs from *Sfpi1*^{lck}-/- mice have similar functional capabilities

when compared to wild type mice. However, transfer experiments will help determine if the persistence of disease in *Sfpi1*^{lck}-/- mice is due to impaired Treg function in *Sfpi1*^{lck}-/- mice. To corroborate our findings from these transfer studies we could also cross Sfpi1$^{fl/fl}$ mice with the Foxp3-Cre mice and induce EAE in these mice.

In addition, we observed increased expression of CD40L in the spleens of *Sfpi1*^{lck}-/- EAE mice, looking for CD40L expression in the spinal cord or brain by immunofluorescence may provide further support for a role for CD40L in promoting exacerbated disease in *Sfpi1*^{lck}-/- mice. $CD4^+$ $CD40L^+$ T-cells and $CD40^+$ monocytes have been detected in CNS tissue from human autopsy patients (154). If increased CD40L expression is observed in the CNS of *Sfpi1*^{lck}-/- EAE mice, we could also investigate the maturation state of APCs. APCs play a pivotal role in licensing myelin specific T-cells to enter the parenchyma of the brain (155). In addition, CD40L-CD40 interaction on APCs leads to increased cytokine production and MHC II presentation (156), which would promote T-cell activation. We also detected increased immunoglobulin in the serum of *Sfpi1*^{lck}-/- EAE mice, which could have contributed to the worsened disease course. Blocking CD40L at different stages of disease progression and transferring serum from *Sfpi1*^{lck}-/- EAE mice to naïve wild type mice may be beneficial in helping to elucidate the mechanism of exacerbated disease observed in *Sfpi1*^{lck}-/- EAE mice.

The role of PU.1 in Tfh and Germinal Center Activity

PU.1 is a transcription factor that plays a prominent role in many different cell types. PU.1 is a master transcription factor for cells in the myeloid lineage and PU.1 can preferentially induce the expression of IL-9 in Th9 cells. The ability of PU.1 to regulate gene expression in two divergent cell types suggests that PU.1 may be operating in very distinct transcription factor networks allowing for specialized gene expression in the different cell types involved. PU.1 has been shown to interact with a variety of proteins. In B cells, BCL6 can repress the expression of the *Igκ* locus by interacting with PU.1 (157). Conversely, interactions between PU.1 and IRF4 can promote the expression of the *Igκ* gene in B cells but limit the expression of Th2 cytokines (76, 158). As BCL6 and IRF4 are required for Tfh development it is possible that the increase in CD40L and IL-21 expression in *Sfpi1*^{lck}-/- Tfh cells is a result of increased activity of BCL6 and IRF4. We did observe an increase in BCL6 mRNA in *Sfpi1*^{lck}-/- Tfh cells however IRF4 levels were significantly decreased. Deciphering if PU.1 can interact with BCL6 or IRF4 in Tfh cells could be challenging because of the low numbers of Tfh cells that develop in vivo. However, Tfh cells have been expanded ex vivo and a culturing system for the differentiation of “Tfh-like” cells in vitro has been developed (159). Although, using the in vitro culturing system would not be ideal it could help us generate preliminary evidence for the interaction between PU.1 and other transcription factors which may help use determine the how the absence of PU.1 leads to increased CD40L and IL-21 expression by *Sfpi1*^{lck}-/- Tfh cells. Alternatively, we could also investigate how PU.1 regulates CD40L and

IL-21 expression in activated T-cells and Th17 cells, respectively. It is not known if PU.1 can regulate CD40L and IL-21 expression by binding directly to DNA. Through the use of chromatin immunoprecipitation (ChIP) assays we can determine if PU.1 binds directly to the CD40L and IL-21 loci. In addition, investigating if PU.1 regulates the human Tfh cell activity will be important. The finding of the ETS motif as a major enhancer motif suggests that PU.1 could have some impact on the human Tfh cell phenotype.

REFERENCES

1. Bieber T (2008) Atopic Dermatitis. *N. Engl. J. Med.* 358(14):1483-1494.
2. Reinholz M, Ruzicka T, & Schauber J (2012) Cathelicidin LL-37: An Antimicrobial Peptide with a Role in Inflammatory Skin Disease. *Annals of Dermatology* 24(2):126-135.
3. Nicod LP (2005) Lung defences: an overview. *European Respiratory Review* 14(95):45-50.
4. Swidsinski A, Sydora BC, Doerffel Y, Loening-Baucke V, Vaneechoutte M, Lupicki M, Scholze J, Lochs H, & Dieleman LA (2007) Viscosity gradient within the mucus layer determines the mucosal barrier function and the spatial organization of the intestinal microbiota. *Inflammatory Bowel Diseases* 13(8):963-970.
5. Merad M, Sathe P, Helft J, Miller J, & Mortha A (2013) The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting. *Annual Review of Immunology* 31(1):563-604.
6. Kang EM, Marciano BE, DeRavin S, Zarember KA, Holland SM, & Malech HL (2011) Chronic granulomatous disease: Overview and hematopoietic stem cell transplantation. *Journal of Allergy and Clinical Immunology* 127(6):1319-1326.
7. Buckley RH (2004) The multiple causes of human SCID. *The Journal of Clinical Investigation* 114(10):1409-1411.

8. Kay AB (2001) Allergy and allergic diseases. First of two parts. *The New England journal of medicine* 344(1):30-37.

9. Sakaguchi S, Yamaguchi T, Nomura T, & Ono M (2008) Regulatory T Cells and Immune Tolerance. *Cell* 133(5):775-787.

10. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, & Coffman RL (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *The Journal of Immunology* 136(7):2348-2357.

11. Zhu J & Paul WE (2008) CD4 T cells: fates, functions, and faults. *Blood* 112(5):1557-1569.

12. Zhu S & Qian Y (2012) IL-17/IL-17 receptor system in autoimmune disease: mechanisms and therapeutic potential. *Clinical science* 122(11):487-511.

13. Baxter AG (2007) The origin and application of experimental autoimmune encephalomyelitis. *Nat Rev Immunol* 7(11):904-912.

14. Zheng W-p & Flavell RA (1997) The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells. *Cell* 89(4):587-596.

15. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, & Glimcher LH (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100(6):655-669.

16. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, & Littman DR (2006) The Orphan Nuclear Receptor RORγt

Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells. *Cell* 126(6):1121-1133.

17. Hori S, Nomura T, & Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299(5609):1057-1061.

18. Fontenot JD, Gavin MA, & Rudensky AY (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature Immunology* 4(4):330-336.

19. Khattri R, Cox T, Yasayko SA, & Ramsdell F (2003) An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nature Immunology* 4(4):337-342.

20. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, Dent AL, Craft J, & Crotty S (2009) Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325(5943):1006-1010.

21. Chang H-C, Sehra S, Goswami R, Yao W, Yu Q, Stritesky GL, Jabeen R, McKinley C, Ahyi A-N, Han L, Nguyen ET, Robertson MJ, Perumal NB, Tepper RS, Nutt SL, & Kaplan MH (2010) The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol* 11(6):527-534.

22. Zhu J, Yamane H, & Paul WE (2010) Differentiation of Effector CD4 T Cell Populations*. *Annual Review of Immunology* 28(1):445-489.

23. Kanno Y, Vahedi G, Hirahara K, Singleton K, & O'Shea JJ (2012) Transcriptional and Epigenetic Control of T Helper Cell Specification: Molecular Mechanisms Underlying Commitment and Plasticity. *Annual Review of Immunology* 30(1):707-731.

24. Lazarevic V, Glimcher LH, & Lord GM (2013) T-bet: a bridge between innate and adaptive immunity. *Nat Rev Immunol* 13(11):777-789.

25. Schroder K, Hertzog PJ, Ravasi T, & Hume DA (2004) Interferon-γ: an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology* 75(2):163-189.

26. Kennedy R & Celis E (2008) Multiple roles for CD4+ T cells in anti-tumor immune responses. *Immunological reviews* 222(1):129-144.

27. Jager A, Dardalhon V, Sobel RA, Bettelli E, & Kuchroo VK (2009) Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *Journal of immunology* 183(11):7169-7177.

28. Pettinelli CB & McFarlin DE (1981) Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. *The Journal of Immunology* 127(4):1420-1423.

29. Feng T, Qin H, Wang L, Benveniste EN, Elson CO, & Cong Y (2011) Th17 Cells Induce Colitis and Promote Th1 Cell Responses through IL-17 Induction of Innate IL-12 and IL-23 Production. *The Journal of Immunology* 186(11):6313-6318.

30. Luger D, Silver PB, Tang J, Cua D, Chen Z, Iwakura Y, Bowman EP, Sgambellone NM, Chan C-C, & Caspi RR (2008) Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *The Journal of Experimental Medicine* 205(4):799-810.

31. Paul WE & Zhu J (2010) How are TH2-type immune responses initiated and amplified? *Nat Rev Immunol* 10(4):225-235.

32. Zhu J, Cote-Sierra J, Guo L, & Paul WE (2003) Stat5 Activation Plays a Critical Role in Th2 Differentiation. *Immunity* 19(5):739-748.

33. Cote-Sierra J, Foucras G, Guo L, Chiodetti L, Young HA, Hu-Li J, Zhu J, & Paul WE (2004) Interleukin 2 plays a central role in Th2 differentiation. *Proceedings of the National Academy of Sciences of the United States of America* 101(11):3880-3885.

34. Korn T, Bettelli E, Oukka M, & Kuchroo VK (2009) IL-17 and Th17 Cells. *Annual Review of Immunology* 27(1):485-517.

35. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, Lucian L, To W, Kwan S, Churakova T, Zurawski S, Wiekowski M, Lira SA, Gorman D, Kastelein RA, & Sedgwick JD (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421(6924):744-748.

36. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, & Cua DJ (2005) IL-23 drives

a pathogenic T cell population that induces autoimmune inflammation. *The Journal of Experimental Medicine* 201(2):233-240.

37. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, & Stockinger B (2006) TGFβ in the Context of an Inflammatory Cytokine Milieu Supports De Novo Differentiation of IL-17-Producing T Cells. *Immunity* 24(2):179-189.

38. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, & Kuchroo VK (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441(7090):235-238.

39. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, & Weaver CT (2006) Transforming growth factor-[beta] induces development of the TH17 lineage. *Nature* 441(7090):231-234.

40. Crotty S (2011) Follicular Helper CD4 T Cells (TFH). *Annual Review of Immunology* 29(1):621-663.

41. Dugas B, Renauld JC, Pène J, Bonnefoy JY, Peti-Frère C, Braquet P, Bousquet J, Van Snick J, & Mencia-Huerta JM (1993) Interleukin-9 potentiates the interleukin-4-induced immunoglobulin (IgG, IgM and IgE) production by normal human B lymphocytes. *European Journal of Immunology* 23(7):1687-1692.

42. Crotty S (T Follicular Helper Cell Differentiation, Function, and Roles in Disease. *Immunity* 41(4):529-542.

43. Weber JP, Fuhrmann F, Feist RK, Lahmann A, Al Baz MS, Gentz L-J, Vu Van D, Mages HW, Haftmann C, Riedel R, Grün JR, Schuh W, Kroczek RA, Radbruch A, Mashreghi M-F, & Hutloff A (2015) ICOS maintains the T follicular helper cell phenotype by down-regulating Krüppel-like factor 2. *The Journal of Experimental Medicine* 212(2):217-233.

44. Lee J-Y, Skon Cara N, Lee You J, Oh S, Taylor Justin J, Malhotra D, Jenkins Marc K, Rosenfeld MG, Hogquist Kristin A, & Jameson Stephen C (The Transcription Factor KLF2 Restrains CD4+ T Follicular Helper Cell Differentiation. *Immunity* 42(2):252-264.

45. Ma CS, Deenick EK, Batten M, & Tangye SG (2012) The origins, function, and regulation of T follicular helper cells. *The Journal of Experimental Medicine* 209(7):1241-1253.

46. Foy TM, Laman JD, Ledbetter JA, Aruffo A, Claassen E, & Noelle RJ (1994) gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. *The Journal of Experimental Medicine* 180(1):157-163.

47. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, Corcoran LM, Godfrey DI, Toellner K-M, Smyth MJ, Nutt SL, & Tarlinton DM (2010) IL-21 regulates germinal center B cell differentiation and proliferation through a B cell–intrinsic mechanism. *The Journal of Experimental Medicine* 207(2):365-378.

48. Vignali DAA, Collison LW, & Workman CJ (2008) How regulatory T cells work. *Nat Rev Immunol* 8(7):523-532.

49. Tanchot C, Terme M, Pere H, Tran T, Benhamouda N, Strioga M, Banissi C, Galluzzi L, Kroemer G, & Tartour E (2013) Tumor-Infiltrating Regulatory T Cells: Phenotype, Role, Mechanism of Expansion In Situ and Clinical Significance. *Cancer Microenvironment* 6(2):147-157.

50. Kearley J, Erjefalt JS, Andersson C, Benjamin E, Jones CP, Robichaud A, Pegorier S, Brewah Y, Burwell TJ, Bjermer L, Kiener PA, Kolbeck R, Lloyd CM, Coyle AJ, & Humbles AA (2011) IL-9 governs allergen-induced mast cell numbers in the lung and chronic remodeling of the airways. *American journal of respiratory and critical care medicine* 183(7):865-875.

51. Temann U-A, Laouar Y, Eynon EE, Homer R, & Flavell RA (2007) IL9 leads to airway inflammation by inducing IL13 expression in airway epithelial cells. *International Immunology* 19(1):1-10.

52. Ma L, Xue HB, Guan XH, Shu CM, Zhang JH, & Yu J (2014) Possible pathogenic role of T helper type 9 cells and interleukin (IL)-9 in atopic dermatitis. *Clinical and experimental immunology* 175(1):25-31.

53. Goswami R & Kaplan MH (2011) A Brief History of IL-9. *The Journal of Immunology* 186(6):3283-3288.

54. Kaplan MH (2013) Th9 cells: differentiation and disease. *Immunological reviews* 252(1):104-115.

55. Anuradha R, George PJ, Hanna LE, Chandrasekaran V, Kumaran P, Nutman TB, & Babu S (2013) IL-4-, TGF-beta-, and IL-1-dependent expansion of parasite antigen-specific Th9 cells is associated with clinical

pathology in human lymphatic filariasis. *Journal of immunology* 191(5):2466-2473.

56. Niedbala W, Besnard AG, Nascimento DC, Donate PB, Sonego F, Yip E, Guabiraba R, Chang HD, Fukada SY, Salmond RJ, Schmitt E, Bopp T, Ryffel B, & Liew FY (2014) Nitric oxide enhances Th9 cell differentiation and airway inflammation. *Nature communications* 5:4575.

57. Yao W, Zhang Y, Jabeen R, Nguyen ET, Wilkes DS, Tepper RS, Kaplan MH, & Zhou B (2013) Interleukin-9 is required for allergic airway inflammation mediated by the cytokine TSLP. *Immunity* 38(2):360-372.

58. Murugaiyan G, Beynon V, Pires Da Cunha A, Joller N, & Weiner HL (2012) IFN-gamma limits Th9-mediated autoimmune inflammation through dendritic cell modulation of IL-27. *Journal of immunology* 189(11):5277-5283.

59. Ye Z-J, Yuan M-L, Zhou Q, Du R-H, Yang W-B, Xiong X-Z, Zhang J-C, Wu C, Qin S-M, & Shi H-Z (2012) Differentiation and Recruitment of Th9 Cells Stimulated by Pleural Mesothelial Cells in Human <italic>Mycobacterium tuberculosis</italic> Infection. *PLoS ONE* 7(2):e31710.

60. Wong MT, Ye JJ, Alonso MN, Landrigan A, Cheung RK, Engleman E, & Utz PJ (2010) Regulation of human Th9 differentiation by type I interferons and IL-21. *Immunology and cell biology* 88(6):624-631.

61. Singh TP, Schön MP, Wallbrecht K, Gruber-Wackernagel A, Wang X-J, & Wolf P (2013) Involvement of IL-9 in Th17-Associated Inflammation and Angiogenesis of Psoriasis. *PLoS ONE* 8(1):e51752.

62. Elyaman W, Bradshaw EM, Uyttenhove C, Dardalhon V, Awasthi A, Imitola J, Bettelli E, Oukka M, van Snick J, Renauld JC, Kuchroo VK, & Khoury SJ (2009) IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proceedings of the National Academy of Sciences of the United States of America* 106(31):12885-12890.

63. Smith SE, Hoelzinger DB, Dominguez AL, Van Snick J, & Lustgarten J (2011) Signals through 4-1BB inhibit T regulatory cells by blocking IL-9 production enhancing antitumor responses. *Cancer immunology, immunotherapy : CII* 60(12):1775-1787.

64. Tan C, Aziz MK, Lovaas JD, Vistica BP, Shi G, Wawrousek EF, & Gery I (2010) Antigen-specific Th9 cells exhibit uniqueness in their kinetics of cytokine production and short retention at the inflammatory site. *Journal of immunology* 185(11):6795-6801.

65. Licona-Limon P, Henao-Mejia J, Temann AU, Gagliani N, Licona-Limon I, Ishigame H, Hao L, Herbert DR, & Flavell RA (2013) Th9 Cells Drive Host Immunity against Gastrointestinal Worm Infection. *Immunity* 39(4):744-757.

66. Zhou Y, Sonobe Y, Akahori T, Jin S, Kawanokuchi J, Noda M, Iwakura Y, Mizuno T, & Suzumura A (2011) IL-9 Promotes Th17 Cell Migration into

the Central Nervous System via CC Chemokine Ligand-20 Produced by Astrocytes. *The Journal of Immunology* 186(7):4415-4421.

67. Nowak EC, Weaver CT, Turner H, Begum-Haque S, Becher B, Schreiner B, Coyle AJ, Kasper LH, & Noelle RJ (2009) IL-9 as a mediator of Th17-driven inflammatory disease. *J Exp Med* 206(8):1653-1660.

68. Li H, Nourbakhsh B, Cullimore M, Zhang GX, & Rostami A (2011) IL-9 is important for T-cell activation and differentiation in autoimmune inflammation of the central nervous system. *Eur J Immunol* 41(8):2197-2206.

69. Elyaman W, Bassil R, Bradshaw EM, Orent W, Lahoud Y, Zhu B, Radtke F, Yagita H, & Khoury SJ (2012) Notch receptors and Smad3 signaling cooperate in the induction of interleukin-9-producing T cells. *Immunity* 36(4):623-634.

70. Zhang DE, Hetherington CJ, Chen HM, & Tenen DG (1994) The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor. *Molecular and Cellular Biology* 14(1):373-381.

71. Hohaus S, Petrovick MS, Voso MT, Sun Z, Zhang DE, & Tenen DG (1995) PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. *Molecular and Cellular Biology* 15(10):5830-5845.

72. Carotta S, Dakic A, D'Amico A, Pang SHM, Greig KT, Nutt SL, & Wu L (2010) The Transcription Factor PU.1 Controls Dendritic Cell Development

and Flt3 Cytokine Receptor Expression in a Dose-Dependent Manner. *Immunity* 32(5):628-641.

73. Kitamura N, Yokoyama H, Yashiro T, Nakano N, Nishiyama M, Kanada S, Fukai T, Hara M, Ikeda S, Ogawa H, Okumura K, & Nishiyama C (2012) Role of PU.1 in MHC class II expression through transcriptional regulation of class II transactivator pI in dendritic cells. *Journal of Allergy and Clinical Immunology* 129(3):814-824.e816.
74. Carotta S, Wu L, & Nutt SL (2010) Surprising new roles for PU.1 in the adaptive immune response. *Immunological reviews* 238(1):63-75.
75. Carotta S, Willis SN, Hasbold J, Inouye M, Pang SHM, Emslie D, Light A, Chopin M, Shi W, Wang H, Morse HC, Tarlinton DM, Corcoran LM, Hodgkin PD, & Nutt SL (2014) The transcription factors IRF8 and PU.1 negatively regulate plasma cell differentiation. *The Journal of Experimental Medicine* 211(11):2169-2181.
76. Pongubala JM, Nagulapalli S, Klemsz MJ, McKercher SR, Maki RA, & Atchison ML (1992) PU.1 recruits a second nuclear factor to a site important for immunoglobulin kappa 3' enhancer activity. *Mol Cell Biol* 12(1):368-378.
77. Chang H-C, Han L, Jabeen R, Carotta S, Nutt SL, & Kaplan MH (2009) PU.1 Regulates TCR Expression by Modulating GATA-3 Activity. *The Journal of Immunology* 183(8):4887-4894.

78. Chang H-C, Zhang S, Thieu VT, Slee RB, Bruns HA, Laribee RN, Klemsz MJ, & Kaplan MH (2005) PU.1 Expression Delineates Heterogeneity in Primary Th2 Cells. *Immunity* 22(6):693-703.

79. Suzuki S, Kumatori A, Haagen I-A, Fujii Y, Sadat MA, Jun HL, Tsuji Y, Roos D, & Nakamura M (1998) PU.1 as an essential activator for the expression of gp91(phox) gene in human peripheral neutrophils, monocytes, and B lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America* 95(11):6085-6090.

80. Rosenbauer F, Wagner K, Kutok JL, Iwasaki H, Le Beau MM, Okuno Y, Akashi K, Fiering S, & Tenen DG (2004) Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *Nat Genet* 36(6):624-630.

81. Mueller BU, Pabst T, Osato M, Asou N, Johansen LM, Minden MD, Behre G, Hiddemann W, Ito Y, & Tenen DG (2002) Heterozygous PU.1 mutations are associated with acute myeloid leukemia. *Blood* 100(3):998-1007.

82. Gerlach K, Hwang Y, Nikolaev A, Atreya R, Dornhoff H, Steiner S, Lehr HA, Wirtz S, Vieth M, Waisman A, Rosenbauer F, McKenzie AN, Weigmann B, & Neurath MF (2014) TH9 cells that express the transcription factor PU.1 drive T cell-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. *Nat Immunol* 15(7):676-686.

83. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, Mitsdoerffer M, Strom TB, Elyaman W, Ho IC, Khoury S, Oukka M, &

Kuchroo VK (2008) IL-4 inhibits TGF-[beta]-induced Foxp3+ T cells and, together with TGF-[beta], generates IL-9+ IL-10+ Foxp3- effector T cells. *Nat Immunol* 9(12):1347-1355.

84. Dakic A, Metcalf D, Di Rago L, Mifsud S, Wu L, & Nutt SL (2005) PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis. *The Journal of Experimental Medicine* 201(9):1487-1502.

85. Jabeen R, Goswami R, Awe O, Kulkarni A, Nguyen ET, Attenasio A, Walsh D, Olson MR, Kim MH, Tepper RS, Sun J, Kim CH, Taparowsky EJ, Zhou B, & Kaplan MH (2013) Th9 cell development requires a BATF-regulated transcriptional network. *The Journal of Clinical Investigation* 123(11):4641-4653.

86. Mo C, Chearwae W, O'Malley JT, Adams SM, Kanakasabai S, Walline CC, Stritesky GL, Good SR, Perumal NB, Kaplan MH, & Bright JJ (2008) Stat4 isoforms differentially regulate inflammation and demyelination in experimental allergic encephalomyelitis. *J Immunol* 181(8):5681-5690.

87. Collison LW & Vignali DAA (2011) In Vitro Treg Suppression Assays. *Methods in molecular biology (Clifton, N.J.)* 707:21-37.

88. Suto A, Kashiwakuma D, Kagami S-i, Hirose K, Watanabe N, Yokote K, Saito Y, Nakayama T, Grusby MJ, Iwamoto I, & Nakajima H (2008) Development and characterization of IL-21–producing CD4+ T cells. *The Journal of Experimental Medicine* 205(6):1369-1379.

89. Arend WP, Malyak M, Guthridge CJ, & Gabay C (1998) INTERLEUKIN-1 RECEPTOR ANTAGONIST: Role in Biology. *Annual Review of Immunology* 16(1):27-55.

90. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, & Yoshie O (1999) Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *International Immunology* 11(1):81-88.

91. Morimoto Y, Bian Y, Gao P, Yashiro-Ohtani Y, Zhou X-Y, Ono S, Nakahara H, Kogo M, Hamaoka T, & Fujiwara H (2005) Induction of surface CCR4 and its functionality in mouse Th2 cells is regulated differently during Th2 development. *Journal of Leukocyte Biology* 78(3):753-761.

92. Mikhak Z, Fukui M, Farsidjani A, Medoff BD, Tager AM, & Luster AD (Contribution of CCR4 and CCR8 to antigen-specific TH2 cell trafficking in allergic pulmonary inflammation. *Journal of Allergy and Clinical Immunology* 123(1):67-73.e63.

93. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, & Napolitani G (2007) Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 8(6):639-646.

94. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, Parente E, Filì L, Ferri S, Frosali F, Giudici F, Romagnani P, Parronchi P,

Tonelli F, Maggi E, & Romagnani S (2007) Phenotypic and functional features of human Th17 cells. *The Journal of Experimental Medicine* 204(8):1849-1861.

95. Vulcano M, Albanesi C, Stoppacciaro A, Bagnati R, D'Amico G, Struyf S, Transidico P, Bonecchi R, Del Prete A, Allavena P, Ruco LP, Chiabrando C, Girolomoni G, Mantovani A, & Sozzani S (2001) Dendritic cells as a major source of macrophage-derived chemokine/CCL22 in vitro and in vivo. *European Journal of Immunology* 31(3):812-822.
96. Mackay F & Schneider P (2009) Cracking the BAFF code. *Nat Rev Immunol* 9(7):491-502.
97. Zhou X, Xia Z, Lan Q, Wang J, Su W, Han Y-P, Fan H, Liu Z, Stohl W, & Zheng SG (2011) BAFF Promotes Th17 Cells and Aggravates Experimental Autoimmune Encephalomyelitis. *PLoS ONE* 6(8):e23629.
98. Huard B, Schneider P, Mauri D, Tschopp J, & French LE (2001) T Cell Costimulation by the TNF Ligand BAFF. *The Journal of Immunology* 167(11):6225-6231.
99. Lai Kwan Lam Q, King Hung Ko O, Zheng B-J, & Lu L (2008) Local BAFF gene silencing suppresses Th17-cell generation and ameliorates autoimmune arthritis. *Proceedings of the National Academy of Sciences* 105(39):14993-14998.
100. Sutherland APR, Ng LG, Fletcher CA, Shum B, Newton RA, Grey ST, Rolph MS, Mackay F, & Mackay CR (2005) BAFF Augments Certain Th1-

Associated Inflammatory Responses. *The Journal of Immunology* 174(9):5537-5544.

101. Hirota K, Duarte JH, Veldhoen M, Hornsby E, Li Y, Cua DJ, Ahlfors H, Wilhelm C, Tolaini M, Menzel U, Garefalaki A, Potocnik AJ, & Stockinger B (2011) Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol* 12(3):255-263.
102. Lee YK, Turner H, Maynard CL, Oliver JR, Chen D, Elson CO, & Weaver CT (2009) Late Developmental Plasticity in the T Helper 17 Lineage. *Immunity* 30(1):92-107.
103. Nakayamada S, Takahashi H, Kanno Y, & O'Shea JJ (2012) Helper T cell diversity and plasticity. *Current Opinion in Immunology* 24(3):297-302.
104. Schmitt E, Germann T, Goedert S, Hoehn P, Huels C, Koelsch S, Kühn R, Müller W, Palm N, & Rüde E (1994) IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. *The Journal of Immunology* 153(9):3989-3996.
105. Helmby H & Grencis RK (2004) Interleukin 1 plays a major role in the development of Th2-mediated immunity. *European Journal of Immunology* 34(12):3674-3681.
106. Schmitt E, Beuscher HU, Huels C, Monteyne P, van Brandwijk R, van Snick J, & Ruede E (1991) IL-1 serves as a secondary signal for IL-9 expression. *The Journal of Immunology* 147(11):3848-3854.

107. Vegran F, Berger H, Boidot R, Mignot G, Bruchard M, Dosset M, Chalmin F, Rebe C, Derangere V, Ryffel B, Kato M, Prevost-Blondel A, Ghiringhelli F, & Apetoh L (2014) The transcription factor IRF1 dictates the IL-21-dependent anticancer functions of TH9 cells. *Nat Immunol* 15(8):758-766.

108. Angkasekwinai P, Chang SH, Thapa M, Watarai H, & Dong C (2010) Regulation of IL-9 expression by IL-25 signaling. *Nat Immunol* 11(3):250-256.

109. Beriou G, Bradshaw EM, Lozano E, Costantino CM, Hastings WD, Orban T, Elyaman W, Khoury SJ, Kuchroo VK, Baecher-Allan C, & Hafler DA (2010) TGF-beta induces IL-9 production from human Th17 cells. *Journal of immunology* 185(1):46-54.

110. Kohm AP, Carpentier PA, Anger HA, & Miller SD (2002) Cutting Edge: CD4+CD25+ Regulatory T Cells Suppress Antigen-Specific Autoreactive Immune Responses and Central Nervous System Inflammation During Active Experimental Autoimmune Encephalomyelitis. *The Journal of Immunology* 169(9):4712-4716.

111. McGeachy MJ, Stephens LA, & Anderton SM (2005) Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. *Journal of immunology* 175(5):3025-3032.

112. Huan J, Culbertson N, Spencer L, Bartholomew R, Burrows GG, Chou YK, Bourdette D, Ziegler SF, Offner H, & Vandenbark AA (2005) Decreased

FOXP3 levels in multiple sclerosis patients. *Journal of neuroscience research* 81(1):45-52.

113. de Andres C, Aristimuno C, de Las Heras V, Martinez-Gines ML, Bartolome M, Arroyo R, Navarro J, Gimenez-Roldan S, Fernandez-Cruz E, & Sanchez-Ramon S (2007) Interferon beta-1a therapy enhances CD4+ regulatory T-cell function: an ex vivo and in vitro longitudinal study in relapsing-remitting multiple sclerosis. *Journal of neuroimmunology* 182(1-2):204-211.

114. Feger U, Luther C, Poeschel S, Melms A, Tolosa E, & Wiendl H (2007) Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients. *Clinical and experimental immunology* 147(3):412-418.

115. Haas J, Hug A, Viehover A, Fritzsching B, Falk CS, Filser A, Vetter T, Milkova L, Korporal M, Fritz B, Storch-Hagenlocher B, Krammer PH, Suri-Payer E, & Wildemann B (2005) Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. *Eur J Immunol* 35(11):3343-3352.

116. Kumar M, Putzki N, Limmroth V, Remus R, Lindemann M, Knop D, Mueller N, Hardt C, Kreuzfelder E, & Grosse-Wilde H (2006) CD4+CD25+FoxP3+ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis. *Journal of neuroimmunology* 180(1-2):178-184.

117. Hug A, Korporal M, Schroder I, Haas J, Glatz K, Storch-Hagenlocher B, & Wildemann B (2003) Thymic export function and T cell homeostasis in patients with relapsing remitting multiple sclerosis. *Journal of immunology* 171(1):432-437.

118. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, Yusuf I, & Crotty S (2011) IL-21 and IL-6 Are Critical for Different Aspects of B Cell Immunity and Redundantly Induce Optimal Follicular Helper CD4 T Cell (Tfh) Differentiation. *PLoS ONE* 6(3):e17739.

119. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, Schluns K, Tian Q, Watowich SS, Jetten AM, & Dong C (2007) Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448(7152):480-483.

120. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, & Kuchroo VK (2007) IL-21 initiates an alternative pathway to induce proinflammatory TH17 cells. *Nature* 448(7152):484-487.

121. Ozaki K, Spolski R, Feng CG, Qi C-F, Cheng J, Sher A, Morse HC, Liu C, Schwartzberg PL, & Leonard WJ (2002) A Critical Role for IL-21 in Regulating Immunoglobulin Production. *Science* 298(5598):1630-1634.

122. Han S, Hathcock K, Zheng B, Kepler TB, Hodes R, & Kelsoe G (1995) Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *The Journal of Immunology* 155(2):556-567.

123. Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, Verma NK, Smyth MJ, Rigby RJ, & Vinuesa CG (2010) IL-21 acts directly

on B cells to regulate Bcl-6 expression and germinal center responses. *The Journal of Experimental Medicine* 207(2):353-363.

124. Allen R, Armitage R, Conley M, Rosenblatt H, Jenkins N, Copeland N, Bedell M, Edelhoff S, Disteche C, Simoneaux D, & et a (1993) CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* 259(5097):990-993.

125. Aruffo A, Farrington M, Hollenbaugh D, Li X, Milatovich A, Nonoyama S, Bajorath J, Grosmaire LS, Stenkamp R, Neubauer M, Roberts RL, Noelle RJ, Ledbetter JA, Francke U, & Ochs HD (1993) The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell* 72(2):291-300.

126. Korthauer U, Graf D, Mages HW, Briere F, Padayachee M, Malcolm S, Ugazio AG, Notarangelo LD, Levinsky RJ, & Kroczek RA (1993) Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature* 361(6412):539-541.

127. DiSanto JP, Bonnefoy JY, Gauchatt JF, Fischer A, & Saint Basile Gd (1993) CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM. *Nature* 361(6412):541-543.

128. Fuleihan R, Ramesh N, Loh R, Jabara H, Rosen RS, Chatila T, Fu SM, Stamenkovic I, & Geha RS (1993) Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. *Proceedings of the National Academy of Sciences of the United States of America* 90(6):2170-2173.

129. Luthje K, Kallies A, Shimohakamada Y, Belz GT, Light A, Tarlinton DM, & Nutt SL (2012) The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. *Nat Immunol* 13(5):491-498.

130. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, Ma L, Watowich SS, Jetten AM, Tian Q, & Dong C (2009) Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* 30(4):576-587.

131. Lloyd CM, Delaney T, Nguyen T, Tian J, Martinez-A C, Coyle AJ, & Gutierrez-Ramos J-C (2000) Cc Chemokine Receptor (Ccr)3/Eotaxin Is Followed by Ccr4/Monocyte-Derived Chemokine in Mediating Pulmonary T Helper Lymphocyte Type 2 Recruitment after Serial Antigen Challenge in Vivo. *The Journal of Experimental Medicine* 191(2):265-274.

132. Conroy DM, Jopling LA, Lloyd CM, Hodge MR, Andrew DP, Williams TJ, Pease JE, & Sabroe I (2003) CCR4 blockade does not inhibit allergic airways inflammation. *J Leukoc Biol* 74(4):558-563.

133. Coquet JM, Chakravarti S, Smyth MJ, & Godfrey DI (2008) Cutting Edge: IL-21 Is Not Essential for Th17 Differentiation or Experimental Autoimmune Encephalomyelitis. *The Journal of Immunology* 180(11):7097-7101.

134. Piao WH, Jee YH, Liu RL, Coons SW, Kala M, Collins M, Young DA, Campagnolo DI, Vollmer TL, Bai XF, La Cava A, & Shi FD (2008) IL-21 Modulates CD4+ CD25+ Regulatory T-Cell Homeostasis in Experimental

Autoimmune Encephalomyelitis. *Scandinavian Journal of Immunology* 67(1):37-46.

135. Vollmer TL, Liu R, Price M, Rhodes S, La Cava A, & Shi FD (2005) Differential effects of IL-21 during initiation and progression of autoimmunity against neuroantigen. *Journal of immunology* 174(5):2696-2701.

136. Piao WH, Jee YH, Liu RL, Coons SW, Kala M, Collins M, Young DA, Campagnolo DI, Vollmer TL, Bai XF, La Cava A, & Shi FD (2008) IL-21 modulates CD4+ CD25+ regulatory T-cell homeostasis in experimental autoimmune encephalomyelitis. *Scand J Immunol* 67(1):37-46.

137. Iezzi G, Sonderegger I, Ampenberger F, Schmitz N, Marsland BJ, & Kopf M (2009) CD40–CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4+ T cells. *Proceedings of the National Academy of Sciences* 106(3):876-881.

138. Skov S, Bonyhadi M, Odum N, & Ledbetter JA (2000) IL-2 and IL-15 regulate CD154 expression on activated CD4 T cells. *Journal of immunology* 164(7):3500-3505.

139. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, & Kuchroo VK (2009) The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH -17 cells. *Nature Immunology* 10(2):167-175.

140. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, Wang Y-H, & Dong C (2009) Bcl6 Mediates the Development of T Follicular Helper Cells. *Science* 325(5943):1001-1005.

141. Choi Y, Kageyama R, Eto D, Escobar T, Johnston R, Monticelli L, Lao C, & Crotty S (2011) ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6. *Immunity* 34(6):932-946.

142. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, Wang Yh, Watowich SS, Jetten AM, Tian Q, & Dong C (2008) Generation of T Follicular Helper Cells Is Mediated by Interleukin-21 but Independent of T Helper 1, 2, or 17 Cell Lineages. *Immunity* 29(1):138-149.

143. Tubo NJ, Pagán AJ, Taylor JJ, Nelson RW, Linehan JL, Ertelt JM, Huseby ES, Way SS, & Jenkins MK (2013) Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell* 153(4):785-796.

144. Fazilleau N, McHeyzer-Williams LJ, Rosen H, & McHeyzer-Williams MG (2009) The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol* 10(4):375-384.

145. Hikami K, Kawasaki A, Ito I, Koga M, Ito S, Hayashi T, Matsumoto I, Tsutsumi A, Kusaoi M, Takasaki Y, Hashimoto H, Arinami T, Sumida T, & Tsuchiya N (2011) Association of a functional polymorphism in the 3′-untranslated region of SPI1 with systemic lupus erythematosus. *Arthritis & Rheumatism* 63(3):755-763.

146. Weinstein JS, Lezon-Geyda K, Maksimova Y, Craft S, Zhang Y, Su M, Schulz VP, Craft J, & Gallagher PG (2014) Global transcriptome analysis and enhancer landscape of human primary T follicular helper and T effector lymphocytes. *Blood* 124(25):3719-3729.

147. Yao W, Tepper RS, & Kaplan MH (2011) Predisposition to the development of IL-9-secreting T cells in atopic infants. *J Allergy Clin Immunol* 128(6):1357-1360 e1355.

148. Goswami R, Jabeen R, Yagi R, Pham D, Zhu J, Goenka S, & Kaplan MH (2012) STAT6-Dependent Regulation of Th9 Development. *The Journal of Immunology* 188(3):968-975.

149. Wang A, Pan D, Lee YH, Martinez GJ, Feng XH, & Dong C (2013) Cutting edge: Smad2 and Smad4 regulate TGF-beta-mediated Il9 gene expression via EZH2 displacement. *Journal of immunology* 191(10):4908-4912.

150. Tamiya T, Ichiyama K, Kotani H, Fukaya T, Sekiya T, Shichita T, Honma K, Yui K, Matsuyama T, Nakao T, Fukuyama S, Inoue H, Nomura M, & Yoshimura A (2013) Smad2/3 and IRF4 play a cooperative role in IL-9-producing T cell induction. *Journal of immunology* 191(5):2360-2371.

151. Goswami R & Kaplan MH (2012) Gcn5 is required for PU.1-dependent IL-9 induction in Th9 cells. *Journal of immunology* 189(6):3026-3033.

152. Xiao X, Balasubramanian S, Liu W, Chu X, Wang H, Taparowsky EJ, Fu YX, Choi Y, Walsh MC, & Li XC (2012) OX40 signaling favors the

induction of T(H)9 cells and airway inflammation. *Nat Immunol* 13(10):981-990.

153. Richard A, Tan C, Hawley E, Gomez-Rodriguez J, Goswami R, Yang X, Cruz A, Penumetcha P, Hayes E, Pelletier M, Gabay O, Walsh M, Ferdinand J, Keane-Myers A, Choi Y, O'Shea J, Al-Shamkhani A, Kaplan M, Gery I, Siegel R, & Meylan F (2014) The TNF family member TL1A promotes Th9 differentiation and Th9-mediated immunopathology (LYM3P.733). *The Journal of Immunology* 192(1 Supplement):64.67.

154. Gerritse K, Laman JD, Noelle RJ, Aruffo A, Ledbetter JA, Boersma WJ, & Claassen E (1996) CD40-CD40 ligand interactions in experimental allergic encephalomyelitis and multiple sclerosis. *Proceedings of the National Academy of Sciences of the United States of America* 93(6):2499-2504.

155. Ransohoff RM & Engelhardt B (2012) The anatomical and cellular basis of immune surveillance in the central nervous system. *Nat Rev Immunol* 12(9):623-635.

156. Quezada SA, Jarvinen LZ, Lind EF, & Noelle RJ (2004) CD40/CD154 interactions at the interface of tolerance and immunity. *Annu Rev Immunol* 22:307-328.

157. Wei F, Zaprazna K, Wang J, & Atchison ML (2009) PU.1 Can Recruit BCL6 to DNA To Repress Gene Expression in Germinal Center B Cells. *Molecular and Cellular Biology* 29(17):4612-4622.

158. Ahyi AN, Chang HC, Dent AL, Nutt SL, & Kaplan MH (2009) IFN regulatory factor 4 regulates the expression of a subset of Th2 cytokines. *Journal of immunology* 183(3):1598-1606.

159. Lu KT, Kanno Y, Cannons JL, Handon R, Bible P, Elkahloun AG, Anderson SM, Wei L, Sun H, O'Shea JJ, & Schwartzberg PL (2011) Functional and epigenetic studies reveal multistep differentiation and plasticity of in vitro-generated and in vivo-derived follicular T helper cells. *Immunity* 35(4):622-632.

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Awards

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Abstracts

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Peer reviewed publications

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2014 Glosson-Byers NL, Sehra S, Stritesky GL, Yu Q, Awe O, Pham D, Bruns HA, & Kaplan MH (2014) Th17 Cells Demonstrate Stable Cytokine Production in a Proallergic Environment. *The Journal of Immunology* 193(6):2631-2640.

2015 Ebel ME, Awe O, Kaplan MH and Kansas GS. (2015) Diverse inflammatory cytokines induce selectin ligand expression on murine CD4 T cells via p38 alpha MAP kinase. J. Immunol. In press

Awe O, Wu H, Pham D, Chang, HC, Jabeen, R, Dent A, Kaplan, MH. (2015) PU.1 expression in T follicular helper cells limits CD40L-dependent germinal center B-cell development. J. Immunol. In Revision.

Awe O, Kaplan MH. (2015) Th9 Cells as Targets for Immunotherapy. Translational Immunology: Mechanisms and Pharmacologic Approaches. In press.