TRANSCRIPTION FACTORS IN THE DEVELOPMENT OF TH9 CELLS

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Microbiology and Immunology,
Indiana University

October 2012

Accepted by the Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

I would like to dedicate this thesis in the memory of my mother.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor Dr. Mark Kaplan who has been an outstanding mentor, a great motivator and a fantastic person with a great sense of humor. Insightful discussions with him have been pivotal throughout my research. I would also like to thank my committee members Dr. Janice Blum, Dr. Alexander Dent, Dr. Robert Tepper, and Dr. Nadia Carlesso. They have been instrumental in giving suggestions on my research and helping me to ask questions in science. Additionally, both present and past Kaplan lab members Duy Pham, Nicole Glosson, Rukhsana Jabeen, Gretta Stritesky, Qing Yu, Weiguo Yao, Hua-Chen Chang, Sarita Sehra, Raji Muthukrishnan, Evelyn Nguyen, Kemi Awe, Melody Zeng, Jared Travers have played an important part in training me as a research scientist.

Finally, I would like to thank my father for being a constant source of support, inspiration, guidance and encouragement.

ABSTRACT

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Cytokines are extracellular proteins that mediate communication between cells. T helper cell subsets secrete specific cytokines that promote the development of inflammation. Naïve CD4+ T cells activated and primed in the presence of TGF-β and IL-4 predominantly secrete IL-9, a cytokine that acts as a growth factor for T cells and mast cells, and promotes allergic inflammation. The transcription factors downstream of TGF-β- and IL-4-induced signaling, and that are required for expression of IL-9, have not been previously examined. IL-4 signaling induces the expression of IRF4, a transcription factor required for the development of Th9 cells. IL-4 and the downstream-activated factor STAT6 also interfere with the expression of the transcription factors T-bet and Foxp3 that inhibit IL-9 production from Th9 cells. The TGF-β pathway induces the expression of PU.1, another transcription factor required for Th9 development. In the absence of PU.1 there is increased association of a subset of histone deacetylases to the *Il9* promoter. In developing Th9 cells, PU.1 can bind to the *Il9* promoter and recruit specific histone acetyltransferases, including Gcn5 to the *Il9* gene. Gcn5 functionally contributes to *Il9* expression as IL-9 production is diminished when Gcn5 expression is reduced, although other cytokines expressed by Th9 cells are not affected. While Gcn5 is not required for PU.1 or IRF4 binding to *Il9*, it is important for controlling histone

acetylation at the *Il9* gene promoter. Together these data define the STAT6-dependent transcription factor network in Th9 cells and the mechanism of PU.1-dependent IL-9 induction in Th9 cells and might indicate that targeting IL-9 regulation is a viable approach for treating inflammatory disease.

Mark H. Kaplan, Ph.D.-Chair

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LIST OF ABBREVIATIONS

Ada	Adaptor
AHR	Aryl hydrocarbon receptor
AP-1	Activator protein 1
APC	Antigen presenting cells
ATAC	Ada Two-A Containing Complex
ATF	Activating transcription factor
BAL	Bronchoalveolar lavage
BATF	Basic leucine zipper transcription factor ATF like
Bcl-6	B-cell lymphoma 6 protein
CBF-β	Core binding factor, beta subunit
CBP	CREB-binding protein
CCL	C-C chemokine ligand
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation
CNS	Conserved non-coding sequence
CREB	cAMP response element-binding
CTLA	Cytotoxic T lymphocyte antigen
DAPA	DNA affinity precipitation assay
EAE	Experimental autoimmune encephalomyelitis
Eomes	Eomesodermin
ERK	Extracellular signal-regulated kinase
ERM	ETS-related molecule

ETS	E-twenty six
Fgl2	Fibrinogen-like protein 2
Foxp3	Forkhead box protein 3
GATA3	GATA binding protein 3
Gcn5	General control non-derepressible 5
GITR	Glucocorticoid-induced TNFR-related protein
Gfi-1	Growth factor independence 1
GRAIL	Gene related to anergy in lymphocytes
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
Hlx	H2.0-like homeobox-1
HSS	Hypersensitive site
IFN-γ	Interferon gamma
IκB	Inhibitor of κB
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, X-linked
IRF	Interferon regulatory factor
JAK	Janus family tyrosine kinase
LCR	Locus control region
Maf	Musculoaponeurotic fibrosarcoma
MAP	Mitogen-activated protein kinase
MHC	Major histocompatibility complex

MOZ	Monocytic leukemia zinc finger protein
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
P300	E1A binding protein p300
PAMP	Pathogen-associated molecular pattern
PARP	Poly ADP ribose polymerase
PCAF	p300/CBP-associated factor
PEST	Proline, glutamic acid, serine, threonine
PI3	Phosphatidylinositol 3
PMN	Polymorphonuclear neutrophil
PRR	Pattern recognition receptor
pSTAT	Phospho-STAT
RHS	Retrotransposon hot spot
ROR	Retinoid-acid-related orphan receptor
RSV	Respiratory syncytial virus
Runx	Runt-related transcription factor
SAGA	Spt-Ada-Gcn5-Acetyltransferase
SCF	Stem cell factor
Sfpi1	Spleen focus forming virus proviral integration site-1
SH2	Src-homology domain
Spt	Suppressor of Ty
STAGA	Spt3-TAF9-Gcn5-Acetyltransferase

STAT	Signal transducer and activator of transcription
TAF	TBP-associated factor
T-bet	T-box expressed in T cells
Tbx21	T-box transcription factor 21
TBP	TATA-binding protein
TCR	T cell receptor
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
Tip60	Tat interacting protein 60
Treg	T regulatory
TRRAP	Transformation/transcription domain-associated protein
TSA	Trichostatin A
WT	Wild type
XLAAD	X-linked autoimmunity allergic dysregulation

INTRODUCTION

Innate and adaptive immunity

Humans are exposed to pathogens every day. However, the human body is protected from the potential damaging effects by a plethora of effector cells and molecules which make up the immune system. The innate and adaptive immune systems, the two arms of the immune system provide immunity. The innate immune system acts as the first line of defense helping in faster clearance of pathogens. The adaptive immune system is critical for providing long-lasting immunity against subsequent infections. Together, both innate and adaptive immune systems act as sentinels against encountered pathogens by mounting immune responses.

The innate immune system is non-specific; all the pathogens are attacked equally. Physical and chemical barriers are employed by the innate immune system as defense mechanisms. The skin is a very important barrier as it cannot be penetrated by most organisms unless its physical integrity is lost. The gastrointestinal and respiratory tracts, lined with sticky mucus, act as a trap for many microorganisms. The stomach releases hydrochloric acid and protein-digesting enzymes, which can destroy many pathogens. Saliva, sweat, and tears contain lysozyme, an enzyme capable of lysing the cell wall of Gram-positive bacteria. Microorganisms can be removed from lungs by ciliary action and sloughing off skin expels pathogens as well. However, pathogens can breach the barriers and form infection which is fought by the innate immune system. The effector functions required to mount host response to the invading pathogens include opsonization,

activation of complement system of blood proteins, and initiation of controlled pro-inflammatory immune responses.

Specialized cell types including granulocytes (mast cells, neutrophils, basophils, and eosinophils), phagocytes (dendritic cells, macrophages, neutrophils) and natural killer (NK) cells mediate innate immune effector functions. The granulocytes and the macrophages release cytokines upon activation. Macrophages also secrete proteins known as chemokines that attract phagocytes such as neutrophils and monocytes bearing specific chemokine receptors. Upon activation macrophages and eosinophils can release reactive oxygen species, cytokines including IL-13, and TNF-α and enzymes such as elastase (Hogan et al., 2008; Rothenberg and Hogan, 2006).

NK cells contain small granules in their cytoplasm, which contain proteins such as perforins and proteases such as granzymes. When released near a target cell, perforin forms pores in the target cell membrane. NK cells are activated by macrophage-derived cytokines and play critical roles in antibody-dependent cell-mediated cytotoxicity. NK cells also play role in tumor cell detection and tumor immunosurveillance activates NK cells (Zamai et al., 2007).

NKT cells, a subset T of cells that express NK1.1, the marker for NK cells, as well as αβ T cell receptor. These cells are divided into two classes: the classical NKT and the non-classical NKT cells (Godfrey et al., 2004). The T cell receptor repertoire on NKT cells is limited; NKT cells recognize lipids and glycolipids presented by CD1d molecules instead

of peptide-MHC complexes. Activated NKT cells can secrete various cytokines including IL-4, IFN-γ, IL-17 and GM-CSF (Kronenberg, 2005; Rachitskaya et al., 2008).

γδ T cells are another limited subset of T cells, which express γ chain and δ chain on the surface of T cells, and can function in both innate and adaptive immunity. The restricted T cell receptor of γδ T cells can be used as pattern recognition receptors (PRRs) to mount defense against microbes. However, γδ T cells have been linked to adaptive immunity as well (Holtmeier and Kabelitz, 2005).

Phagocytes are the critical innate immune cells which protect our body by phagocytosing harmful foreign particles. These cells fight infections and also clear dead cell debris from our body. Professional phagocytes such as macrophages, mast cells, dendritic cells, and neutrophils have receptors on their surfaces to detect pathogens such as bacteria. The receptors on phagocytes include Toll-like receptors, scavenger receptors and opsonin receptors. Phagocyte-mediated pathogen killing can occur intracellularly or extracellularly (Dale et al., 2008). Intracellular killing is both oxygen-dependent (by superoxide production, use of the enzyme myeloperoxidase) and oxygen-independent (lysozymes, proteases). The complement system which is activated by antibody-dependent and independent mechanisms, enhances engulfment of microbes by phagocytes. Phagocytes also act as antigen presenting cells (APCs). Professional APCs include macrophages and dendritic cells. After engulfment by phagocytes, proteins from pathogens are broken down into small peptides which are then bound to major histocompatibility complex (MHC) molecules. MHC-peptide complex moves to the cell

surface to activate the lymphocytes and begin adaptive immune response. Eosinophils have also been found to act as APCs.

The innate immune system is capable of recognizing certain conserved structures found in a large body of pathogens. The system with its hundreds of receptors or PRRs can identify structures of invading microorganisms known as pathogen-associated molecular patterns (PAMPs). PRRs share structural characteristics such as calcium-dependent leucine domains and leucine rich domains. These receptors are conserved and cannot recognize self thereby posing no danger to the host. PAMPs include peptidoglycans, bacterial polysaccharide, bacterial DNA, double stranded RNA. Once PRRs recognize PAMPs and get activated, innate immune cells are capable of secreting various cytokines and chemokines and process and present antigen to adaptive immune cells (Thompson et al., 2011).

The features of adaptive immune system are the specificity, memory, adaptability, and discrimination between self and non-self antigens. The B and T lymphocytes are the major cell types involved in the adaptive immune response. According to clonal selection theory, prior to contact with antigen, B and T cells exist with all antigenic specificities. The highly diverse receptors on B and T cells are capable of identifying a large number of diverse antigens. T cell receptor is composed of αβ or γδ chains while B cell receptor is membrane-bound form of the antibody. Upon encountering its cognate antigen and receiving signals from T cells, B cells differentiate into plasma cells which make antibodies in response to foreign proteins including bacteria and viruses. During germinal

center reaction B cells hypermutate the variable regions of the immunoglobulin gene and undergo class switching (Teng and Papavasiliou, 2007). Some activated B cells become memory B cells, and are important for fighting subsequent infections.

T cells are divided into two types based on the presence of the surface glycoproteins CD4 and CD8. CD8+ T cells, also known as cytotoxic T lymphocytes are important for destroying intracellular pathogens. CD8+ T cells play important role in immunosurveillance and kill infected cells exhibiting viral proteins in the surface of class I MHC molecules. CD4+ T cells are referred to as T helper cells which are critical for both antibody-mediated and cell-mediated immunity. Macrophages and dendritic cells act as APCs in cell-mediated immunity while B cells present antigens to CD4+ T cells during antibody-mediated immunity. CD4+ T cells are critical for providing immunity as infections such as HIV and AIDS are associated with depletion of CD4+ T cells. T helper cells direct other immune cells to execute effector functions.

JAK-STAT pathway

Naïve CD4+ T cells proliferate and polarize towards different effector CD4+ T cells including pro-inflammatory Th1, Th2, Th17 and immunoregulatory Treg cells based on their interaction with cognate antigen and also on the cytokine milieu. Cytokine-mediated T helper cell differentiation depends upon the activation of the Janus family tyrosine kinases (JAKs)/Signal Transducer and Activator of Transcription (STAT) pathway. STAT molecules control CD4+ T cell stability and plasticity. The Janus kinase family consists of 4 members, JAK1, JAK2, JAK3, and, TYK2; while the STAT family of

transcription factors includes, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Cytokine stimulation by JAKs through type I and II cytokine receptors results in the activation of STAT proteins. After cytokine binding, receptor chains associate and elicit JAK auto-phosphorylation or trans-phosphorylation and activation. Subsequently, JAKs phosphorylate specific tyrosine residues on the receptor cytokine tail which serve as STATs docking sites. In turn, phosphorylated tyrosine residues allow STAT molecules to form homo or heterodimers through the interaction of SH2 domain. The dimerization facilitates STAT protein translocation and binding to gene-specific promoter elements (O'Shea and Murray, 2008; Shuai and Liu, 2003). Each T helper cell lineage is dependent on distinct STAT molecules for its differentiation and effector functions, and activation is dependent on the cytokine environment in which the cell develops.

Transcriptional regulation of Th1 cells

Th1 cells are characterized by the production of IFN-γ and lymphotoxin-α. Naïve CD4+ T cells when cultured in the presence of IL-12 and blocking IL-4 antibodies polarize into Th1 cells (Hsieh et al., 1993; Manetti et al., 1993). IFN-γ can activate and enhance microbicidal activity of macrophages (Suzuki et al., 1988). Th1 cells are critical for immunity against intracellular bacteria, fungi, protozoa (Fieschi et al., 2004; Fieschi et al., 2003; Hsieh et al., 1993).

Role of STAT molecules in Th1 cell differentiation

Th1 cells, which secrete high levels of IFN-γ, require IL-12 and IFN-γ for their differentiation. IL-12 signals through activated JAK2 and Tyk2, and activates STAT4,

the key transcription factor for Th1 commitment (Zhu and Paul, 2010). STAT4-deficient CD4+ T cells fail to produce IFN-γ after IL-12 or *Listeria monocytogenesis* stimulation and STAT4-deficient mice die of uncontrolled worm burden in response to *Toxoplasma gondii* (Cai et al., 2000; Kaplan et al., 1996b; Thierfelder et al., 1996). IL-12 synergizes with IL-18 for driving Th1 differentiation which is STAT4-dependent (Barbulescu et al., 1998; Ouyang et al., 1999; Yang et al., 1999). IFN-γ signals through STAT1 and STAT1-deficient mice have impaired Th1 differentiation (Afkarian et al., 2002). JAK3 and STAT5 activation is required for optimal IFN-γ secretion. JAK3-deficient T cells fail to produce IFN-γ and IL-2 activated STAT5 is required for optimal IFN-γ secretion (Shi et al., 2008b).

Lineage-specific transcription factors and other factors required for Th1 differentiation

Downstream of specific STAT family members, other transcription factors are required for Th1 cell development. T-bet (*Tbx21*), a T-box family transcription factor has been shown to be the master regulator of Th1 cell differentiation. *Tbx21* expression is inducible upon TCR stimulation. T-bet is preferentially expressed in Th1 cells, compared to Th2 cells. Ectopic expression of T-bet in Th2 cells can induce IFN-γ secretion (Mullen et al., 2002; Szabo et al., 2000). *In vitro*, T-bet-deficient Th1 cells have diminished IFN-γ production and *in vivo* T-bet-deficient mice elicit impaired Th1 response after antigen immunization (Szabo et al., 2002). Even though IFN-γ/STAT1 is a key pathway of T-bet induction, STAT4 also controls *Tbx21* expression and collaborates with T-bet to induce *IL-12Rβ2* and *IL-18Rα* expression (Afkarian et al., 2002; Lighvani et al., 2001; Thieu et al., 2008; Usui et al., 2003; Usui et al., 2006). STAT1-deficient T cells have attenuated T-

bet levels after *T. gondii* infection; however, IFN-γ secretion remains unchanged (Lieberman et al., 2004). The synergistic effects of IL-12 and IL-18 in driving Th1 differentiation are in part via T-bet induction (Zhu and Paul, 2010). H2.0-like homeobox-1 or Hlx, is a Th1-specific transcription factor, induced by T-bet. Hlx, a homeobox gene interacts with T-bet to induce heritable Th1 gene expression (Mullen et al., 2002). Transgenic mice constitutively expressing Hlx in CD4+ T cells generated increased numbers of Th1 cells in response to keyhole limpet hemocyanin immunization (Zheng et al., 2004). Runx3, a Runt-related transcription factor is selectively upregulated in Th1 cells. In Th1 cells, Runx3 interacts with T-bet and enhances IFN-γ production while reducing *Il4* expression via binding of Runx3/T-bet complex to the *Ifng* promoter and *Il4* silencer, respectively (Djuretic et al., 2007; Yagi et al., 2010). Ectopic Runx3 expression in CD4+ T cells or Runx3-expressing transgenic mice have increased Th1 responses (Kohu et al., 2009). Runx3-mediated *Ifng* expression is suppressed by GATA3 (Yagi et al., 2010). The Ets family transcription factor, ERM which is specifically expressed in Th1 cells is an IL-12-inducible and STAT4-dependent gene. However, ectopic ERM expression in STAT4-deficient Th1 cells fails to rescue IFN-γ production suggesting it may have functions in Th1 cells that are not yet defined (Ouyang et al., 1999). The helix-loop-helix transcription repressor Twist1 is expressed in Th1 cells but not in Th2 or Th17 cells (Niesner et al., 2008). Twist1 is induced by IL-12/Stat4 signaling but not IFN-γ/Stat1 signaling (Niesner et al., 2008). Ectopic expression of Twist1 diminishes the production of Th1 cytokines *in vitro* correlated with a decrease in Th1-mediated inflammation model *in vivo* (Niesner et al., 2008). Twist1 reduces the expression of T-bet, Runx3 and IL-12Rβ2 and regulates *Ifng* gene expression by forming complex with

Runx3 (Pham et al., 2012). Ectopically expressed Runx3, but not T-bet or IL-12Rβ2 can compensate for the Twist1 effect on IFN-γ production (Pham et al., 2012). The Ca^{2+} dependent transcription factors, nuclear factor activator of T cells (NFATs) are also important for the polarization of effector CD4+ T cells. STAT1 along with TCR-induced transcription factors AP-1, NF-κB bind to the *Tbx21* promoter thereby facilitating its transcription in Th1 cells (Afkarian et al., 2002). Therefore expression and interaction of several Th1-associated transcription factors are required for optimal Th1 responses (Figure 1).

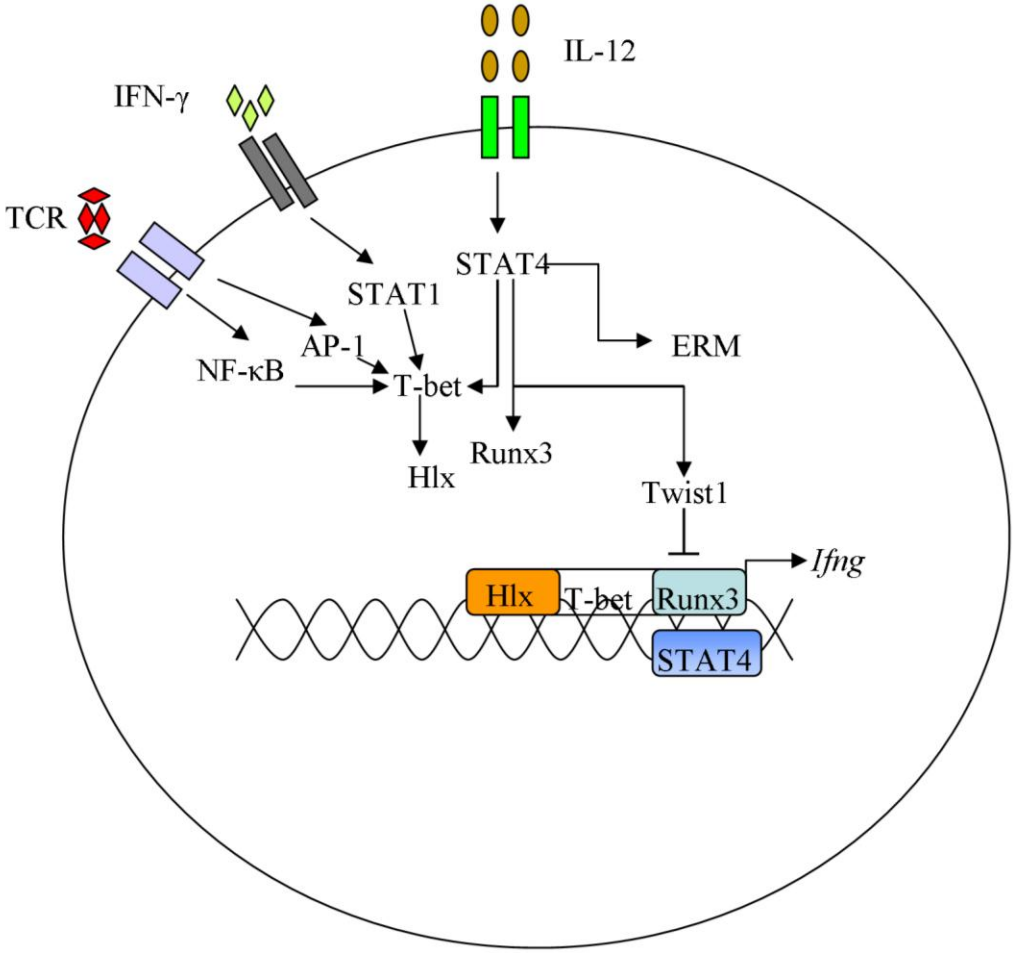

Figure 1: Transcription factor network of Th1 cells

Epigenetic regulation of Th1 cells

Chromatin structure modification plays an important role in polarizing subset-specific gene expression. Studies to elucidate the mechanism of Th1-specific *IL12Rβ2* expression was addressed in human Th1 cells. Two DNase I hypersensitive sites (HS) are present in the 5′-upstream region of the *IL12RB2* gene. HS1 contains IFN-γ activated sequence (GAS) element, consensus STAT protein binding sequences. HS2 is the core promoter of the *IL12RB2* gene (Letimier et al., 2007). In mice, interferon regulatory factor1 (IRF1) can directly activate the β1 chain of IL-12R by binding to an IFN-stimulated response element (ISRE) in the promoter of *IL12Rβ1* gene (Kano et al., 2008). STAT4 binding promotes hyperacetylation of *Il18r1* thereby preventing the recruitment of DNA methyltransferases and repression of *Il18r1* locus (Yu et al., 2007). A scanning of the *Tbx21* locus for STAT1 and STAT4 binding yielded an enhancer element 13Kb upstream of transcription start site containing 2 binding sites (Yang et al., 2007b). The *Ifng* gene contains specific DNase I hypersensitive sites in the 1st and 3rd introns (Agarwal and Rao, 1998). Histones 3 and 4 undergo hyperacetylation at the *Ifng* promoter (Avni et al., 2002; Fields et al., 2002). In Th1 cells, H4 acetylation and H3K4 methylation, both positive chromatin marks, span a 50kb region of the *Ifng* gene (Hamalainen-Laanaya et al., 2007; Schoenborn and Wilson, 2007; Zhou et al., 2004). Conserved non-coding sequences (CNSs) are evolutionary conserved and are associated with transcription factor binding sites as well as *cis*-acting elements. There are two CNSs, one 6kb upstream and another 18kb downstream of the transcription start site of the *Ifng* gene (Bream et al., 2004; Lee et al., 2004; Shnyreva et al., 2004). The CNS-6 site, contains a DNase I hypersensitivity in naïve T cells and the transcription factors T-bet, STAT5, and NFAT1 can bind to this

site (Bream et al., 2004; Lee et al., 2004; Shi et al., 2008b). Furthermore, enhancer elements of the *Ifng* gene were identified at CNS-54, CNS-34, and CNS-22 sites (Hatton et al., 2006). Th1-specific H4 acetylation and T-bet binding occur at CNS-34, and CNS-22 sites. In addition to histone acetylation, the *Ifng* locus also undergoes DNA methylation (Schoenborn et al., 2007). Multiple CNS of the *Ifng* locus can bind STAT4, T-bet or both (Chang and Aune, 2005; Hatton et al., 2006). In Th2 cells, the *Ifng* locus is characterized by high level of di-and tri-methylation of H3K27, repressive chromatin modifications while DNA is demethylated at CpG residues at CNS-54, intron, and CNS+18-20 regions during Th1 development (Chang and Aune, 2007; Schoenborn et al., 2007). The significance of the presence of H3K9Me2, another repressive chromatin mark at the *Ifng* locus in Th1 cells is not well understood (Chang and Aune, 2007). Therefore histone modifications which remodel the chromatin at the *Ifng* locus during Th1 differentiation define the Th1 cell responses (Figure 2).

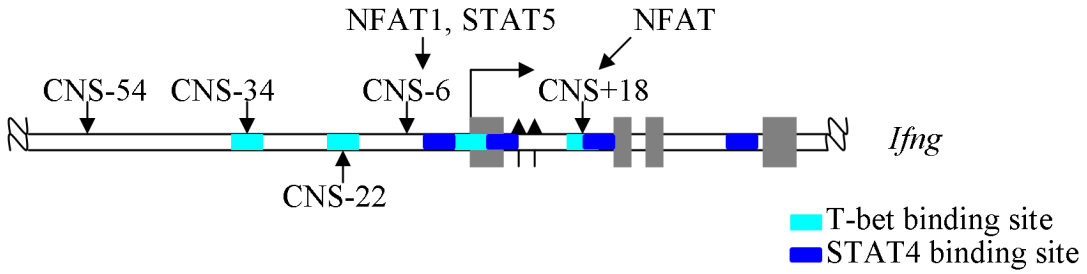

Figure 2: Epigenetic regulation in Th1 cells

Transcriptional regulation of Th2 cells

Naïve T cells activated by antigen in the presence of IL-4 differentiate into Th2 cells. These cells produce IL-4, IL-5, and IL-13. Th2 cells are critical for providing immunity

against extracellular parasites (Zhu and Paul, 2008). Production of these cytokines also enable B cell help towards antibody production, a function which has also been attributed to T follicular helper cells (Crotty, 2011). IL-4 acts as B-cell switch factor and as a positive feedback loop in Th2 differentiation (Kopf et al., 1993; Le Gros et al., 1990; Swain et al., 1990). IL-5 is the key eosinophil development and recruitment cytokine, while IL-13 is responsible for reducing worm burden and inducing airway inflammation and hypersensitivity (Coffman et al., 1989; Urban et al., 1998; Wynn, 2003).

STAT molecules in Th2 differentiation

IL-4 signaling is transmitted through STAT6, and STAT6 is required for Th2 development *in vitro* (Kaplan et al., 1996a; Shimoda et al., 1996). Mice injected with neutralizing IL-4 antibodies or *Stat6-/-* mice have defective worm clearance after nematode infection, while CD4+ T cells from mice expressing constitutively active STAT6 (STAT6VT) have a bias towards Th2 cell lineage (Bruns et al., 2003; Else et al., 1994; Sehra et al., 2008; Takeda et al., 1996). However, the development of some Th2-cytokine secreting cells can occur *in vivo* in the absence of Stat6 (Jankovic et al., 2000; van Panhuys et al., 2008). IL-4R/STAT6 signaling is shown to be required for generating memory IL-4 responses and stabilization of Th2 phenotype (Finkelman et al., 2000; Jankovic et al., 2000). STAT6 is also required for the expression of Th2 transcription factors *Gfi-1* and *Gata3* (Lee and Rao, 2004; Zhu et al., 2002). In addition to the IL-4/STAT6 pathway, IL-2-mediated STAT5 activation induces the production of IL-4, IL-5, and IL-13 by Th2 cells (Zhu et al., 2003). STAT5 is required in priming for IL-4 production which is enhanced by IL-2 *in vivo* possibly by regulating IL-4Rα (Cote-Sierra

et al., 2004; Liao et al., 2008). Th2 cell development is impaired in *Stat5A-/-* mice in spite of normal STAT6 phosphorylation by IL-4 (Kagami et al., 2001). Ectopic expression of either Stat5A or Stat5B restores Th2 differentiation in *Stat5A-/-* mice (Kagami et al., 2001). Constitutively active Stat5A expression can induce restricted Th2 cytokine production and allergy airway inflammation in the absence of STAT6 (Takatori et al., 2005; Zhu et al., 2003). STAT3 which is required for the differentiation and effector function of Th17 and Tfh cells is activated in Th2 cells (Nurieva et al., 2008; Stritesky et al., 2011; Yang et al., 2007a). STAT3 deficiency leads to attenuated Th2 cytokine production and decreased Th2-specific transcription factor expression (Stritesky et al., 2011). STAT3-deficient T cells show significantly lower allergic inflammation and cooperates with STAT6 to promote Th2 cells development (Stritesky et al., 2011).

Additional transcription factors required for Th2 differentiation

Along with multiple STAT molecules that signal during Th2 cell differentiation, other transcription factors are required for Th2 development. IL-4-activated STAT6 induces GATA3, the master regulator of Th2 cells (Zheng and Flavell, 1997). GATA3 is specifically expressed in Th2 cells and directly regulates the expression of Th2 cytokines (Agarwal et al., 2000; Kishikawa et al., 2001; Siegel et al., 1995; Yamashita et al., 2002; Zhang et al., 1997; Zhang et al., 1998a). GATA3 is able to autoactivate its own transcription and can induce Th2 differentiation in Th1-polarizing conditions (Ouyang et al., 2000). *In vivo*, a dominant negative form of GATA3 results in diminished allergic inflammation, while T-cell specific deletion of GATA3 has impaired initiation and maintenance of Th2 responses (Pai et al., 2004; Zhang et al., 1999; Zhu et al., 2004). In

addition to GATA3, the transcription factor c-Maf is required for IL-4 expression. c-Maf is expressed in Th2 cells and is a potent transactivator of IL-4 (Ho et al., 1996; Ho et al., 1998). c-Maf transgenic mice have enhanced Th2 responses *in vivo* (Ho et al., 1998). Mice deficient in c-Maf display diminished IL-4 but unaltered IL-13 production from Th2 cells and c-Maf fails to transactivate *Il5* or *Il10* promoters (Kim et al., 1999). IRF4, a member of the interferon regulatory factor family is also associated with Th2 differentiation. IRF4-deficient T cells have impaired Th2 differentiation (Lohoff et al., 2002; Rengarajan et al., 2002). Synergistic effects of IRF4 and NFATc2 to augment transcriptional activation of IL-4 are dependent upon physical interaction between IRF4 and NFATc2 (Rengarajan et al., 2002). *Irf4* expression can be traced to a subpopulation of Th2 cells which express high levels of IL-10 (Ahyi et al., 2009). IRF4 over expression enhances IL-4 and IL-10 production without any changes in IL-5 and IL-13 expression (Ahyi et al., 2009). IRF4 can bind directly to the *Il10* gene and can transactivate *Il10* control elements in reporter assay (Ahyi et al., 2009). IL-4/STAT6 signaling activates the transcription factor Growth factor independence 1 (Gfi-1), which favors the growth of $GATA3^{hi}$ cells (Zhu et al., 2002). CD4+ T cell specific deletion of Gfi-1 attenuates Th2 proliferation *in vitro* and impairs Th2 responses *in vivo* (Zhu et al., 2006b). Gfi-1 plays important role in regulating IL-5 and IFN-γ in Th2 cells by controlling GATA3 protein stability via repression of ubiquitin/proteasome-dependent GATA3 degradation (Shinnakasu et al., 2008). The transcription factor JunB, which is selectively expressed in Th2 cells, can activate and bind to the *Il4* promoter synergistically with c-Maf (Li et al., 1999). JunB-deficient Th2 cells have reduced IL-4, IL-5 but not IL-13 production *in vitro* and impaired allergic airway inflammation *in vivo* (Hartenstein et al., 2002). Dec2, a

basic helix-loop-helix family transcription factor is specifically expressed in Th2 cells and its expression is regulated by STAT6 (Liu et al., 2009). Dec2 binds and activates the expression of *Gata3* and *Junb* (Yang et al., 2009). Dec2 transgenic mice demonstrate strong Th2 responses, while knockdown of *Dec2* expression or Dec2-deficient mice have impaired Th2 differentiation both *in vitro* and *in vivo* (Liu et al., 2009; Yang et al., 2009). Mechanistically Dec2 induces *IL-2Rα* expression and synergistically controls IL-2 signaling with IL-4 to promote Th2 differentiation (Liu et al., 2009). Batf, a member of basic leucine zipper transcription factor family, has been shown to be important for Th17 differentiation; however, Batf is also required for the development of functional Th2 cells (Betz et al., 2010). A hematopoietic transcription factor Ikaros, is also a regulator of Th2 cells. Ikaros-deficient Th2 cells have impaired IL-4, IL-5, and IL-13 but enhanced IFN-γ production (Quirion et al., 2009). In naïve T cells, Ikaros is able to associate with Th2 locus regulatory regions while Ikaros-deficient T cells display diminished *Gata3* and *c-Maf* with increased *Tbx21* and *Stat1* expression (Quirion et al., 2009). In Th2-polarizing conditions, Ikaros is required to suppress *Ifng* expression (Thomas et al., 2010). The ETS family transcription factor, PU.1 controls the heterogeneity of Th2 cells (Chang et al., 2005). PU.1 is expressed in the $CCL22^{hi}IL\text{-}4^{lo}$ population of Th2 cells (Chang et al., 2005). Enforced expression of PU.1 reduces Th2 cytokine production while RNAi-mediated reduced PU.1 expression results in augmented Th2 cytokine production (Chang et al., 2009; Chang et al., 2005). Mechanistically PU.1 interferes with the binding of GATA3 in $IL\text{-}4^{lo}$ cells without altering GATA3 protein levels (Chang et al., 2009; Chang et al., 2005). NFATs regulate the transcription of several cytokines and their receptors, and are also important for Th2 differentiation. IL-4-activated STAT6 together with AP-1,

NF-κB, and NFAT drive *Il4* transcription (Chuvpilo et al., 1993; Macian et al., 2000). NFATc2 binds to the *Il4*, *Il5*, and *Il13* promoters and many enhancer elements within the *Il4* locus (Ansel et al., 2006). IRF4 synergizes with NFATc2 and c-Maf to enhance *Il4* promoter activity (Rengarajan et al., 2002). IRF4 and NFATc2 also synergize to augment Th2-specific enhancer activity of CNS-9, a distal cis-regulatory element upstream of *Il10* gene loci (Lee et al., 2009). Thus the expression of these transcription factors is required for optimal Th2 differentiation (Figure 3).

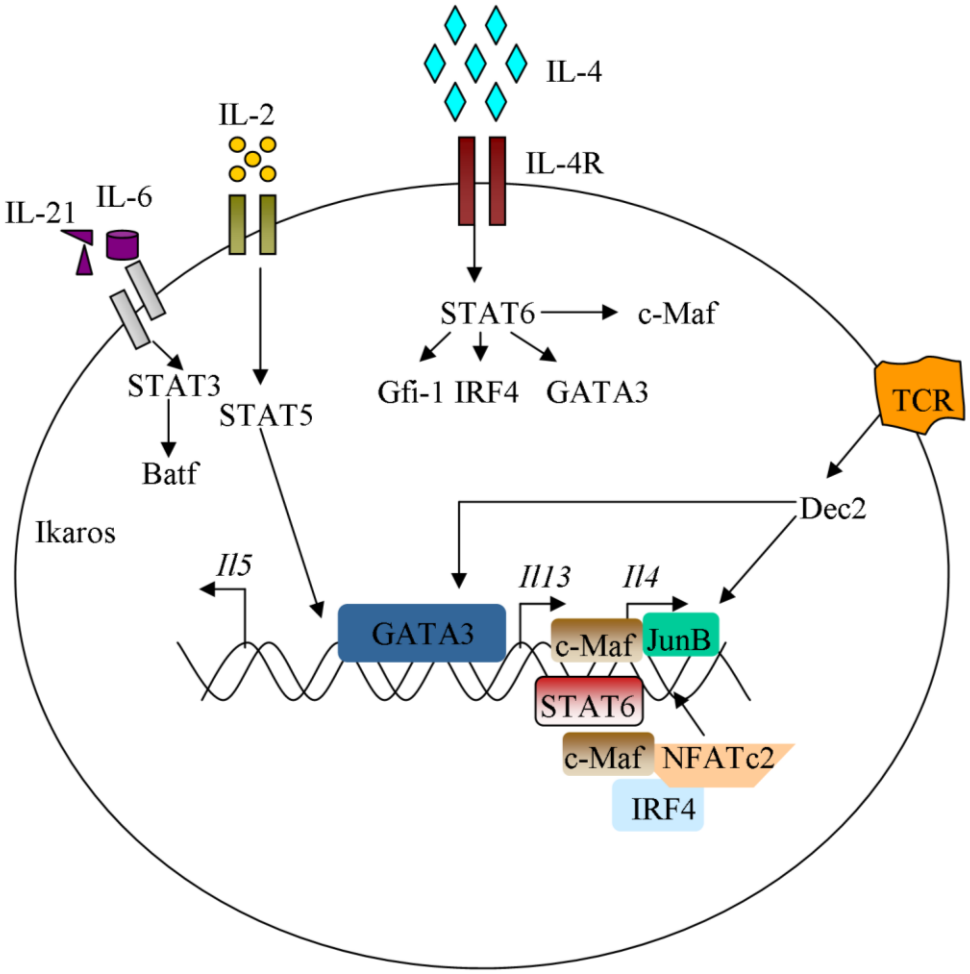

Figure 3: Transcriptional regulation of Th2 cells

Chromatin modifications of Th2 cells

The *Il4*, *Il5*, and *Il13* loci are closely linked on mouse chromosome 11, and on human chromosome 5. The locus control region (LCR) of *Il4-Il13* lies in 25kb region of *Rad50* at the 5'end of *Il13* (Fields et al., 2004). The expression of *Il5,* which lies on the other side of *Rad50,* may not be controlled by this LCR. During Th2 differentiation chromatin modifications occur at the *Il4-Il13* loci. Several distinct clusters of DNase I hypersensitive sites have been identified in the *Il4* locus (Agarwal et al., 2000; Agarwal and Rao, 1998; Fields et al., 2004; Lee and Rao, 2004; Takemoto et al., 1998). Naïve T cells and Th1 cells display identical patterns of HSS across the *Il4* locus; however, additional HSSs are present in Th2 cells including *Il4 HSI, II, III,* and *V* and *Il13 HSI, II,* and *III, HSS2* and *HSS3* and *RHS1, 4* and *7* within the *Il5* and *Rad50* genes (Agarwal et al., 2000; Fields et al., 2004; Takemoto et al., 1998). Deletion of *RHS7* reduces the production of IL-4 and IL-13 in Th2 cells (Lee et al., 2005). *Il4 HSII, HSIII, Il13 HSI,* and a conserved GATA response element function as enhancers in Th2 cells (Monticelli et al., 2005; Yamashita et al., 2002). *HS IV* which is accessible in both Th1 and Th2 cells is a potential target for IL-4 silencing in Th1 cells (Ansel et al., 2004). During the early stages of Th2 differentiation GATA3 binds to the regulatory regions within the *Il4* locus to create a transcriptionally permissive chromatin structure. However, during the later stages of Th2 differentiation GATA3 is important for *Il5,* and *Il13* but not *Il4* expression. In Th2 cells GATA3 binding to HSS3 within the *Il4* locus allows recruitment of histone acetyltransferases CBP and p300 which mediate histone acetylation (Baguet and Bix, 2004; Yamashita et al., 2002). GATA3 also binds to *RHS7/Rad50* of Th2 locus control region (Spilianakis and Flavell, 2004). GATA3 is also recruited to the *Ifng* promoter in

Th2 cells to silence its expression (Chang and Aune, 2007). Modifications of histone tails also contribute to gene expression. Acetylation of H3K9 and H3K14 residues are present at *Il4* promoter but not at the *HSIV* region in Th2 cells (Ansel et al., 2006; Avni et al., 2002). H3K4Me3 is found at the *Ifng* gene promoter in Th2 cells, but not Th1 cells (Wei et al., 2009). Histone methyltransferases MLL and EZH2 are required for permissive H3K4Me3 modification in Th2 cells and repressive H3K27Me3 modification in Th1 cells, respectively (Koyanagi et al., 2005; Yamashita et al., 2006). GATA3 regulates modifications of mono-and di-methylation of H3K4 and H3K27Me3 at the enhancer regions of the *Il4* and *Ifng* genes in Th2 cells (Wei et al., 2011). The key histone modifications which regulate Th2 cytokine gene expression is depicted below (Figure 4).

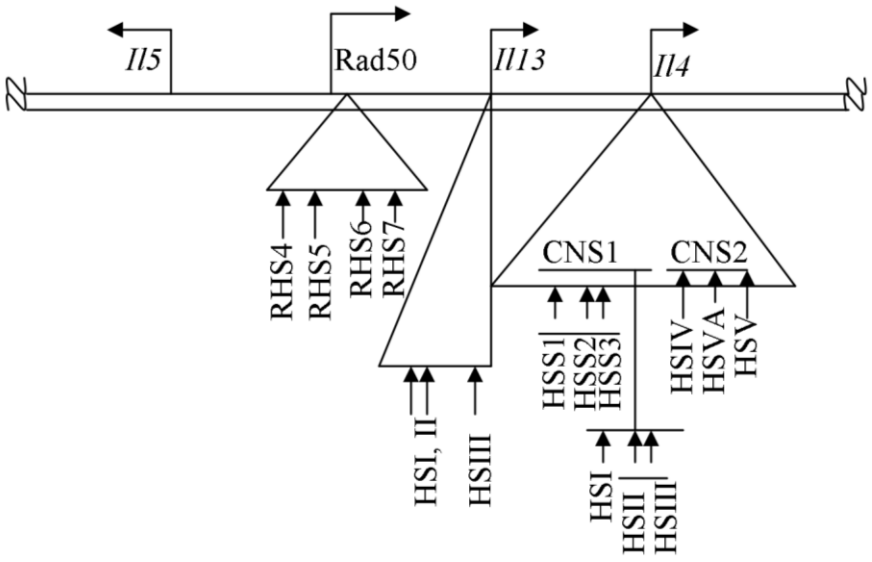

Figure 4: Chromatin modifications of Th2 cells

Cross regulation between Th1 and Th2 cells

T helper cell differentiation requires positive signal from cytokines and cross-inhibition of other effector phenotypes. Cross talk between IFN-γ-producing Th1 cells and IL-4-producing Th2 cells has been documented. TGF-β can suppress both Th1 and Th2 responses (Gorelik et al., 2000). IL12Rβ2 induction by T-bet, which is dependent on IFN-γ/STAT1 signaling, is inhibited by IL-4 (Szabo et al., 1997). Ectopic expression of T-bet represses *Gata3* expression (Usui et al., 2006). Conversely, GATA3 and IL-4 are upregulated in the absence of T-bet even in a strong Th1 environment (Yagi et al., 2010). T-bet suppresses GATA3 function by binding to it (Hwang et al., 2005). Runx3 co-operates with T-bet to promote IFN-γ production while silencing the *Il4* gene in Th1 cells by directly binding to the *Il4* locus (Djuretic et al., 2007; Naoe et al., 2007; Yagi et al., 2010). Runx3 inhibits GATA3 function by interacting with GATA3 protein (Kohu et al., 2009). In Th2 cells, GATA3 blocks the expression of STAT4, and Th2 cells from $GATA3^{fl/fl}$CD4-Cre mice exhibit increased levels of STAT4 compared to wild-type Th2 cells (Usui et al., 2006). GATA3-deficiency results in IL-12 and IFN-γ-independent IFN-γ production, likely by the increased activity of Runx3 (Yagi et al., 2010; Zhu et al., 2004). Runx3 induces *Eomes* expression and ectopic Runx3 expression in Th2 cells induces T-bet-independent *Ifng* expression (Kohu et al., 2009; Yagi et al., 2010). However, GATA3 and T-bet double-deficient Th1 cells do not express *Eomes* (Yagi et al., 2010). IL-4 can possibly induce *Eomes* expression in GATA3-limiting conditions. Ectopic expression of constitutively active of STAT5 in Th1 cells can induce Th2 polarization without altering *Gata3* expression. But, *Tbx21* expression is repressed in these cells implying that the relative amount of GATA3/T-bet regulated by STAT5

signaling affects Th1 and Th2 differentiation (Zhu et al., 2003). The transcription factor Gfi-1 suppresses Th1 differentiation, while Ikaros limits Th1 responses by inhibiting *Tbx21* expression (Quirion et al., 2009; Zhu et al., 2006b).

Transcriptional regulation of Th17 cells

Th17 cells, which have been described in the last decade, are pro-inflammatory cells which secrete IL-17A, IL-17F, IL-21, and IL-22. Th17 cells provide immunity to several extracellular pathogens including defense against infections from Candida, Citrobacter and Klebsiella (Happel et al., 2005; Huang et al., 2004; Mangan et al., 2006). TGF-β, IL-6, and IL-21 are required for the differentiation of Th17 cells (Bettelli et al., 2006; Korn et al., 2007; Mangan et al., 2006; Nurieva et al., 2007; Veldhoen et al., 2006a; Zhou et al., 2007). However; an alternative mode of pathogenic Th17 cells differentiation without TGF-β signaling has been recently documented (Ghoreschi et al., 2010). IL-23 is required for Th17 cell maintenance but not development (McGeachy et al., 2007; Stritesky et al., 2008). The cytokine IL-1β augments IL-23 responsiveness and promotes Th17 differentiation (Cho et al., 2006; Cua et al., 2003; Veldhoen et al., 2006b).

STAT family members in the development of Th17 cells

The cytokines IL-6, IL-21, and IL-23, essential for Th17 differentiation, activate STAT3 (Nurieva et al., 2007; Yang et al., 2007a; Zhou et al., 2007). Subsequently the importance of STAT3 in Th17 cell development was shown in several studies (Mathur et al., 2007; Yang et al., 2007a). STAT3-deficient T cells have impaired IL-17A, IL-17F, IL-22, and IL-23R expression (Yang et al., 2007a). STAT3 deficiency leads to attenuated expression

of *RORγt* and *RORα*, the Th17 cell lineage-specific transcription factors (Yang et al., 2007a; Yang et al., 2008b). IL-6 activated STAT3 represses the expression of *Foxp3*, the transcription factor which is required for Treg differentiation. Foxp3 down regulation is associated with decreased Treg differentiation and increased Th17 differentiation (O'Malley et al., 2009; Yang et al., 2007a; Yao et al., 2007). Patients with Job's syndrome have STAT3 mutations and impaired Th17 differentiation (Ma et al., 2008; Milner et al., 2008). In Th17 cells IL-23 signals through both STAT3 and STAT4. IL-17 production from Th17 cells stimulated with IL-23 and IL-18 require STAT4 (Mathur et al., 2007).

Role of additional transcription factors in Th17 development

Two retinoic acid-related orphan receptor family members, RORγt (an isoform of RORγ) and RORα have been shown to regulate Th17 cell differentiation (Ivanov et al., 2006; Yang et al., 2008b). RORγt, required for lymphoid organogenesis and thymocyte survival is specifically expressed in cells of immune system (Eberl and Littman, 2003; Sun et al., 2000). Over expression of RORγt promotes Th17 differentiation while RORγt-deficient T cells have impaired Th17 differentiation (Ivanov et al., 2006; Nurieva et al., 2007; Zhou et al., 2007). RORγt and STAT3 act in concert to induce *Il17* expression (Zhou et al., 2007). RORα, similar to RORγt can be induced by TGF-β, and IL-6 in a STAT3-dependent fashion (Yang et al., 2008b). RORα-deficiency leads to selective decrease in *Il17* and *Il23r* expression, while double deficiency of RORα and RORγt results in total inhibition of IL-17 production and EAE development (Yang et al., 2008b). IRF4, an inducer of GATA3 in Th2 cells is also required for Th17 development. Mice deficient in

IRF4 show defective Th17 differentiation in response to TGF-β and IL-6 (Brustle et al., 2007). Even though *RORγt* expression is diminished in *Irf4-/-* Th17 cells, ectopic expression of RORγt in IRF4-deficient T cells can partially restore Th17 differentiation. IRF4-deficient T cells display impaired IL-21-mediated autocrine response, although the precise mechanisms are not well understood (Chung et al., 2009; Huber et al., 2008). IRF4 may be responsible for early Th17 differentiation (Chung et al., 2009). IRF4 can interact with NFATp and possibly modulates NFATp-dependent IL-2 production that inhibits IL-17 production (Chen et al., 2007). Studies also demonstrate aryl hydrocarbon receptor (AHR), a type I nuclear receptor to be critical in Th17 cell differentiation (Quintana et al., 2008; Veldhoen et al., 2008a). Th17 cells have the highest expression of *Ahr* compared to other Th cells (Veldhoen et al., 2008a). AHR-deficient Th17 cells display activated levels of STAT1 and STAT5, negative regulators of Th17 development (Kimura et al., 2008). Th17 cells from AHR-deficient mice do not express IL-22 (Veldhoen et al., 2008a). Administration of the AHR ligand FICZ augments IL-17 production from Th17 cells *in vitro* and Th17-specific genes expression during EAE *in vivo* (Quintana et al., 2008; Veldhoen et al., 2008a). Batf, which is expressed in both Th2 and Th17 cells, is required for Th17 development (Betz et al., 2010; Schraml et al., 2009). Batf-deficient CD4+ and CD8+ T cells fail to produce IL-17 with increased Treg differentiation *in vivo*, and these mice are protected from EAE (Schraml et al., 2009). In Th17 cells, Batf forms heterodimer with JunB and binds to the *Il17*, *Il21*, and *Il22* promoters (Schraml et al., 2009). Batf synergizes with RORγt to induce *Il17* expression, but the precise role of Batf in Th17 cells is not fully understood (Schraml et al., 2009). The transcription factor Bcl6 which plays critical role in inhibiting Th2 cell

differentiation and promoting Tfh differentiation is also required for Th17 development (Johnston et al., 2009; Kusam et al., 2003; Mondal et al., 2010; Nurieva et al., 2009; Yu et al., 2009). *Bcl6* expression is up regulated under Th17 conditions and Bcl6-deficient mice display impaired Th17 differentiation (Mondal et al., 2010). Ectopic expression of Bcl6 in CD4+ T cells suppresses *Il4* but enhances *Il17* expression (Mondal et al., 2010). Bcl6-deficient T cells develop normal Th17 responses *in vivo*, possibly because Bcl6-deficient macrophages display augmented expression of IL-6, IL-23, and TGF-β. Thus Bcl6 may be required for optimal Th17 differentiation but it plays a compensatory role in Th17 development in the macrophage lineage (Mondal et al., 2010). c-Maf, Th2-related transcription factor is expressed in Th17 cells. TGF-β synergizes with IL-6 and IL-27 for *Maf* expression and c-Maf regulation of IL-10 production in Th17 cells (Xu et al., 2009). c-Maf regulated IL-10 production in Th17 cells is not STAT6/GATA3 dependent (Xu et al., 2009). c-Maf is also able to bind and activate the *Il21* promoter (Hiramatsu et al., 2010). c-Maf is critical for memory Th17 responses. IL-23R, important for Th17 responses is a potential target of c-Maf, and over expression of c-Maf does not enhance early stages of Th17 differentiation but expands the memory population (Sato et al., 2011). NF-κB family member IκBζ synergizes with RORγt and RORα to regulate Th17 differentiation (Okamoto et al., 2010). Another NF-κB family member, c-Rel also plays key role in Th17 differentiation (Chen et al., 2010). Therefore, in addition to RORγt and RORα which co-ordinate the diverse cytokine-induced signals, additional transcription factors are required to control Th17 cell differentiation (Figure 5).

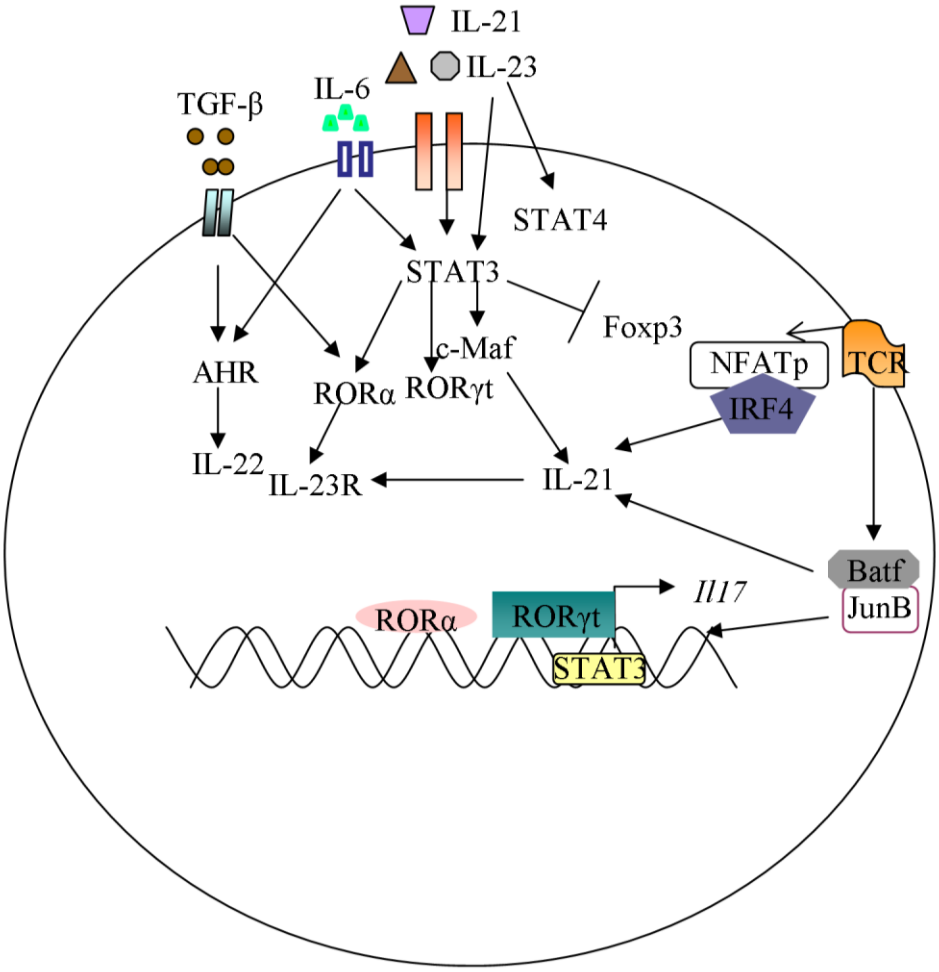

Figure 5: Transcriptional regulation of Th17 cells

Epigenetic regulation of Th17 cells

The *Il17a* gene in mice is linked to the *Il17f* gene on chromosome 1. Even though the expression of *Il17a* and *Il17f* is linked there can be specific expression of one or the other gene (Liang et al., 2007). During Th17 differentiation, both the *Il17a* and *Il17f* promoters undergo histone H3 acetylation and H3K4Me3 methylation suggesting enhanced accessibility of the locus (Akimzhanov et al., 2007). Eight CNS regions have been identified in the locus and four of them residing in the intergenic region, undergo specific histone acetylation (Akimzhanov et al., 2007). STAT3 can bind to this intergenic region

and permissive histone marks are STAT3-dependent (Durant et al., 2010). There are STAT3-dependent p300 binding sites at the *Il17* locus (O'Shea et al., 2011). CNS2, a *cis* element in the *Il17-Il17f* locus can physically interact with the *Il17* and *Il17f* promoters (Wang et al., 2012). CNS2-deficient T cells demonstrate attenuated IL-17 and IL-17F production which can be attributed to impaired chromatin architecture in the *Il17-Il17f* locus because of defective recruitment of histone modifying enzymes p300 and JMJD3 (Wang et al., 2012). How RORγt contributes to chromatin modifications in Th17 cells is not understood. As we appreciate the flexibility of Th cells, it is not surprising that the histone methylation pattern of transcription factor genes required for lineage decisions display bivalent modifications of genes, even when they are not expressed (Wei et al., 2009). *In vitro* generated Th17 cells have been shown to be unstable while memory Th17 cells exhibit a more stable phenotype associated with specific chromatin modifications (Bending et al., 2009; Lexberg et al., 2008; Mukasa et al., 2010; Shi et al., 2008a). Thus, the presence of multiple CNS regions in the *Il17* locus is required for its epigenetic regulation (Figure 6).

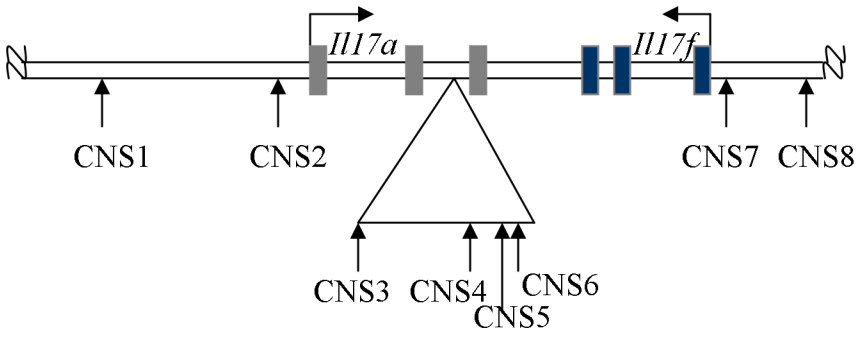

Figure 6: Epigenetic regulation of Th17 cells

Transcriptional regulation of T regulatory (Treg) cells

Regulatory T cells are a distinct T cell subset which control pro-inflammatory responses of effector Th cells and promote self-tolerance (Sakaguchi et al., 1995). Tregs suppress T cell activation by weak stimuli and protect elimination of commensal bacteria by the immune system (Baecher-Allan et al., 2002; Dembic, 2008). Naturally occurring Treg (nTregs) cells are derived from thymus and have suppressive functions. Naïve CD4+ T cells exposed to TGF-β gives rise to inducible Tregs (iTregs) in the periphery, and these cells have similar properties of nTregs (DiPaolo et al., 2007). Retinoic acid can augment Treg differentiation (Benson et al., 2007; Coombes et al., 2007; Mucida et al., 2007; Sun et al., 2007). Naïve T cells differentiated to iTregs produce the suppressive cytokine TGF-β (Li et al., 2006b). The markers used to identify Tregs include CD25, cytotoxic T lymphocyte-associated antigen 4 (CTLA4), glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), and CD127 (Liu et al., 2006; McHugh et al., 2002; McNeill et al., 2007; Read et al., 2000; Sakaguchi et al., 1995; Seddiki et al., 2006; Takahashi et al., 2000). However, these markers are not Treg-specific and controversy exists on whether Helios, an Ikaros family transcription factor can be used to differentiate nTregs from iTregs (Lan et al., 2012; Thornton et al., 2010).

Role of STAT proteins in Treg cell development

IL-2/IL-2R plays an important role Treg development as IL-2Rβ-deficient mice lose functional Tregs (Malek and Bayer, 2004). IL-2 signaling is suggested to be required for Treg activation, function and survival but not for homeostasis even though debate persists on the actual role of IL-2/IL-2R signaling (Burchill et al., 2007; D'Cruz and Klein, 2005;

Davidson et al., 2007; Fontenot et al., 2005; Furtado et al., 2002; Setoguchi et al., 2005). However, IL-2-activated STAT5 is required for the development of Treg cells (Burchill et al., 2007; Davidson et al., 2007). STAT5-deficient T cells blocks Treg development while reconstitution of *IL2rb-/-* mice with activated STAT5 reinstates Treg development (Burchill et al., 2007). STAT5 binds directly to the *Foxp3* gene, the master transcription factor of Treg cells (Burchill et al., 2007). Thus STAT5 may directly induce *Foxp3* expression (Burchill et al., 2007; Yao et al., 2007). STAT3, required for Th17 development has been shown to be important for the maintenance of nTreg phenotype and *in vivo* Treg differentiation (Pallandre et al., 2007). Other STAT proteins including STAT4 and STAT6 repress Treg development (Chapoval et al., 2010; O'Malley et al., 2009; Takaki et al., 2008; Xu et al., 2011).

Role of additional transcription factors in Treg cell differentiation

The transcription factor Foxp3 has been identified as a reliable marker for Treg cells in both human and mice (Bennett et al., 2001; Chatila et al., 2000; Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Wildin et al., 2001). These studies revealed that Foxp3 is able to convert CD4+CD25- T cells into suppressive CD4+CD25+ T cells. Foxp3 has both transcriptional activator and repressor functions (Marson et al., 2007; Zheng et al., 2007). Even though *Foxp3* expression is CD4+ T cell-specific, some CD8+ T cells express Foxp3 (Fontenot et al., 2003). Mutations in Foxp3 result in two severe autoimmune syndromes in human, namely IPEX and XLAAD (Bennett et al., 2001; Chatila et al., 2000; Wildin et al., 2001). T cell-specific Foxp3 deletion results in lymphoproliferative autoimmune disease as observed in Foxp3-deficient mice. Mice

lacking CTLA-4, TGF-β, and TGF-βR on T cells display similar phenotypes as observed in Foxp3-deficient mice. Although Foxp3 is critical for the development of Tregs, its role as lineage-specific transcription factor of Tregs has been challenged recently (Gavin et al., 2007; Lin et al., 2007). Foxp3 may be important to enhance and stabilize already established features of Treg cells mediated by IL-2 signaling (Gavin et al., 2007). Additionally, Foxp3 augments the expression of Fgl2, CD39, CD73, GRAIL or CTLA4, which are usually up regulated by conventional T cells following TCR activation (Gavin et al., 2007). The suppressive nature of mature Tregs in the periphery may require persistent expression of *Foxp3* (Williams and Rudensky, 2007).

The NFAT signaling cascade is required for Treg differentiation. In the absence of AP-1, signaling through calcium-calcineurin activates NFAT, which in turn upregulates *Cbl-b* expression, thereby inducing *Foxp3* expression in a positive feedback loop by down regulating TCR signals (Heissmeyer et al., 2004; Sundrud and Rao, 2007; Wohlfert et al., 2006). Apart from activating the *Foxp3* promoter, NFAT and Smad3 induce *Foxp3* expression through its 120-bp enhancer of the *Foxp3* intron between +2079 and +2198 nucleotides (Tone et al., 2008; Wu et al., 2006). NFAT and Foxp3 co-operate with each other and bind to the *Il2*, *Cd25*, and *Ctla4* promoters to induce their expression (Hu et al., 2007; Wu et al., 2006). TCR induced NF-κB responsive element is located in the *Foxp3* promoter and upon TCR activation c-Rel, a NF-κB family member binds to the *Foxp3* enhancer region (Long et al., 2009). A Runt-related family member transcription factor, Runx1 is also required for Treg differentiation. Runx1 and Foxp3 can interact physically and functionally (Ono et al., 2007). In Treg cells Runx1 co-operates with Foxp3/NFAT

complex to activate *Cd25*, *Ctla4*, and *Gitr* expression (Hu et al., 2007). Th2 master regulator GATA3 has been shown to be important for the function of Treg cells (Wang et al., 2011; Wohlfert et al., 2011). GATA3 is expressed in Foxp3+ Tregs from barrier sites and Treg-specific GATA3 deletion leads to spontaneous inflammation in mice and the Tregs do not accumulate in the inflamed tissues (Wang et al., 2011; Wohlfert et al., 2011). GATA3 binds to the *cis*-acting elements of *Foxp3* thereby controlling its expression (Wang et al., 2011). Thus, multiple transcription factors cooperate with Foxp3 to regulate the transcription of immunoregulatory Treg cells (Figure 7).

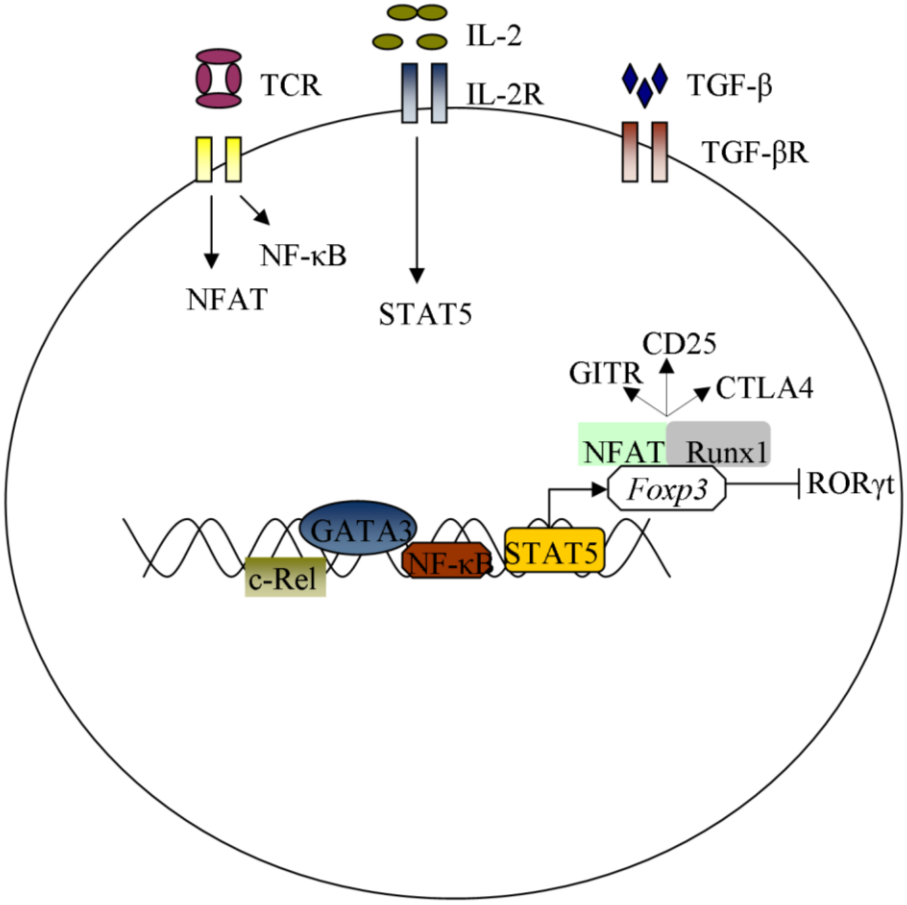

Figure 7: Transcriptional regulation of Treg cells

Epigenetic regulation of Treg cells

Expression of Foxp3 is stabilized by chromatin modifications during Treg development. A 30.8 kb genomic fragment containing *Foxp3* gene can rescue the lymphoproliferative disorder of scurfy mice (Brunkow et al., 2001). An intronic enhancer between non-coding exons 2b and 1 of the *Foxp3* gene is highly conserved and is responsible for regulation of *Foxp3* expression. The CNS3 in the first intron of *Foxp3* has functional ATF and CREB binding sites (Kim and Leonard, 2007). There is selective demethylation of CpG islands within the *Foxp3* promoter in CD4+CD25+ Treg cells (Floess et al., 2007). CD4+CD25+ Treg cells have increased histone H3 acetylation compared to CD4+CD25- conventional T cells within the *Foxp3* locus (Floess et al., 2007). In humans, *FOXP3* demethylation acts as a stable mark of natural Treg expression (Baron et al., 2007). In the Treg-specific demethylated region (TSDR), DNA demethylation is apparent in *in vivo* generated Foxp3+ Treg cells (Polansky et al., 2008). TSDR methylation results in reduced transcriptional activity of Foxp3, while inhibition of DNA methylation leads to induction and stabilization of Foxp3 during priming and restimulation (Polansky et al., 2008). An upstream enhancer region of the *Foxp3* gene with CpG residues recruits various DNMTs and other transcriptional repressors in naïve CD4+ T cells and iTregs but not in nTregs (Lal et al., 2009). Histone H3 acetylation of the upstream *Foxp3* enhancer is observed in nTregs but not iTregs suggesting differences between nTreg and iTreg enhancer structure (Lal et al., 2009). The various epigenetic modifications that regulate the expression of *Foxp3* are summarized below (Figure 8).

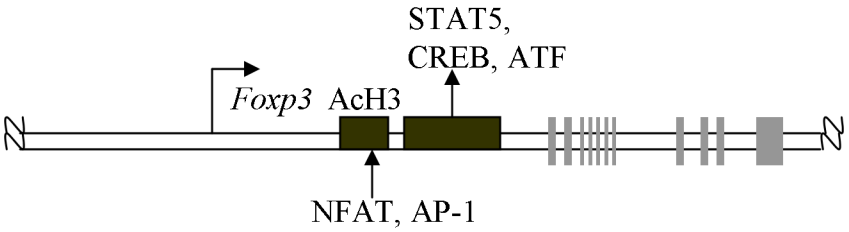

Figure 8: Chromatin modification of Treg cells

Cross regulation of Th17, Tregs, Th1 and Th2 cells

Ectopic RORγt expression inhibits STAT4 activation and T-bet induction when Th17 cells are treated with IL-12 (Mukasa et al., 2010). IFN-γ and IL-27 inhibit Th17 differentiation, the later by a STAT1-dependent mechanism (Batten et al., 2006; Harrington et al., 2005; Stumhofer et al., 2006). Deletion of T-bet in both CD4 and CD8+ T cells induces IL-17 production (Harrington et al., 2005; Intlekofer et al., 2008; Lazarevic et al., 2011; Mathur et al., 2006; Park et al., 2005; Yang et al., 2008a). IL-4-induced Gfi-1, which promotes Th2 differentiation, blocks polarization of Th17 and iTregs (Zhu et al., 2009). Thus it is evident that Th1 and Th2 cell signaling pathway inhibit Th17 responses. However, whether Th17 signals restrict Th1 and Th2 responses need to be determined. TGF-β is the key cytokine for the differentiation of Th17 and Tregs, in the presence of IL-6 and IL-2, respectively. Mice without TGF-β do not develop Th17 and Treg cells and suffer from Th1-mediated autoimmune responses (Li et al., 2006a; Veldhoen et al., 2006b). IL-6-activated STAT3 suppresses *Foxp3* expression in developing Treg cells (Korn et al., 2007; Xu et al., 2007; Yang et al., 2008a; Yang et al., 2007a; Zhou et al., 2008). Foxp3-mediated RORγt-induced IL-17 suppression may occur in a cell-intrinsic fashion. While naïve cells transduced with RORγt induces IL-17, co-transduction of RORγt and Foxp3 reduces the induction of IL-17 (Zhou et al., 2008). The

RORγt-Foxp3 balance can be tipped to a Treg phenotype in the presence of retinoic acid and IL-2 which reduce *RORγt* expression and enhance TGF-β-induced *Foxp3* expression (Coombes et al., 2007; Laurence et al., 2007; Mucida et al., 2007; Sun et al., 2007). The deficiency in IRF4, the transcription factor required for Th17 differentiation leads to increased *Foxp3* expression and diminished *RORγt* expression. Treg-specific deletion of IRF4, also a target of Foxp3 leads to impaired abrogation of Th2 cell responses (Zheng et al., 2009). Foxp3 and Runx1 interaction in Tregs is required for IFN-γ inhibition and up regulation of Treg-specific markers (Ono et al., 2007). Pro-inflammatory cytokines block TGF-β-induced *Foxp3* expression in a STAT3-dependent fashion. Therefore, mounting evidence from recent studies suggest that helper T cell subsets are not probably terminally differentiated and thus Th17 and Treg cells both regulated by TGF-β can cross-regulate with Th1 and Th2 cells.

Transcriptional regulation of T follicular helper (Tfh) cells

Tfh cells are CD4+ T cells are required to provide B cell help leading to antibody production, somatic hypermutation and class switching for long-lasting humoral immunity. Tfh cells were identified in human tonsils with CD4+ T cells expressing high levels of CXCR5 (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000). The cytokines IL-6 and IL-21 promote for Tfh cell differentiation (Chtanova et al., 2004; Eddahri et al., 2009; Nurieva et al., 2008; Suto et al., 2008). IL-6 has been demonstrated to induce IL-21 production (Eddahri et al., 2009; Nurieva et al., 2008; Suto et al., 2008). IL-21 signaling is required for increased CXCR5 expression on Tfh cells (Vogelzang et al., 2008). One study suggests that even though the absence of one of these cytokines

does not affect Tfh cell numbers, combined deficiency of both IL-6 and IL-21 leads to significantly reduced Tfh cell numbers (Eto et al., 2011). Tfh cells are characterized by the expression and production of CXCR5, inducible co-stimulator (ICOS), programmed death-1 (PD-1), and IL-21 (Breitfeld et al., 2000; Good-Jacobson et al., 2010; Hutloff et al., 2004; Schaerli et al., 2000). Mice with disrupted ICOS-ICOS ligand interaction or patients with *ICOS* mutations have decreased Tfh cell numbers (Akiba et al., 2005; Bauquet et al., 2009; Bossaller et al., 2006). Studies suggest that ICOS signaling via PI3K is important to increase expression of Tfh-specific genes such as c-Maf, and IL-21 (Bauquet et al., 2009; Gigoux et al., 2009; Rolf et al., 2010), however whether ICOS is required to maintain Tfh differentiation is not completely understood. Studies in mice deficient in PD-1 or its ligand PD-L1 indicate that PD-1/PD-L1 interactions may control long-lived plasma cells either positively or negatively (Good-Jacobson et al., 2010; Hamel et al., 2010; Hams et al., 2011).

STAT family members in the development of Tfh cells

Both IL-6 and IL-21 signal through STAT3 and STAT3-deficient CD4+ T cells demonstrate impaired differentiation of Tfh cells (Nurieva et al., 2008). T cell specific STAT3-deficient mice have defective germinal center B cell formation (Nurieva et al., 2008). However, one report suggests normal CXCR5+ CD4+ T cells in $Stat3^{CD4-/-}$ mice (Eddahri et al., 2009). IL-12 and IL-12 induced STAT4 induces genes that contribute to both Tfh and Th1 cell phenotypes during early Th1 differentiation (Nakayamada et al., 2011).STAT5 negatively regulates Tfh cell differentiation and function (Johnston et al., 2012; Nurieva et al., 2012). STAT5 inhibits the expression of CXCR5, c-Maf, and IL-21

in Tfh cells but positively regulates the expression of Blimp-1 (Nurieva et al., 2012). In the absence of STAT5 CD4+ T cells have increased germinal center B cell numbers and Tfh cell development (Johnston et al., 2012; Nurieva et al., 2012).

Additional transcription factors required for Tfh cell development

Bcl6, a transcriptional repressor, is the master regulator of Tfh cells (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Tfh cells express high level of *Bcl6* and Bcl6 over expression leads to *in vivo* Tfh cell differentiation (Johnston et al., 2009).Bcl6 regulates germinal center B cell differentiation by suppressing key signaling pathways (Basso et al., 2010; Crotty, 2011). A cluster of micro-RNAs that negatively regulate CXCR5, a key Tfh molecule, is suppressed by Bcl6 (Yu et al., 2009). Transcription factors associated with other T helper subsets are suppressed by Bcl6 (Nurieva et al., 2009). Bcl6 suppresses the expression of Blimp-1, associated with non-Tfh cells to restrict non-Tfh differentiation (Crotty et al., 2010).

The transcription factor Batf, required for Th2 and Th17 differentiation is also important for Tfh cell development (Betz et al., 2010). Batf-deficient mice have reduced number of Tfh cells and defects in germinal center development (Betz et al., 2010). Tfh cells produce IL-21, which is regulated by c-Maf (Bauquet et al., 2009). c-Maf-deficient mice have defective Tfh differentiation *in vivo* which may result from impaired induction or maintenance of IL-21 in the absence of c-Maf (Bauquet et al., 2009). IL-6 activates *Maf* expression and c-Maf directly binds to the *Il21* promoter (Hiramatsu et al., 2010; Pot et al., 2009). Therefore c-Maf plays an important role in the regulation of IL-21, a cytokine

produced by Tfh cells. The role of IRF4 in Tfh cell development is not well understood. IRF4-deficient mice show defect in Tfh cells but it is not clear whether this effect is due to defect in CD4+ T cell activation (Kwon et al., 2009). IRF4 induces Blimp-1 in CD4+ T cells (Kwon et al., 2009) while IRF4 and Bcl6 repress each other in germinal center B cells (Saito et al., 2007) making it difficult to elucidate the role of IRF4 in Tfh cell development. Therefore multiple transcription factors contribute to the development of Tfh cells.

Epigenetic regulation of Tfh cells

Studies have indicated plasticity between Tfh cells and other effector T cells (O'Shea and Paul, 2010). It is possible that Tfh cells may differentiate into Th1 cells while Treg and Th2 cells can become Tfh cells (O'Shea and Paul, 2010). In *ex vivo* Tfh cells and Tfh-like cells, the *Bcl6* locus bears H3K4Me3 marks (Lu et al., 2011). Th1, Th2, and Th17 cells also display positive H3K4Me3 marks at the *Bcl6* locus suggesting that these effector T cells have the ability to become Tfh cells (Lu et al., 2011). Both positive H3K4Me3 and negative H3K27Me3 marks are observed in *Prdm1* gene which encodes the transcriptional repressor Blimp-1 in all effector T cells suggesting possible plasticity between Tfh and other effector T cells (Lu et al., 2011). Further studies need to be performed to understand the epigenetic regulation between Tfh and non-Tfh cells.

Transcriptional regulation of Th9 cells

The newest member of CD4+ T cell family is the Th9 cell. Culturing Th2 cells with TGF-β or treating naïve CD4+ T cells with TGF-β and IL-4 gives rise to predominantly IL-9-secreting Th9 cells (Dardalhon et al., 2008; Veldhoen et al., 2008b). Th9 cells make very low amounts of Th2 cytokines IL-4, IL-5, and IL-13. The levels of IL-10 produced by Th9 cells vary among a number of reports (Chang et al., 2010; Dardalhon et al., 2008; Veldhoen et al., 2008b). Th9 cells promote allergic airway inflammation (Chang et al., 2010; Staudt et al., 2010). Th9 cells play a protective role in worm expulsion as transgenic mice expressing a dominant negative form of TGF-βR fail to mount protective immune responses leading to increased worm burden (Veldhoen et al., 2008b). Adoptive transfer of Th9-polarized cells can promote the development of EAE and EAU, though the inflammation that develops is distinct from Th1- or Th17-mediated immunity (Jager et al., 2009; Tan et al., 2010). Contrary to these studies, IL-9 from Th9 cells could promote regulatory function (Elyaman et al., 2009). IL-9 can synergize with TGF-β to differentiate naïve CD4+ T cells into Th17 cells (Elyaman et al., 2009).

Transcription factors in Th9 cell development

Differentiation of Th9 cells requires both TGF-β and IL-4 signals. IL-4 signaling activated STAT6 is required in the development of Th9 cells similar to its requirement in Th2 cells (Dardalhon et al., 2008). The role of the other STAT molecules has not been examined. Although one report has demonstrated that downstream molecules of the TGF-β signaling pathway, Smad2 and Smad3 are probably not required for *Il9* expression, another study has documented the requirement of Notch receptors and Smad3 signaling

in the induction of Th9 cells (Elyaman et al., 2012; Takimoto et al., 2010). Notch signaling mediated by Jagged2 ligand but not Delta-like 1 promotes Th9 differentiation by activating the *Il9* gene (Elyaman et al., 2012).

The transcription factor, PU.1, promotes the development of IL-9-secreting T cells. PU.1-deficient T cells exhibit attenuated IL-9 production, while ectopic expression of PU.1 in Th2 and Th9 cells can further induce IL-9 production (Chang et al., 2010). PU.1 is able to act directly on *Il9* gene as it can bind to the *Il9* promoter in Th9 cells (Chang et al., 2010). PU.1 is also required for IL-9 production in human T cells as inhibiting *PU.1* expression in human T cells is associated with diminished IL-9 production (Chang et al., 2010). The transcription factor IRF4, required for the development of Th2 and Th17 cells is also essential for Th9 cell development (Staudt et al., 2010). IRF4-deficient CD4+ T cells display impaired Th9 cell differentiation (Staudt et al., 2010). IRF4 is able to transactivate *Il9* gene by binding directly to its promoter (Staudt et al., 2010). Multiple transcription factor binding sites are identified at the *Il9* promoter including PU.1, IRF4, AP-1, and NF-κB (Perumal and Kaplan, 2011). TCR-activated transcription factors NFAT1 and NF-κB (p65) synergize to induce IL-9 in Th9 cells (Jash et al., 2012). Both NFAT1 and p65 can bind to the *Il9* promoter and NFAT1-deficient mice exhibit impaired IL-9 production resulting from inaccessible chromatin at the *Il9* promoter (Jash et al., 2012). Multiple transcription factors including AP-1, NF-κB, and c-Jun play important roles in the activation of human IL-9 in T cells (Zhu et al., 1996). In mast cells GATA1 promotes IL-9 production and *Il9* promoter activation which is p38MAPK-dependent (Stassen et al., 2007).

Epigenetic regulation of Th9 cells

Il9 gene undergoes chromatin modification in Th9 cells. Three CNS regions have been identified in the *Il9* gene (Perumal and Kaplan, 2011). The CNS1 and CNS0 regions have been shown to harbor binding sites of several transcription factors (Chang et al., 2010). The CNS2 region identified between human and mouse sequences is not conserved among other species (Perumal and Kaplan, 2011). Total acetylation of histone H3 and H4 and acetylation of histone H3K9 and H3K18, all permissive chromatin marks are highest in Th9 cells at CNS1 and CNS2 of the *Il9* gene when compared to other T helper subsets (Chang et al., 2010). Th9 cells have the lowest histone trimethylated H3K27, a repressive chromatin mark compared to the other Th subsets (Chang et al., 2010). In contrast, even though Tregs have acetylation marks similar to Th9 cells, the *Il9* locus in Tregs has more trimethylated H3K27 compared to Th9 cells (Chang et al., 2010). This suggests that active *Il9* expression is associated with a combination of high H3 acetylation and low H3K27 methylation. In the absence of PU.1, total H3 acetylation is reduced; however, total H4 acetylation remains unchanged (Chang et al., 2010). PU.1-deficiency does not alter H3 acetylation of *Il9* in either Th2 or Th17 cells thereby implying Th9-specific histone modification (Chang et al., 2010). Whether IRF4 regulates histone modification of *Il9* locus has not been examined in detail. NFAT1-deficiency results in transcriptionally repressive histone modifications at the *Il9* promoter. The recruitment of acetylation of H3, H4 and methylation of H3K4Me2, associated with transcriptionally active chromatin, are attenuated in the absence of NFAT1 (Jash et al., 2012). Significantly diminished RNA polymerase II binding is observed in *Nfat1-/-* Th9 cells, correlated with reduced binding of active histone marks (Jash et al., 2012). Potential

Smad3 and RBP-Jκ binding motifs have been identified at the *Il9* promoter (Elyaman et al., 2012). Th9 cells demonstrated enhanced permissive H3K4Me1 and attenuated restricted H3K27Me3 modifications in the Smad3 and RBP-Jκ binding sites of the *Il9* promoter (Elyaman et al., 2012). Therefore, the *Il9* locus undergoes active histone modifications which are required for optimal *Il9* expression.

Biology of Interleukin-9 (IL-9)

IL-9, IL-9 receptor cloning, signaling and expression

IL-9 is a mast cell and T cell growth factor; however, IL-9 is functionally different from other growth factors as IL-9 can provide long term IL-2- and IL-4-independent growth of specific helper T cell lines but cannot stimulate the activity of cytolytic T cell lines (Hultner et al., 1990; Uyttenhove et al., 1988; Van Snick et al., 1989; Yang et al., 1989). The IL-9 gene encodes a 14 kD peptide, which has 144-amino acids including a 18-amino acid signal peptide (Renauld et al., 1990). The mouse *Il9* gene is located on chromosome 13 while human *IL9* gene is located on chromosome 5 (Mock et al., 1990). The murine and human IL-9 genes are 69% identical at the nucleotide level and 55% at the protein level. Murine IL-9 can act on human cells; however, human IL-9 is not active on murine cells (Renauld et al., 1993).

The IL-9 receptor (IL-9R) is constituted of two subunits: the alpha chain (IL-9Rα) and the common gamma chain receptor shared by the cytokines IL-2, IL-4, and IL-7 (Renauld et al., 1992; Russell et al., 1994). The mouse receptor contains 468-amino acids while human receptor cDNA encodes 522-amino acids with 53% identity with the mouse. Due

to alternative splicing, IL-9R mRNA lacks the sequences encoding the cytoplasmic and transmembrane domains (Renauld et al., 1992). The IL-9Rα chain is a member of the hematopoietin superfamily due to the presence of WSXWS motif in the extracellular domain, and the Box1 and Box2 motifs in the intracellular domain. IL-9/IL-9R interaction leads to the activation of STAT1, STAT3, and STAT5 (Bauer et al., 1998). Amino acids between 338 and 422 including a YLPQ motif in the Box1 intracellular domain is critical for IL-9-induced cell growth, activation of STAT3, and induced gene expression (Zhu et al., 1997). IL-9-mediated signaling results in formation of STAT5 homodimers, STAT1 and STAT3 homodimers and heterodimers (Demoulin et al., 1996; Hornakova et al., 2009). A single tyrosine residue (Tyr407) in IL-9Rα is phosphorylated after ligand binding to the receptor leading to activation of associated Jak1. Mutation of this residue demonstrates its importance for IL-9-dependent responses (Demoulin et al., 1996). IL-9R signaling also activates the MAP kinase and Insulin Receptor Substrate-PI3 kinase pathways (Demoulin et al., 2003; Demoulin et al., 1996; Yin et al., 1995). IL-9R is expressed on T cell lines, and on effector T cells but not on naïve T cells (Cosmi et al., 2004; Druez et al., 1990). In asthma patients, IL-9R is found on mast cells and PMNs in the lung and IL-9R is also expressed in non-hematopoietic cells (Abdelilah et al., 2001; Kearley et al., 2011).

IL-9-secreting cells besides Th9 cells

T cells are one of the sources of IL-9 since it can be produced by long-term T cell lines, antigen-specific T cell lines and naïve murine T cells (Schmitt et al., 1989). Among the effector T cells, Th2 cells have been shown to produce IL-9 (Gessner et al., 1993). Other

Th cells including Th17 cells can also produce IL-9 (Elyaman et al., 2009; Nowak et al., 2009). Human Th17 cells can secrete IL-9, long-term Th17 cultures have the ability to co-express IL-17A and IL-9, and Th17 cells from patients with autoimmune diabetes have enhanced IL-9 production (Beriou et al., 2010). In some instances Treg cells may also produce IL-9. Both natural Tregs (nTregs) and inducible Tregs (iTregs) can secrete IL-9 according to a study where mast cells have been linked to peripheral tolerance (Lu et al., 2006). Whether human Treg cells produce IL-9 is not properly understood (Beriou et al., 2010). Recently, a specialized subset of T cells has been shown to produce IL-9 (Dardalhon et al., 2008; Veldhoen et al., 2008b). Naïve CD4+ T cells primed in the combination of TGF-β and IL-4 selectively, produce IL-9 abundantly (Dardalhon et al., 2008; Schmitt et al., 1994; Veldhoen et al., 2008b). Mast cells in the presence of LPS and IL-1 can produce IL-9 (Hultner et al., 2000; Stassen et al., 2000). iNKT cells can also produce IL-9 (Coquet et al., 2008).

Cell-type specific functions of IL-9

IL-9 is a cytokine that has biological functions on a number of cell types in addition to T cells. IL-9 plays roles on B cell development and function. Transgenic expression of IL-9 results in enhanced peritoneal CD11b+ B1 B cells, and can recover the B1 cell numbers, but not natural IgM production, in *xid* mice (Knoops et al., 2004; Vink et al., 1999). From human B cells IL-9 can augment IL-4-mediated IgE and IgG but not IgM production (Dugas et al., 1993; Petit-Frere et al., 1993). IL-9 plays a similar role on germinal center B cells which have greater *IL-9Rα* expression than other B cell types (Fawaz et al., 2007). IL-9 promotes mast cell growth and function in response to pathogens (Townsend et al.,

2000). IL-9, alone or in combination with Stem Cell Factor (SCF) or FcεRI, promotes the expression of mast cell proteases and pro-allergic cytokines in cultured mast cells (Eklund et al., 1993; Hultner et al., 1990; Lora et al., 2003; Wiener et al., 2004). Together with SCF, IL-9 can induce mast cell growth from bone marrow and mast cell progenitors (Matsuzawa et al., 2003). Hematopoiesis is also regulated by IL-9. Apart from synergizing with IL-3 in promoting the growth of IL-3-dependent cell lines, IL-9 can also enhance BFU-E, CFU-E and multi-lineage colony formation in combination with other factors including SCF from cord blood and bone marrow precursors (Donahue et al., 1990; Holbrook et al., 1991; Lemoli et al., 1994; Williams et al., 1990). Human IL-9 is also an activator of megakaryocyte progenitor cells (Fujiki et al., 2002). IL-9 transgenic expression in the lung alters airway epithelial cell gene expression (Temann et al., 1998). The effects of IL-9 in the lung can occur due to indirect effects of IL-13 (Steenwinckel et al., 2009; Steenwinckel et al., 2007; Temann et al., 2007; Whittaker et al., 2002). The effects of IL-9 on human primary airway epithelial cells and cell lines can result through direct induction of mucus genes (Longphre et al., 1999; Louahed et al., 2000; Reader et al., 2003; Vermeer et al., 2003). IL-9 also acts on airway smooth muscle cells in the lung. Human airway smooth muscle cells express both IL-9 receptors and IL-9 can potentiate the ERK-dependent release of CCL11 and IL-8 from these cells (Baraldo et al., 2003; Gounni et al., 2004). Thus, IL-9 functions on multiple cell types and is a pleiotropic cytokine (Figure 9).

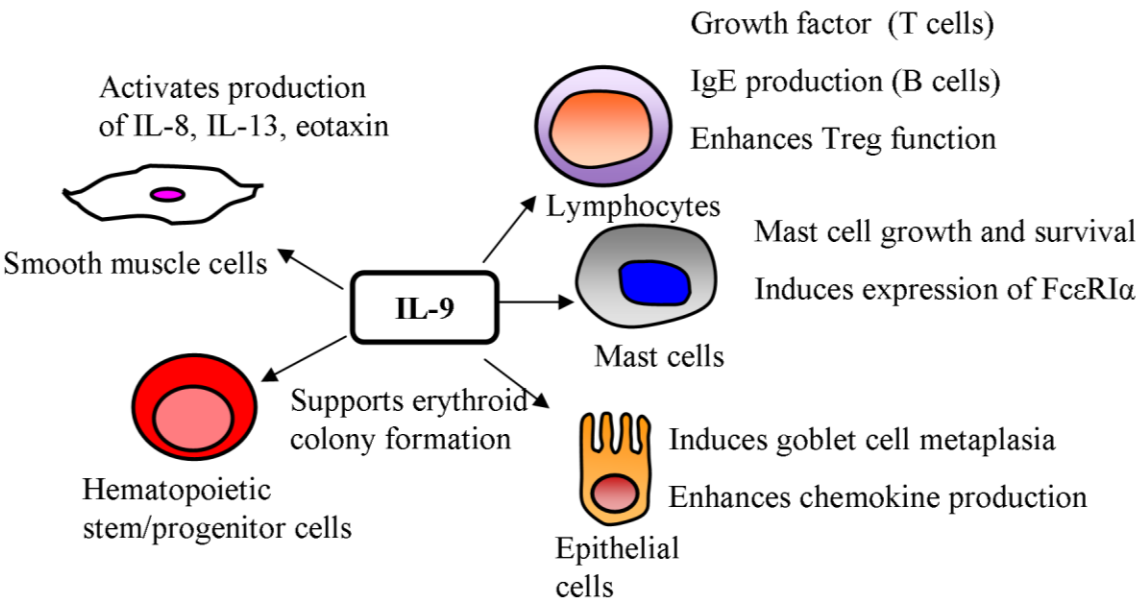

Figure 9: IL-9 is a pleiotropic cytokine

IL-9-mediated inflammation

IL-9 exhibits pro-inflammatory activity in several mouse models of inflammation. Genetic studies have provided evidence that IL-9 is a key player in asthma (Nicolaides et al., 1997). Transgenic expression of IL-9 in the lung results in allergic inflammation that is at least partially dependent on the presence of Th2 cytokines (Temann et al., 1998; Temann et al., 2002). IL-25, a cytokine that shares sequence similarity with IL-17 also induces allergic inflammation through an IL-9-dependent mechanism (Angkasekwinai et al., 2010). Though blocking of IL-9 reduces allergic airway inflammation in standard sensitization-challenge model, IL-9-deficient mice still can develop allergic inflammation (Cheng et al., 2002; Kung et al., 2001; McMillan et al., 2002). In a chronic model of lung inflammation, blocking IL-9 inhibits mastocytosis and airway remodeling. Intratracheal administration of IL-9 in naïve C57BL/6 mice leads to lung eosinophilia, *Il5Rα*

expression and elevated serum IgE levels (Levitt et al., 1999). In humans, cells in the bronchial mucosa of atopic asthmatics express enhanced *Il9* mRNA; correlated with the numbers of eosinophils and mast cells at the inflammation site (Shimbara et al., 2000; Ying et al., 2002). In the bronchoalveolar lavage fluid of atopic asthmatics segmental allergen challenge can enhance IL-9 production from lymphocytes (Erpenbeck et al., 2003).

In addition to the lung, IL-9 can mediate inflammation in the intestine (Forbes et al., 2008; Knoops et al., 2005; Osterfeld et al., 2010). Transgenic IL-9 expression or IL-9 administered by injection can enhance the susceptibility of both active and passive anaphylaxis (Knoops et al., 2005). IL-9 may be important for anaphylaxis caused by allergen challenge at mucosal surfaces but not parenterally administered allergen as systemic anaphylaxis can occur in both *Il9-/-* and *Il9r-/-* mice (Forbes et al., 2008; Knoops et al., 2005).

The IL-9-mediated pro-inflammatory environment plays protective role in immunity to intestinal parasites. Mice bearing transgenic expression of IL-9 can clear worms more efficiently after infection with *Trichuris muris* and *Trichinella spiralis* (Faulkner et al., 1997; Faulkner et al., 1998). Adoptive transfer of IL-9 transgenic bone marrow derived DC increases immunity to *T. spiralis*, while anti-IL-9 blocks *T. muris* immunity (Leech and Grencis, 2006; Richard et al., 2000). IL-9-deficient mice are able to control *Giardia lamblia* and *Nippostrongylus brasiliensis* infection, although neutralization of IL-9 results in increased immunity to *Leishmania major*, owing to blockade of Th2 immunity

(Arendse et al., 2005; Li et al., 2004; Townsend et al., 2000). IL-9, though important for mastocytosis and goblet cell metaplasia is not required for granuloma formation following injection with *Schistosoma mansoni* eggs (Townsend et al., 2000). IL-9 also plays a role in regulating immunity to infectious disease. IL-9 neutralization can be therapeutic in the clearance of RSV (Dodd et al., 2009). IL-9 can diminish septic shock symptoms by reducing inflammatory cytokines and respiratory burst in the monocytes (Grohmann et al., 2000; Pilette et al., 2002).

The exact roles of IL-9 in EAE remain controversial. IL-9 can promote the development of peripheral nervous system lesions (Elyaman et al., 2009). IL-9Rα-deficiency or anti-IL-9 treatment results in delayed EAE development (Li et al., 2011; Nowak et al., 2009). However, in a separate report there was increased severity of EAE from *Il9r-/-* mice owing to decreased IL-9 effect on Treg cells (Elyaman et al., 2009). The anti-inflammatory role of IL-9 is consistent with a described role for IL-9 in promoting mast cell dependent tolerance to transplanted allografts and nephritis (Eller et al., 2011; Lu et al., 2006). IL-9 certainly has effects in the EAE models on T and non-T cells and the effect of blocking IL-9/IL-9R may depend upon the cells present in the microenvironment (Nowak et al., 2009).

T helper cells: Overall functions

Each T helper subset is characterized by the expression of individual STAT molecules and lineage-specific transcription factors. The effector T cells execute immune functions to protect from different kind of invading pathogens. The summary of the transcription factors and the functions associated with each T helper subset is depicted below (Figure 10).

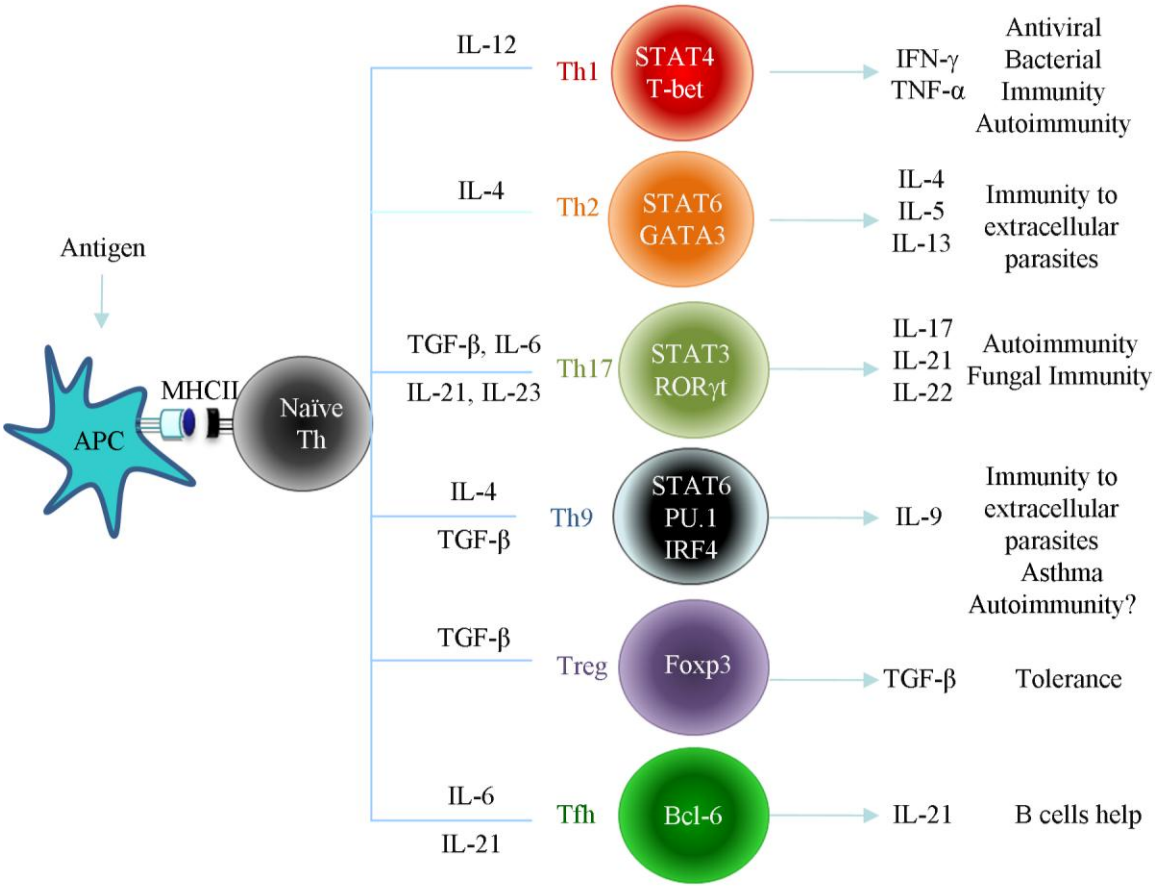

Figure 10: Schematic diagram of various T helper subsets. Each T helper cells is characterized by the cytokines they produce and the transcription factor they express. These T helper cells play a role in mounting defense against various pathogens.

Research goals

The focus of our research is to understand the transcriptional regulation of IL-9-secreting Th9 cells. We have three specific goals for our research. The first goal is to characterize the phenotype of IL-9-secreting T cells. Our second goal is to elucidate the transcriptional network governing Th9 cells. This aim will specifically look into the role of STAT family members and the transcription factors downstream of TGF-β and IL-4 signals required for the development of Th9 cells. We have previously shown that the transcription factor PU.1 is required for Th9 cell development. Our final goal therefore is to define the PU.1-dependent mechanism for the regulation of *Il9* gene in Th9 cells.

There has been renewed interest in IL-9 cytokine biology. Initially IL-9 was characterized as a cytokine produced by Th2 cells evident in models of Th2-mediated inflammation. That a specific T helper subset exists for secreting IL-9 has been highlighted recently in two reports (Dardalhon et al., 2008; Veldhoen et al., 2008b). Naïve CD4+ T cells activated and primed in the presence of TGF-β and IL-4 have the capacity to secrete IL-9. Therefore we want to characterize the cytokine profile of the Th9 subset and determine how the subset differs from other T helper subsets.

We hypothesized that PU.1 might play a positive role in Th9 cell development. Th2 cells ectopically expressed with PU.1-expressing retrovirus produce reduced amount of Th2 cytokines. Similarly regular Th9 cells secrete diminished amount of Th2 cytokines. We observed that PU.1-deficient T cells have an impaired ability to produce IL-9 under Th9 differentiating conditions.

In addition to STAT6, the role of other STAT proteins has not been described in the development of Th9 cells. The transcription factors regulated by both TGF-β- and IL-4-induced signaling have not been examined previously. We therefore establish a transcription factor network downstream of TGF-β and IL-4 that regulates IL-9.

PU.1 can bind to the *Il9* promoter and there are PU.1-dependent histone modifications in Th9 cells. Based on multiple techniques, including retroviral gene transduction and ChIP, we define the domains of PU.1 required for *Il9* gene induction, demonstrate association of histone modifying enzymes with PU.1, chromatin modifications which are PU.1-dependent and address the mechanisms by which PU.1 induces IL-9 in Th9 cells.

Together, these studies will elucidate how *Il9* gene is transcriptionally and epigenetically regulated in Th9 cells.

MATERIALS AND METHODS

Mice

Wild-type (WT) mice on both C57BL/6 and Balb/c background were purchased from Harlan Biosciences (Indianapolis, IN). Mice with lymphocyte conditional PU.1 gene deletion (*Sfpi1*$^{lck-/-}$) on C57BL/6 background were previously described and were mated to mice expressing the Cre transgene under the control of the Lck promoter (Dakic et al., 2005). Mice with conditional Stat3 deletion, which were provided by Dr. David Levy (New York University, New York, NY) and described previously, were mated to *Cd4*-cre mice (Chiarle et al., 2005; Stritesky et al., 2011). *Stat6*-deficient mice and *Parp14*-deficient mice were described previously and are on a BALB/c and C57BL/6 background, respectively (Cho et al., 2009; Kaplan et al., 1996a). *Runx3*$^{fl/fl}$ mice were mated to *dLck*-Cre mice (Yagi et al., 2010). *Tbx21-/-* mice were described previously and were on C57BL/6 background (Szabo et al., 2002). *Il4*-deficient mice were purchased from Jackson Laboratories and were bred in the Indiana University animal facility. All mice were maintained in specific pathogen-free conditions and experiments approved by the Indiana University Institutional Animal Care and Use Committee.

Murine T helper cell differentiation

Naive $CD4^+CD62L^+$ T cells were purified from spleens and lymph nodes by magnetic selection (Miltenyi Biotec). Naive CD4+ T cells (1×10^6 cells/ml complete RPMI 1640 medium) were activated with plate-bound anti-CD3 (2 μg/ml; 145-2C11; BioXcell) and soluble anti-CD28 (1 μg/ml; 37.51; BD Biosciences) and cultured under Th9 conditions (IL-4 [20 ng/ml; PeproTech], TGF-β [2 ng/ml; R&D Systems], and anti–IFN-γ [10

μg/ml; XMG; BioXcell]); Th2 conditions (IL-4 and anti–IFN-γ); Th1 conditions (IL-12 [5 ng/ml; R&D Systems], IL-2 [50 U/ml; PeproTech], and anti–IL-4 [10 μg/ml; 11B11; BioXcell]); Th17 conditions (TGF-β, IL-6 [100 ng/ml; R&D Systems], IL-1β [10 ng/ml; eBioscience], IL-23 [10 ng/ml; R&D Systems], anti–IFN-γ, and anti–IL-4); and Treg conditions (TGF-β and anti–IL-4). After 3 days, cultures were expanded with fresh complete RPMI 1640 medium with IL-4 and TGF-β added to the Th9 cells; half the dose of IL-1β, IL-23, and IL-6 added to the Th17 cells; and IL-2 added to Tregs. Following 5 days of differentiation Th9, Th2, and Th17 cells were re-stimulated with plate-bound anti-CD3 (2 μg/ml) for 1 day. Cell-free supernatant was collected and stored at -20^{0}C until further use. In some experiments Th9 cultures were supplemented with neutralizing IL-10 antibody (10 μg/ml; JES5-2A5; Biolegend) and Th2 cultures were treated with recombinant IL-9 (10 ng/ml; PeproTech).

Intracellular cytokine staining

Differentiated cells were stimulated with PMA (phorbol 12-myrisate 13-acetate) (50 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma) for a period of 5 hours. Monensin was added to the cells for the last 3 hours of stimulation. After fixing the cells with paraformaldehyde and permeabilizing with saponin, cells were stained with fluorochrome conjugated anti-mouse IL-9 (RM9A4, Biolegend), anti-mouse IL-4 (11B11, Biolegend), anti-mouse IL-17A (eBio17B7, eBioscience), anti-mouse IL-10 (JES5-16E3, BD Pharmingen) antibodies for 30 minutes at 4^{0}C. Intracellular Foxp3 staining was performed according to manufacturer's instructions (eBioscience). Samples were

analyzed by flow cytometry using FACSCalibur machine (Beckton Dickinson) and were analyzed by WinMDI.

Phospho-STAT protein analysis

Th2 and Th9 cells were harvested each day during the 5 day differentiation, fixed with 1.5% paraformaldehyde for 10 min at room temperature, and permeabilized for 10 min with 100% methanol at 4^0C. Cells were then stained for phospho-STAT3 (4/P-STAT3, BD Pharmingen) and phospho-STAT6 (J71-773.58.11, BD Pharmingen) after 30 min at room temperature. Cells were analyzed by flow cytometry with FACSCalibur.

Cell surface staining

During five days of Th2 and Th9 cell differentiation, cells were collected each day and washed with FACS/ELISA buffer (2% BSA and 0.01% sodium azide in 1X PBS). Cells were then surface stained for ST2 (DJ8, MD Biosciences) and IL-4Rα (mIL4R-M1, BD Pharmingen) using fluorochrome conjugated antibodies at 4^0C for 30 minutes. Following incubation cells were washed with FACS/ELISA buffer and resuspended in FACS/ELISA buffer to analyze by flow cytometry using FACSCalibur. Data were analyzed by WinMDI software.

Intracellular c-Maf and GATA3 staining

Differentiated Th2 and Th9 cells were harvested and were kept unstimulated or stimulated with 2 μg/ml anti-CD3 for 6 hours and were fixed with formaldehyde (1.5% final concentration) for 10 minutes at room temperature. Cells were then permeabilized

with 100% ethanol for 10 minutes at 4^0C. Following permeabilization cells were stained with anti-c-Maf (sc-10019) antibodies for 15 minutes followed by anti-IgG alexa 647 for 15 minutes at room temperature or with anti-GATA3 (L50-823, BD Pharmingen) for 30 minutes.

Retroviral transduction

Bicistronic retroviral vectors encoding mouse GATA3 and human CD4, T-bet, Foxp3, JunB, or c-Maf (kindly provided by I.C. Ho, Brigham and Women's Hospital, Boston, MA) and enhanced GFP, or Runx3 and Thy1.1 (kindly provided by Shreevrat Goenka) were used to generate virus. Myc-tagged PU.1 cDNA or PU.1 mutant cDNAs were cloned into MIEG-eGFP vector (Klemsz and Maki, 1996). The Phoenix-Eco packaging cell line were transiently transfected with 15 μg of each purified plasmid by calcium phosphate precipitation in chloroquine containing DMEM media to generate retroviral supernatants. Cells were cultured at 37^0C and chloroquine containing DMEM media was replaced with fresh DMEM media one day after transfection. After one and two days following transfection, the supernatants containing retrovirus were collected, filtered through a 0.45 μm filter and stored at -80^0C. Following 48 hours of differentiation, T helper cells were transduced with 2-4 ml of retroviral supernatant containing 8 μg/ml polybrene by centrifugation at 2000 RPM at room temperature for 1 hour. After the spin infection the retroviral supernatant was replaced with original RPMI 1640-conditioned media with 50 U/ml human IL-2. On day 5, transduced cells ($eGFP^+$, $Thy1.1^+$, or $hCD4^+$) were analyzed by flow cytometry. In some experiments transduced cells were sorted by

flow cytometry before cytokine production, gene expression analyses and chromatin immunoprecipitation.

In some experiments developing Th9 cells were transduced with control or PU-Estrogen receptor fusion retroviruses with increasing doses (0, 50, 250, and 1000nM) of 4-Hydroxytamoxifen (4-HT), an active metabolite of tamoxifen which binds estrogen receptors each day during 5 day differentiation. The retrovirus supernatants were prepared in DMEM media with charcoal treated serum to remove endogenous estrogens. Since PU.1 expression at the basal level was already high, 4-HT-inducible gene expression was not observed. This may occur due to the presence of phenol red in the media thereby reducing the ability of exogenous estrogen to stimulate responses (Berthois et al., 1986).

Assessing cytokine production by ELISA

T cells differentiated for 5 days were harvested, washed and stimulated (1×10^6 cells/ml) with 2 μg/ml plate-bound anti-CD3 for 24 hours to generate cell-free supernatant. In some experiments, differentiated Th9 cells were re-stimulated with plate-bound anti-CD3 and soluble TGF-β (2 ng/ml) for 24 hours. To detect cytokine secretion 2 μg/ml α-IL-4 (11B11, BD Pharmingen) α-IL-5 (TRFK5, BD Pharmingen), α-IL-10 (JES5-2A5, BD Pharmingen), α-IL-13 (eBio13A, eBioscience), α-IL-17A (TC11-18H10, BD Pharmingen), α-IL-21 (AF594, R&D Systems) and 1 μg/ml α-IL-9 (D8402E8, BD Pharmingen) capture antibodies were dissolved in 0.1M $NaHCO_3$ (pH 9.0) coating buffer. 50 μl of the capture antibodies were coated in each well of 96-well Nunc-immuno plates

and were incubated overnight at 4^0C. Plates were washed in ELISA wash buffer (0.1% Tween-20 in PBS) and blocked for 2 hours in FACS/ELISA buffer at room temperature. Supernatants and cytokine standards were added to the plates after FACS/ELISA buffer was removed, and plates were incubated overnight at 4^0C. Biotinylated [α-IL-4 (BVD6-24G2, BD Pharmingen), α-IL-5 (TRFK4, BD Pharmingen), α-IL-9 (D9302C12, Biolegend), α-IL-10 (SXC-1, BD Pharmingen), α-IL-13 (eBio1316H, eBioscience), α-IL-17A (TC11-8H4.1, BD Pharmingen), and α-IL-21 (BAF594, R&D Systems)] antibodies (1 μg/ml) were dissolved in FACS/ELISA buffer and were added to the plates (100 μl/well) after the plates were washed with ELISA wash buffer. Following 2 hours incubation at room temperature plates were washed again with ELISA wash buffer and incubated with avidin-alkaline phosphatase (100 μl/well)(1:2000 dilution; Sigma) in FACS/ELISA buffer for 1 hour at room temperature. Cytokine levels were assessed after the addition of Sigma 104 phosphatase substrate (5 mg/ml) dissolved in ELISA substrate buffer (10% diethanolamine, 0.05 mM $MgCl_2$, 0.02% NaN_3, pH 9.8) by measuring absorbance at 405 and 450 nm to subtract the background noise (Bio-Rad microplate reader model 680).

Quantitative RT-PCR (qPCR)

Total RNA was isolated from unstimulated or stimulated (plate-bound anti-CD3; 2 μg/ml) cells using TRIzol and reverse transcribed, according to the manufacturer's instructions (Invitrogen Life Technologies). For qPCR, cDNA (2 μl), TaqMan Fast Universal Master Mix (5 μl) and commercially available primers were used for *Bcl6*, *Foxp3*, *Gata3*, *Gcn5*, *Gfi-1*, *Il9*, *Il10*, *Il21*, *Irf4*, *Maf*, *Runx3*, *Sfpi1*, and *Tbx21* (Applied

Biosystems, 0.5 µl each). DEPC water was added to final reaction volume of 10 µl. RNA expression was normalized to the expression of β_2-microglobulin. The relative expression was calculated by the change-in-threshold ($-\Delta\Delta C_T$) method.

Primers	Applied Biosystems catalog number
Bcl6	Mm00477633_m1
Foxp3	Mm00475156_m1
Gata3	Mm00484683_m1
Gcn5	Mm00517402_m1
Gfi-1	Mm00515853_m1
Il9	Mm00434305_m1
Il10	Mm00439614_m1
Il21	Mm00517640_m1
Irf4	Mm00516431_m1
Maf	Mm01546091_s1
Runx3	Mm00450960_m1
Sfpi1	Mm00488140_m1
Tbx21	Mm00450960_m1

Table 1: Catalog numbers of the primers used for real time PCR

Western blot analysis

Total cell lysates were prepared from Th9, Th2, or Th1 cells after 5 days of differentiation. In some experiments Th9 cell extracts were prepared from WT and *Sfpi1*$^{lck-/-}$ mice. Differentiated Th cells were harvested, washed and lysed in 25 µl of lysis buffer (10% glycerol, 1% IGEPAL, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktail (1:100; Sigma)). Cells were incubated on ice for 10 minutes and spinned down at 14,000 RPM at 4^0C. The lysates were resuspended in SDS-PAGE loading buffer (200 mM Tris-HCl, pH 6.8, 40% glycerol, 8% SDS, 4% β-mercaptoethanol, 0.04% bromophenol blue). Samples were boiled at 100^0C for 5 minutes to denature proteins and complexes. Protein samples and Precision Blue marker (Bio-Rad) were loaded and separated on pre-cast 4-12% gradient Bis-tris polyacrylamide gel (Invitrogen). After running the gel at 100V for 2-3 hours proteins from the gel were transferred onto nitrocellulose membrane (Whatman GmbH) at 40 mA overnight at 4^0C. The blots were blocked in 5% nonfat dry milk at room temperature for 1-2 hours. The membrane was immunoblotted using primary antibodies dissolved in PBST with 5% nonfat dry milk to detect Gcn5 (sc-20698, Santa Cruz), IRF4 (sc-6059, Santa Cruz), p300 (sc-584, Santa Cruz), PCAF (sc-8999, Santa Cruz), PU.1 (sc-352, Santa Cruz), and Runx3 (sc-130014, Santa Cruz) overnight at 4^0C. The membrane was washed with ELISA wash buffer for 5 minutes 3 times. Secondary antibody diluted in PBST with 5% nonfat dry milk was added to the membrane for 1 hour at room temperature. The membrane was washed 5 minutes 3 times following incubation. The final reaction was developed using Western Lighting Chemiluminescence reagent (Perkin Elmer Life Sciences, Wellesley, MA) and classic blue autoradiography film BX (Molecular

Technologies, MO) was used to detect the signal. The membrane was stripped in stripping buffer (10% SDS, 62.5 mM Tris-HCl, 0.7% β-mercaptoethanol) and reprobed with anti-β-actin as loading control.

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed (with 5-10×10^6 cells), as previously described with minor modifications (Yu et al., 2007). Similar numbers of differentiated cells were kept unstimulated, fixed with 1% formaldehyde, resuspended in cell lysis buffer and incubated on ice for 10 minutes. Following lysis of the nuclei and shearing the genomic DNA, cell lysates were diluted in ChIP dilution buffer, pre-cleared with salmon sperm DNA and BSA. Immunocomplex was precipitated with protein A or protein G-agarose beads at 4^0C for 1 hour. The supernatant was incubated in the presence of rabbit polyclonal antibodies anti-PU.1 (sc-352 X, anti-Gcn5 (sc-20698, Santa Cruz), anti-Stat6 (sc-1698 X, Santa Cruz) anti-Histone H3 (06-755, Upstate), anti-acetyl-H3 lysine 9/18 (07-593, Millipore), anti-acetyl-H3 lysine 14 (07-353, Millipore), anti-acetyl-H3 lysine 27 (07-360, Millipore), anti-Histone H4 (ab7311, Abcam), anti-acetyl-H4 lysine 5 (07-327, Millipore), anti-acetyl-H4 lysine 8 (ab15823, Abcam), anti-acetyl-H4 lysine 16 (07-329, Millipore), anti-acetyl-H3 lysine 36 (07-540, Millipore), rabbit IgG (12-370, Millipore), mouse monoclonal anti-HDAC1 (sc-8410, Santa Cruz), mouse IgG1 (sc-3877, Santa Cruz), goat polyclonal anti-IRF4 (sc-6059 X, Santa Cruz), anti-HDAC2 (sc-6296, Santa Cruz), and goat IgG (sc-3887, Santa Cruz)) overnight at 4^0C. The beads were washed consecutively with low salt wash buffer, high salt wash buffer, LiCl wash buffer, and twice in TE buffer. The supernatant was collected, supplemented with 2 mM EDTA, 20

mM Tris-HCl, 10 mg/ml Proteinase K. DNA cross-links were reversed by incubating precipitates overnight at 65^0C, and DNA was purified by phenol-chloroform extraction and ethanol precipitation. DNA quantification was performed with SYBR Green Fast PCR Master Mix via an ABI 7500 Fast Real-time PCR system. A standard curve was generated from serial dilutions of input DNA to quantify immunoprecipitated DNA. The amount of immunoprecipitated DNA from the IgG control was subtracted from the amount of immunoprecipitated DNA from the specific antibody ChIP and normalized against the amount of input DNA to calculate percentage input.

Primers	Sequence
Gata3 For	5'CGTATATGATGAGTCTTCTCTGGGACTTG 3'
Gata3 Rev	5'AAATCTCAGAACACACACATTTCCAGGG 3'
Il9 CNS1 For	5'CAGTCTACCAGCATCTTCCAGTCTAGC 3'
Il9 CNS1 Rev	5'GTGGGCACTGGGTATCAGTTTGATGTC 3'
Il9 CNS0 For	5'GAGCTGAACGCAGGCCAAGAACGA 3'
Il9 CNS0 Rev	5'CTTGGAACTAGTTATCTCTCCACTG 3'
Irf4 For	5'CCGACAGACAGAAGGTGTTCAAAGAAT 3'
Irf4 Rev	5'TTCCATTGTCAGAGCCCTGGTAGTAAAC 3'

Table 2: Primer sequences used for ChIP.

DNA Oligo Pull-down experiment

Nuclear lysates were prepared from wild-type and *Sfpi1*$^{lck-/-}$ Th9 cells. Differentiated Th9 cells were washed in cold PBS and centrifuged at 1500 RPM for 5 min at 4^0C. Cell

pellets were resuspended in ice cold cell lysis buffer (100 mM Tris-HCl, 100 mM NaCl, 3% $MgCl_2$) for 10 minutes and were incubated for another 10 minutes after adding 20% NP-40. Cells were centrifuged 5000 RPM for 5 min at 4^0C and were lysed in lysis buffer for 20 minutes at 4^0C. Nuclear extracts were collected to fresh tubes after spinning at 14,000 RPM for 10 minutes at 4^0C. Streptavidin-agarose beads were incubated with consensus or mutated PU.1-binding oligonucleotides in pull-down buffer (25 mM HEPES, 15 mM NaCl, 0.5 mM DTT, 0.5% NP-40, 0.1 mM EDTA pH 7.5, 10% Glycerol) for 30 minutes at 4^0C. Following incubation the beads were washed for 3 times in pull-down buffer (1 minute each). Extracts were then incubated with the beads and salmon sperm DNA for 2 hours at 4^0C. The consensus PU.1-binding site was previously described (Chang et al., 2010). The sequences for the mutated PU.1-binding sites are biotin-CTTTTCTGATCGCTGGTATT and its reverse complement. Protein-DNA complexes were separated by SDS-PAGE and immunoblots were probed with anti-Gcn5, anti-PCAF, and anti-PU.1 antibodies.

Gcn5 small interfering RNA (siRNA) transfection

Differentiated Th9 cells from wild-type mice were transiently transfected with control or Gcn5-specific siRNA (0.2 μM, sc-37947, Santa Cruz) using Amaxa Nucleofector (Lonza). Cells were rested overnight in 5% CO_2 incubator with hIL-2 (50 U/ml). Cells were then either kept unstimulated for ChIP assay or stimulated with 2 μg/ml plate bound anti-CD3 for 6 hours for RNA isolation or for 24 hours to collect cell-free supernatant for ELISA.

RESULTS

PART I: Phenotype of IL-9-secreting T cells

Naïve T cells differentiated with TGF-β and IL-4 produce IL-9

There has been a recent surge of interest towards the understudied cytokine IL-9, a factor which has been previously demonstrated to be involved in multiple inflammatory responses. More than 15 years ago, one study demonstrated that when T cells are treated with TGF-β and IL-4 they secrete IL-9 (Schmitt et al., 1994); this finding has been reiterated by two recent reports (Dardalhon et al., 2008; Veldhoen et al., 2008b). These primed cells are presently known as Th9 cells. Our initial goal was to further define cytokine production by Th9 cells, and to determine the specificity of IL-9 expression among Th subsets. To determine if other T helper subsets are capable of secreting IL-9, we isolated naïve CD4+ T cells from WT mice and differentiated them under Th1, Th2, Th17, Treg, and Th9 (TGF-β+IL-4) cell conditions for 5 days. *Il9* expression was measured by real time PCR from differentiated cells after anti-CD3 reactivation. Expression of *Il9* was observed only in Th9 cell conditions (Figure 11A). As the cytokine IL-4 is required for the development of both Th2 and Th9 cells, and IL-9 has been shown to be secreted in various Th2-mediated inflammation models, we wanted to define the production of IL-9 by Th2 cells in detail. To test this, WT naïve CD4+ T cells were cultured under Th2 and Th9 cell conditions for 5 days. The percentages of IL-9-secreting cells assessed by intracellular staining and the total IL-9 production assessed using ELISA and *Il9* expression measured by real time PCR, was significantly lower in Th2 cells (Figure 11B-D). There were very few IL-4-secreting cells (about 3 fold less than Th2 cells) produced by Th9 cells (Figure 11B). Th2 and Th9 cells had about 20 % of IL-

4+IL-9+ double-positive cells (Figure 11B). Published reports had shown that Th17 cells can produce IL-9 and conversely, Th9 cells can produce IL-17 (Elyaman et al., 2009; Nowak et al., 2009). We observed minimal IL-9 produced by Th17 cells compared to Th9 cells by intracellular cytokine staining and ELISA (Figure 11E, F). This observation can be attributed to variability in the length of the differentiation and the concentration of cytokines used for polarization. In contrast, Th9 cells produced detectable IL-17 but significantly less than Th17 cells (Figure 11F). TGF-β-induced Treg cells have also been suggested to produce IL-9 (Lu et al., 2006). Thus we compared the levels of Foxp3 and IL-9 between Th9 and Treg cells. Th9 cells expressed Foxp3 at a level lower than Tregs (Figure 11G). When compared with Th9 cells, Treg cells secreted very low or undetectable levels of IL-9 assessed by both intracellular staining and ELISA (Figure 11G, H). Therefore among various T helper cells, Th9 cells are the predominant producers of IL-9.

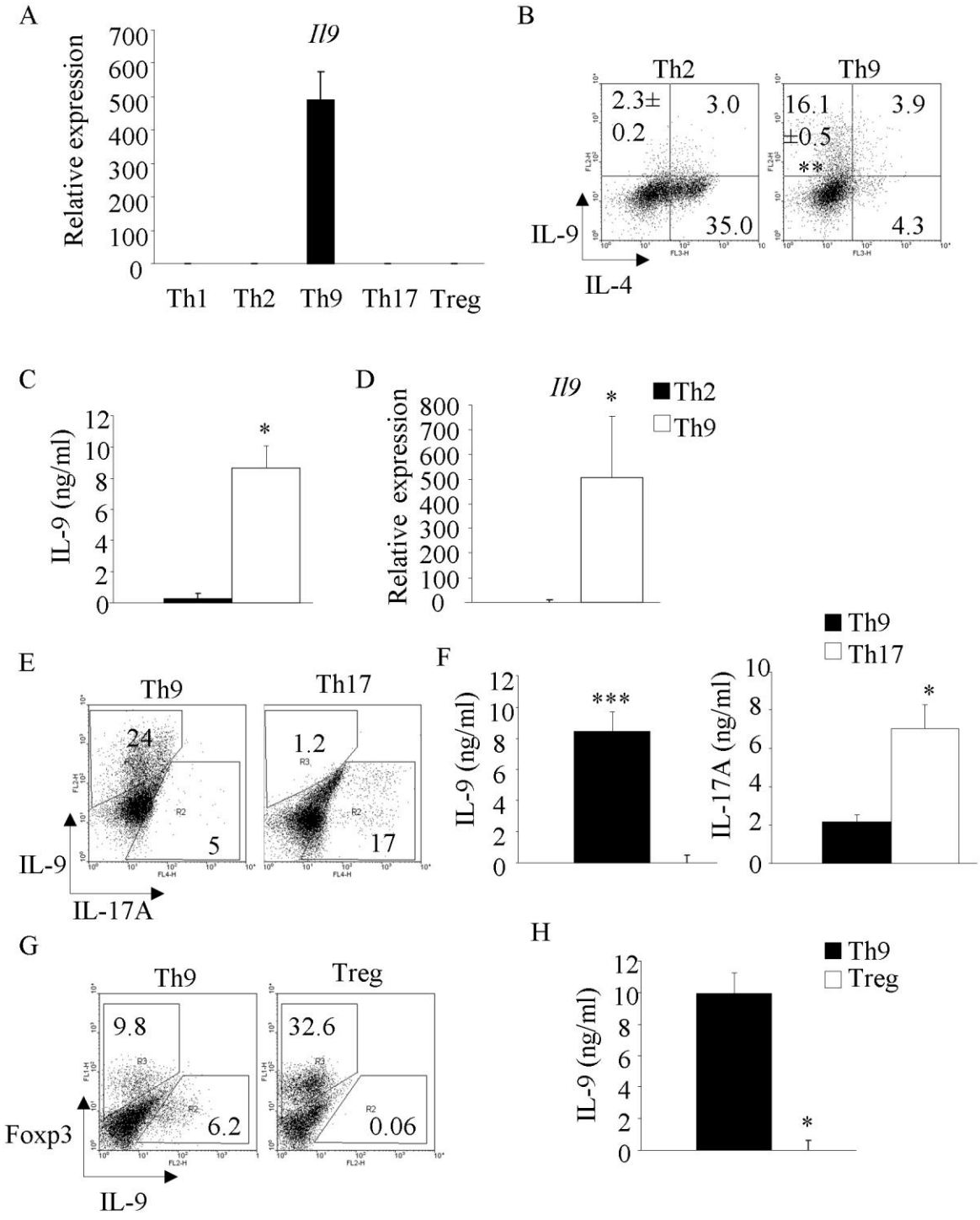

Figure 11: Naïve T cells cultured with IL-4 and TGF-β predominantly produce IL-9. (A) Naïve CD4+ T cells from WT mice were differentiated under Th1 (IL-12+ anti-IL-4), Th2 (IL-4+ anti-IFN-γ), Th9 (IL-4+ TGF-β+ anti-IFN-γ), Th17 (TGF-β+ IL-6+ IL-21+ IL-1β+ anti-IFN-γ+ anti-IL-4), and Treg (TGF-β+ anti-IL-4) cell conditions for 5 days. On day 5, cells were re-stimulated with anti-CD3 for 6 hours to isolate RNA from cell pellet. *Il9* gene expression was assessed by real time PCR. (B-D) WT naïve CD4+ T cells

were differentiated under Th2 and Th9 cell conditions (as in A) for 5 days. On day 5, cells were stimulated with PMA and ionomycin for 5 hours before intracellular staining for IL-9 and IL-4 (B), differentiated cells were also stimulated with anti-CD3 for 24 hours to collect cell-free supernatant to assess IL-9 production by ELISA (C) and for 6 hours to measure *Il9* by real time PCR (D). (E, F) WT naïve CD4+ T cells were differentiated under Th9 and Th17 cell conditions (as in A) for 5 days. On day 5, cells were stimulated with PMA and ionomycin for 5 hours before staining intracellularly with IL-9 and IL-17A (E), the cells were also stimulated with anti-CD3 for 24 hours to measure IL-9 and IL-17A by ELISA (F). (G, H) Naïve CD4+ T cells from WT mice were differentiated under Th9 and Treg cell conditions (as in A). On day 5, cells were harvested and stained with Foxp3 and IL-9 (G), the differentiated cells were also stimulated with anti-CD3 for 24 hours to assess IL-9 by ELISA (H). Data are average ± S.D of 6-8 animals from 3-4 experiments (A-D, F-H) or replicates representative of 3 independent experiments (E). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Th9 cells produce lower levels of Th2 cytokines than Th2 cells

As Th2 and Th9 cells are linked together by the IL-4 requirement for differentiation, we wanted to determine the levels of Th2 cytokines produced by Th9 cells. To test this, naïve CD4+ T cells were differentiated under Th2 and Th9 cell conditions for 5 days. On day 5, polarized cells reactivated with anti-CD3 for 24 hours and IL-4, IL-5, and IL-13 production was assessed using ELISA. Th9 cells secreted significantly lower levels of IL-4, IL5, and IL-13 compared to Th2 cells (Figure 12). Studies have suggested that Th9 cells retain their 'Th2-ness' by producing IL-10 abundantly (Dardalhon et al., 2008; Veldhoen et al., 2008b). Therefore, IL-10 production was compared between Th2 and Th9 cultures in detail. Th2 cells produced significantly higher amount of IL-10 assessed by intracellular cytokine staining and ELISA (Figure 13A, B). *Il10* was also measured at the transcript level when 5 day differentiated Th2 and Th9 cells were reactivated by anti-CD3 at different time points. The peak of *Il10* expression was observed after 6 hours of stimulation. Th2 cells had higher *Il10* expression than Th9 cells for all the time points

measured (Figure 13C). Overall these data show that Th9 cells secrete low levels of IL-4, IL-5, IL-10, and IL-13 while they produce IL-9 abundantly.

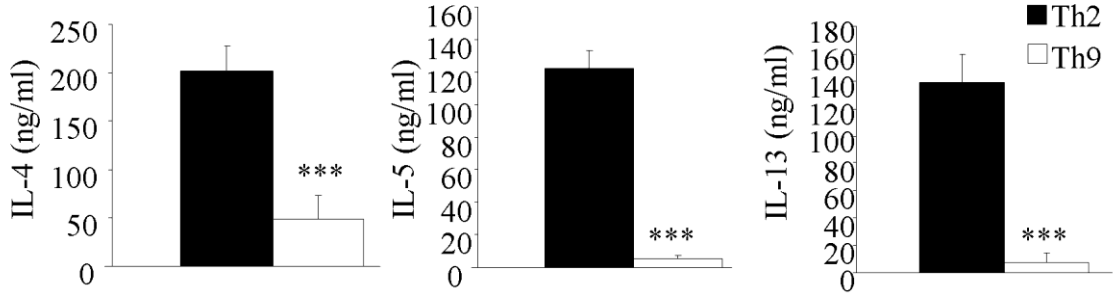

Figure 12: Th9 cells produce lower levels of Th2 cytokines. Naïve CD4+ T cells from WT mice were cultured with Th2 and Th9 cell conditions for 5 days. Differentiated cells were stimulated with anti-CD3 for 24 hours. Cell-free supernatant was used to measure IL-4, IL-5, and IL-13 using ELISA. Data are average ± S.D of 6-8 animals from 3-4 experiments. *** $p<0.001$.

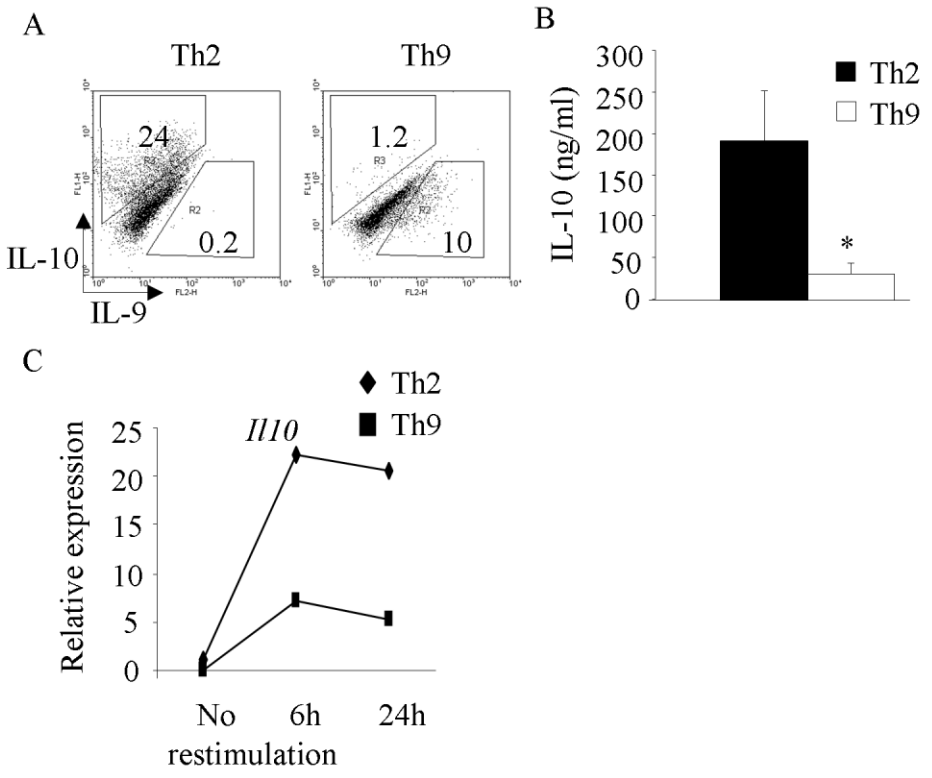

Figure 13: Th9 cells secrete less IL-10 than Th2 cells. (A) WT naïve CD4+ T cells were cultured under Th2 and Th9 cell conditions for 5 days. On day 5, cells were stimulated with PMA and ionomycin for 5 hours before IL-9 and IL-10 intracellular cytokine staining was performed. (B) Differentiated Th2 and Th9 cells were re-stimulated

with anti-CD3 for 24 hours and cell-free supernatant was used to assess IL-10 using ELISA. (C) Differentiated Th2 and Th9 cells were also activated with anti-CD3 for 0, 6, and 24 hours to measure *Il10* expression by real time PCR. Data are replicate representative of 3 independent experiments (A, C) or average ± S.D of 4 animals from 2 independent experiments (B). * $p<0.05$.

IL-9 and IL-10 are not coordinately regulated

We had earlier demonstrated reciprocal regulation of IL-9 and IL-10 in Th2 cells (Ahyi et al., 2009). In a model where Th9 cells were activated by peptide and APCs, the IL-9 and IL-10 expression kinetics differed greatly (Tan et al., 2010). One report has suggested that IL-10 can promote IL-9 production in mouse T cells (Monteyne et al., 1997) while another study has observed that neutralizing IL-10 receptor in primary Th9 cells or Th2 cells 'reprogrammed' to Th9 cells does not negatively regulate *Il9* gene expression (Veldhoen et al., 2008b) . To further determine the regulation of IL-9 and IL-10 in Th9 cells, Th9 cells were treated with neutralizing IL-10 antibody which has been shown to decrease IL-10 production (Riemann et al., 2005). IL-9 production was increased when Th9 cells were treated with anti-IL-10 with a concomitant reduction in IL-10 production (Figure 14A). Neutralizing IL-10 in IL-10^{hi} Th2 cells results in modest increase in *Il9* mRNA (Ahyi et al., 2009). When TGF-β was added to differentiated Th9 cultures during anti-CD3 restimulation it resulted in more IL-9 production (Figure 14B). In contrast, IL-10 production was reduced (Figure 14B). Since Th2 cells express IL-9 receptor (Nowak et al., 2009), we wanted to test if Th2 cells treated with IL-9 altered IL-10 production. The addition of IL-9 to Th2 cells did not alter IL-10 production (Figure 14C). Therefore, even though Th9 cells produce IL-10, IL-9 and IL-10 are not coordinately regulated.

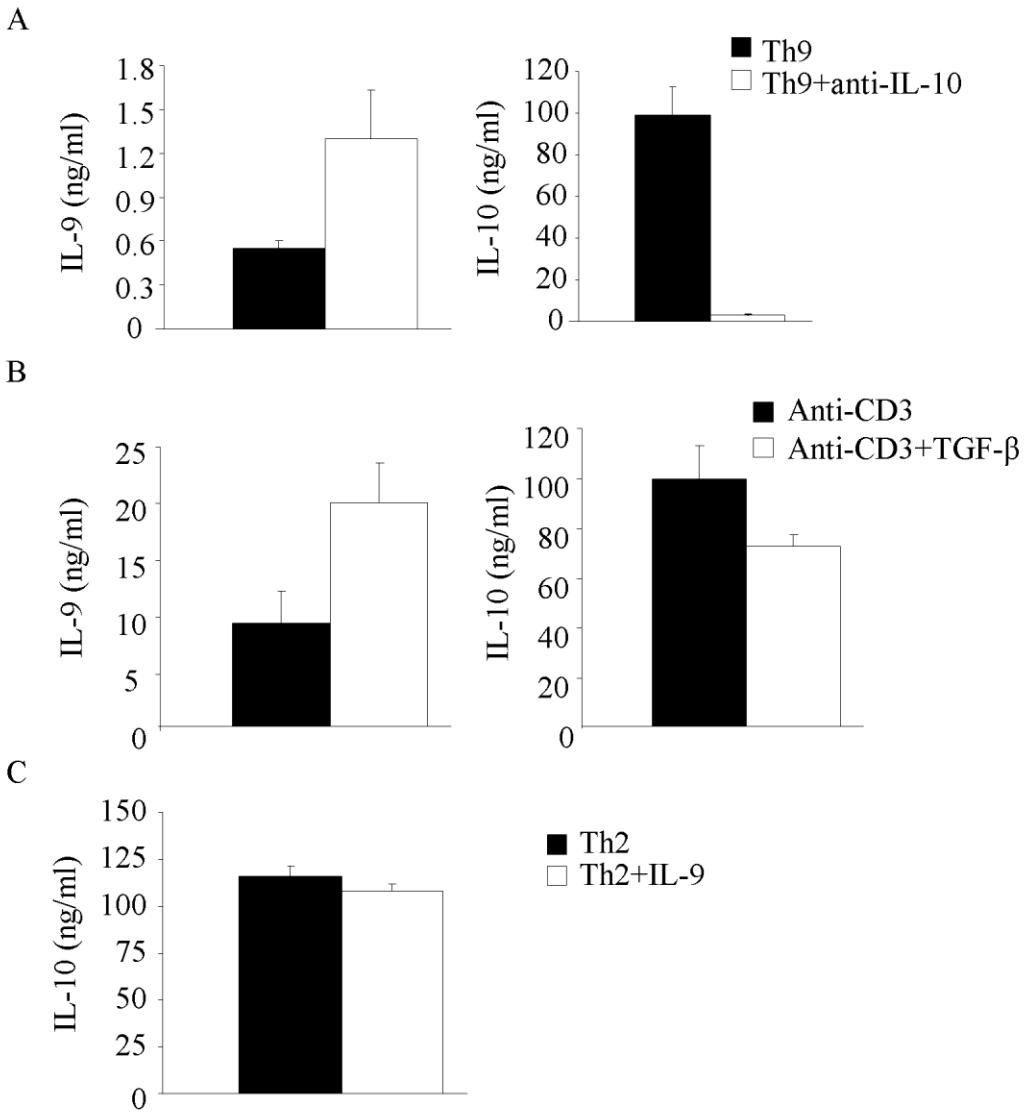

Figure 14: IL-9 and IL-10 are not coordinately regulated in Th9 cells. (A) Naïve CD4+ T cells from WT mice were cultured under Th9 cell conditions in the presence or absence of blocking IL-10 antibody for 5 days. On day 5, cells were re-stimulated with anti-CD3 for 24 hours. Cell-free supernatant was collected to assess IL-9 and IL-10 production using ELISA. (B) WT naïve CD4+ T cells were cultured under Th9 cell conditions for 5 days. On day 5, cells were stimulated with either plate-bound anti-CD3 or plate-bound anti-CD3 and soluble TGF-β (2 ng/ml) for 24 hours. Cell-free supernatant was used to assess IL-9 and IL-10 using ELISA. (C) Naïve CD4+ T cells from WT mice were cultured under Th2 cell conditions in the presence or absence of recombinant IL-9 (10 ng/ml). On day 5, anti-CD3 stimulated cell-free supernatant was used to measure IL-10 using ELISA. Data are replicates representative of 2-3 independent experiments with 2 mice in each experiment.

Foxp3 expression is limited by IL-4 in Th9 cells

IL-4 is required for the differentiation of Th9 cells as Treg cells cultured in the presence of TGF-β and neutralized IL-4, secrete very low amounts of IL-9. To determine the role IL-4 plays in Th9 cells, naïve CD4+ T cells from WT mice were cultured with increasing doses of TGF-β in the presence (Th9) or absence (Tregs) of IL-4. Increasing doses of TGF-β resulted in augmented production of Foxp3+ cells (Figure 15A). However, addition of IL-4 attenuated the production of Foxp3+ cells with a concomitant increase in the production of IL-9 (Figure 15A). A small percentage of dim IL-9+ cells were observed in Treg cells, however no IL-9 was detectable by ELISA from Treg cells (Figure 15A, B). Thus one of the roles of IL-4 in Th9 cultures is to suppress *Foxp3* expression.

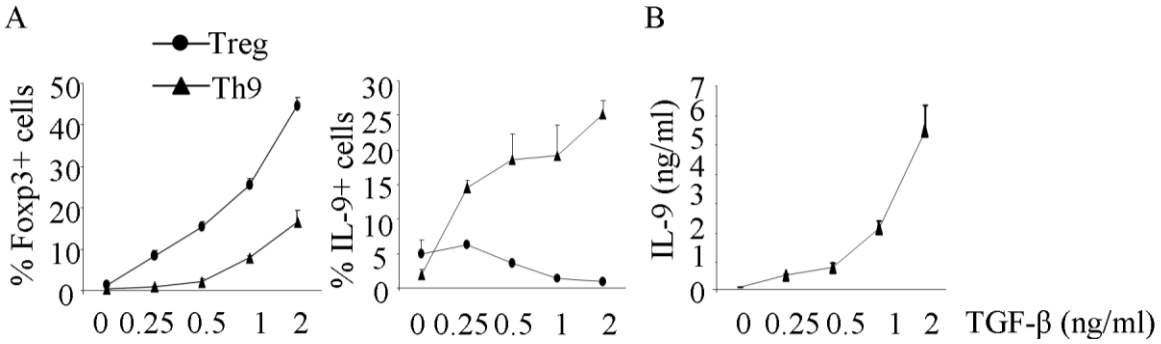

Figure 15: IL-4 limits *Foxp3* expression from Th9 cells. (A) WT naïve CD4+ T cells were cultured with increasing doses of TGF-β in the presence (Th9) or absence (Treg) of IL-4 for 5 days. On day 5, cells were then intracellularly stained for Foxp3 (left panel) and IL-9 (right panel). (B) Differentiated cells from (A) were stimulated with anti-CD3 for 24 hours. IL-9 production was measured using ELISA. IL-9 was not detected in supernatants of Treg cultures. Data are replicates representative of 2-3 independent experiments with 2 mice in each experiment.

IL-13 cannot substitute for IL-4 in the induction of IL-9-producing T cells

IL-4 shares some overlapping functions with IL-13 (Wynn, 2003). IL-4 signals via either type I IL-4R (IL-4Rα and the common γ chain) or the type II IL-4R (IL-4Rα and IL-13Rα1). IL-13 receptor is composed of IL-13Rα1 and IL-4Rα, with IL-13Rα2 being a negative regulatory receptor. Most T cells do not express *Il13Rα1*, although reports have shown receptor expression is increased in Th17 cells (Newcomb et al., 2009). Since IL-4 and IL-13 share similar signaling cascades, we wanted to determine if IL-13 could replace IL-4 and secrete IL-9 in the presence of TGF-β. Naïve CD4+ T cells were treated with TGF-β and either a fixed amount of IL-4 or increasing doses of IL-13 in the absence of IL-4. T cells cultured in the presence of IL-4 and TGF-β produced IL-9 (Figure 16A). In contrast, T cells differentiated with TGF-β and IL-13 did not produce IL-9 (Figure 16A). We wanted to determine if the lack of IL-9 production was due to the lack of *Il13Rα1* expression. To this end naïve CD4+ T cells were cultured with TGF-β and IL-13 and *Il13Rα1* expression was assessed by real time PCR. T cells treated with TGF-β and IL-13 did not express *Il13Rα1*, consistent with the result that IL-13 failed to induce IL-9 in the presence of TGF-β (Figure 16B). Since IL-4 attenuated TGF-β-mediated Foxp3 induction we wanted to determine if IL-13 has a similar effect on Foxp3. In contrast to IL-4, IL-13 failed to diminish the TGF-β-mediated Foxp3-producing T cells (Figure 16C). Thus, although IL-4 and IL-13 have some redundant functions and share signaling pathways, IL-4 but not IL-13 promotes the differentiation of Th9 cells.

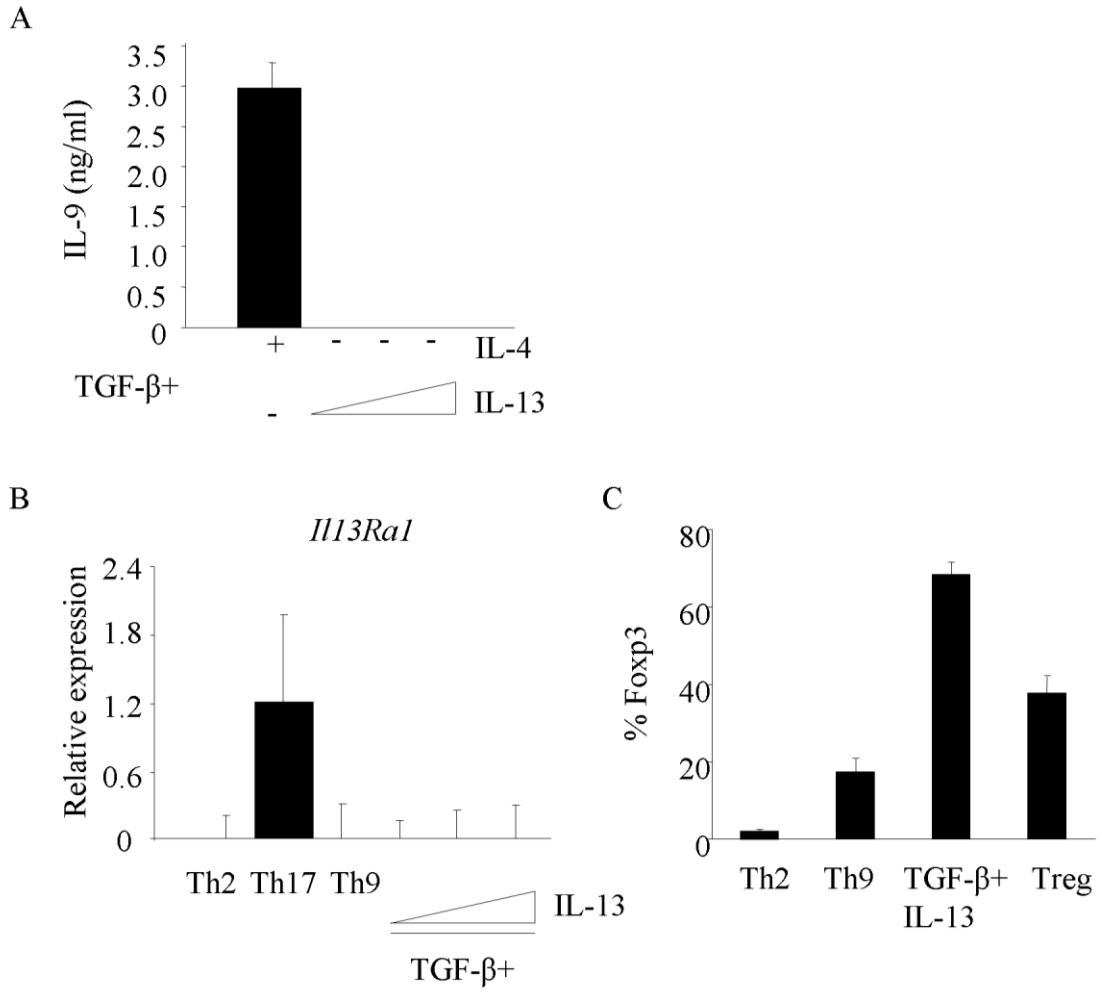

Figure 16: IL-13 cannot substitute for IL-4 to induce IL-9-secreting cells. (A) Naïve CD4+ T cells from WT mice were cultured with TGF-β and IL-4 or with TGF-β and increasing doses of IL-13 (5, 10, 20 ng/ml) for 5 days. Cell-free supernatant was used to assess IL-9 using ELISA. (B) Naïve CD4+ T cells were differentiated as in (A), and under Th2 and Th17 culture conditions for 5 days. *Il13Rα1* expression was measured by real time PCR. (C) Naïve CD4+ T cells were cultured under Th2, Treg, Th9 culture conditions, and with TGF-β in the presence or absence of IL-13 for 5 days. Differentiated cells were then intracellularly stained for Foxp3. Data are average ± S.D of 4 mice. Experiments were done in collaboration with Jared Travers.

IL-23 negatively regulates IL-9 production from Th9 cells

IL-9 had been shown to synergize with TGF-β to polarize naïve T cells into IL-17-secreting cells (Elyaman et al., 2009). IL-23 which is required for the maintenance of

Th17 cells had been reported to inhibit IL-9 production from Th17 cells; conversely IL-9 production from IL-23R-deficient Th17 cells was elevated (Elyaman et al., 2009). We therefore wanted to define the role of IL-23 in Th9 cells in regulating IL-9 production. Th9 cells cultured in the presence of IL-23 resulted in diminished the percentages of IL-9-producing cells (Figure 17A). This was confirmed using ELISA as well (Figure 17B). However, IL-23 did not have a potent effect on the production of IL-17-secreting cells (Figure 17A). Whether Th9 cells treated with IL-23 results in increased IL-10 production is not known although Th17 cells incubated with IL-23 do not express IL-10 (McGeachy et al., 2007). Thus, the data suggests that IL-23 attenuates IL-9 production from Th9 cells.

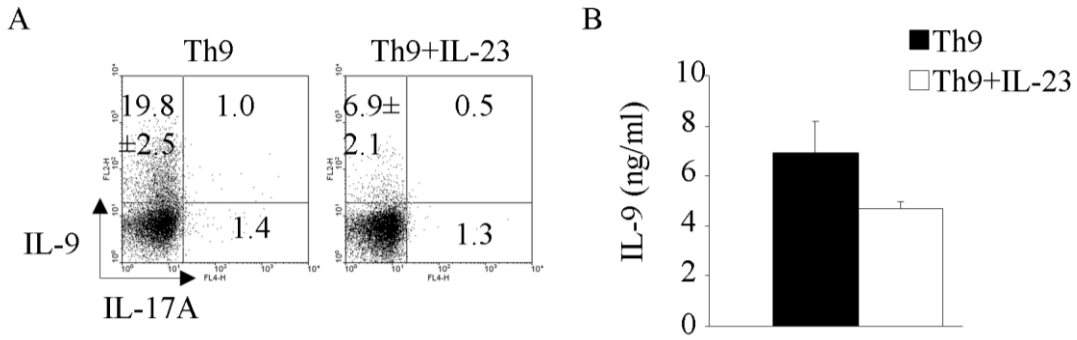

Figure 17: IL-23 attenuates IL-9 production from Th9 cells. (A) WT naïve CD4+ T cells were differentiated under Th9 cell conditions for 5 days in the presence or absence of recombinant IL-23 (10 ng/ml). On day 5 cells, were stimulated with PMA and ionomycin before intracellular staining for IL-9 and IL-17A. (B) IL-9 production was assessed from anti-CD3 stimulated cells using ELISA. Data are average of two mice.

Type I and type II interferons inhibit IL-9 production from Th9 cells

In murine studies, IFN-γ reduces IL-4 and TGF-β-mediated IL-9 production (Schmitt et al., 1994). However, in human Th9 cells, type I interferons, IFN-α and IFN-β augment IL-9 production (Wong et al., 2010). Therefore we wanted to define the role of type I and

type II interferons in murine Th9 cell differentiation. Therefore to define the role of type I and type II interferons in Th9 cell development, WT Th9 cells were cultured in the presence of IFN-α, IFN-β, IFN-γ, and also IL-12 which promotes Th1 differentiation. Th9 cells cultured with IFN-γ reduced IL-9 production consistent with the published report (Figure 18A, B). Even though there was modest reduction in the percentages of IL-9-secreting cells when Th9 cells were treated with IFN-α and IFN-β, the decrease in IL-9 production by type I interferons was more profound when assessed by ELISA (Figure 18A, B). Though IL-12 reduced IL-9 production from Th9 cells, the effect was not as profound as from IFN-β or IFN-γ (Figure 18A, B). As IFN-γ activates STAT1, we wanted to determine the role of STAT1 in interferon-mediated IL-9 regulation. STAT1-deficiency did not affect IL-9 production when Th9 cells were treated with type I interferons or IL-12 (Figure 18A, B). However, STAT1 is required for IFN-γ-mediated IL-9 suppression in Th9 cells (Figure 18A, B).

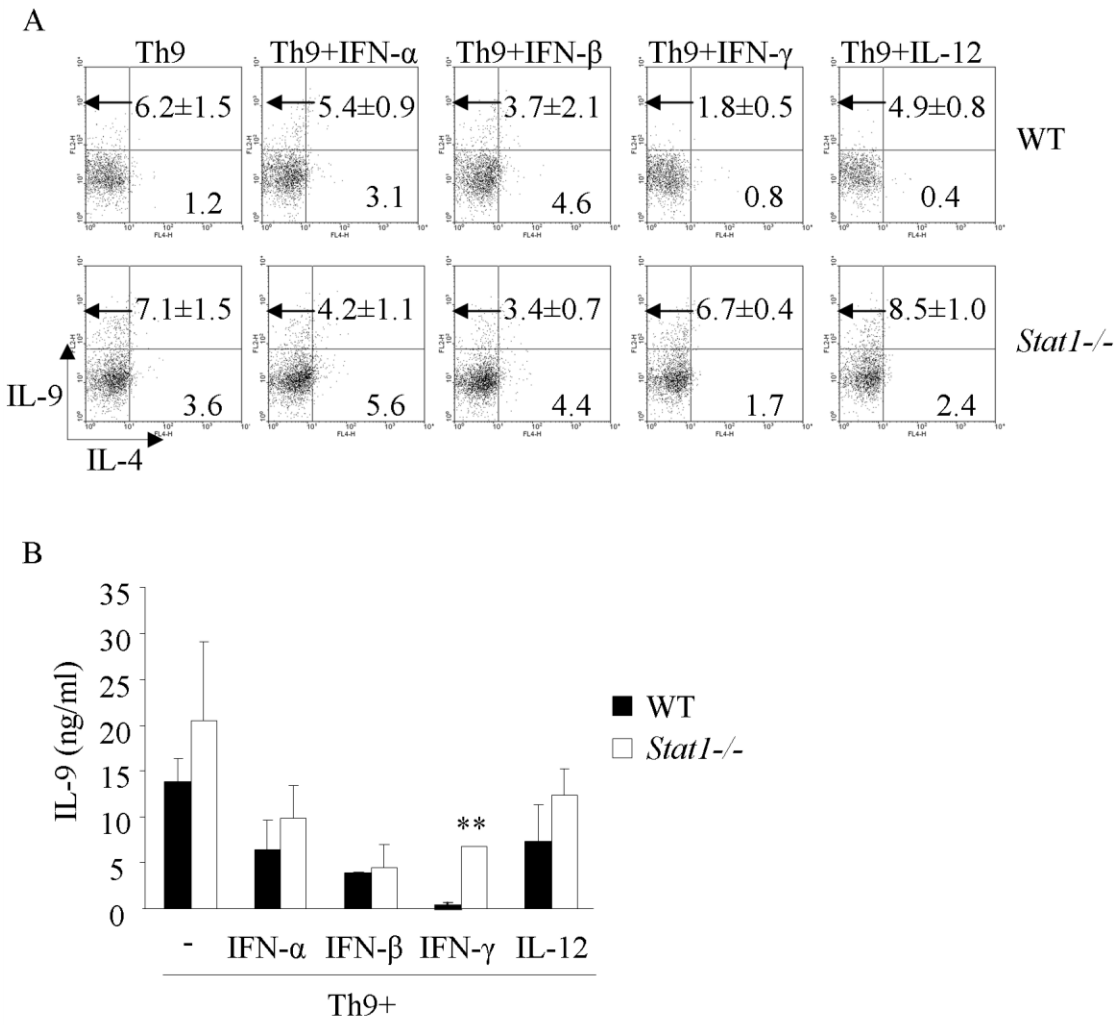

Figure 18: Type I and type II interferons inhibit IL-9 production. (A) Naïve CD4+ T cells from WT and *Stat1-/-* mice were cultured under Th9 cell conditions in the presence of IFN-α (10,000 U/ml), or IFN-β (10,000 U/ml), or IFN-γ (100 ng/ml), or IL-12 (5 ng/ml) for 5 days. On day 5, cells were harvested and re-stimulated with PMA and ionomycin before intracellular staining with IL-9 and IL-4. (B) Cell-free supernatant was used from anti-CD3 stimulated cultures (as in A) to measure IL-9 using ELISA. Data are average ± S.D of 4 mice. ** $p<0.01$

As IL-12 activates STAT4, we wanted to define the role of STAT4 in interferon-mediated regulation of IL-9 in Th9 cells. To test this, Th9 cells from WT and STAT4-deficient mice were treated with IFN-α, IFN-β, IFN-γ, and IL-12. As demonstrated earlier Th9 cells treated with either type I or type II interferons reduced IL-9 production, with

IFN-γ having the most profound effect (Figure 19A, B). However, STAT4 was not required for regulation of IL-9 production by interferons (Figure 19A, B). STAT4 deficiency did not significantly affect IL-9 production under standard Th9 conditions or when Th9 cells were treated with IL-12 (Figure 19A, B). Overall, these data suggest that both type I and type II interferons but not IL-12 negatively regulate IL-9 production.

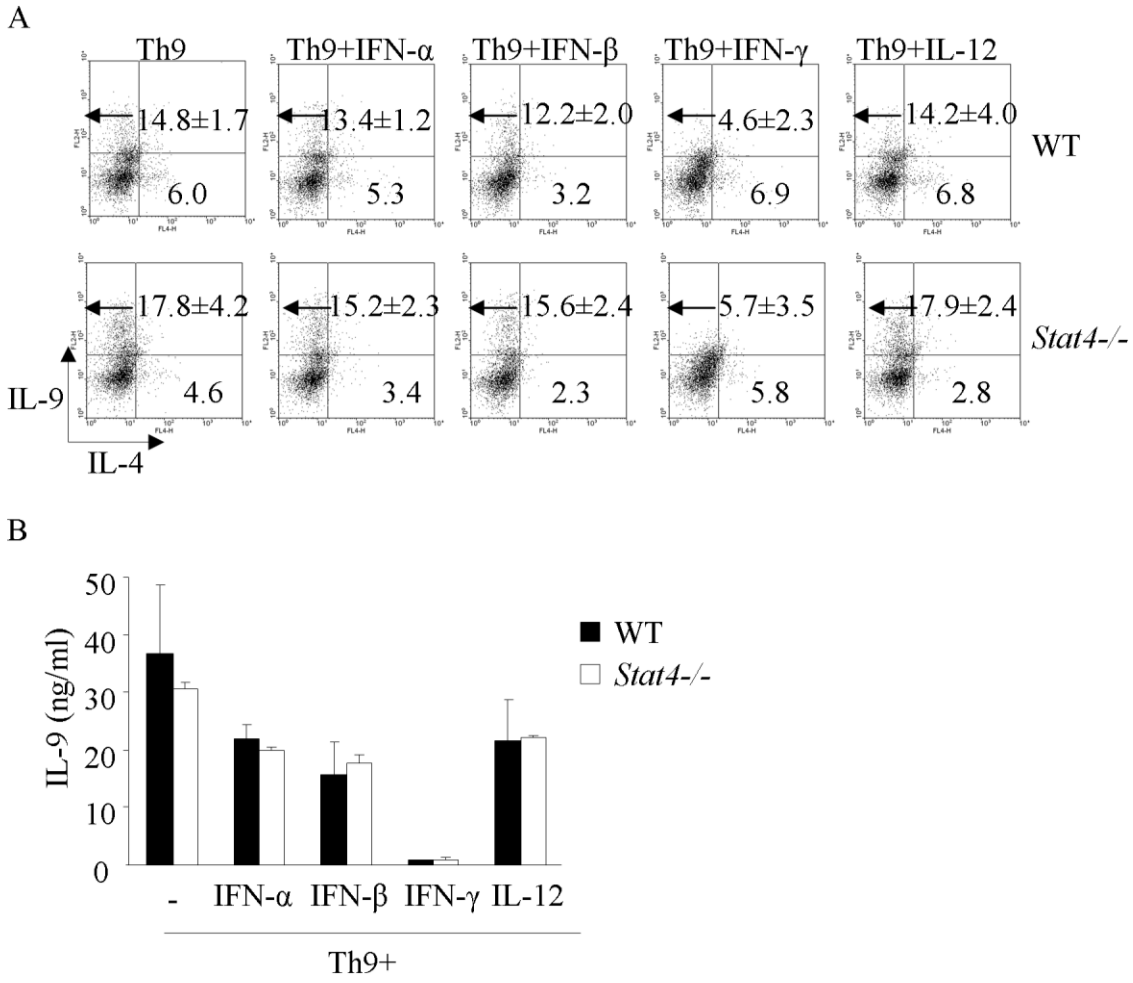

Figure 19: STAT4 signal is not required for IL-9 inhibition by interferons. (A) Naïve CD4+ T cells from WT and *Stat4-/-* mice were cultured under Th9 cell conditions in the presence of IFN-α, or IFN-β, or IFN-γ, or IL-12 for 5 days as in Figure 18. On day 5, cells were re-stimulated with PMA and ionomycin before intracellular staining with IL-9 and IL-4. (B) IL-9 production was assessed using ELISA from stimulated cells. Data are average ± S.D of 4 mice.

Cytokines regulating IL-9 production from Th9 cells: Summary

Our findings on the effects of cytokines on IL-9 production are summarized below.

Cytokine	IL-9 Regulation
IL-12	No effect
IL-23	Negative
IFN-α	Negative
IFN-β	Negative
IFN-γ	Negative
IL-10	Negative
IL-9	No effect?
TGF-β	Positive

Table 3: Summary of IL-9 production from Th9 cells by different cytokines

PART II: A STAT6-dependent transcriptional network in Th9 cells

STAT6 and STAT3 are activated in Th9 cells

STAT6 is required for the development of Th2 cells (Kaplan et al., 1996a; Shimoda et al., 1996; Takeda et al., 1996). However, recently STAT3 has been demonstrated to cooperate with STAT6 in promoting Th2 cell development (Stritesky et al., 2011). Both STAT6 and STAT3 molecules were activated during Th2 differentiation (Stritesky et al., 2011). We therefore wanted to determine if both STAT6 and STAT3 were activated during Th9 cell differentiation. To define STAT6 and STAT3 activation during Th9 differentiation, naïve CD4+ T cells from WT mice were polarized under Th2 and Th9 cell conditions for 5 days and were assessed for intracellular phospho-STAT6 and phospho-STAT3 each day during differentiation. Phospho-STAT6 was identically activated between Th2 and Th9 cells (Figure 20A, B). The mean fluorescence intensity of phospho-STAT6 positive cells was similar between Th2 and Th9 cells (Figure 20A, B). The peak of STAT6 activation was observed on day 2 of differentiation with a second peak on day 4 after cell expansion (Figure 20A, B). STAT3 was also phosphorylated in Th9 cells albeit with lower percentages and mean fluorescence intensity of phospho-STAT3-positive cells than Th2 cells (Figure 20A, B). However, both Th2 and Th9 cells have similar kinetics of phosphorylated STAT3 (Figure 20 A, B).Together, these data suggest that similar to Th2 cells both STAT6 and STAT3 are activated in Th9 cells.

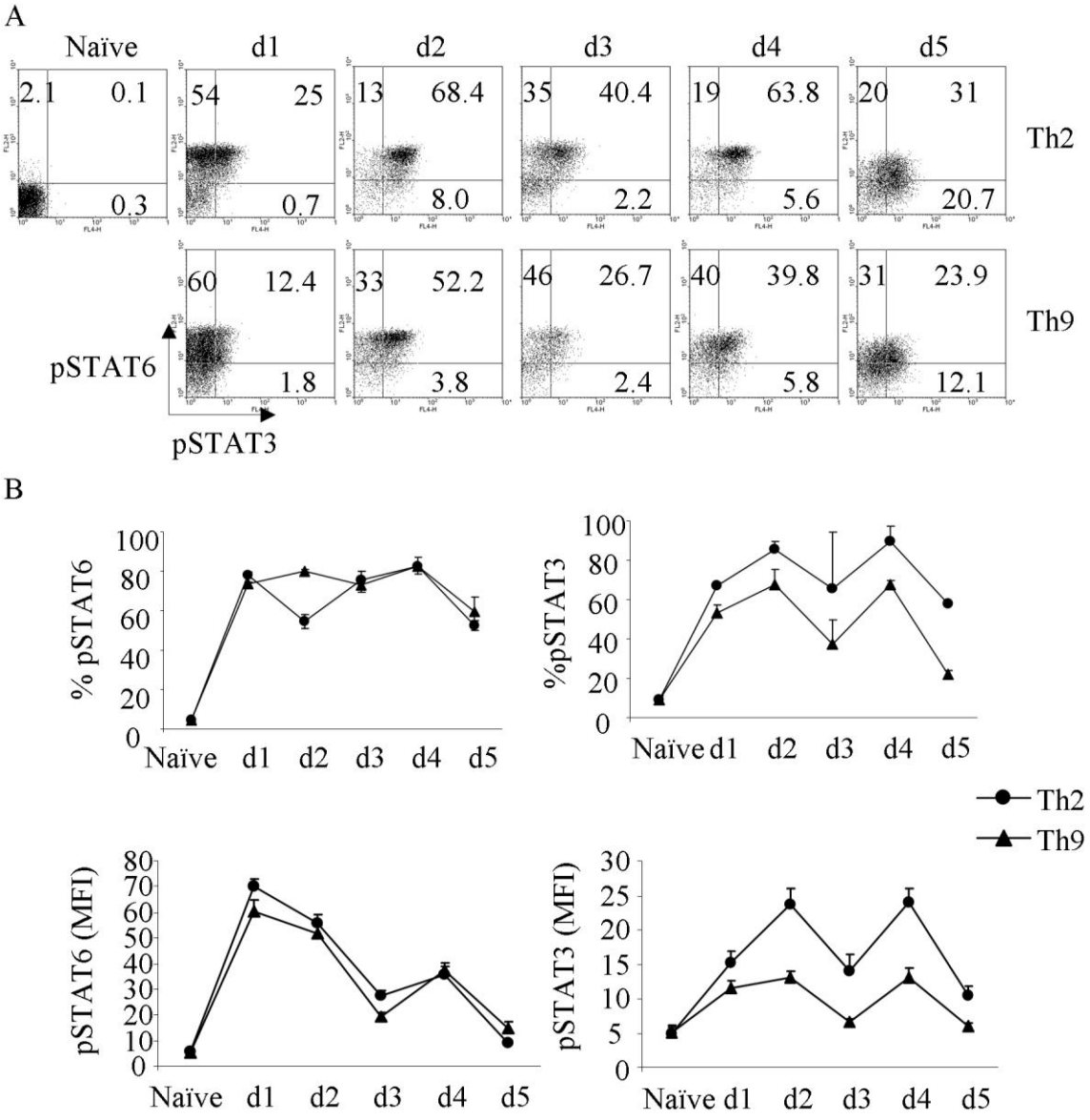

Figure 20: STAT6 and STAT3 are activated in Th9 cells. (A) Naïve CD4+ T cells from WT mice were cultured under Th2 and Th9 cell conditions for 5 days. Each day during differentiation cells were stained for intracellular phospho-STAT6 and phospho-STAT3. (B) Graphical representation of the percentages (top panel) and mean fluorescence intensity (MFI) (bottom panel) of the cells. Data are average ± S.D of 6 mice.

Differential requirement of STAT6 and STAT3 in Th2 and Th9 cells

As both STAT6 and STAT3 were activated during Th9 cell differentiation we then wanted to define the requirement of each of these molecules in the development of Th9 cells. To determine the role of STAT6, naïve CD4+ T cells were isolated from WT and STAT6-deficient mice and were cultured under Th9 cell conditions. IL-9 production was completely abolished in the absence of STAT6 assessed by intracellular cytokine staining and ELISA, which was consistent with previous reports (Dardalhon et al., 2008; Veldhoen et al., 2008b)(Figure 21A, B). This data demonstrates that the STAT6 signaling pathway is required for Th9 cell development. To define the role of STAT3 in Th9 development, naïve CD4+ T cells from WT and *Stat3*$^{CD4-/-}$ mice were cultured under Th9 cell conditions and IL-9 production was assessed by intracellular cytokine staining and ELISA. Even though STAT3 was activated throughout Th9 differentiation, STAT3 was dispensable for the development of IL-9-secreting T cells (Figure 21C, D).

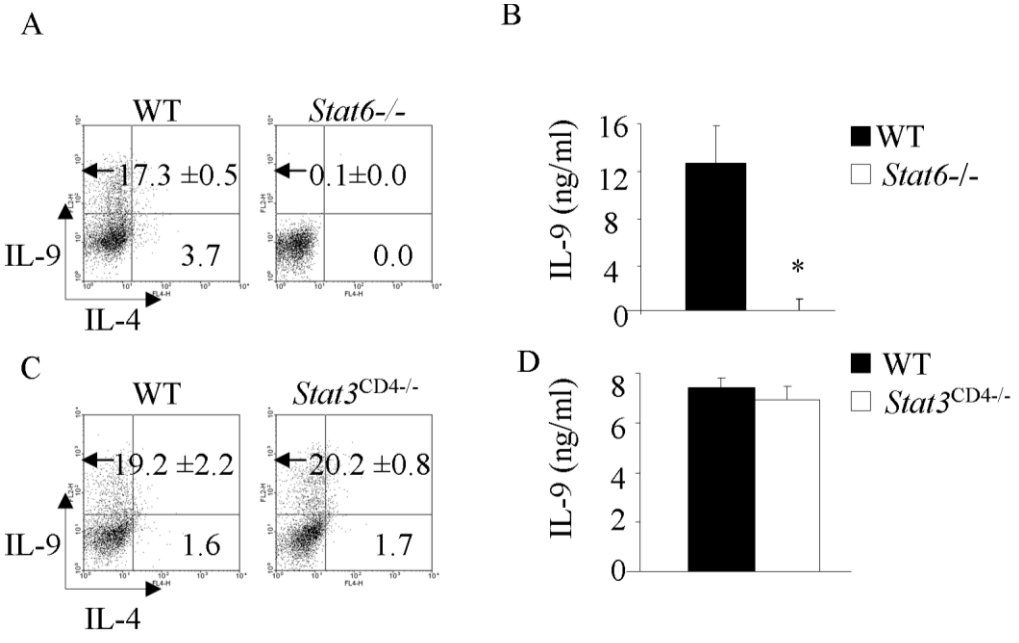

Figure 21: STAT6 but not STAT3 is required for Th9 cell development. (A, B) Naïve CD4+ T cells from WT and *Stat6-/-* mice were cultured under Th9 cell conditions for 5

days. On day 5, cells were harvested and stimulated with PMA and ionomycin before intracellular staining with IL-9 and IL-4 (A) or were stimulated with anti-CD3 for 24 hours to assess IL-9 production using ELISA (B). (C, D) Naïve CD4+ T cells from WT and *Stat3*$^{CD4-/-}$ mice were cultured under Th9 conditions for 5 days. Intracellular staining for IL-9 and IL-4 (C) and IL-9 production using ELISA (D) was measured as in (A) and (B). Data are average ± S.D of 6 mice from 3 independent experiments. * $p<0.05$.

Since STAT6 is critical for the development of Th9 cells we then wanted to determine the role of STAT6 co-factors in Th9 cell development. STAT6 recruits various co-activators at the IgE promoter in B cells. The STAT6 co-activator PARP14, a poly-ADP ribosyltransferase augments and acts as a switch in STAT6-dependent transcription by facilitating the clearance of repressive molecules from gene promoters and increasing binding of STAT6 (Goenka and Boothby, 2006; Mehrotra et al., 2011). To define the role of PARP14 in the development of Th9 cells we compared IL-9 production from WT and *Parp14-/-* Th9 cells. Consistent with the role of PARP14 as a co-activator, diminished production of IL-9 was observed in the absence of PARP14 (Figure 22). However, the production of IL-10 was not altered in the absence of PARP14 (Figure 22). Together these data suggest that STAT6 and its co-factor PARP14 are required for the optimal development of Th9 cells.

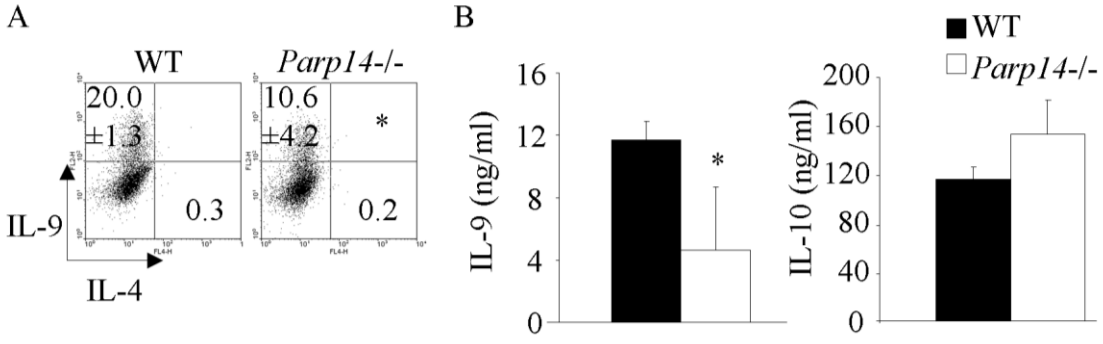

Figure 22: Absence of STAT6 co-activator PARP14 results in diminished IL-9 production. (A, B) Naïve CD4+ T cells from WT and *Parp14-/-* mice were cultured

under Th9 cell conditions for 5 days. Differentiated cells were stimulated with PMA and ionomycin before intracellular staining with IL-9 and IL-4 (A) or were stimulated with anti-CD3 for 24 hours to assess IL-9 and IL-10 production using ELISA (B). Data are average ± S.D of 4-5 mice from 2-3 independent experiments. * $p<0.05$.

The Il9 promoter is not directly targeted by STAT6

Since STAT6 is indispensable for the development of Th9 cells, we wanted to examine if STAT6 was directly regulating the *Il9* gene. The *Il9* locus was compared among different species. The evolutionary conserved region (ECR) browser was used to scan the *Il9* gene which showed that the mouse *Il9* gene consisted of 5 exons (Figure 23A). rVISTA and JASPAR software was used to predict transcription factor binding sites. Two conserved non-coding sequences, CNS1 and CNS0 were predicted to be multiple transcription factor binding sites of the *Il9* gene (Perumal and Kaplan, 2011). CNS1 is located at the *Il9* promoter while CNS0 is about 7 kb upstream of the transcription start site (Figure 23A). STAT binding sites were predicted at the CNS1 and CNS0 regions of the *Il9* gene. To determine if STAT6 was regulating IL-9 production by directly binding to the *Il9* gene, chromatin immunoprecipitation was performed testing the association of STAT6 to CNS1 and CNS0 of the *Il9* gene in Th2 and Th9 cells. STAT6 binding was detectable at the CNS1, more than the CNS0 (Figure 23B). However, the percent input values were about 2% of the binding of STAT6 to the *Maf* and *Irf4* promoters in Th2 and Th9 cells (Figure 23B). This was consistent with a previous study demonstrating that STAT6 binding was undetectable in the *Il9* locus in Th2 cells by analyzing published ChIP-Seq datasets (Wei et al., 2010). Overall, these data suggest that STAT6 is not directly targeting the *Il9* promoter; however, it may be affecting the binding of other factors to the *Il9* gene, or binding to regulatory elements that are not yet identified.

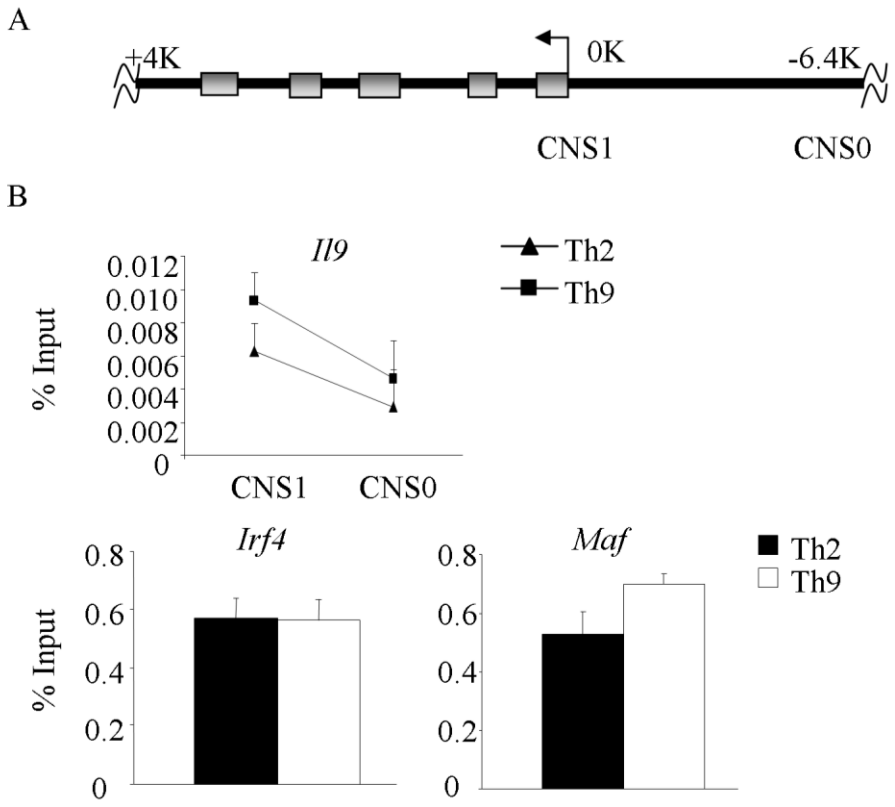

Figure 23: *Il9* promoter is not a direct target of STAT6. (A) Schematic diagram of *Il9* gene with conserved non-coding sequences (CNS). (B) WT naïve CD4+ T cells were cultured under Th2 and Th9 cell conditions for 5 days. Chromatin immunoprecipitation (ChIP) was performed for STAT6 before qPCR for two *Il9* CNS sites, or for the *Irf4* or *Maf* promoters, to determine the amount of Stat6 binding. We performed control Ig ChIPs for all ChIP assays and subtracted the control Ig values from the specific Ab values. All ChIP data in the thesis indicate specific % input. Data are average ± S.D of four mice from two experiments.

IRF4 is a relevant transcription factor downstream of the IL-4/STAT6 pathway

The transcription factor IRF4 has been shown to bind and transactivate *Il9* gene (Staudt et al., 2010). The absence of IRF4 leads to diminished IL-9 production from Th9 cells (Staudt et al., 2010). IRF4 is downstream of IL-4/STAT6 pathway in Th2 cells. Therefore we wanted to determine if IRF4 was dependent on the IL-4/STAT6 pathway in Th9 cells. To test this, Th9 cells from WT and *Stat6-/-* mice were assessed for *Irf4* expression. In the absence of the STAT6 signal there was decreased *Irf4* expression in Th9 cells (Figure

24A). We then wanted to examine if STAT6 deficiency affects IRF4 binding to the *Il9* gene. Association of IRF4 to the *Il9* CNS1 and CNS0 regions was attenuated in *Stat6-/-* Th9 cultures (Figure 24B). These data suggest that IRF4 is a potentially important transcription factor downstream of IL-4/STAT6 signal in Th9 cells.

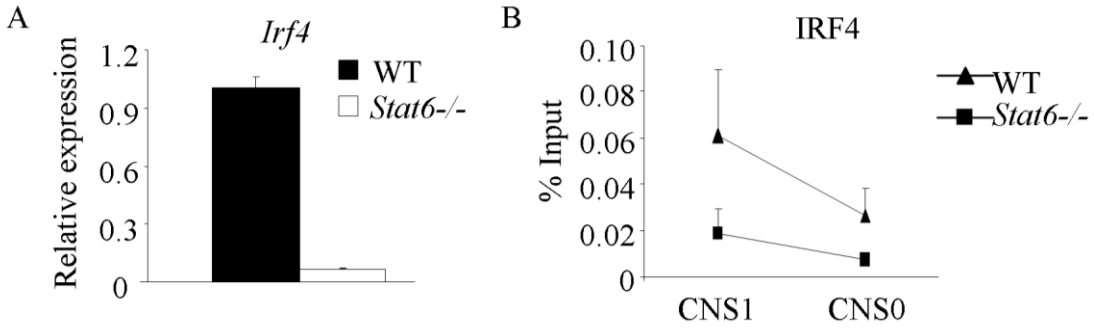

Figure 24: IRF4 is a relevant transcription factor downstream of IL-4/STAT6 pathway. (A, B) Naïve CD4+ T cells isolated from WT and *Stat6-/-* mice were cultured under Th9 cell conditions for 5 days. From differentiated cells *Irf4* expression by real time PCR (A) and IRF4 binding to the *Il9* gene (B) by ChIP assay was performed. Data are average ± S.D of 4 mice from two experiments.

IRF4 alone cannot restore IL-9 production in the absence of STAT6

Since *Irf4* expression was diminished in STAT6-deficient Th9 cells, we wanted to determine if ectopic expression of IRF4 was sufficient to rescue IL-9 production. To test this, naïve CD4+ T cells from WT and *Stat6-/-* mice were activated under Th9 cell conditions and were ectopically expressed with control (hCD4) or IRF4-expressing (IRF4-hCD4) retroviruses. After 5 days of differentiation, cells were magnetically sorted based on hCD4-expression and IL-9 production was assessed using ELISA and real time PCR after anti-CD3 restimulation. WT Th9 cells transduced with IRF4-expressing retrovirus showed an increase in IL-9 production both at the protein and at the transcript level (Figure 25A, B). However, ectopic IRF4 expression was not able to induce IL-9 in

Stat6-/- Th9 cells (Figure 25A, B). To define the mechanism of the inability of IRF4 to rescue IL-9 in the absence of STAT6 signal we wanted to determine if ectopic IRF4 expression altered the expression of *Foxp3* which is suppressed by IL-4 signal. Ectopic IRF4 expression in *Stat6-/-* Th9 cells did not alter Foxp3 production (Figure 25C). In the absence of STAT6, T-bet-deficient CD4+ T cells have the ability to produce IFN-γ (Yang et al., 2008c). Therefore we determined the level of T-bet after ectopic IRF4 expression in *Stat6-/-* Th9 cells. Ectopic IRF4 expression modestly affected T-bet production (Figure 25C). These data suggest that IRF4 alone is not sufficient to recover IL-9 production in the absence of STAT6 signal and additional factors are required for the development of IL-9-secreting T cells.

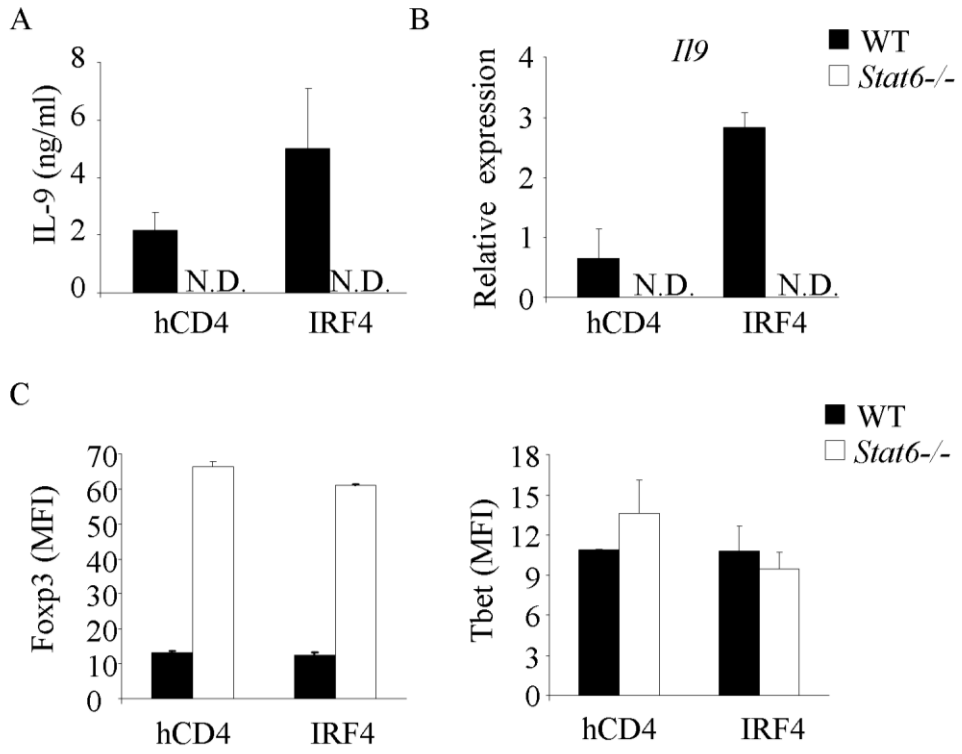

Figure 25: IRF4 is not sufficient to recover IL-9 production in the absence of STAT6. (A, B) Naïve CD4+ T cells from WT and *Stat6-/-* mice were cultured under Th9 cell conditions. On day 2, cells were transduced with control (hCD4) or IRF4-expressing (IRF4-hCD4) retroviruses. On day 5, cells were collected and magnetically sorted for

hCD4+ population. Sorted cells were re-stimulated with anti-CD3 for 24 hours (A) or 6 hours (B) to assess IL-9 production using ELISA and real time PCR, respectively. (C) Naïve cells were cultured and transduced as in (A). Cells were harvested on day 5 and stained intracellularly for Foxp3 and T-bet, based on hCD4+ population. Data are average ± S.D of 4 mice from two experiments. N.D.: Not detected.

IL-4R and ST2 are expressed similarly in Th2 and Th9 cells

IL-4R is expressed on Th2 cells and the transmembrane form of T1/ST2 is a specific marker of murine Th2 cells that is part of the IL-33 receptor (Lohning et al., 1998; Schmitz et al., 2005; Xu et al., 1998a). To determine if these factors are also expressed in Th9 cells, WT naïve CD4+ T cells were differentiated into Th2 and Th9 cell conditions. IL-4R and ST2 expression was compared between Th2 and Th9 cells on each day during 5 day differentiation. The percentages and the mean fluorescence intensity of IL-4R-expressing cells were similar between Th2 and Th9 cells (Figure 26A, B). IL-4R expression reached the peak on day 4 after the cells were expanded (Figure 26A, B). The percentages and the mean fluorescence intensity of ST2-expressing cells were also similar between Th2 and Th9 cells (Figure 26A, B). However, unlike IL-4R expression the peak of ST2 expression was on days 2 and 3 after which the levels drop (Figure 26A, B). Together, these data indicate no subset-specific difference in IL-4R and ST2 expression between Th2 and Th9 cells.

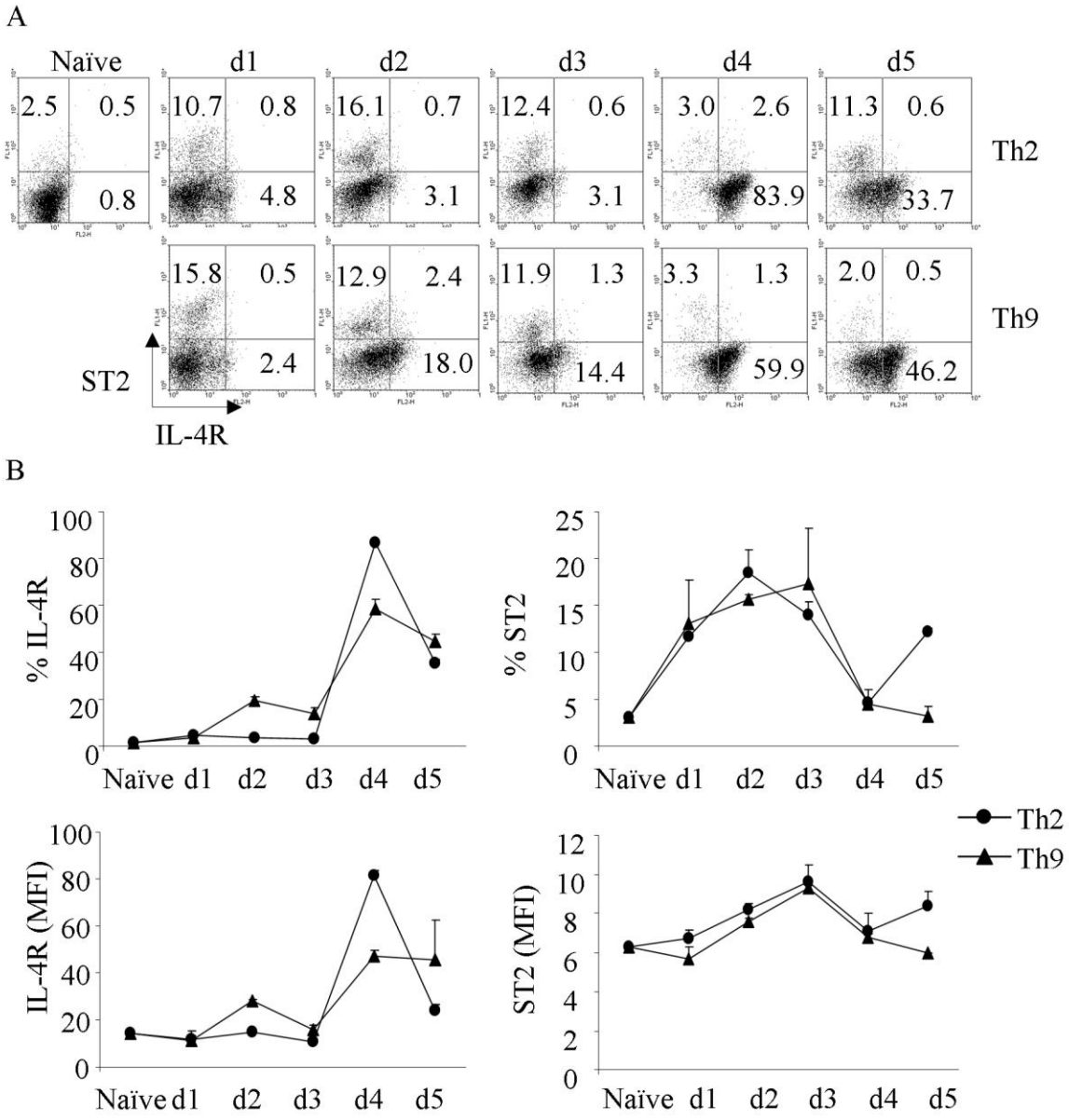

Figure 26: IL-4R and ST2 are expressed similarly in Th2 and Th9 cells. (A) Naïve CD4+ T cells from WT mice were cultured under Th2 and Th9 cell conditions for 5 days. Each day during differentiation cells were stained for IL-4R and ST2. (B) Graphical representation of the percentages (top panel) and mean fluorescence intensity (bottom panel) of the cells. Data are average ± S.D of 4 mice.

The transcription factors Gfi-1 and Bcl6 are expressed at lower levels in Th9 cells

We next wanted to determine if additional transcription factors that regulate Th2 cells are also expressed in Th9 cells. The transcription factor Gfi-1 is important for IL-2 mediated

Th2 cell expansion (Zhu et al., 2006a). Bcl6, a transcriptional repressor, limits Th2 development and inflammation (Dent et al., 1998). To test expression in Th9 cells, naïve CD4+ T cells were cultured under T helper cell polarizing conditions. *Gfi-1* expression was lower in Th9 cells compared to Th2 cells (Figure 27). Among Th1, Th2, Th9 and Th17 cells, Th17 cells had the highest expression of *Bcl6*, consistent with a previously published report (Mondal et al., 2010)(Figure 27). *Bcl6* expression was not different between Th2 and Th9 cells, and was expressed at a lower level than in Th17 cells (Figure 27).

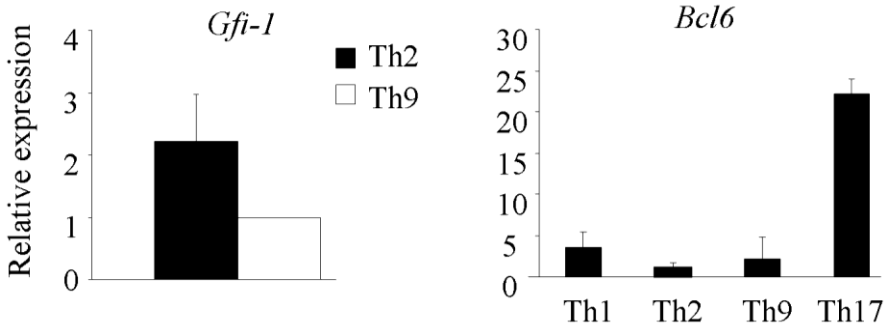

Figure 27: The transcription factors Gfi-1 and Bcl6 are expressed at lower levels in Th9 cells compared to other T helper subsets. WT naïve CD4+ T cells were cultured under Th2 and Th9 (left) or Th1, Th2, Th9, and Th17 (right) culture conditions for 5 days. RNA was isolated from cell pellet and was used to measure *Gfi-1* and *Bcl6* expression by real time PCR. Data are replicates representative of 2-3 independent experiments with 2 mice in each experiment.

Th2-specific transcription factors are expressed in Th9 cells

Since STAT6 was required for the development of both Th2 and Th9 cells, we wanted to determine if the expression of transcription factors downstream of STAT6 signaling promote Th9 cell development. GATA3 was demonstrated to be critical for the development of Th9 cells; however GATA3 was expressed at a lower level in Th9 cells

than Th2 cells (Dardalhon et al., 2008). Expression and the role of other Th2-specific transcription factors have not been examined carefully in Th9 cells. Therefore we examined the expression of *Gata3* and *Maf* in Th9 cells and compared it with other T helper cells by real time PCR. *Gata3* expression was highest in Th2 cells; however Th9 cells also expressed *Gata3* at a decreased level (Figure 28A). We confirmed this observation by analyzing GATA3 protein expression via intracellular staining. GATA3 protein levels were identical between Th2 and Th9 cells before stimulation (Figure 28B). When Th2 and Th9 cells were activated by anti-CD3, the amount of GATA3 protein was doubled in Th2 cells (Figure 28B). In contrast, GATA3 protein expression was unchanged in stimulated Th9 cells (Figure 28B). *Maf*, which is critical for IL-4 production in Th2 cells and IL-10 and IL-21 production in Th17 cells (Hiramatsu et al., 2010; Ho et al., 1996; Ho et al., 1998; Xu et al., 2009), was expressed abundantly in Th9 cells (Figure 28C). We also confirmed the results by examining Maf protein expression by intracellular staining in Th2 and Th9 cells. Th9 cells had higher Maf protein compared to Th2 cells (Figure 28D).

We wanted to further determine if there were differences in kinetics of the Th2 transcription factors between Th2 and Th9 cells. *Gata3* and *Maf* expression patterns were similar between Th2 and Th9 cells with greater induction of *Gata3* and *Maf* in Th2 and Th9 cells, respectively, towards the end of the differentiation (Figure 28E). We also tested the induction of *Irf4* in Th2 and Th9 cells and observed it was expressed consistently higher in Th9 cells throughout differentiation (Figure 28E). Overall these

data suggest that expression of *Gata3*, *Irf4* and *Maf* is distinct between Th2 and Th9 cells.

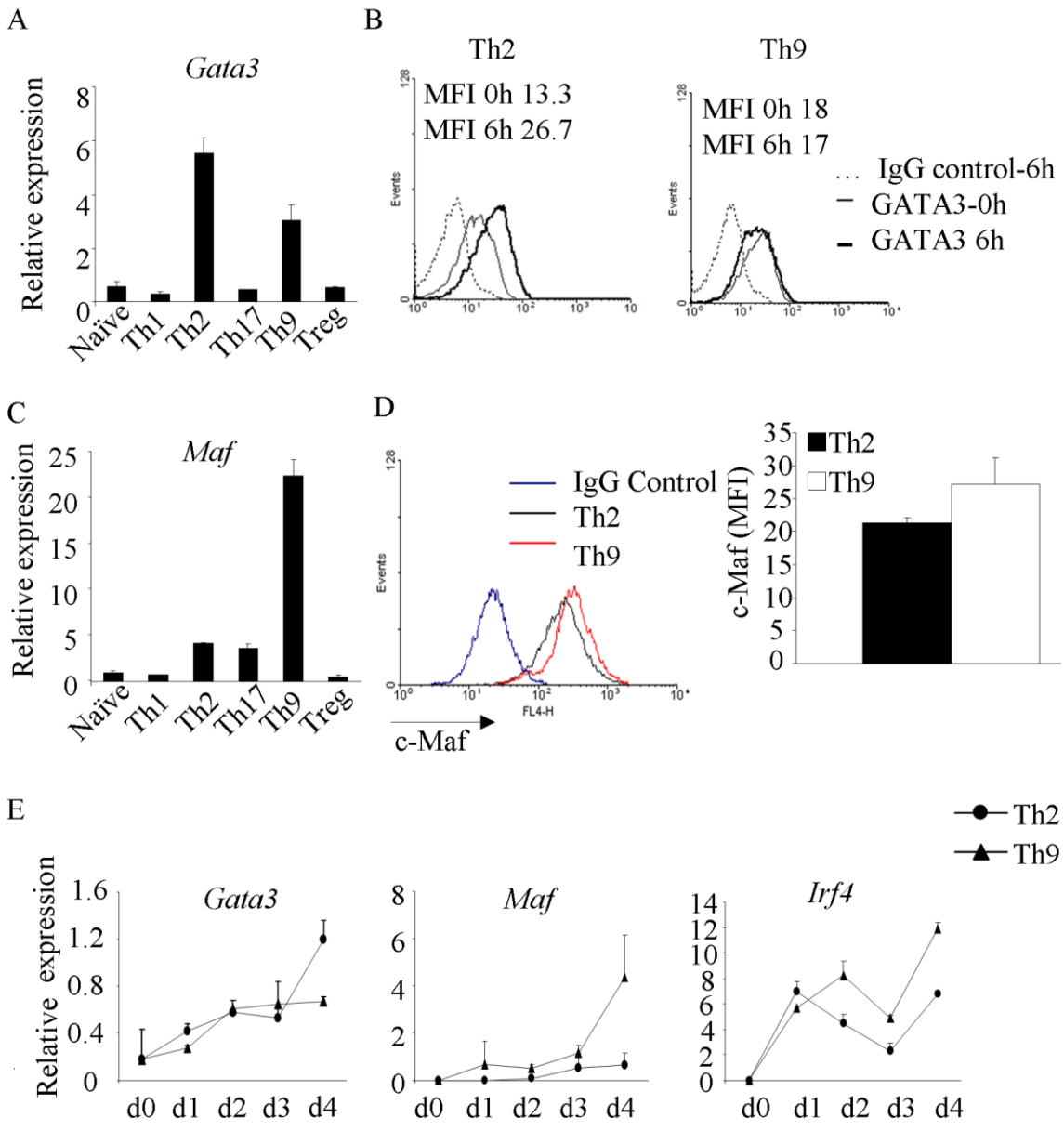

Figure 28: Th2-specific transcription factors are expressed in Th9 cells. (A, C) Naïve CD4+ T cells from WT mice were cultured under Th1, Th2, Th9, Th17, Treg conditions or directly *ex vivo* (naïve) for 5 days. RNA was isolated from cell pellet and real time PCR was performed to measure *Gata3* (A) and *Maf* (C) expression. (B, D) WT naïve CD4+ T cells were differentiated under Th2 and Th9 cell conditions for 5 days. Cells were harvested on day 5 and were kept unstimulated or stimulated with anti-CD3 for 6 hours before intracellular staining for GATA3 (B) and c-Maf (D) was performed. (E) WT

naïve CD4+ T cells were cultured under Th2 and Th9 conditions for 5 days. RNA was isolated from cells on each day during differentiation and examined for the expression of the indicated genes by real time PCR. Data are replicates representative of 2-3 independent experiments with 2 mice in each experiment. Panel A-C was performed in collaboration with Dr. Rukhsana Jabeen.

Gata3 and Maf expression are STAT6-dependent in Th9 cells

IRF4 was earlier shown to be dependent on IL-4/STAT6 pathway but not sufficient to induce IL-9 in Th9 cells. We therefore wanted to determine if either Gata3 or Maf might account for the IL-4/STAT6-dependent induction of IL-9. To test this, differentiated Th9 cultures from WT and *Stat6-/-* mice were measured for *Gata3* and *Maf* expression by real time PCR. Expression of both *Gata3* and *Maf* was decreased in the absence of STAT6 signal (Figure 29). These data suggest similar to Th2 cells, GATA3 and c-Maf are downstream of IL-4/STAT6 signaling cascade in Th9 cells.

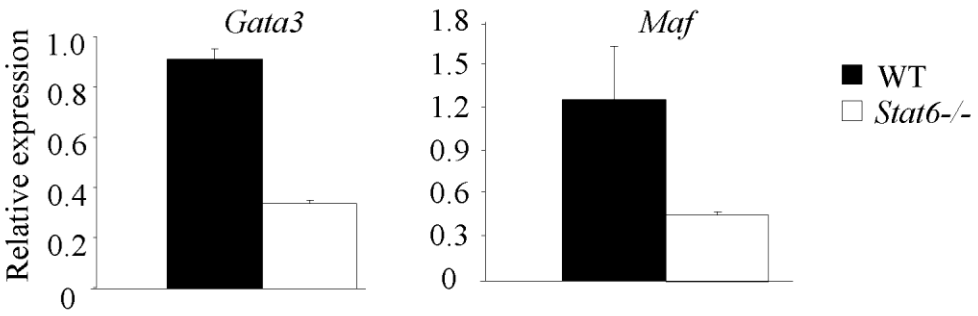

Figure 29: *Gata3* and *Maf* expression in Th9 cells are IL-4/STAT6-dependent. Naïve CD4+ T cells isolated from WT and *Stat6-/-* mice were cultured under Th2 and Th9 cell conditions for 5 days. Cell pellet was harvested on day 5 and *Gata3* and *Maf* expression was assessed by real time PCR. Data are replicates representative of 3 independent experiments with 2 mice in each experiment.

IL-4-induced transcription factors repress IL-9 production in Th9 cells

Since both Gata3 and c-Maf were expressed in Th9 cells we wanted to determine if either of the factors directly promoted IL-9 production. To test this, developing Th9 cells were transduced with bicistronic retroviruses containing either GATA3 or c-Maf. Following 5 days of differentiation, IL-9 production was assessed by flow cytometry. Compared to Th9 cultures transduced with control retrovirus, transduction of Th9 cells by GATA3 resulted in a reduction of IL-9-producing cells (Figure 30A). Similarly, a decrease in IL-9-secreting cells was observed when Th9 cells were transduced with c-Maf-expressing retrovirus (Figure 30B). We hypothesized that since both GATA3 and c-Maf can induce the production of IL-4, these factors might be augmenting IL-4-secreting cells at the expense of IL-9-producing cells. However, neither of these transcription factors enhanced the percentages of IL-4-secreting cells (Figure 30A, B). We speculated that increases in endogenous IL-4 during the culture were possibly affecting the differentiation of IL-9-secreting cells. To test this, Th9 cells from *Il4-/-* mice were analyzed for IL-9 production. Exogenous IL-4 was sufficient to induce IL-9 from *Il4-/-* Th9 cells (Figure 30A, B). We then wanted to determine if either GATA3 or c-Maf ectopic expression was affecting IL-9 production from *Il4-/-* Th9 cells. Transduction of GATA3 or c-Maf into *Il4-/-* Th9 cultures was as effective at reducing IL-9 production as transduction into wild-type cells (Figure 30A, B). Therefore, even though GATA3 and c-Maf are expressed in Th9 cells, ectopic expression attenuates IL-9 production through effects that are independent from the induction of IL-4.

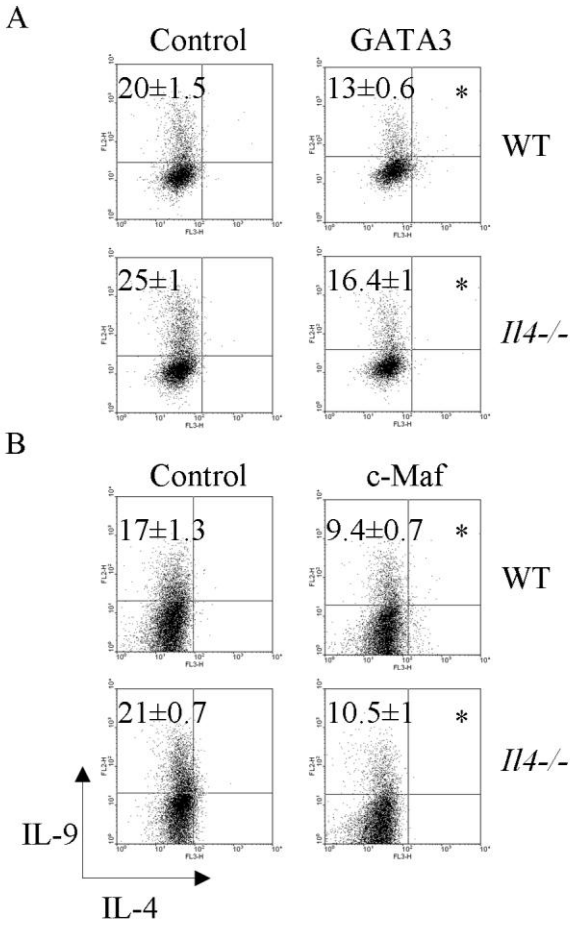

Figure 30: Th2 transcription factors repress IL-9 production in Th9 cells. (A, B) Naïve CD4+ T cells from WT or *Il4-/-* mice were cultured under Th9 cell conditions. On day 2, cells were transduced with control and GATA3 (A) or control and c-Maf-expressing retroviruses (B). On day 5, cells were stimulated with PMA and ionomycin for intracellular staining of IL-9 and IL-4. Data are average ± S.D of 4-6 mice from 2-3 independent experiments. Experiments were performed in collaboration with Dr. Rukhsana Jabeen. * $p<0.05$.

Th1-associated transcription factors repress IL-9 production

Apart from promoting Th2 differentiation, IL-4 and STAT6 interfere with the expression and function of transcription factors T-bet and Runx3 which promote IFN-γ expression and Th1 differentiation (Afkarian et al., 2002; Djuretic et al., 2007). Therefore we wanted to determine if Th1-specific transcription factors negatively regulate IL-9 production

from Th9 cells. To test this, *Tbx21* and *Runx3* expression was assessed among different T helper cells. As expected *Tbx21* expression was highest in Th1 cells; however, Th9 cells expressed similar levels of *Tbx21* compared to other T helper cells (Figure 31A). Runx3 was expressed in most T helper cells with highest expression in Th1 cells (Figure 31A). A detectable level of Runx3 was observed in Th9 cells at the protein level (Figure 31B).

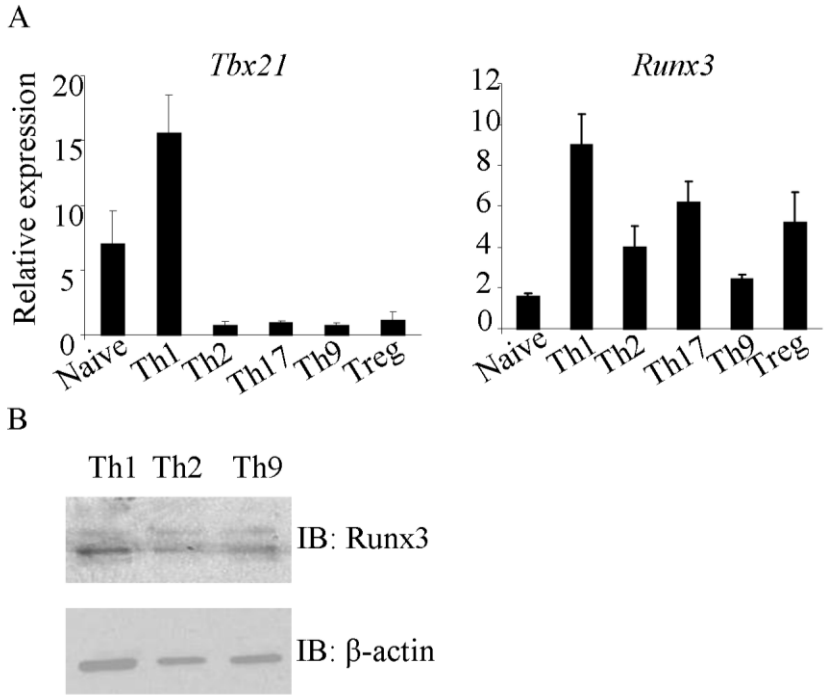

Figure 31: Th1 transcription factors, T-bet and Runx3 are expressed in Th9 cells. (A) Naïve CD4+ T cells from WT mice were cultured under Th1, Th2, Th9, Th17, Treg conditions or directly *ex vivo* (naïve) for 5 days. RNA was isolated from cell pellet and real time PCR was performed to measure the indicated genes. (B) Total cell lysates were prepared from Th1, Th2, and Th9 cells, and immunoblot was performed for Runx3. Data are replicates representative of 3 independent experiments with 2 mice in each experiment. Panel A was performed in collaboration with Dr. Rukhsana Jabeen.

To determine if T-bet and Runx3 were limiting Th9 development, developing Th9 cells were transduced with either T-bet or Runx3-expressing retroviruses. Transduction of Th9 cells with either of the factors resulted in attenuated percentages of IL-9-secreting cells

(Figure 32A). To further define the inhibitory roles of T-bet and Runx3, naïve CD4+ T cells were differentiated under Th9 cell conditions from WT and T-bet-deficient or WT and Runx3-deficient mice. The percentages of IL-9-producing cells were increased from both T-bet and Runx3-deficient mice (Figure 32B). However, there was no difference in the percentages of IL-4-secreting cells between the wild-type and the gene-deficient Th9 cells (Figure 32B). These data are complementary to the findings observed in the retroviral expression studies. Overall these data suggest that Th1-associated transcription factors are inhibitors of IL-9 production from Th9 cells.

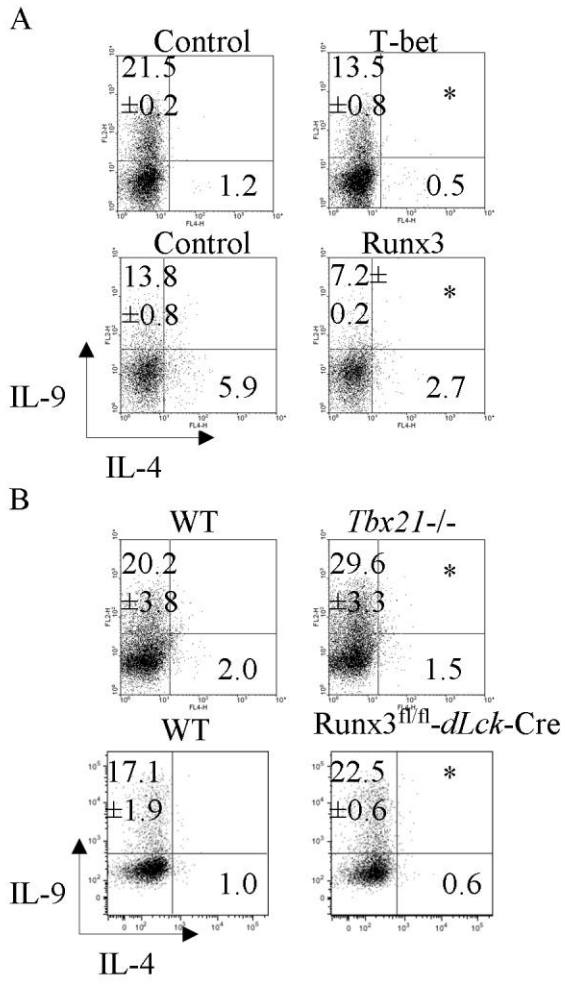

Figure 32: T-bet and Runx3 limit IL-9 production. (A) WT naïve CD4+ T cells were cultured under Th9 cell conditions for 48 hours before being transduced with control and

T-bet-expressing (top panel), or control and Runx3-expressing retroviruses (bottom panel). On day 5, cells were harvested and stimulated with PMA and ionomycin before intracellular IL-9and IL-4 staining. (B) Naïve CD4+ T cells from WT and *Tbx21-/-* mice (top panel) or WT and Runx3$^{fl/fl}$–*dLck*–Cre mice (bottom panel) were cultured under Th9 cell conditions for 5 days. On day 5, cells were harvested and stimulated with PMA and ionomycin before intracellular IL-9 and IL-4 staining. Data are average ± S.D of 4 mice from 2 independent experiments. Panel B (bottom panel) was performed in collaboration with Drs. Jinfang Zhu and Ryogi Yagi. * $p<0.05$.

T-bet but not Runx3 expression is repressed by STAT6 signal in Th9 cells

Since the STAT6 signal is indispensable for Th9 cell development, we wanted to determine if either of T-bet or Runx3 contributed to the lack of IL-9 production in *Stat6-/-* Th9 cultures. To test this, *Tbx21* and *Runx3* expression was measured in differentiated Th9 cells from WT and *Stat6-/-* mice by real time PCR. *Tbx21* expression was enhanced in the absence of STAT6 (Figure 33). In contrast, *Runx3* expression was diminished in *Stat6-/-* Th9 cultures (Figure 33). Together, these data suggest that both T-bet and Runx3 can limit IL-9 production; however, Runx3 is probably not a contributing factor for the lack of IL-9 production in *Stat6-/-* Th9 cells. Therefore, one of the roles of IL-4/STAT6 signaling is to limit T-bet-mediated inhibition of IL-9 in Th9 cells.

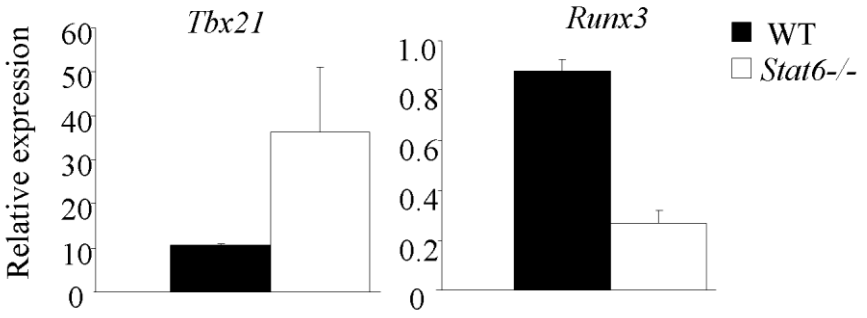

Figure 33: STAT6 signal represses T-bet but not Runx3 expression in Th9 cells. Naïve CD4+ T cells from WT and *Stat6-/-* mice were cultured under Th9 cell conditions for 5 days. RNA was isolated from cell pellet and was used to measure the indicated

genes by real time PCR. Data are average ± S.D of 4 mice from 2 independent experiments.

STAT6 signal inhibits Foxp3 expression in Th9 cells

As one of the functions of IL-4 in the Th9 cell development is to inhibit Foxp3, we wanted to test if Foxp3 negatively regulates IL-9 production. To test this, we first examined the expression of *Foxp3* among different T helper cell subsets by real time PCR. As expected Treg cells had the highest *Foxp3* expression (Figure 34A). Th9 cells also expressed Foxp3; however, at a lower level than Treg cells (Figure 34A). To test if Foxp3 was a negative regulator of IL-9 in Th9 cells, developing Th9 cells were transduced with control or Foxp3-expressing retroviruses and IL-9 production was measured by intracellular staining. Ectopic expression of Foxp3 attenuated the percentages of IL-9-producing cells without affecting IL-17A induction (Figure 34B). To test if STAT6 regulated the expression of Foxp3 in Th9 cells, mRNA from WT and *Stat6-/-* Th9 cultures were examined. In the absence of STAT6 there was enhanced expression of *Foxp3* (Figure 34C). This suggests that an indirect role of STAT6 during Th9 cell development is to suppress *Foxp3* expression which would otherwise diminish IL-9 production.

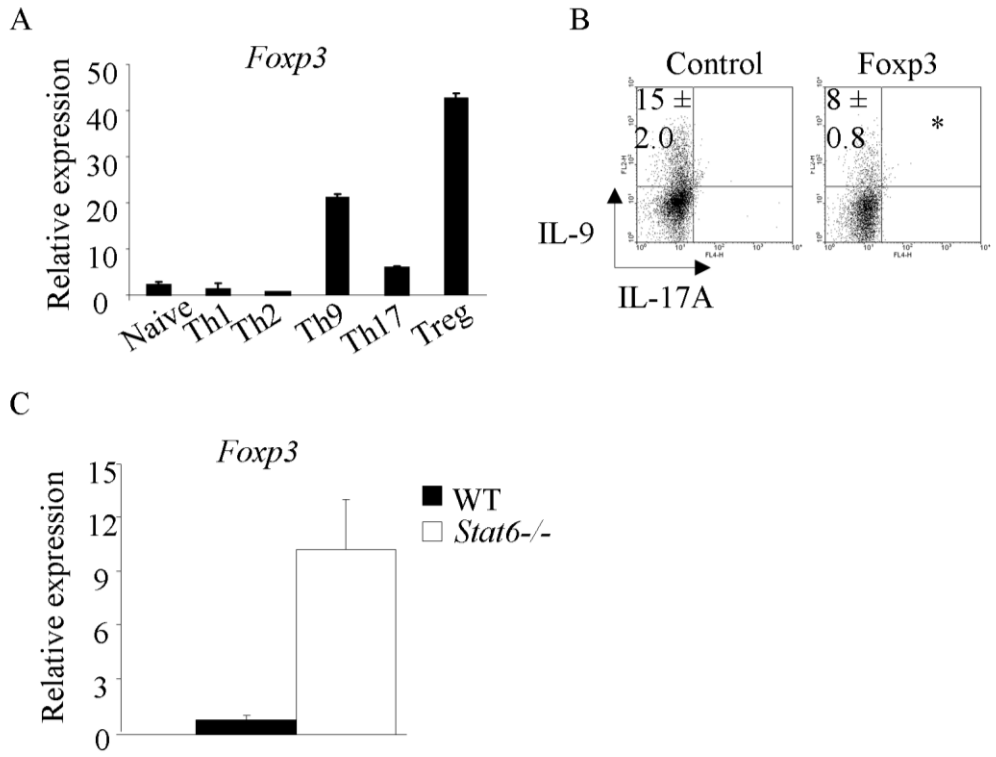

Figure 34: STAT6 limits Foxp3 inhibition of IL-9. (A) Naïve CD4+ T cells from WT mice were cultured under Th1, Th2, Th9, Th17, Treg conditions or directly ex vivo (naïve) for 5 days. *Foxp3* expression was assessed by real time PCR. (B) Developing Th9 cells from WT mice were transduced with control or Foxp3-expressing retroviruses. After 5 days cells were harvested and stimulated with PMA and ionomycin before intracellular IL-9and IL-17A staining. (C) Naïve CD4+ T cells from WT and *Stat6-/-* mice were differentiated under Th9 cell conditions for 5 days. RNA was isolated from cell pellet and real time PCR was performed to measure *Foxp3* expression. Data are average ± S.D of 4-6 mice from 2-3 independent experiments. Panel B was performed in collaboration with Dr. Rukhsana Jabeen. * $p<0.05$.

Overall these data indicate that the IL-4 signal regulates the expression of both positive and negative regulators of IL-9 production (Figure 35).

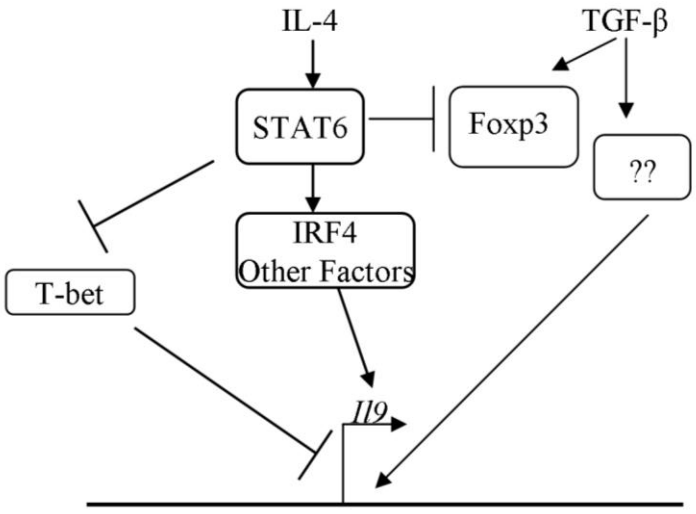

Figure 35: Model of Th9 transcriptional network. Schematic diagram showing the summary of STAT6-dependent transcription factor network in Th9 cells.

PART III: PU.1 promotes IL-9 expression through a Gcn5-dependent mechanism

PU.1 is abundantly expressed in Th9 cells

In the previous section we have described the transcription factor network downstream of IL-4 signal required for the development of Th9 cells. We therefore wanted to define the transcription factor network downstream of TGF-β signal. Based on the similar ability of TGF-β and PU.1 to decrease Th2 cytokine expression (Chang et al., 2005; O'Malley et al., 2009), we examined the expression of PU.1 in Th9 cells and compared it among different T helper subsets. Naïve CD4+ T cells were polarized under Th1, Th2, Th9, and Th17 cell conditions for 5 days and *Sfpi1* (the gene which encodes PU.1) expression was assessed by real time PCR. Expression of *Sfpi1* mRNA was lowest among Th1 and Th17 cells, while Th2 cells had 3-5 fold more abundant *Sfpi1* expression (Figure 36). Th9 cells had expressed 2-3 fold higher *Sfpi1* than Th2 cells (Figure 36).

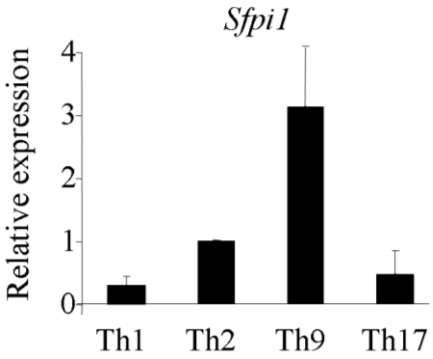

Figure 36: PU.1 is abundantly expressed in Th9 cells. WT naïve CD4+ T cells were cultured under Th1, Th2, Th9, and Th17 cell conditions for 5 days. On day 5, cells were harvested and real time PCR was performed to measure *Sfpi1* expression. Data are replicates representative of 3 independent experiments.

We then wanted to determine if *Il9* and *Sfpi1* expression were induced similarly during Th9 cell differentiation. To test this, naïve CD4+ T cells from WT mice were cultured

under Th2 and Th9 cell conditions and RNA was isolated during each day of differentiation. A peak of *Sfpi1* expression preceded *Il9* induction during differentiation, with Th9 cells expressing higher amounts of *Il9* and *Sfpi1* than Th2 cells (Figure 37A). At the end of the differentiation Th9 cells expressed higher levels of *Il9* concomitant with increased *Sfpi1* expression, compared to Th2 cells (Figure 37B). Therefore *Sfpi1* is expressed consistently higher in Th9 cells than Th2 cells.

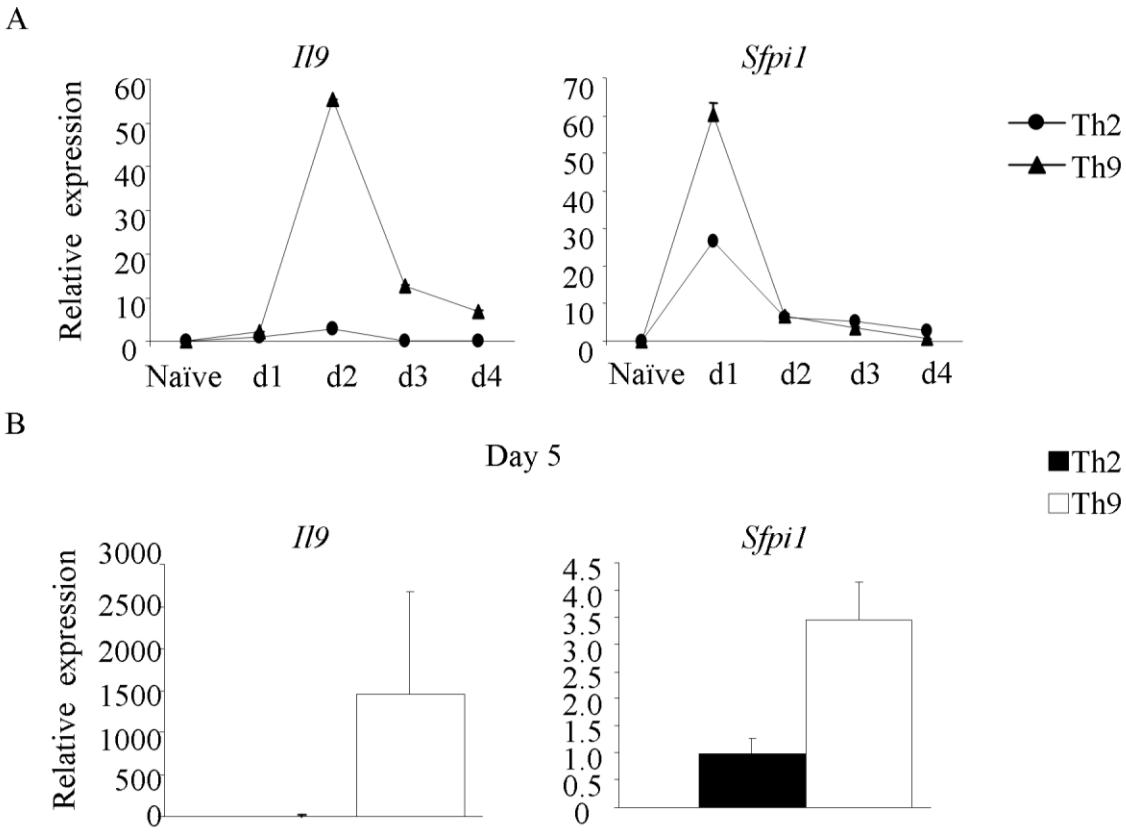

Figure 37: IL-9 and PU.1 expression follow similar kinetics with a transient early peak during Th9 differentiation. (A) WT naïve CD4+ T cells were cultured under Th2 and Th9 cell conditions. Cells were collected on each day during differentiation. *Il9* and *Sfpi1* expression was assessed by real time PCR. (B) On day 5, *Il9* and *Sfpi1* expression was measured. Data are replicates representative of 3 independent experiments.

PU.1-deficient Th9 cells have impaired IL-9 production

As PU.1 was predominantly expressed in Th9 cells compared to other Th subsets we wanted to determine if PU.1 was required for the development of Th9 cells. To test this, naïve CD4+ T cells from WT and PU.1 conditional gene-deficient mice (*Sfpi1*$^{lck-/-}$) were polarized under Th9 culture conditions for 5 days. On day 5, differentiated cells were analyzed for IL-9 production. In the absence of PU.1, Th9 cultures had significantly reduced percentages of IL-9-secreting cells compared to WT Th9 cultures (Figure 38A). There was a corresponding increase in IL-4-producing cells (Figure 38A). Similar to the cytokine staining data, IL-9 production was impaired in *Sfpi1*$^{lck-/-}$ Th9 cultures measured using ELISA and real time PCR (Figure 38B, C). To determine if PU.1 also regulated IL-10 production from Th9 cells, cell-free supernatant from re-stimulated WT and *Sfpi1*$^{lck-/-}$ Th9 cultures was assessed for IL-10 production. Even though PU.1-deficiency resulted in defective IL-9 production, IL-10 production remained unchanged in Th9 cells (Figure 38D). Since Th9 cells also produce IL-21 we wanted to determine if PU.1 regulated IL-21 production from Th9 cells. We observed unchanged IL-21 production from PU.1-deficient Th9 cells compared to wild-type Th9 cells (Figure 38D). Overall these data suggest that the transcription factor PU.1 is required for IL-9 production in Th9 cells.

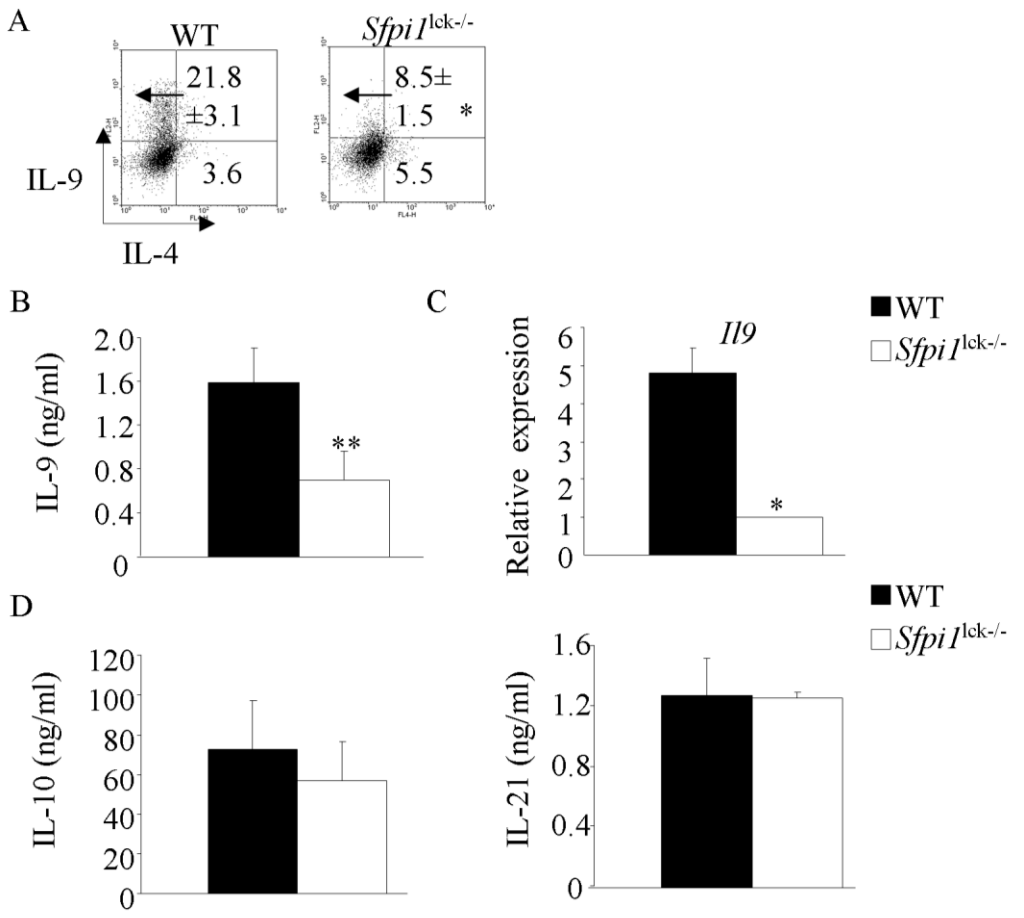

Figure 38: PU.1-deficient Th9 cells have impaired IL-9 production. (A) Naïve CD4+ T cells from WT and *Sfpi1*$^{lck-/-}$ mice were differentiated under Th9 cell conditions for 5 days. Cells were re-stimulated with PMA and ionomycin on day 5 before intracellular IL-9 and IL-4 staining was performed. (B, C) Cells cultured as in (A) were re-stimulated with anti-CD3 for 24 hours (B) and 6 hours (C) to assess IL-9 production using ELISA and real time PCR, respectively. (D) Stimulated cells as in (B) were used to measure IL-10 and IL-21 production using ELISA. Data are average ± S.D of 4-6 mice from 2-3 independent experiments. * $p<0.05$, ** $p<0.01$.

PU.1 ectopic expression enhances IL-9 production

As a complementary approach to the gene knock-out model of PU.1 we wanted to determine if ectopically expressed PU.1 can induce IL-9 production in Th9 cells. To test this, developing Th9 cells were transduced with control or PU.1-expressing retroviruses and IL-9 production was measured by flow cytometry. Ectopic expression of PU.1

increased both the percentages and the mean fluorescence intensity of IL-9-secreting cells (Figure 39A, B). In contrast, IL-4 production was reduced when Th9 cells were transduced with PU.1 (Figure 39A). When PU.1 was ectopically expressed in Th0 cells there was a modest increase in the percentages of IL-9-secreting cells (Figure 39C). Overall these data suggest that while *Sfpi1*$^{lck-/-}$ Th9 cultures have impaired IL-9 production, ectopic PU.1 expression enhances IL-9 production.

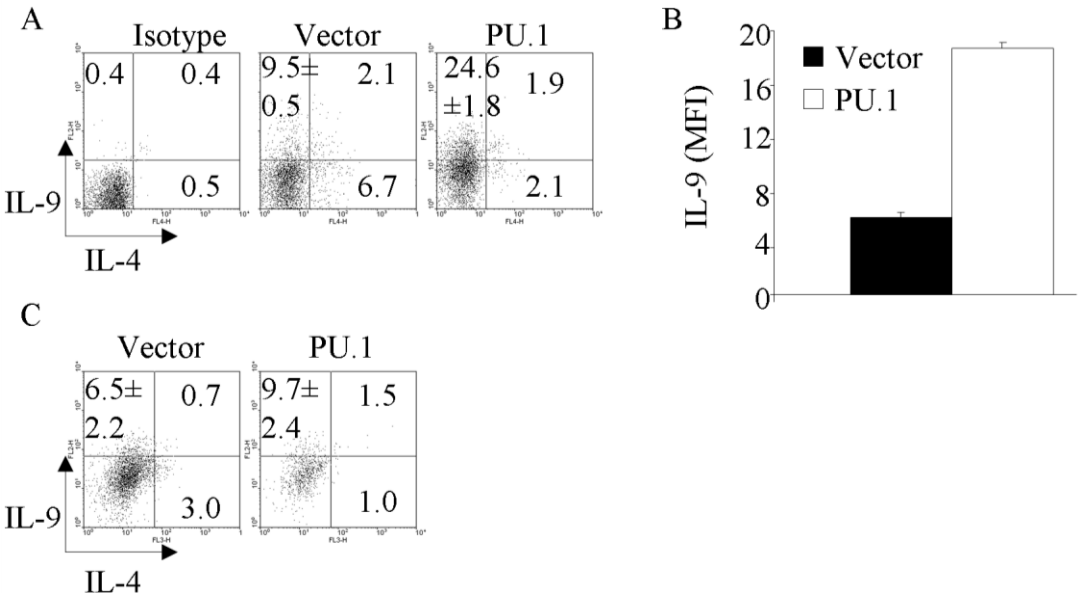

Figure 39: Ectopic PU.1 expression induces IL-9 production. (A) WT naïve CD4+ T cells were cultured under Th9 cell conditions for 48 hours before being transduced with control or PU.1-expressing retroviruses. Cells were harvested on day 5 and stimulated with PMA and ionomycin before staining intracellularly for IL-9 and IL-4 based on eGFP+ population. (B) IL-9 MFI from the cells cultured as in (A). (C) WT naïve CD4+ T cells were activated with anti-CD3 and anti-CD28 for 48 hours before being transduced with control or PU.1-expressing retroviruses. Cells were harvested on day 5 and stimulated with PMA and ionomycin before staining intracellularly for IL-9 and IL-4 based on eGFP+ population. Data are replicates representative of 3 independent experiments (A, B) or average of 2 mice (C).

Ectopic IRF4 expression does not rescue IL-9 production in PU.1-deficient Th9 cells

Since IRF4 was shown to be important for the development of Th9 cells, we wanted to determine the role of IRF4 in PU.1-mediated IL-9 production. To test this, first we determined the expression of *Irf4* in WT and PU.1-deficient Th9 cells. *Irf4* expression was decreased in PU.1-deficient Th9 cells compared to WT Th9 cells (Figure 40A). We then wanted to determine if ectopic IRF4 expression was able to recover IL-9 production in PU.1-deficient Th9 cells. We therefore transduced developing Th9 cells from WT and *Sfpi1*$^{lck-/-}$ mice with control or IRF4-expressing retroviruses. Even though IRF4 was able to increase the percentages of IL-9-secreting cells from WT cultures, it did not enhance the percentages of IL-9-secreting cells from PU.1-deficient Th9 cultures to a level similar to WT Th9 cultures transduced with control retroviruses suggesting that IRF4 was not sufficient to recover IL-9 production in PU.1-deficient Th9 cells (Figure 40B).

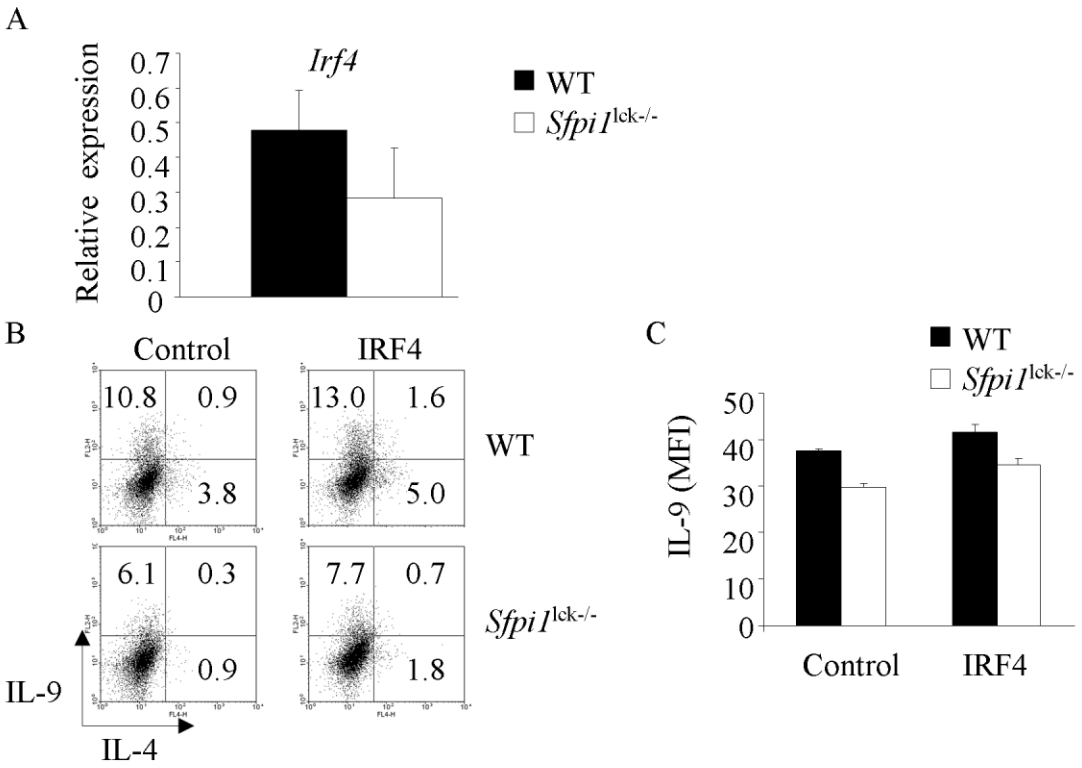

Figure 40: Ectopic IRF4 expression does not recover IL-9 production from PU.1-deficient Th9 cells. (A) Naïve CD4+ T cells from WT and *Sfpi1*$^{lck-/-}$ mice were cultured for 5 days under Th9 cell conditions. RNA was isolated from differentiated cells and *Irf4* expression was assessed by real time PCR. (B, C) Naïve CD4+ T cells from WT and *Sfpi1*$^{lck-/-}$ mice were cultured under Th9 cell conditions for 48 hours before being transduced with control or IRF4-expressing retroviruses. Cells were re-stimulated on day 5 with PMA and ionomycin before intracellular staining for IL-9 and IL-4 was performed (B) or IL-9 MFI was assessed (C) based on hCD4+ population.

TGF-β but not IL-4 regulates PU.1 expression

As we established that PU.1 is one of the transcription factors required for Th9 cell development we wanted to determine if PU.1 is downstream of either IL-4 or TGF-β signal. When we compared *Sfpi1* expression between Th9 and Treg cultures we observed that Tregs expressed *Sfpi1* 4-5 fold higher than Th9 cells (Figure 41A). Since TGF-β is the cytokine required for the development of both Th9 and Treg cells, we speculated that TGF-β might be regulating PU.1. To test this, we cultured naïve CD4+ T cells with

increasing doses of TGF-β in the presence (Th9) or absence (Treg) of a constant dose of IL-4 (20 ng/ml). TGF-β dose-dependent induction of *Sfpi1* was observed in both Treg and Th9 cultures assessed by real time PCR; however, Tregs had higher *Sfpi1* expression (Figure 41B). In contrast, when Th9 cells were cultured with fixed TGF-β dose (2 ng/ml) and in the presence of decreasing IL-4 doses, *Sfpi1* expression was not affected by the IL-4 dose (Figure 41B). However, IL-9 production measured using ELISA from Th9 cells was reduced in IL-4 dose-dependent manner when TGF-β was held constant (Figure 41C). To directly determine if *Sfpi1* expression was regulated by IL-4/STAT6 signal we assessed *Sfpi1* expression in WT and *Stat6-/-* Th9 cells by real time PCR. There was no difference in *Sfpi1* expression in the absence of STAT6 (Figure 41D). To test whether PU.1 association to the *Il9* gene was altered in the absence of STAT6, we performed a ChIP assay of PU.1 association at the *Il9* locus in WT and *Stat6-/-* Th9 cultures. In the absence of STAT6, PU.1 binding to CNS1 and CNS0 regions of *Il9* gene was not altered (Figure 41E). These data suggest that TGF-β but not IL-4 controls *Sfpi1* expression and the fine balance of TGF-β and IL-4 is required for optimal IL-9 production.

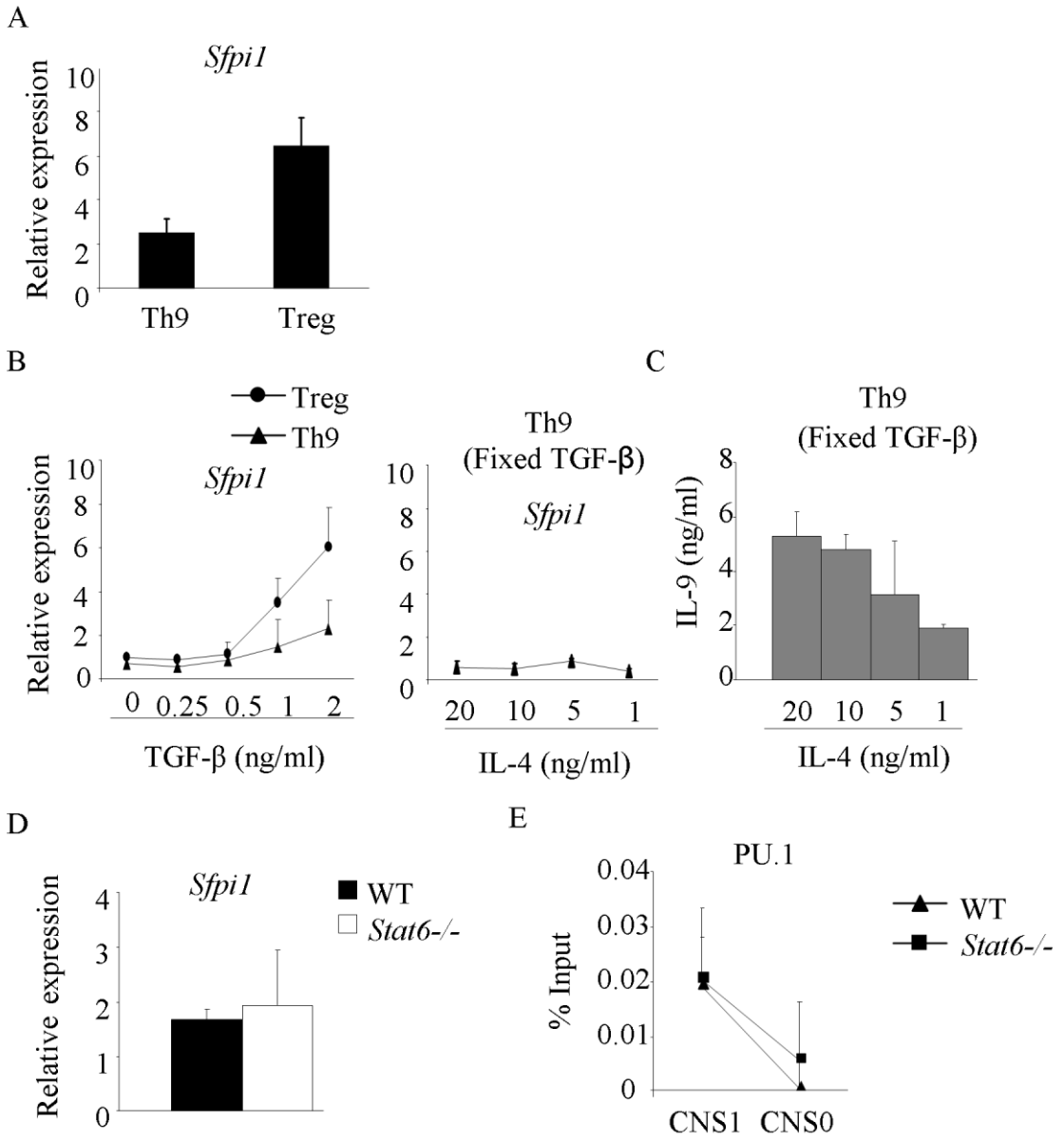

Figure 41: TGF-β but not IL-4 regulates PU.1 expression. (A) WT naïve CD4+ T cells were cultured under Th9 and Treg cell conditions for 5 days. *Sfpi1* expression was measured on day 5 by real time PCR. (B, C) WT naïve CD4+ T cells were cultured with increasing doses of TGF-β in the presence (Th9) or absence (Treg) of IL-4 (20 ng/ml, left panel). On day 5, *Sfpi1* expression was measured. Naïve CD4+ T cells were also cultured Th9 cell conditions with fixed dose of TGF-β (2 ng/ml) and decreasing doses of IL-4 for 5 days. Cells were harvested on day 5 and were kept unstimulated or re-stimulated with anti-CD3 to assess *Sfpi1* expression by real time PCR (B) or IL-9 production using ELISA (C), respectively. (D, E) Naïve CD4+ T cells from WT and *Stat6-/-* mice were cultured under Th9 cell conditions for 5 days. Following differentiation cells were used to measure *Sfpi1* expression by real time PCR (D). PU.1 binding to the *Il9* locus was

assessed by chromatin immunoprecipitation (E). Data are replicates representative of 3 independent experiments.

Blocking endogenous TGF-β production in Th2 cells reduces Il9 expression

PU.1 is expressed in the IL-4^{lo} population of Th2 cells (Chang et al., 2010). Therefore we wanted to determine whether the IL-4^{lo}, PU.1-expressing Th2 cells are really Th9 cells. To test this, Th2 cultures from WT mice were treated with neutralizing TGF-β antibody and *Sfpi1* expression was assessed by real time PCR. Th2 cells treated with anti-TGF-β resulted in two-fold decreased *Sfpi1* expression with a concomitant reduction in *Il9* expression (Figure 42). Therefore endogenous TGF-β production in a Th2 culture can promote the development of Th9 cells which is consistent with the report demonstrating that developed Th2 cells treated with TGF-β can switch to a Th9 phenotype (Veldhoen et al., 2008b).

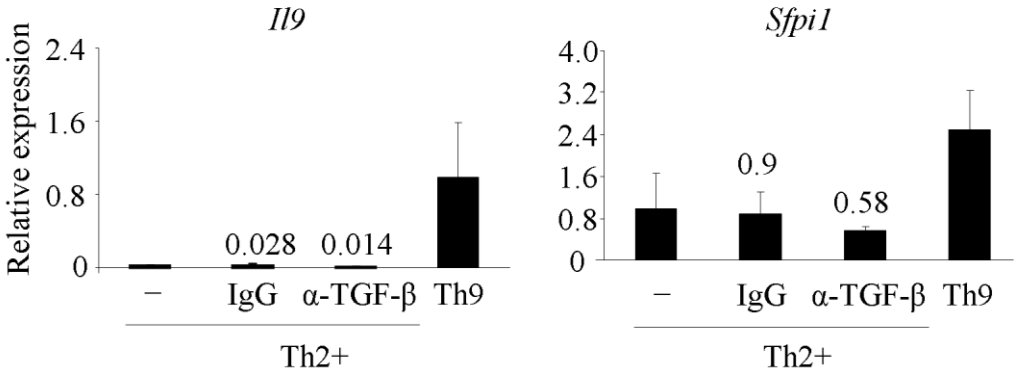

Figure 42: Blocking endogenous TGF-β production in Th2 cells leads to reduced *Il9* expression. Naïve CD4+ T cells were cultured under Th2 cell conditions in the presence of control or TGF-β neutralizing antibody and under Th9 cell conditions for 5 days. Cells were harvested on day 5 and were either re-stimulated with anti-CD3 for 6 hours (left panel) or kept unstimulated (right panel) to measure *Il9* and *Sfpi1*, respectively, by real time PCR. Data are replicates representative of 2 independent experiments.

Increased histone deacetylase binding to the Il9 locus in the absence of PU.1

PU.1 has been shown to bind directly to the *Il9* promoter in Th9 cells (Chang et al., 2010)(Figure 41E). However, mechanisms for PU.1-dependent *Il9* expression in Th9 cells have not been defined. Global gene expression results from a balance between histone acetylation and deacetylation controlled by a group of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. Global hyperacetylated states can be generated through the use of HDAC inhibitors which induce expression of a subset of genes (Luo and Dean, 1999; Van Lint et al., 1996). Therefore we wanted to determine if HDAC association to the *Il9* locus was PU.1-dependent. We examined the binding of the class I HDACs, HDAC1 and HDAC2, in WT and PU.1-deficient Th9 cultures at the two previously described CNS regions of the *Il9* locus by ChIP assay. Increased association of both the HDAC molecules was observed at the CNS1 in the absence of PU.1 (Figure 43). In contrast, HDAC binding at CNS0 was lower than CNS1 and was unaffected in the absence of PU.1 (Figure 43). This suggests that enhanced HDAC binding to the *Il9* promoter in the absence of PU.1 could possibly contribute to reduced *Il9* transcription.

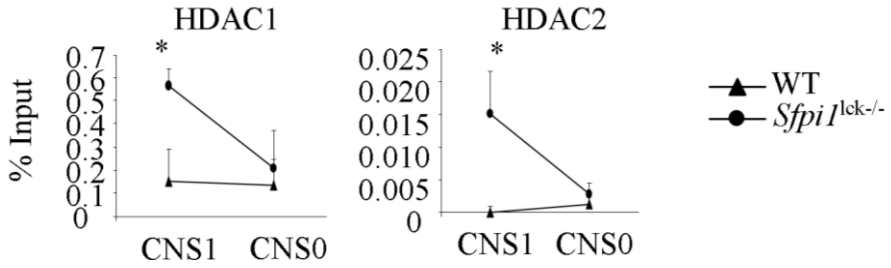

Figure 43: Increased histone deacetylase binding at *Il9* locus in the absence of PU.1. Naïve CD4+ T cells from wild-type and *Sfpi1*$^{lck-/-}$ mice were cultured under Th9 cell conditions for 5 days. ChIP assay was performed for HDAC1 and HDAC2 before quantitative PCR for two *Il9* CNS sites. Data are average ± S.D of 4 mice from 2 independent experiments.* $p<0.05$.

HDAC inhibitor enhances IL-9 production

As HDAC association was increased to the *Il9* promoter in the absence of PU.1, we wanted to determine if HDAC inhibition reverses repressive chromatin modification and leads to augmented IL-9 production. To test this, naïve CD4+ T cells from WT mice were cultured under Th9 polarizing conditions in the presence of trichostatin A (TSA), an antifungal antibiotic that selectively inhibits class I and class II HDACs. Th9 cells treated with TSA had enhanced percentages of IL-9-secreting cells and increased *Il9* gene expression (Figure 44A, B). To determine if TSA treatment enhanced the induction of other cytokines produced by Th9 cells we examined the expression of *Il10* and *Il21*. In contrast to *Il9* expression, TSA treatment did not affect the expression of *Il10* or *Il21* (Figure 44B). Overall these data indicate that HDAC inhibition specifically enhanced *Il9* induction in Th9 cells.

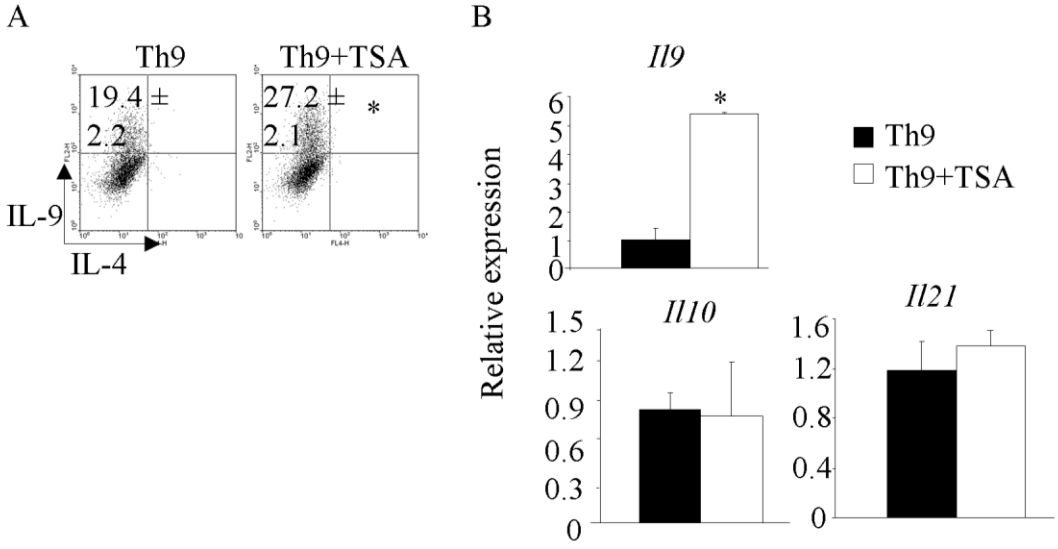

Figure 44: HDAC inhibitor only enhances IL-9 production. (A) WT naive CD4+ T cells were cultured under Th9 conditions for 5 days in the presence or absence of trichostatin A (TSA). On day 5, cells were re-stimulated with PMA and ionomycin before intracellular staining for IL-9 and IL-4 was performed. (B) Differentiated Th9 cells were stimulated with anti-CD3 for 6h before RNA isolation. *Il9*, *Il10*, and *Il21* gene expression

was assessed by real time PCR. Data are average ± S.D of 4-6 mice from 2-3 independent experiments. * $p<0.05$.

HDAC-mediated IL-9 induction is PU.1-dependent

Since TSA specifically enhanced IL-9 production in Th9 cells we wanted to determine if TSA treatment altered the expression of Th9-specific transcription factors, PU.1 and IRF4. Differentiated Th9 cells treated with TSA were examined for *Sfpi1* and *Irf4* expression by real time PCR. We observed an increase in *Sfpi1*, but not *Irf4* expression after TSA treatment (Figure 45A). This was confirmed by immunoblots of total cell lysates prepared from Th9 cells treated with TSA (Figure 45B). As the expression of *Sfpi1* was enhanced after TSA treatment we wanted to determine if TSA-mediated enhanced IL-9 production was PU.1-dependent. To test this, WT and PU.1-deficient Th9 cultures were treated with TSA and IL-9 production was measured using ELISA. Even though TSA was able to enhance IL-9 production significantly from WT Th9 cells, addition of TSA in PU.1-deficient Th9 cultures did not augment IL-9 production (Figure 45C). Together, these data suggest HDAC inhibition-induced IL-9 production in Th9 cells is PU.1-dependent.

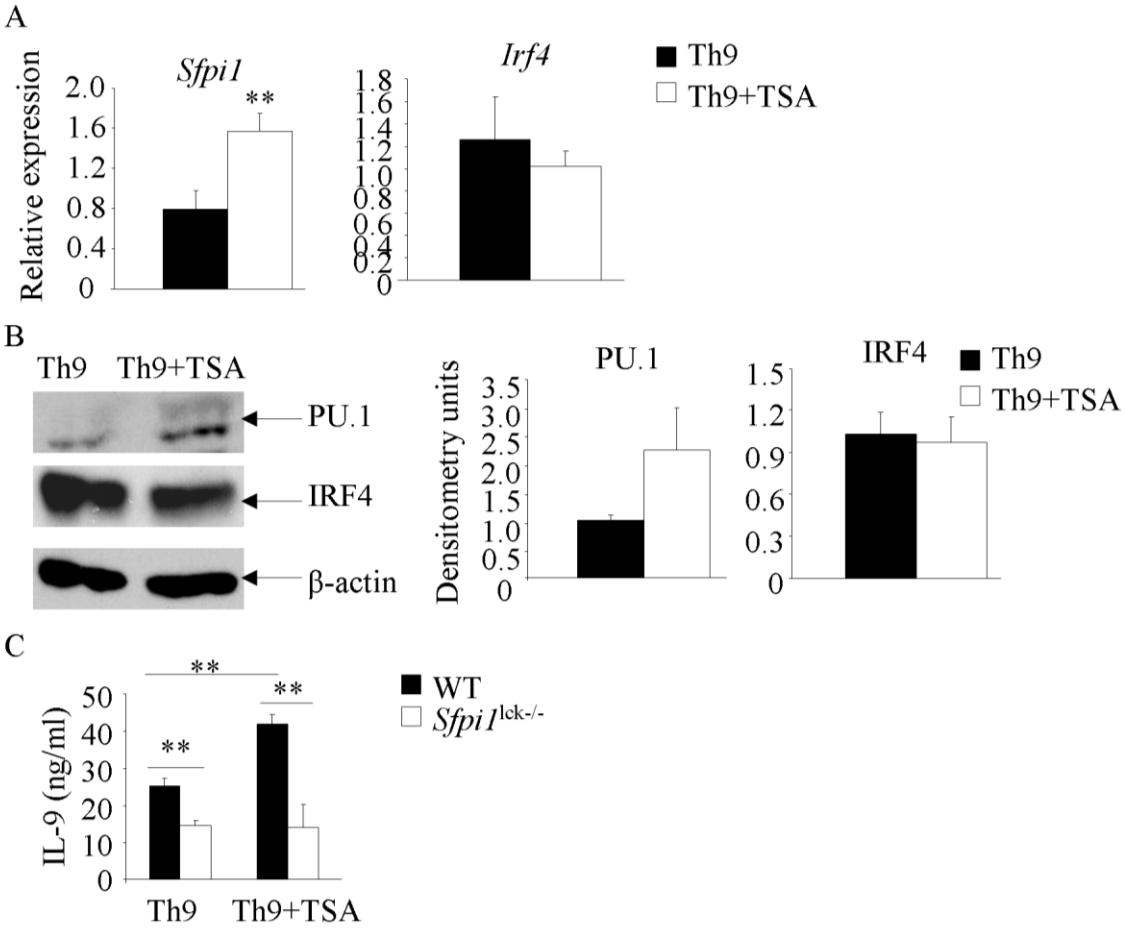

Figure 45: HDAC-mediated IL-9 induction is PU.1-dependent. (A) Naïve CD4+ T cells from WT mice were cultured under Th9 cell conditions for 5 days in the presence or absence of TSA. Cells were harvested on day 5 to assess *Sfpi1* and *Irf4* expression by real time PCR. (B) Total cell extracts were immunoblotted for PU.1, IRF4, and β-actin as a control (left panel), blots were scanned and presented in densitometry units (right panel). (C) Naïve CD4+ T cells from wild-type and *Sfpi1*$^{lck-/-}$ mice were cultured under Th9 cell conditions in the presence or absence of TSA for 5 days. On day 5, cells were re-stimulated with anti-CD3 for 24 hours and cell-free supernatant was used to measure IL-9 production using ELISA. Data are average ± S.D of 4 mice from 2 independent experiments. ** $p<0.01$ (A, paired t-test; C, One way ANOVA post hoc Bonferroni correction).

TSA treatment enhances histone acetylation and Th9-associated transcription factors binding to the Il9 promoter

Since HDAC inhibition increases histone acetylation we wanted to determine if TSA-induced *Il9* expression correlated with altered histone acetylation at the *Il9* gene. To test this, ChIP assay was performed to assess histone acetylation of H3K14 and H4K5 residues at the *Il9* promoter when WT Th9 cells were treated with TSA. We observed enhanced acetylation of both histone lysine residues after TSA treatment (Figure 46). We further tested if TSA treatment altered PU.1 and IRF4 binding to the *Il9* gene. An increase in PU.1 and IRF4 association was observed at the *Il9* promoter, despite TSA not affecting *Irf4* expression (Figure 46). Together, these data suggest that increased IL-9 production after HDAC inhibition correlated with enhanced histone acetylation and increased PU.1 and IRF4 binding to the *Il9* promoter.

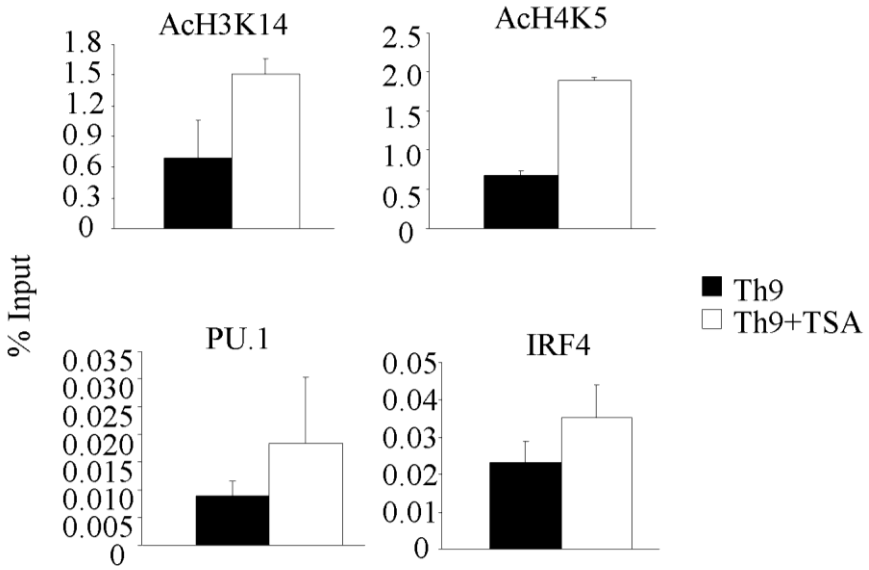

Figure 46: TSA treatment enhances histone acetylation and PU.1, IRF4 binding at the *Il9* promoter. WT naïve CD4+ T cells were harvested under Th9 cell conditions for 5 days in the presence or absence of TSA. On day 5, ChIP assay was performed for AcH3K14, AcH4K5, PU.1 and IRF4 before quantitative PCR for *Il9* promoter. Data are replicates representative of 3 independent experiments.

The activation domains of PU.1 induce IL-9 in Th9 cells

Transcription factors are composed of different functional domains. PU.1 also has multiple functional modules including N-terminal activation domain and C-terminal DNA binding domain (Figure 47A). Activation and DNA-binding domains are broadly required for transactivation. As PU.1 is required for the development of Th9 cells we wanted to define the structural requirements for PU.1-mediated IL-9 induction. To test this, we transduced developing WT Th9 cells with control retrovirus, full-length PU.1-expressing retrovirus (PU.1) and PU.1 mutant-expressing retroviruses that lacked the transactivation domain (Δ33-100), the PEST domain (Δ118-160) or had a point mutation in the DNA-binding domain (R232G) that results in diminished DNA binding. Intact PU.1 enhances both the percentages and the mean fluorescence intensity of IL-9-secreting T cells (Figure 47B, C). In contrast, cells transduced with either the PU.1 transactivation mutant or with the DNA-binding mutant retroviruses failed to induce IL-9 (Figure 47B, C). The PEST mutant was able to modestly increase IL-9-producing cells; however, it was not as efficient as the intact PU.1 in transduced cells (Figure 47B, C) Overall, these data suggest that the activation domain and DNA-binding domain of PU.1 are required for the induction of IL-9.

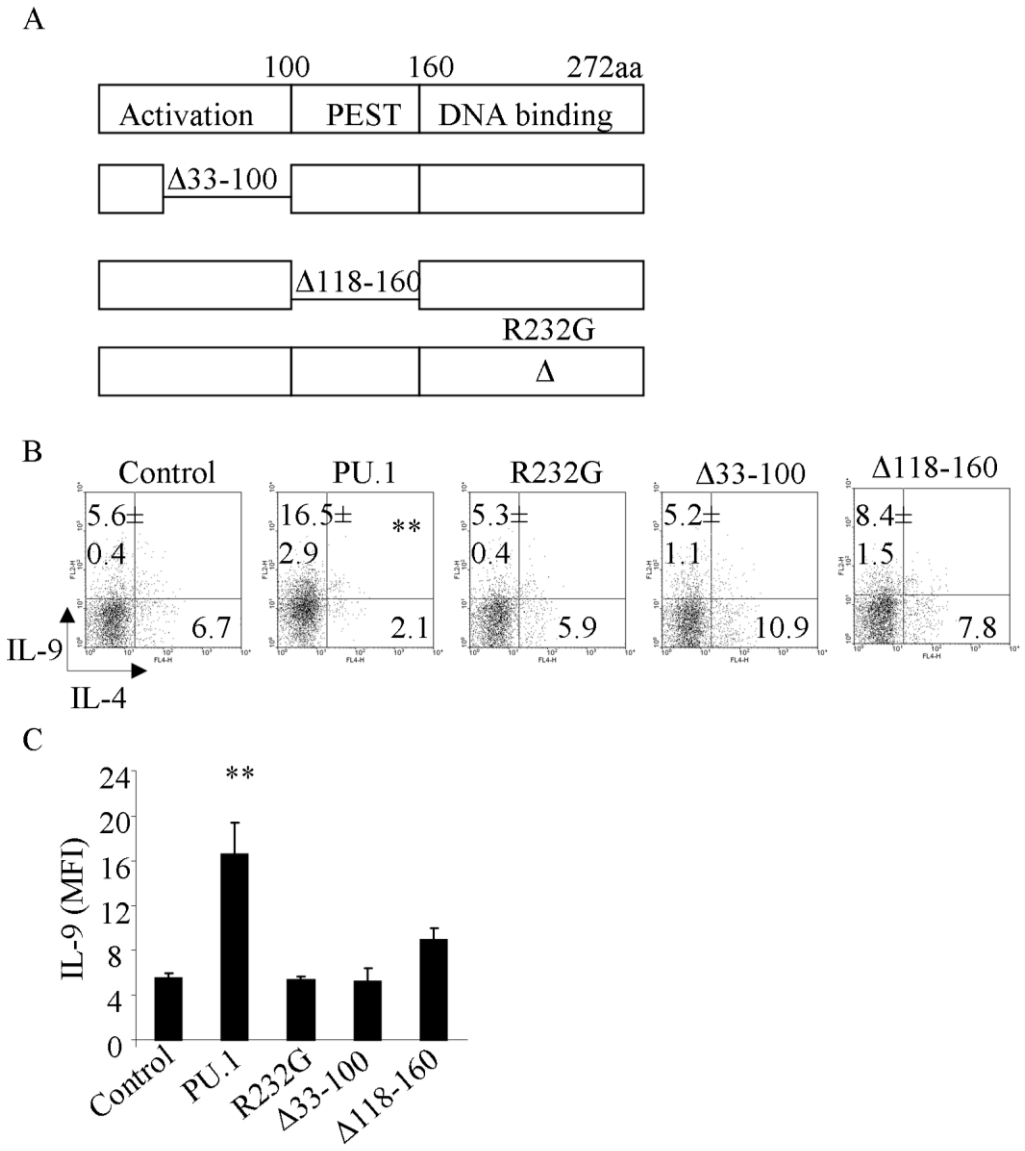

Figure 47: Activation and DNA-binding domains of PU.1 are important for IL-9 induction. (A) Schematic diagram of PU.1 domain structure along with the mutants used in the PU.1 structure function analysis. (B, C) WT naïve CD4+ T cells were cultured under Th9 cell conditions for 48 hours before being transduced with control or full-length PU.1 (PU.1) or PU.1 mutant-expressing retroviruses. On day 5, cells were re-stimulated with PMA and ionomycin. Percentages of IL-9-secreting cells (B) and IL-9 mean fluorescence intensity (C) were analyzed based on eGFP+ cells. Data are average ± S.D of 4 mice from 2 independent experiments. ** $p<0.01$.

To further demonstrate the requirement of the activation domain in IL-9 expression, we transduced developing PU.1-deficient Th9 cultures with retroviruses expressing full-

length PU.1 or activation domain mutant PU.1 and control retrovirus. WT Th9 cells were transduced with control retrovirus only. As expected PU.1-deficient Th9 cells transduced with control retrovirus had significantly attenuated IL-9-secreting cells by flow cytometry (Figure 48A) or IL-9 production using ELISA compared to WT Th9 cells (Figure 48B). However, full-length PU.1was able to restore IL-9 production in PU.1-deficient Th9 cells to a level similar to WT Th9 cells transduced with control retrovirus (Figure 48A, B). In contrast, the activation domain mutant had significantly impaired ability to recover IL-9 in PU.1-deficient Th9 cells (Figure 48A, B). Overall, these data suggest that the activation domain of PU.1 is important for the induction of IL-9.

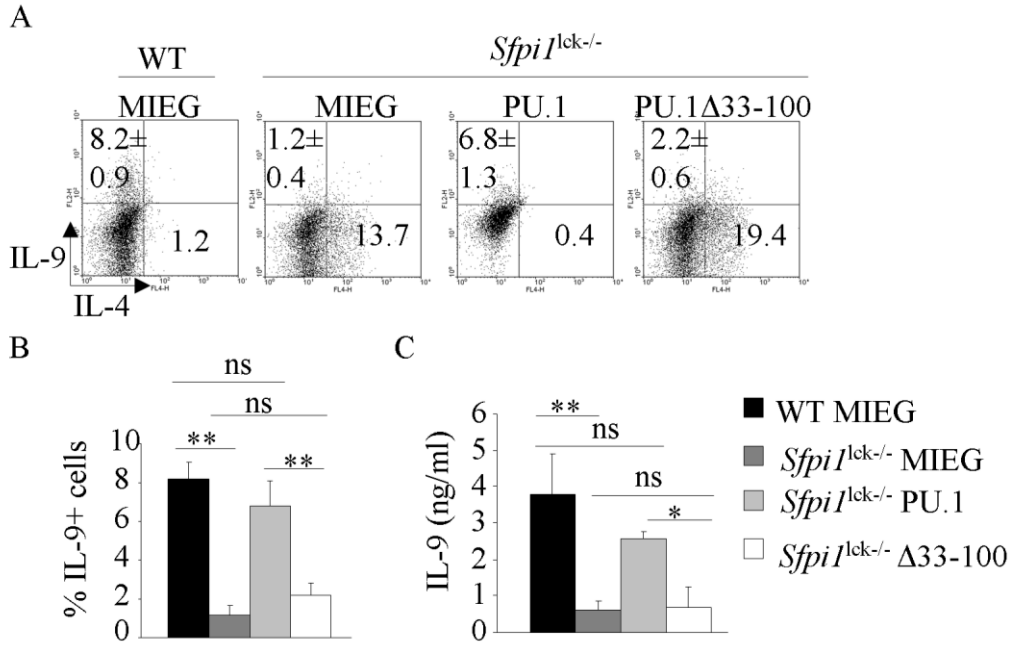

Figure 48: Activation domain mutant of PU.1 fails to rescue IL-9 in PU.1-deficient Th9 cells. (A-C) Naïve CD4+ T cells from wild-type and *Sfpi1*$^{lck-/-}$ mice were cultured under Th9 cell conditions and transduced with control, full-length or PU.1 activation domain mutant-expressing retroviruses. After 5 days, cells were stimulated with PMA and ionomycin for 5 hours and flow cytometry was performed based on eGFP+ cells (A, B) or eGFP+ cells were sorted and re-stimulated with anti-CD3 for 24 hours. Cell-free supernatant was collected to measure IL-9 using ELISA (C). Data are average ± S.D of 3 mice. * $p<0.05$, ** $p<0.01$ (One way ANOVA post hoc Bonferroni correction).

Altered histone acetyltransferase binding at the Il9 locus

As PU.1 can bind to the *Il9* promoter in Th9 cells (Chang et al., 2010), we hypothesized that PU.1 was also recruiting other factors such as histone modifying enzymes including histone acetyltransferases to the *Il9* locus for transactivation. HATs-mediated acetylation of lysine residues reduces electrostatic interactions between histone and the phosphodiester backbone of DNA thereby facilitating the accessibility of *trans*-acting factors to the gene (Strahl and Allis, 2000). To test this, we examined the recruitment of three commonly described HATs; Gcn5, PCAF, and p300 in WT and PU.1-deficient Th9 cells to *Il9* locus by chromatin immunoprecipitation. Significantly diminished association of both Gcn5 and PCAF at the CNS1 and CNS0 region of the *Il9* locus was observed in the absence of PU.1 (Figure 49A). In contrast to a previous report demonstrating that PU.1 recruited p300 to the IgK locus in B cells (Bai et al., 2005), an enhanced binding of p300 at the *Il9* promoter was observed in the absence of PU.1 (Figure 49A). Therefore, PU.1 is required for the association of specific HATs to the *Il9* promoter.

It is possible that PU.1-deficient Th9 cells express a reduced amount of the HAT molecules which would contribute to decreased specific HATs association to the *Il9* promoter. To test this, total cell extracts from WT and PU.1-deficient Th9 cells were prepared for immunoblots for Gcn5, PCAF, and p300. There was no difference in the expression of these HAT molecules between WT and PU.1-deficient Th9 cells (Figure 49B). Overall, these data imply that differential recruitment of the HAT molecules to the *Il9* locus was not because of their altered expression in the absence of PU.1.

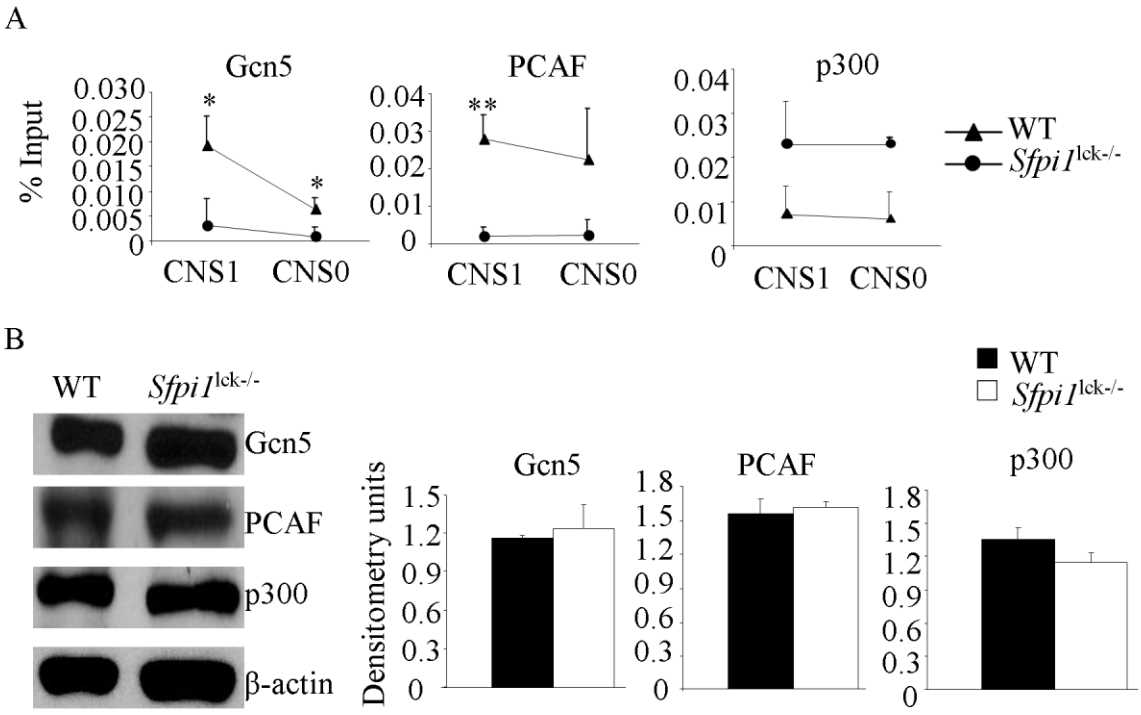

Figure 49: Altered histone acetyltransferase binding at the *Il9* gene. (A) Naïve CD4+ T cells from wild-type and *Sfpi1*$^{lck-/-}$ mice were cultured under Th9 cell conditions for 5 days. Cells were then fixed and ChIP assay was performed for Gcn5, PCAF, and p300 before quantitative PCR for two *Il9* CNS sites. (B) Total cell lysates were prepared from WT and PU.1-deficient Th9 cells and were immunoblotted for Gcn5, PCAF, and p300. β-actin was used as loading control (left panel). Blots were scanned and presented as densitometry units (right panel). Data are average ± S.D of 6-8 mice from 3-4 experiments (A) or representative of two independent experiments (B). * $p<0.05$, ** $p<0.01$.

Altered histone acetylation at the Il9 locus

Since there was differential recruitment of HATs to the *Il9* locus in the absence of PU.1, we wanted to define the functional consequences of altered HAT binding. Previously we demonstrated decreased total H3 acetylation at the *Il9* promoter in PU.1-deficient Th9 cultures (Chang et al., 2010). We therefore examined the acetylation of specific histone residues in WT and PU.1-deficient Th9 cells. Significantly diminished histone acetylation of histone H3K14 was observed at the *Il9* promoter in PU.1-deficient Th9 cells (Figure

50A). Acetylation of H3K9/18, H4K5, H4K8, and H4K16 was diminished significantly at both CNS1 and CNS0 of the *Il9* gene (Figure 50A). In contrast, there was no difference in acetylation of H3K27 and H3K36 between WT and PU.1-deficient Th9 cells (Figure 50A). As a control we compared total H3 and H4 at the *Il9* promoter between WT and PU.1-deficient Th9 cells and observed no difference in total histone content at the locus (Figure 50B). Collectively, these data indicate that PU.1 regulates specific histone acetylation at the *Il9* locus.

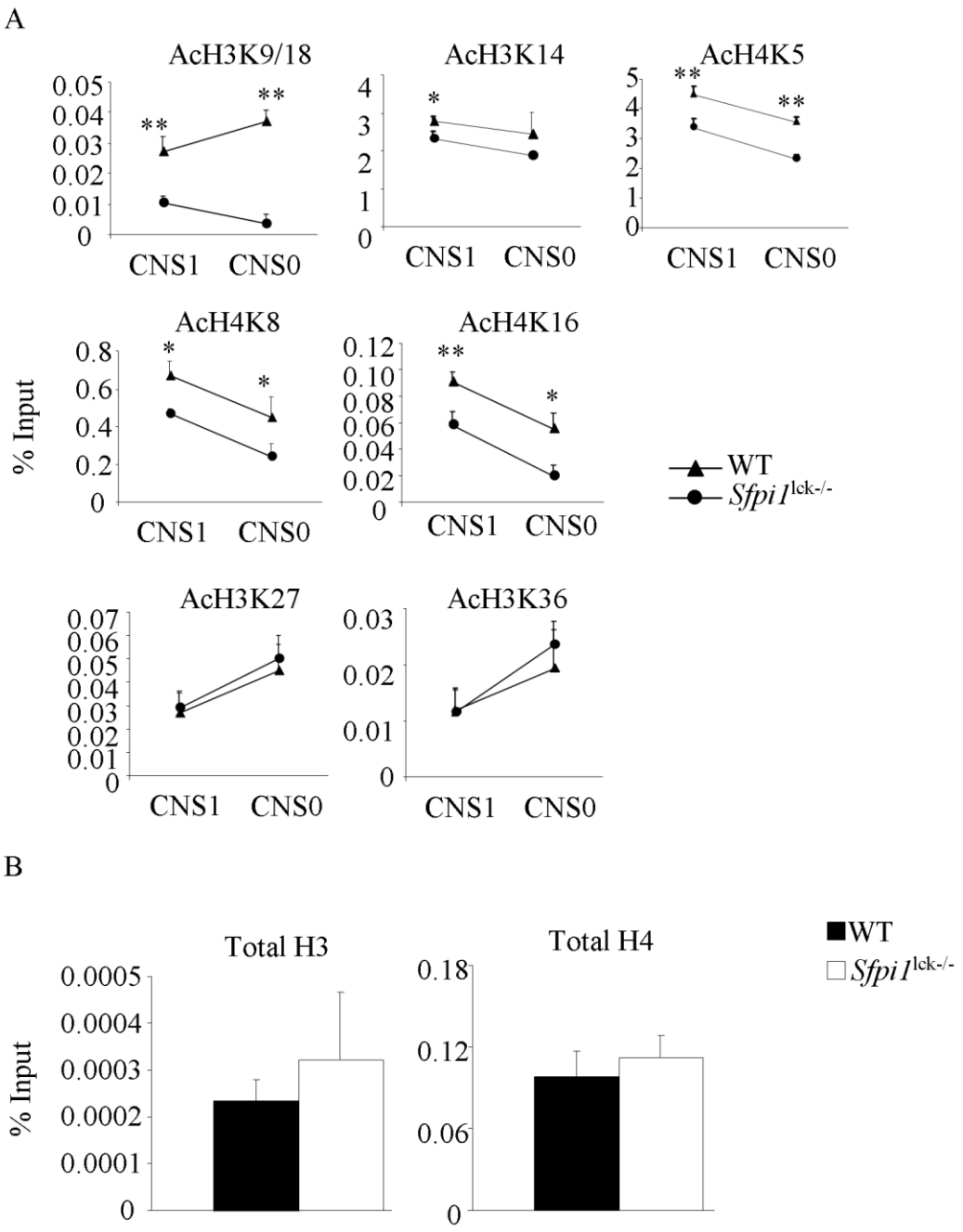

Figure 50: Altered histone acetylation at the *Il9* gene. (A-B), Naïve CD4+ T cells from wild-type and *Sfpi1*$^{lck-/-}$ mice were cultured under Th9 cell conditions for 5 days. On day 5, cells were harvested and subjected to ChIP assay for AcH3K9/18, AcH3K14, AcH4K5, AcH4K8, AcH4K16, AcH3K27, and AcH3K36 before quantitative PCR for *Il9* CNS1 and *Il9* CNS0 (A) or for total H3 and H4 before quantitative PCR for *Il9* CNS1 (B). Data are average ± S.D of 6-8 mice from 3-4 experiments. * $p<0.05$, ** $p<0.01$.

PU.1 associates directly with Gcn5

Since we observed reduced Gcn5 and PCAF recruitment to the *Il9* locus in the absence of PU.1, we wanted to determine if PU.1 was able to directly associate with either of these HAT molecules. To test this, we first examined association of PU.1 with each HAT by co-immunoprecipitation. However, we were unable to observe any significant association. We therefore hypothesized that PU.1 interacts with HATs more efficiently when bound to DNA. To test this, DNA-affinity precipitation assay (DAPA) was performed with nuclear lysates prepared from WT and PU.1-deficient Th9 cells incubated with a biotinylated WT oligonucleotide (oligonucleotide containing a consensus PU.1 binding site) or mutant oligonucleotide (oligonucleotide containing a mutated PU.1 binding site) in the presence of streptavidin-agarose. Following incubation, immunoblot was performed to assess Gcn5 and PCAF precipitation. WT DNA-bound PU.1 was able to precipitate Gcn5 from wild-type lysates (Figure 51A). In contrast, Gcn5 was not precipitated when WT oligonucleotide was used with PU.1-deficient Th9 extracts or the mutated oligonucleotide was used either WT or PU.1-deficient Th9 extracts (Figure 51A). PCAF was not precipitated from wild-type or PU.1-deficient Th9 lysates using either oligonucleotide (Figure 51A). Together, these data suggest that DNA-bound PU.1 is able to directly associate with Gcn5, but not PCAF.

We then wanted to determine if the requirement for the activation domain in the induction of IL-9 correlated with a requirement for the activation domain to interact with Gcn5. To test this, phoenix-GP packaging cells were transfected with control, full-length or Δ33-100 mutant PU.1 plasmids and DAPA was performed. We observed Gcn5

precipitate when cells were transfected with full-length plasmid (Figure 51B). In contrast, the precipitate was not observed when the cells were transfected with the PU.1 activation mutant plasmid (Figure 51B). This suggests that DNA-bound PU.1 requires the activation domain to precipitate Gcn5 which correlates with the requirement of activation domain for inducing IL-9.

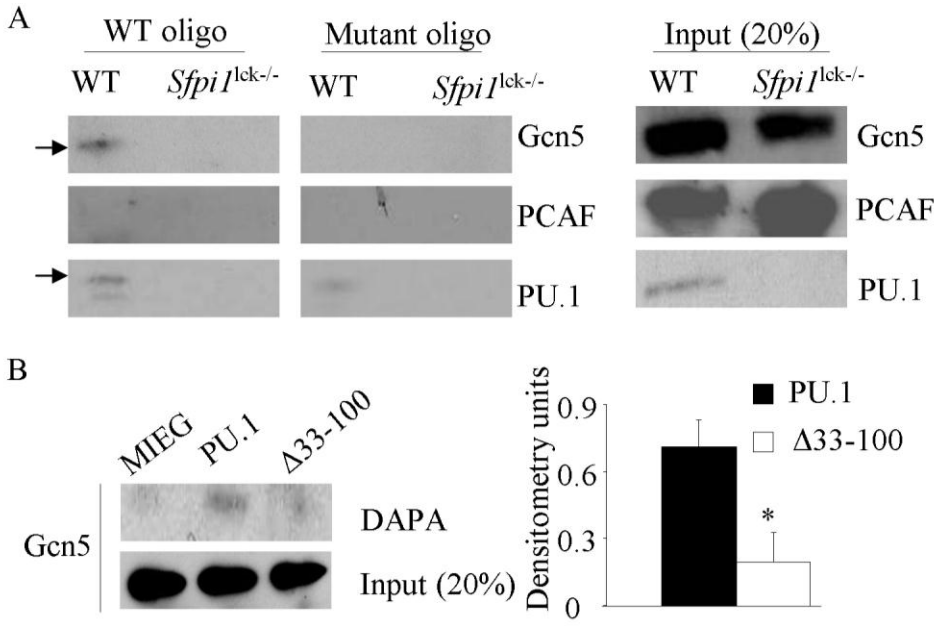

Figure 51: PU.1 associates with Gcn5. (A) Naïve CD4+ T cells from WT and *Sfpi1*$^{lck-/-}$ mice were cultured under Th9 cell conditions for 5 days. On day 5, nuclear lysates were prepared from differentiated cells and were incubated with WT oligonucleotide (oligonucleotide with consensus PU.1-binding site) or mutant oligonucleotide (oligonucleotide with mutated PU.1-binding site) and streptavidin-agarose. Lysates were then subjected to immunoblot for Gcn5, PCAF and PU.1. Input samples were prepared from untreated nuclear lysates. (B) Phoenix-GP packaging cell lines were transfected with control (MIEG), full-length PU.1 (PU.1) or activation domain mutant PU.1 (Δ33-100)-expressing plasmids. Nuclear extracts were then prepared and subjected to DAPA using WT oligonucleotide before immunoblot for Gcn5 (left panel). Densitometry of the immunoblots was performed where the background signal (MIEG) was subtracted from the full-length or activation domain mutant PU.1 immunoblot signal and data are presented as arbitrary densitometry units from 3 experiments (right panel). * $p<0.05$.

Ectopic expression of functional PU.1 restores Gcn5 binding to the Il9 promoter

We next wanted to determine if transduced PU.1 recruited Gcn5 to the *Il9* promoter in the absence of endogenous PU.1. To test this, we examined whether ectopic expression of PU.1 recovered Gcn5 binding to the *Il9* promoter in PU.1-deficient Th9 cells. Developing WT Th9 cells were transduced with control retrovirus while PU.1-deficient Th9 cells were transduced with control, full-length and PU.1 activation mutant-expressing retroviruses. We observed significant decrease in Gcn5 binding to the *Il9* promoter when PU.1-deficient Th9 cells were transduced with control retrovirus compared to WT Th9 cells (Figure 52). However, full-length PU.1 was able to rescue Gcn5 binding in PU.1-deficient Th9 cells (Figure 52). In contrast, cells transduced with the activation domain mutant failed to rescue Gcn5 binding (Figure 52). This data further underlines the importance of the activation domain, which is critical for Gcn5 binding to the *Il9* promoter.

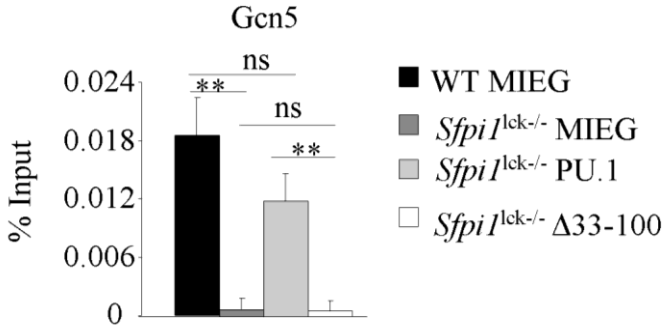

Figure 52: Ectopic expression of PU.1 rescues Gcn5 binding to the *Il9* promoter. Naïve CD4+ T cells from wild-type and *Sfpi1*$^{lck-/-}$ mice were cultured under Th9 conditions and after 48 hours transduced with control, full-length or PU.1 activation domain mutant-expressing retroviruses. After 5 days, eGFP+ cells were sorted and were subjected to ChIP for Gcn5 before quantitative PCR for *Il9* promoter. Data are average ± S.D of 3 mice. ** $p<0.01$ (One way ANOVA post hoc Bonferroni correction).

Gcn5 inhibition leads to diminished Il9 expression

Since PU.1 associates with and recruits Gcn5 binding to *Il9*, we wanted to determine if reduced *Gcn5* expression affects *Il9* gene expression. Gcn5-null embryos die during embryogenesis. Therefore we transfected differentiated Th9 cells with control or *Gcn5*-specific siRNA. Th9 cells transfected with *Gcn5*-specific siRNA resulted in significantly decreased Gcn5 mRNA and decreased Gcn5 binding to the *Il9* promoter (Figure 53A, B). Concomitantly, IL-9 production was significantly reduced when *Gcn5* expression was inhibited (Figure 53C). To determine if altered *Gcn5* expression affected the production of other cytokines we measured the amount of IL-10 and IL-21 produced by Th9 cells with diminished Gcn5 expression. Reduced *Gcn5* expression did not alter the production of IL-10 and IL-21, suggesting that the *Il9* locus was a specific target of Gcn5 (Figure 53C). This was also consistent with our previous data that Th9 production of IL-10 and IL-21 was PU.1-independent (Figure 38D)(Chang et al., 2010). These data suggest that Gcn5 is recruited by PU.1, and is required specifically for the production of IL-9 but not other cytokines produced by Th9 cells.

To define a mechanism for how Gcn5 functionally contributed to *Il9* expression, we examined the binding to PU.1 and IRF4 to the *Il9* promoter following transfection of differentiated Th9 cells with control or *Gcn5*-specific siRNA. Decreased *Gcn5* expression did not affect PU.1 or IRF4 binding to the *Il9* promoter (Figure 53D). To examine if reduced *Gcn5* expression in Th9 cells decreased histone acetylation we assessed histone acetylation of specific lysine residues mediated by Gcn5. Gcn5 acetylates H3K9/18, H4K8, and under some circumstances H4K5 (Kouzarides, 2007;

Kuo et al., 1996; Zhang et al., 1998b). Acetylation of histone H3K9/18, H4K5 and H4K8 at the *Il9* promoter was significantly attenuated when Gcn5 was inhibited (Figure 53E).

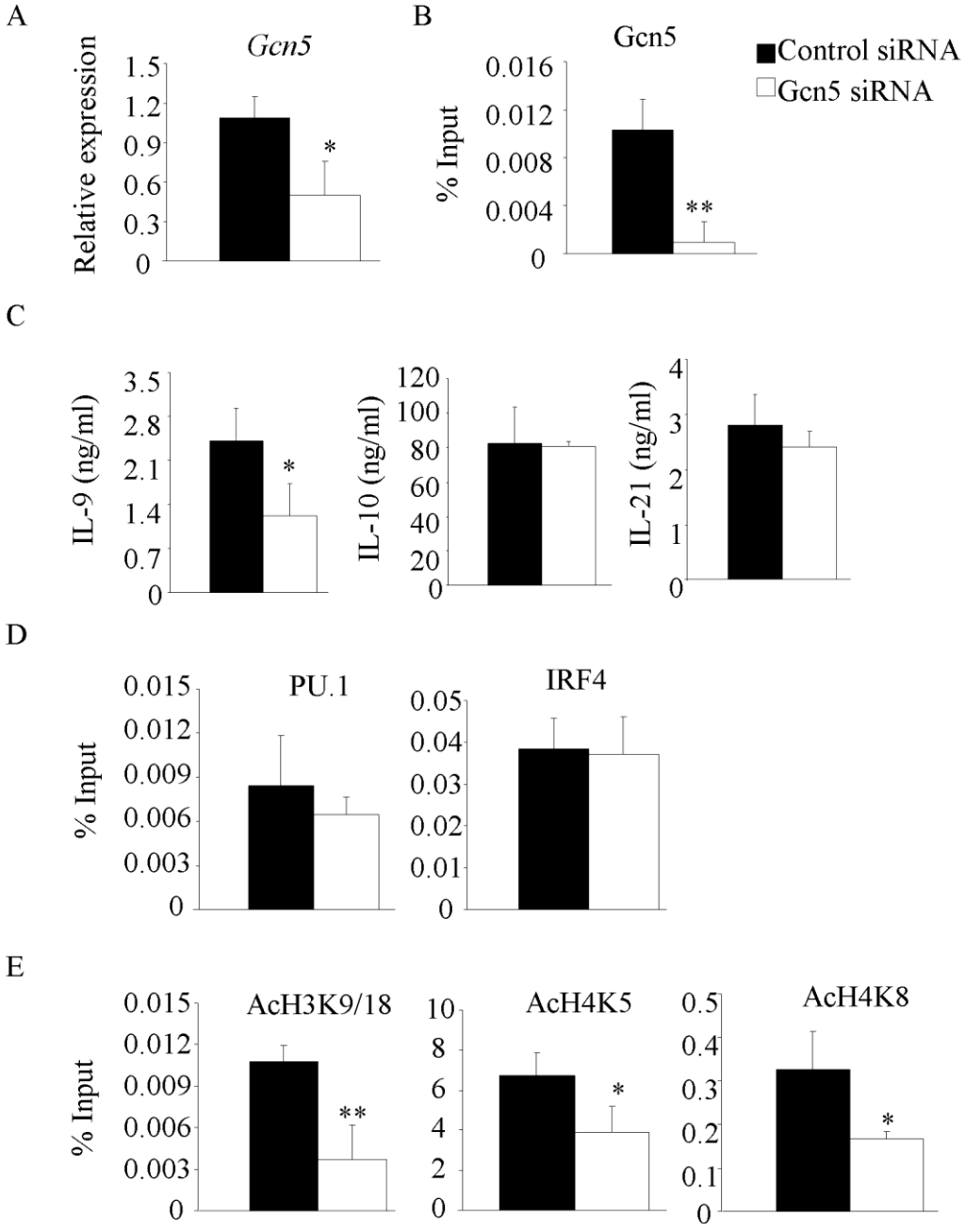

Figure 53: Gcn5 inhibition reduces IL-9 production. (A-C), WT naive CD4+ T cells were cultured under Th9 conditions for 5 days. Differentiated cells were then transfected with control or *Gcn5*-specific siRNA. After transfection cells were rested overnight with IL-2 and were then stimulated with anti-CD3 for 6h for isolating RNA or for 24h to harvest cell free supernatant for ELISA (A, C) or kept unstimulated for ChIP (B). (D-E),

ChIP assay was performed from Th9 cells transfected with Gcn5 siRNA as in (A) for Gcn5, PU.1, IRF4, AcH3K9/18, AcH4K5 and AcH4K8 followed by quantitative PCR with primer for *Il9* CNS1. Data are average ± S.D of 4-6 mice from 2-3 experiments. * $p<0.05$, ** $p<0.01$.

Together, these data suggest that Gcn5 inhibition leads to reduced IL-9 production concomitant with decreased specific histone acetylation of *Il9* although it is not required for PU.1 and IRF4 association to the *Il9* gene. The schematic of PU.1-dependent IL-9 induction in Th9 cells is shown below (Figure 54).

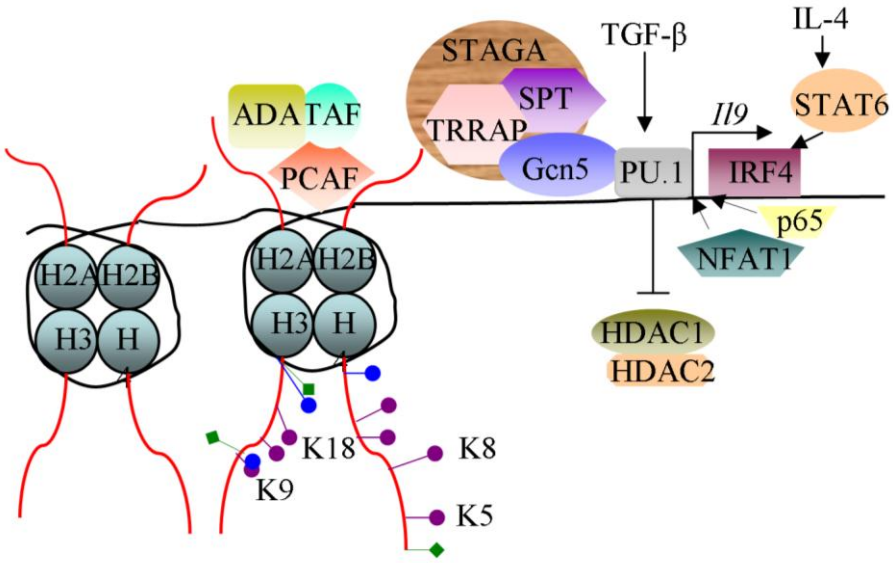

Figure 54: Mechanism of PU.1-dependent IL-9 induction in Th9 cells

PART IV: Alternative pathways of IL-9 regulation

Th2 cells treated with pro-inflammatory cytokines can induce IL-9

The pro-inflammatory cytokines IL-1β, IL-6, and IL-21 have been shown to enhance IL-9 production in human T cell cultures (Putheti et al., 2010; Wong et al., 2010). We therefore wanted to determine if these cytokines can augment IL-9 production in murine T cells. To test this, we cultured Th2 and Th9 cells in the presence or absence of IL-1β, IL-6, and IL-21 individually or in combinations. As demonstrated previously, IL-9 production was higher in Th9 cells than Th2 cells (Figure 55A). However, when Th2 cells were cultured with the combination of three cytokines IL-9 production was enhanced dramatically which was not observed when Th2 cells were cultured with individual cytokines (Figure 55A). There was a modest increase in IL-9 production when Th9 cells were treated with IL-1β, IL-6, and IL-21 compared to standard Th9 conditions (Figure 55A). As PU.1 has been shown to be required for the induction of IL-9 in Th9 cells we wanted to determine if PU.1 was required for IL-9 induction when Th2 cells were treated with the pro-inflammatory cytokines. We observed PU.1-dependent IL-9 production when Th9 cells were cultured under standard IL-9 culture conditions (Figure 55B). However, PU.1 was dispensable for Th2 cell mediated IL-9 production by the pro-inflammatory cytokines (Figure 55B). In the absence of PU.1 there was enhanced IL-9 production when Th9 cells were cultured with IL-1β, IL-6, and IL-21 (Figure 55B).

STAT3 is activated by IL-6, and IL-21. To define if the pro-inflammatory cytokine mediated IL-9 induction was dependent on STAT3 we cultured Th2 and Th9 cells in the presence or absence of IL-1β, IL-6, and IL-21 from WT and *Stat3*$^{CD4-/-}$ mice. As

demonstrated previously STAT3-deficient Th9 cells did not have defective IL-9 production (Figure 55C). STAT3 was also dispensable for IL-9 production when Th9 cells were treated with IL-1β, IL-6, and IL-21 (Figure 55C). In contrast, IL-9 production was severely impaired in the absence of STAT3 when Th2 cells were cultured with IL-1β, IL-6, and IL-21 (Figure 55C). Together these data demonstrate that IL-9 can be alternatively induced when Th2 cells are exposed to an inflammatory cytokine environment, and this process is STAT3-dependent.

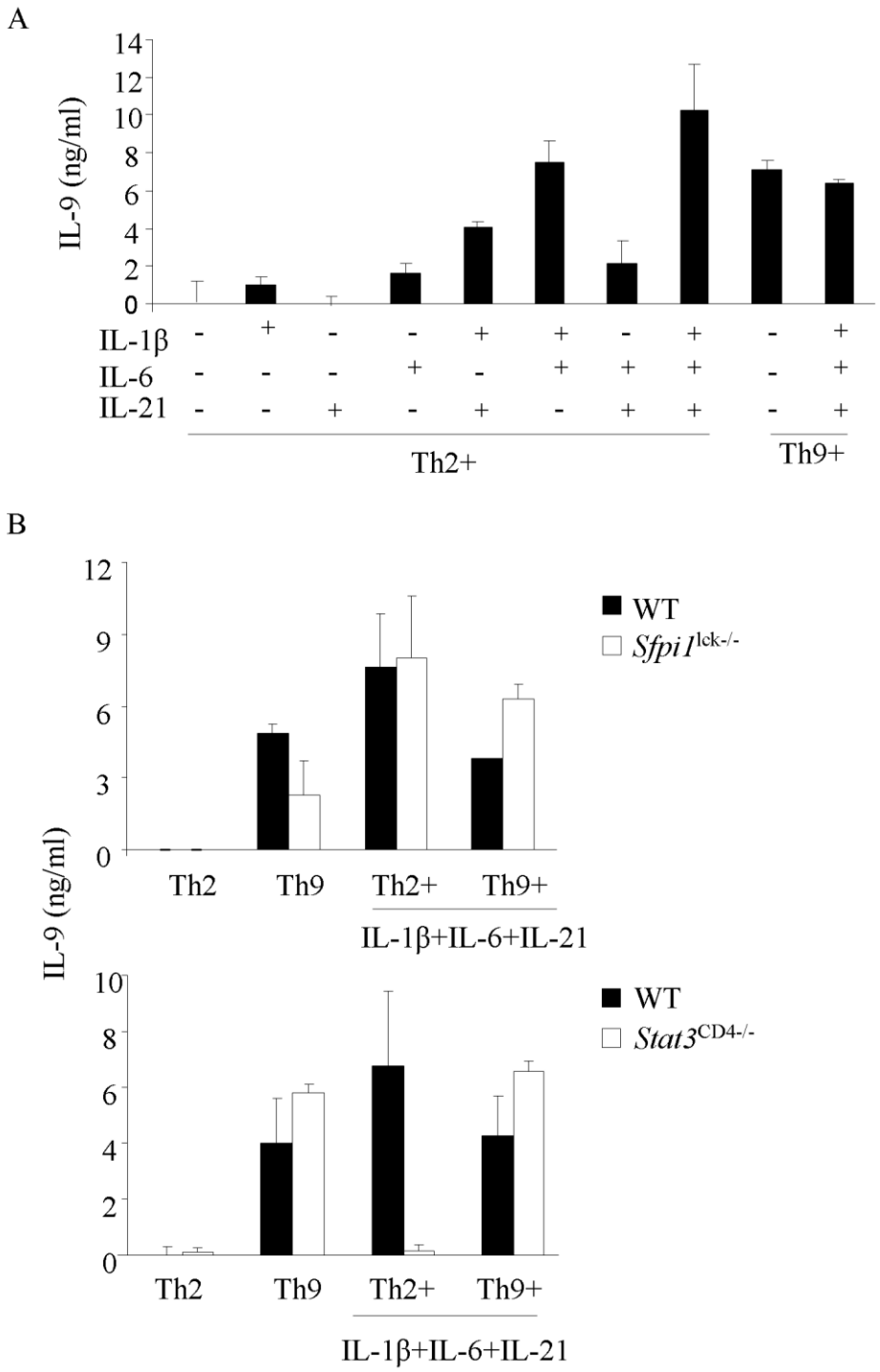

Figure 55: Th2 cells treated with pro-inflammatory cytokines can induce IL-9. (A) WT naïve CD4+ T cells were cultured under Th2 and Th9 cell conditions in the presence or absence of pro-inflammatory cytokines IL-1β, IL-6, and IL-21 individually or in combination for 5 days. On day 5, cells were re-stimulated with anti-CD3 to assess IL-9 production using ELISA. (B) Naïve CD4+ T cells from WT and PU.1-deficient (top panel) or WT and *Stat3*$^{CD4-/-}$ mice (bottom panel) were cultured as in (A). On day 5, cells

were re-stimulated with anti-CD3 for 24 hours to measure IL-9 production using ELISA. Data are replicates representative of 2 independent experiments.

DISCUSSION

One of the focuses of this thesis is characterization of Th9 cells, which predominantly produce IL-9. While defining the phenotype of Th9 cells we observed that other Th cell lineages produce very modest amount of IL-9. Even though Th9 cells are related to Th2, Th17, and Treg cells because of shared cytokines that promote their development, Th9 cells secrete low level of Th2, and Th17-associated cytokines. IL-13, related to IL-4 in both function and signaling pathway cannot replace IL-4 to synergize with TGF-β to produce IL-9. IL-23, the cytokine required for the maintenance of Th17 cells negatively regulates IL-9 production. Type I and type II interferons also inhibit IL-9 production from Th9 cells.

Next we defined the transcription factor network downstream of IL-4 and TGF-β signals in Th9 cells. PU.1, the transcription factor critical for the development of Th9 cells is downstream of the TGF-β signal. Even though both STAT6 and STAT3 are activated during Th9 cell differentiation only STAT6 is required for Th9 cell development. IRF4, another transcription factor required for Th9 cells is downstream of IL-4/STAT6 pathway. We also show that Th2-associated transcription factors, including GATA3, c-Maf, are IL-4/STAT6-dependent in Th9 cells; however, they inhibit IL-9 production. We further observed that Th1-specific transcription factors T-bet and Runx3 inhibit IL-9 production from Th9 cells.

As PU.1 is required for the development of Th9 cells we defined the PU.1-dependent mechanism for induction of IL-9. We observed that PU.1 is required for differential

recruitment of specific histone acetyltransferases to the *Il9* locus in Th9 cells. Functionally this leads to PU.1-dependent specific histone modifications of the *Il9* gene. Furthermore, we observed that PU.1 is able to form a complex with Gcn5, one of the HATs. Reduced Gcn5 expression resulted in attenuated IL-9 production.

Finally, we provide evidence of an alternative pathway for IL-9 induction. Th2 cells cultured in the presence of IL-1β, IL-6, and IL-21 can promote PU.1-independent but STAT3-dependent induction of IL-9.

PART I: Defining the phenotype of IL-9-secreting T cells

Naïve T cells primed with TGF-β and IL-4 produce IL-9

Renewed interest had been generated in the biology of the understudied cytokine IL-9. The work on IL-9 had been mostly focused on its role as a pro-inflammatory cytokine and as T cell and mast cell growth factor. IL-9 is produced in different Th2-mediated inflammatory models. Recent studies have demonstrated that T cells cultured in the presence of TGF-β and IL-4 can secrete IL-9 and are known as Th9 cells (Dardalhon et al., 2008; Veldhoen et al., 2008b). IL-4 was able to block the generation of TGF-β-induced Tregs and promote IL-9 and IL-10 production (Dardalhon et al., 2008). TGF-β was able to reprogram polarized Th2 cells into IL-9-secreting T cells (Veldhoen et al., 2008b). As these published reports suggested the presence of an additional member in Th subset, we wanted to optimize and characterize the phenotype of Th9 cells in detail. We observed that naïve CD4+ T cells cultured with TGF-β and IL-4 can produce high amounts of IL-9 (Figure 11A). IL-4 is required for Th2 and Th9 cell differentiation, while TGF-β is associated with Th9, Th17 and Treg cell differentiation. Previous studies have documented the ability of Th17 and Treg cells to produce IL-9 (Elyaman et al., 2009; Lu et al., 2006). One study has shown that TGF-β and IL-9 can prime T cells into IL-17-secreting cells (Elyaman et al., 2009). We observed that Th2, Th17, and Tregs produce very low to undetectable levels of IL-9 (Figure 11B-H). In contrast, Th9 cells secrete significantly reduced amount of IL-4, IL-5, IL-13 and IL-17; cytokines produced by Th2 and Th17 cells (Figures 11E-F, 12). Even though TGF-β was required for the differentiation of Th9 cells, Foxp3 was expressed at a reduced level in Th9 cells compared to Tregs (Figure 11G). We also observed that the amount of IL-10 produced by

Th9 cells is lower than Th2 cells, contrary to published reports (Dardalhon et al., 2008; Veldhoen et al., 2008b)(Figure 13). This observation can be attributed to the variability in differentiation conditions and the length of differentiation used in different studies. Our studies also demonstrate that IL-9 and IL-10 are not coordinately regulated in Th9 cells (Figure 14). This observation is consistent with our previous published report indicating that in Th2 cells IL-9 and IL-10 are reciprocally regulated (Ahyi et al., 2009). Together, these data suggest that TGF-β and IL-4 prime T cells to produce predominantly IL-9; however, Th9 cells have the ability to secrete reduced amounts of other cytokines.

IL-4 is indispensible for Th9 cell differentiation

IL-4 shares functions and signaling pathways with another Th2 cytokine, IL-13. We therefore wanted to determine if IL-4 could be substituted by IL-13 to synergize with TGF-β to induce IL-9. We observed that IL-13 could not synergize with TGF-β to produce IL-9 (Figure 16). As Th17 cells are the only Th subset to express functional *Il13Rα1* (Newcomb et al., 2009) we wanted to determine if *Il13Rα1* was expressed when naïve CD4+ T cells were cultured with TGF-β and IL-13. We could not detect *Il13Rα1* when T cells were primed with IL-13 and TGF-β (Figure 16B) suggesting that lack of functional *Il13Rα1* expression was one of the reasons of naïve CD4+ T cells not being able to produce IL-9 when cultured with TGF-β and IL-13.

PART II: Defining the STAT6-dependent Th9 transcriptional network

Multiple STAT proteins are required for T effector subset development

There has been a shift from the concept that one STAT molecule is required for one T effector subset, to one where multiple STAT molecules control the expression of one T effector subset. Initially STAT4 was shown to be critical for Th1 cell development; while STAT6 was required for the development of Th2 subset (Kaplan et al., 1996a; Kaplan et al., 1996b; Shimoda et al., 1996; Thierfelder et al., 1996). However, this paradigm was modified when STAT1 was shown to be important for Th1 cell development (Afkarian et al., 2002). STAT5 was documented to be important for Treg cell development (Burchill et al., 2007; Yao et al., 2007), after STAT5 was demonstrated to function with STAT6 for Th2 cell development (Cote-Sierra et al., 2004; Zhu et al., 2003). The requirement of STAT3 in addition to STAT6 in the development of Th2 cells further shifted from the paradigm of one STAT-one phenotype to multiple STAT-one phenotype (Stritesky et al., 2011). STAT3 was shown to be critical for the development of Th17 cells and STAT4 was reported to be important for the memory Th17 responses (Mathur et al., 2007). Therefore, in this thesis we wanted to define the requirement of STAT proteins in Th9 cell development.

Differential requirement of STAT proteins in the development of Th9 cells

STAT6 is required for the development of Th2 cells. However, STAT3 also cooperates with STAT6 to promote Th2 cell development (Stritesky et al., 2011). We therefore wanted to define the role of both STAT6 and STAT3 in Th9 cell development. Both STAT6 and STAT3 were activated during Th9 cell differentiation although the

percentages and the mean fluorescence intensity of STAT3-positive Th9 cells were slightly lower than Th2 cells (Figure 20). Consistent with published reports we observed complete impairment of IL-9 production in the absence of STAT6 (Figure 21A, B). This suggests that the IL-4/STAT6 pathway is critical for Th9 cell development. Furthermore, we observed the requirement for the STAT6 co-factor PARP14 in promoting Th9 cell development, consistent with the importance of STAT6 (Figure 22). In contrast, although STAT3 was activated throughout Th9 cells differentiation it was dispensable for the development of Th9 cells (Figure 21C, D). STAT3 could be activated by IL-4 in some conditions (Orchansky et al., 1999), yet, STAT3 remained activated in STAT6-deficient Th2 cells (Stritesky et al., 2011). Similar to Th17 cells, Th9 cells also have the ability to produce IL-21 (Kaplan et al., 2011). IL-21 produced by Th17 cells is STAT3-dependent (Wei et al., 2007). In Th17 cells STAT3 can bind to the *Il21* promoter (Wei et al., 2007). Therefore, STAT3 might be regulating the production of IL-21 but not IL-9 in Th9 cells. We also show that IL-9 can be generated when Th2 cells are primed with IL-6, IL-21 and IL-1β (Figure 55). IL-6 and IL-21 both activate STAT3, and the absence of STAT3 almost abolished IL-9 production when T cells are cultured with IL-4, IL-6, IL-21, and IL-1β (Figure 55). Therefore, STAT3 could play an important role in an alternative mode of IL-9 induction. This might be relevant *in vivo,* where immune cells are exposed to a plethora of cytokines and STAT3 could drive Th2 cells to produce IL-9. The role of STAT5 has not been examined in Th9 cell development. As IL-2-activated STAT5 plays a key role in Th2 cell differentiation and IL-2 is essential for IL-9 production by CD4+ T cells we speculate STAT5 might play an important role to

promote Th9 cell development (Cote-Sierra et al., 2004; Fung et al., 2005; Schmitt et al., 1994; Zhu et al., 2003).

STAT6 targets in Th9 cells

Since IL-4/STAT6 signaling was required for the development of Th9 cells, we wanted to define if STAT6 regulates IL-9 by directly binding to the *Il9* gene. When the *Il9* gene was scanned, potential STAT binding sites were observed at CNS1 and CNS0 regions (Figure 23A). STAT6 binding was observed at the *Il9* promoter (Figure 23B); however, the binding was very low when compared to STAT6 binding to the *Maf* or *Irf4* promoters. STAT6 binding to CNS0 was even lower than binding to the *Il9* promoter (Figure 23B). This observation was consistent with the finding that STAT6 binding was undetectable in the *Il9* locus in Th2 cells (Wei et al., 2010). Importantly, STAT6 could be binding to regions of DNA outside of CNS1 and CNS0. Thus, although STAT6 is required for Th9 cell development, it does not bind proximal regions of the *Il9* gene.

STAT6 is required for the expression of *Gata3, Irf4*, and *Maf* in Th2 cells (Veldhoen, 2010; Zhu et al., 2010). Therefore we speculated that GATA3, IRF4, and c-Maf are targets of STAT6 in Th9 cells. In the absence of STAT6 signal in Th9 cells *Gata3, Irf4*, and *Maf* expression was attenuated (Figures 24A, 29). STAT6 is not only required for *Irf4* expression in Th9 cells but also important for IRF4 binding to the *Il9* gene. The absence of STAT6 results in diminished IRF4 recruitment at the *Il9* locus which could be due to decreased *Irf4* expression (Figure 24B). Therefore, IRF4 a transcription factor required for Th9 cells development is downstream of STAT6 signal. However, IRF4

alone was not sufficient to recover IL-9 production from STAT6-deficient Th9 cells suggesting that additional factors downstream of IL-4/STAT6 pathway are required to induce IL-9 (Figure 25).

The role of IL-4 could be to suppress inhibitory molecules in Th9 cells. IL-4 had been previously shown to repress *Foxp3* expression (Chapoval et al., 2010; Mantel et al., 2007; O'Malley et al., 2009). To further define the function of IL-4 we cultured Th9 cells in the presence of increasing TGF-β doses with a constant dose of IL-4. We observed IL-4 reduced Foxp3-positive cells in Th9 cells compared to Treg cells (Figure 15). Foxp3-expressing Treg cells had barely detectable levels of IL-9-secreting cells while Th9 cells had higher IL-9-producing cells (Figure 15), implying Foxp3 plays an inhibitory role in the development of Th9 cells. We observed ectopically expressed Foxp3 repressed IL-9 production from Th9 cells (Figure 34). That IRF4 over expression failed to reduce *Foxp3* expression was probably one of the reasons for its inability to recover IL-9 production from STAT6-deficient Th9 cells (Figure 25C). Therefore, the IL-4 signal regulates the expression of both positive and negative regulators of IL-9 production and both IL-4 and TGF-β are required for the development of IL-9-producing cells. IL-4-induced Th2 cells where endogenous TGF-β is blocked produce very low amounts of IL-9. Similarly, in TGF-β-induced Treg cells, where IL-4 is not added, there are undetectable amounts of IL-9 produced.

Th2-associated transcription factors negatively regulate IL-9 production

Since Th2-specific transcription factors were dependent on the STAT6 signal in Th9 cells we wanted to determine the role of each of these factors. *Gata3, Maf,* and *Irf4* was expressed throughout Th9 differentiation, with *Gata3* expressed higher in Th2 cells, while *Maf* and *Irf4* were expressed more in Th9 cells (Figure 28E). When either GATA3 or Maf was transduced in Th9 cells, the percentages of IL-9-producing cells were diminished (Figure 30). However, neither of the factors enhanced IL-4 production from Th9 cells (Figure 30). Even in the absence of endogenous IL-4 in Th9 cells, GATA3 or Maf can reduce IL-9-producing cells similar to the decrease in WT Th9 cells (Figure 30). Therefore neither GATA3 nor c-Maf directly regulates the *Il9* gene. It would be interesting to know how siRNA-mediated reduced expression of GATA3 and c-Maf in Th9 cells affect IL-9 production. GATA3 was shown to be required for the development of Th9 cells using conditional mutant cells (Dardalhon et al., 2008). It can be speculated that GATA3 might act as an intermediate in the IL-4/STAT6-dependent reduction of Foxp3 as is demonstrated for GATA3 in Treg cells (Mantel et al., 2007); however, recent studies have indicated that GATA3 is required for the function of Treg cells (Wang et al., 2011; Wohlfert et al., 2011). Whether GATA3 is temporally required early in Th9 differentiation has not been examined in detail. GATA3 might help prevent Th9 cells from switching to a Th17 phenotype, even though one study has demonstrated that cells from GATA3-transgenic mice can induce IL-17 production (van Hamburg et al., 2008). However, cells with transgenic GATA3 expression are protected from EAE. It is possible that ectopic GATA3 expression in Th9 cells enhances IL-17-secreting cells. c-Maf might be involved in the regulation of other genes in Th9 cells. Th9 cells secrete IL-21 and as

IL-21 is a c-Maf target in other Th lineages, there might be a role for c-Maf in promoting IL-21 production in Th9 cells. c-Maf has been shown to regulate IL-10 production in Th17 cells (Xu et al., 2009) and might be important for IL-10 production by Th9 cells as well. Whether GATA3 or c-Maf over expression leads to reduction in PU.1 or IRF4, the transcription factors required for Th9 cell development, has not been examined carefully. There could also be kinetic effects of the expression of each factor during T helper cell differentiation that might be obscured by ectopic expression during *in vitro* culture. Although we did not observe significant differences in expression of these factors until the final days of Th2 and Th9 differentiation, it is still possible that a balance of these factors, at particular times during the differentiation process, is critical for appropriate IL-9 production.

Th1-associated transcription factors repress IL-9 production

IL-4 is a negative regulator of T-bet and Runx3, transcription factors required for Th1 cell differentiation. Therefore we hypothesized that T-bet and Runx3 are negative regulators of IL-9 production. We observed ectopic Runx3 and T-bet expression diminished the production of IL-9-secreting cells (Figure 32A). In contrast, T-bet-deficient and Runx3-deficient Th9 cells have enhanced IL-9 production (Figure 32B). T-bet and Runx3 probably contribute to attenuation of *Il9* expression in Th1 cells, similar to their effect at the *Il4* locus. This could be through both direct and indirect mechanisms. In previously generated ChIP-seq datasets of Runx3/CBF-β and T-bet binding in Th1 cells (Yagi et al., 2010), both factors bind directly to the *Il9* gene; T-bet at a major peak around -22.5 kb and smaller peaks between +7 to +9 kb, and Runx3 at a major peak around +5

kb and minor peaks at -25, -17, -7 and +11 kb. Therefore they likely bind to the *Il9* locus in Th9 cells. They might also have indirect effects by regulating other transcription factors required for IL-9 production. Runx3, however might not be responsible for the lack of IL-9 production in *Stat6*-/- Th9 cultures, since expression of *Runx3* was decreased in the absence of STAT6 (Figure 33). Another contributing factor to the failure of IRF4 to rescue IL-9 in STAT6-deficient Th9 cells was probably of its ability to modestly affect *Tbx21* expression (Figure 25C). Increased expression of *Tbx21* in the absence of STAT6 suggests that the IL-4/STAT6 signal is required to limit T-bet-mediated inhibition in Th9 cells. Overall, IL-4/STAT6 signal regulates the expression of several transcription factors required for the induction of IL-9.

STAT6-dependent transcriptional network in Th9 cells: Summary

We propose the following model of the STAT6-dependent transcriptional network in Th9 cells (Figure 35). Downstream of IL-4/STAT6 pathway is IRF4 which promotes Th9 cell development. However, other factors downstream of STAT6 signal are required as IRF4 alone is not sufficient to recover IL-9 in the absence of STAT6. The IL-4/STAT6 pathway is also critical to inhibit TGF-β-induced *Foxp3* expression, and *Tbx21* expression to promote IL-9 production.

PART III: Gcn5 is required for PU.1-dependent IL-9 production in Th9 cells

The transcription factor PU.1 promotes the development of Th9 cells

PU.1, an ETS family transcription factor, regulates Th2 heterogeneity. PU.1 is specifically expressed in the IL-4^{lo} subpopulation of Th2 cells and ectopic PU.1 expression in Th2 cells attenuates the production of Th2-specific cytokines partly by interacting and interfering with the function of GATA3 and IRF4 (Ahyi et al., 2009; Chang et al., 2005). In contrast, reduced PU.1 expression enhances Th2 cytokine production (Chang et al., 2009; Chang et al., 2005). We observed PU.1 was abundantly expressed in Th9 cells (Figure 36), and speculated that the specific deletion of PU.1 in T cells would have defect in IL-9 production. Impaired IL-9 production was observed in the absence of PU.1 (Figure 38). There was a modest increase in IL-4 production (Figure 38A). Consistent with this finding, ectopic expression of PU.1 in Th9 cells enhanced the percentages of IL-9-secreting cells (Figure 39A, B). In this thesis we wanted to define the mechanism of PU.1-dependent IL-9 induction in Th9 cells.

TGF-β regulates Sfpi1 expression

In the previous sections, we defined the downstream factors of IL-4 signal required for Th9 cell development. We then wanted to determine the factors downstream of TGF-β signal important for promoting Th9 cells. As we established PU.1 as one of the key transcription factors critical for Th9 cell development we wanted to determine the cytokines regulating the expression of PU.1. We observed that *Sfpi1* was expressed higher in Treg cells compared to Th9 cells (Figure 41A). As TGF-β is required for the differentiation of both Th9 and Tregs, we speculated that TGF-β might be regulating

PU.1. When activated T cells were cultured with increasing doses of TGF-β in the presence or absence of constant IL-4, an increase in *Sfpi1* expression was observed (Figure 41B). However, when TGF-β was kept constant and IL-4 doses were varied there was no IL-4-dose-dependent alteration in *Sfpi1* expression (Figure 41B). Consistent with this finding we observed *Sfpi1* expression and PU.1 binding to the *Il9* gene was unaffected in the absence of STAT6 (Figure 41D, E). Moreover, ectopic PU.1 expression in Th2 cells can augment IL-9-secreting cells while decreasing IL-4-secreting cells (Chang et al., 2010). These results suggest that IL-9-secreting cells arise when there is a balance between the TGF-β signal that induces *Sfpi1* expression, and the IL-4 signal that limits *Foxp3* expression (Figures 15, 41). When endogenous TGF-β production is blocked in Th2 cells it reduces *Sfpi1* expression concomitant with a reduction in *Il9* gene expression (Figure 42). This suggests endogenous TGF-β in Th2 cultures can promote IL-9 production and TGF-β regulates PU.1 expression.

HDAC inhibitor-mediated IL-9 induction is PU.1-dependent

Global gene expression results from a balance between histone acetylation and deacetylation regulated by the group of enzymes HATs and HDACs, respectively. Chromatin structure is dependent on strong interactions between positively charged lysine residues of histone and negatively charged phosphate groups of DNA. Acetylation of histone lysine residues by HATs diminishes the electrostatic interaction between histone and DNA thereby allowing accessibility of transcription factors to a gene (Strahl and Allis, 2000). HDACs reverse this process thereby restricting transcription factors access to a gene. However, HDAC inhibition reverses repressive chromatin and induces

the expression of a subset of genes (Luo and Dean, 1999; Van Lint et al., 1996). HDAC activity has been demonstrated to regulate T cell development and function (Shakespear et al., 2011). T cell-specific deletion of HDAC1 results in increased IL-4 production from *in vitro* cultured Th2 cells and enhanced allergic airway inflammation (Grausenburger et al., 2010). HDAC2 has been shown to be required for B cell proliferation (Yamaguchi et al., 2010). We observed that HDAC association at the *Il9* promoter was enhanced in the absence of PU.1 which could contribute to reduced IL-9 production in the absence of PU.1 (Figure 43). In contrast, when Th9 cells were treated with the HDAC inhibitor TSA, IL-9 production was enhanced (Figure 44). However, TSA treatment did not alter the expression of other cytokines produced by Th9 cells including IL-10 and IL-21, suggesting that in Th9 cells the *Il9* locus was a specific target of HDAC inhibition (Figure 44B). TSA treatment enhanced the expression of *Sfpi1* but not *Irf4* indicating that PU.1 might be important TSA-mediated IL-9 induction (Figure 45A, B). We observed that in the absence of PU.1, TSA did not induce IL-9 production (Figure 45C). TSA-induced *Il9* expression was correlated with enhanced histone acetylation at the *Il9* gene and increased PU.1 and IRF4 binding at the *Il9* promoter (Figure 46). A recent study has demonstrated that functional cooperation of NFAT1 and NF-κB augments IL-9 transcription (Jash et al., 2012). In another report it was documented that the binding sites of NFAT and GATA3 at human *IL5* promoter were critical for TSA-mediated enhanced *IL5* expression (Han et al., 2007). Therefore the presence of NFAT binding sites in the *Il9* gene (Perumal and Kaplan, 2011) could be crucial for TSA-mediated enhanced *Il9* gene transcription. It is also possible that TSA could enhance *Il9* promoter activity as one report has demonstrated that TSA treatment enhanced CD154 promoter activity (Pham et

al., 2005). It had been shown that TSA increased RNA polymerase II recruitment to the hLHR gene promoter (Zhang and Dufau, 2002). TSA can potentially enhance the specific recruitment of RNA polymerase II to the *Il9* promoter thereby enhancing *Il9* gene transcription. Though PU.1 is regulated by HDAC inhibition, the effects might be cell-type specific. HDAC inhibition in macrophage and pro-T cell lines resulted in loss of PU.1 expression (Laribee and Klemsz, 2001). Consistent with our observations, the HAT inhibitor, curcumin, reduced PU.1 expression in Th9 cultures (Ramming et al., 2012). Therefore HDAC inhibition may enhance *Il9* transcription by several mechanisms.

Structural requirements of PU.1 to induce IL-9

PU.1, like most transcription factors, consists of multiple functional domains (Figure 47A). It regulates the expression of myeloid and lymphoid genes required for lineage development and function. Multiple functional domains have been identified at the N-terminus of PU.1 (Hagemeier et al., 1993; Klemsz and Maki, 1996; Kominato et al., 1995; Shin and Koshland, 1993). The ETS DNA-binding domain is located at the C-terminus and mediates DNA binding and interactions with multiple proteins (Karim et al., 1990; Klemsz et al., 1990; Nagulapalli et al., 1995). The PEST domain, enriched in proline, glutamic acid, serine and threonine has been shown to control protein stability. The PEST domain has not been generally associated with gene transactivation (Erman and Sen, 1996; Klemsz and Maki, 1996; Kominato et al., 1995). Th2 cells over expressed with PU.1 activation domain or the PEST domain mutant did not alter cytokine production significantly compared to Th2 cells transduced with full-length PU.1; however, PU.1 DNA-binding mutant has reduced ability to regulate Th2 cytokine

production (Chang et al., 2005). PU.1 had been reported to interact and interfere with GATA3 at the *Il4* locus to regulate Th2 heterogeneity (Chang et al., 2005). We had demonstrated the ability of PU.1 to bind to the *Il9* promoter directly while the DNA-binding mutant of PU.1 results in impaired ability to bind to the *Il9* gene (Figure 41E)(Chang et al., 2010) When Th9 cells were ectopically expressed with full-length PU.1 there were enhanced percentages and mean fluorescence intensity of IL-9-secreting cells (Figure 47B, C). In contrast, Th9 cells transduced with either activation domain mutant or DNA-binding domain mutant failed to augment IL-9 production (Figure 47B, C). We observed a modest increase in the percentages of IL-9-secreting cells when Th9 cells were transduced with the PEST domain mutant (Figure 47B, C) which is consistent with a published report showing that the PEST domain plays a negative role in monocyte-specific gene expression (Nishiyama et al., 2004). That the activation domain was required for IL-9 expression was further demonstrated when the PU.1 activation domain mutant had a significantly attenuated ability to recover IL-9 in PU.1-deficient Th9 cells, while the full length PU.1 was able to rescue IL-9 production to a level similar to WT Th9 cells transduced with control retrovirus (Figure 48). Therefore, PU.1 requires multiple domains to regulate the Th9 phenotype.

Altered HAT binding and histone acetylation at the Il9 locus

Since PU.1 binds to the *Il9* promoter in Th9 cells, we speculated one functional role of PU.1 is possibly recruiting other factors such as histone modifying enzymes including HATs to the *Il9* locus. Even though PU.1 is a critical factor in cellular differentiation, little work has extensively focused on the interaction of PU.1 and HAT molecules. A

limited number of studies suggested that PU.1 interacts with HATs including p300, Tip60, and MOZ (Bai et al., 2005; Hlubek et al., 2001; Katsumoto et al., 2006). Contrary to the published report that PU.1 recruited p300 to the Igκ locus in B cells, we observed enhanced p300 association at the *Il9* promoter in the absence of PU.1, suggesting that other transcription factors may mediate p300 recruitment in Th9 cells (Bai et al., 2005)(Figure 49A). However, the association of Gcn5 and PCAF to the *Il9* gene was attenuated in the absence of PU.1 (Figure 49A). Therefore in the absence of PU.1 there was differential recruitment of HAT molecules; despite the expression of these HATs being unaffected by the absence of PU.1 (Figure 49B). There could be potentially differential recruitment of other HATs including CBP, MOZ and Tip60 at the *Il9* locus mediated by PU.1.

The functional consequences of altered HAT binding to *Il9* gene could be altered histone acetylation at the *Il9* locus. The *Il9* locus in Th9 cells is characterized by increased acetylation of total histone H3, histone H4 and specific histone H3K9/18 (Chang et al., 2010). PU.1-deficient Th9 cells had reduced H3 but unchanged H4 acetylation than WT Th9 cells (Chang et al., 2010). The specificity of PU.1 effects on histone acetylation was evident in significantly attenuated acetylation of H3K9/18, H3K14, H4K5, H4K8 and H4K16 in PU.1-deficient Th9 cells, while acetylation of histone H3K27 and H3K36 remained unchanged (Figure 50). In addition to acetylation, histone lysine residues undergo methylation as well. It is possible that PU.1 can also mediate histone lysine methylation. PU.1 has been shown to repress GATA1 to block erythroid differentiation program by recruiting histone methyltransferase Suv39h, thereby causing methylation of

histone H3K9 (Stopka et al., 2005). Therefore PU.1 can potentially recruit different histone methyltransferases at the *Il9* locus. However, histone methylation of H3K27 was PU.1-independent (Chang et al., 2010). Thus, PU.1 regulates specific histone modifying enzyme association and histone modifications at the *Il9* locus.

Gcn5 is required for the induction of IL-9 in Th9 cells

Since the recruitment of Gcn5 and PCAF to the *Il9* gene was attenuated we wanted to define if either of these factors were required for IL-9 expression. We observed DNA-bound PU.1 was able to associate with Gcn5 but not PCAF suggesting that PU.1 specifically associates with Gcn5 (Figure 51A). The requirement for the activation domain for IL-9 induction was also correlated with a requirement for the activation domain to interact with Gcn5 as DNA-bound PU.1 activation mutant failed to precipitate Gcn5 (Figure 51B). The activation domain of PU.1 was also required to recruit Gcn5 binding to the *Il9* promoter as PU.1-deficient Th9 cells transduced with activation domain mutant failed to recover Gcn5 binding (Figure 52).

Gcn5 functionally contributed to *Il9* expression as IL-9 production was diminished when *Gcn5* expression was reduced (Figure 53A-C). However, attenuated *Gcn5* expression did not affect IL-10 and IL-21 production implying that the *Il9* locus in Th9 cells is a specific target of Gcn5 (Figure 53C). Inhibition of Gcn5 expression did not affect PU.1 or IRF4 binding to the *Il9* gene; however, there was significantly reduced acetylation of H3K9/18, H4K5, and H4K8 the target lysine residues of Gcn5 (Figure 53D, E). It is also possible

that Gcn5 inhibition can also lead to reduced acetylation of H3K14 and H4K16, the other known lysine residues acetylated by Gcn5 (Struhl, 1998).

Gcn5 is required for PU.1-dependent IL-9 production in Th9 cells: Summary

We propose the following mechanism for the requirement of Gcn5 in PU.1-dependent IL-9 induction (Figure 54). In developing Th9 cells PU.1 binds to the *Il9* promoter leading to complex formation and recruitment of Gcn5-containing chromatin remodeling complexes. Gcn5 is a subunit of multiprotein complexes including SAGA (Spt-Ada-Gcn5-Acetyltransferase), ATAC (Ada Two-A Containing Complex), and STAGA (Spt3-TAF9-Gcn5-Acetyltransferase) (Lee and Workman, 2007). The domain structure of Gcn5 includes an N-terminal PCAF homology domain, the acetyltransferase domain, and a C-terminal bromodomain (Nagy and Tora, 2007). The bromodomain, an approximately 110-amino acid module is required for recognizing acetylated histone tails to regulate acetylation-dependent chromatin modification. It is hypothesized that Gcn5-containing chromatin acetylates a site on histone tail without binding to it. That specific site is subsequently bound by another Gcn5-containing complex leading to acetylation of an adjacent site, thus propagating the modification (Nagy and Tora, 2007). The metazoan Gcn5-containing complex exists in two distinct forms: a 2 MD Gcn5-containing STAGA complex and a 700 kD ATAC complex (Baker and Grant, 2007; Kusch et al., 2003). Spt (Suppressor of Ty) molecules are involved in facilitating TATA-binding protein (TBP) function and can act as eukaryotic transcription elongation factors (Winston, 2001). Ada or adaptor proteins are critical in forming a catalytic core to confer lysine specificity for nucleosomal acetylation (Balasubramanian et al., 2002; Bhaumik and Green, 2002). TBP-

associated factors (TAFs), which act as co-activators are important for promoter selection during transcription. They are critical for transcription from TATA-less promoters (Lee and Young, 2000). Transformation/transcription domain-associated protein (TRRAP) is a subunit of many HAT complexes. TRRAP is a key player in DNA repair and transcription by recruiting HAT complexes to chromatin (Robert et al., 2006). The mouse Gcn5 sequence is 98% identical with human GCN5 sequence; however, the mouse gene encodes an extended N-terminal domain with high similarity to PCAF-containing domain (Xu et al., 1998b). Full-length mouse Gcn5 protein can acetylate both free and nucleosomal histones H3 and H4, in contrast to yeast and human Gcn5 proteins that selectively acetylate free core histones (Xu et al., 1998b). There is an indirect recruitment of PCAF to the *Il9* locus as well. Overall, the recruitment of specific HATs leads to histone acetylation. In the absence of PU.1, there is enhanced HDAC recruitment at the *Il9* locus. While Gcn5 is not required for PU.1 or IRF4 binding to the *Il9* gene, it is important for controlling histone acetylation at the *Il9* gene promoter. Gcn5-dependent histone acetylation probably enhances binding of TCR-induced factors including NF-κB or NFAT which are important for *Il9* gene expression (Jash et al., 2012). It may also facilitate the progression of general transcription factors like RNA polymerase II (Sanso et al., 2011). PU.1 could also recruit other specific histone modifying enzymes and interact with other transcription factors at the *Il9* locus to contribute to IL-9 production. As histone acetyltransferases emerge as key targets for drugs (Dekker and Haisma, 2009), development of small molecule inhibitors of Gcn5 could become relevant in reducing the level of IL-9 in allergic patients.

FUTURE DIRECTIONS

PART I: Role of STAT6-dependent transcription factors in Th9 cells

STAT6-dependent Th9 regulation

STAT6 is critical for the development of Th9 cells (Figure 21). IRF4 which is downstream of STAT6 in Th9 cells is not sufficient to recover IL-9 production in the absence of STAT6 (Figure 25). The transcription factors GATA3 and c-Maf are also downstream of STAT6 in Th9 cells. However, when over expressed in Th9 cells they attenuate IL-9 production (Figure 30). This suggest that there are other factors downstream of STAT6 which act together to induce IL-9 production in Th9 cells. To examine potential factors downstream of the STAT6 pathway relevant for IL-9 induction we will perform a microarray with Th9 cells from WT and *Stat6-/-* mice. The *Il9* locus has potential binding sites of multiple transcription factors including AP-1, NFAT and NF-κB (Perumal and Kaplan, 2011). We expect expression of some transcription factors associated with AP-1, NFAT, and NF-κB family will be greatly diminished in the absence of STAT6 in Th9 cells. Therefore transducing WT and STAT6-deficient Th9 cells with retroviruses expressing specific transcription factors that are greatly attenuated in the absence of STAT6 signal will be important to define the 'missing' factors downstream of STAT6 signal. If no recovery of IL-9 is observed from the STAT6-deficient Th9 cells ectopically expressed with a single factor, developing Th9 cells will be transduced with a combination of factors and assess IL-9 production. Furthermore it will be relevant to assess whether the expression of T-bet and Foxp3, the negative regulators of IL-9, is altered after developing Th9 cells are transduced with a single or combination of transcription factors.

To define the mechanism how additional transcription factors regulate IL-9 production in Th9 cells it will be important to examine the binding of potential transcription factors to the *Il9* gene by performing chromatin immunoprecipitation using Th9 cells from wild-type mice and determine if the absence of STAT6 affects the association of the factors to the *Il9* locus. Furthermore, it will be critical to determine if any one of those transcription factors can transactivate *Il9* gene using luciferase reporter assay. Together these experiments will provide the insight into the additional factors required for STAT6-dependent *Il9* transcription.

PART II: PU.1-mediated *Il9* gene regulation in Th9 cells

Defining the role of additional HATs in Th9 cells

Numerous HAT molecules regulate histone acetylation of lysine residues. We have investigated the role of some of the well-defined HATs in PU.1-dependent IL-9 induction and observed PU.1-mediated differential recruitment of the HAT molecules to the *Il9* gene (Figure 54). PU.1 has been shown to mediate the recruitment of the HATs MOZ, Tip60, and p300. PU.1 inhibits acetylation mediated by CBP, another HAT molecule (Hong et al., 2002). However, our preliminary data suggests that in the absence of PU.1 there is decreased association of CBP to the *Il9* gene. We will therefore like to expand the relevance of these findings by examining the PU.1 mediated recruitment of other HAT molecules to the *Il9* gene including CBP and define their functional consequences. It will also be important to determine if any one of these additional HAT molecules associate directly with PU.1. If PU.1 can associate with any other HATs in addition to Gcn5 we could alter the expression of the specific HAT molecule and determine its effect in

inducing the *Il9* gene. We will further determine if the altered expression of the HAT molecules associated with PU.1 also affects the expression of other HAT molecules.

Determining PU.1-mediated histone methylation in Th9 cells

We have demonstrated PU.1 mediates specific histone acetylation at the *Il9* locus (Figure 50). However, PU.1 can also regulate histone methylation as is evident from a previous study (Stopka et al., 2005). H3K27 trimethylation was not PU.1-dependent (Chang et al., 2010). Our preliminary data suggests that H3K4 methylation is PU.1-dependent. Histone methylation adds complexity to chromatin modifications as the lysine residues can undergo mono-, di-, or tri-methylation. Methylation of H3K4, H3K36, and H3K79 has been associated with transcription activation (Kouzarides, 2007). H3K4, and H3K36 methylation play roles in transcriptional elongation (Kouzarides, 2007). In contrast, H3K9, H3K27, and H4K20 methylation has been implicated in transcriptional repression (Kouzarides, 2007). Therefore, we could also examine specific histone methylation status at the *Il9* locus in WT and PU.1-deficient Th9 cells. If PU.1 regulates histone methylation at the *Il9* locus then it can speculated that it could possibly regulate the recruitment of histone methyltransferases, the enzymes which catalyze histone methylation. Therefore it will be important to determine if PU.1 regulates recruitment of histone methyltransferases at the *Il9* locus in Th9 cells. These data will define the extensive histone modifications at the *Il9* locus.

Defining the maintenance and commitment of Th9 cells

Th1, Th2 and Th17 cells have been shown to maintain their ability to secrete their characteristic cytokines after multiple rounds of cultures *in vitro*. Whether long term Th9 cells retain their ability to secrete IL-9 has not been examined in detail. Therefore it will be important to define the ability of long term Th9 cultures to secrete IL-9. The amount of IL-17 secreted by Th17 cells decreases over each round during long term cultures. However, *in vitro* cultured long term Th17 cells display restricted plasticity. Furthermore, we will determine if long term Th9 cultures can also produce cytokines signature of other T helper subsets or if they show limited plasticity. It will be informative to determine if long term Th9 cells begin to produce IL-17 and IL-4 as TGF-β and IL-4 are required for the differentiation of Th17 and Th2 cells, respectively. Since PU.1 is one of the transcription factors required for Th9 cell development, the expression of PU.1 during each round of the long term Th9 cultures will be assessed. To test the requirement of PU.1 in maintenance of long term Th9 cells we could take advantage of *Sfpi1*$^{fl/fl}$ mice which are Cre negative. We will transduce either first, second or last round of long term Th9 cultures with Cre-expressing retrovirus. From the sorted cells we will determine if the deletion of PU.1 earlier or later results in any altered phenotype. These studies will provide insight in to the maintenance of Th9 cells.

Though long term Th17 cells have the ability to secrete IL-17, these cells are not committed. Therefore it will be relevant to define if Th9 cells are committed. Long term polarized Th9 cells will be cultured under Th1, Th2, and Th17-skewing conditions for one more round of culture. One aliquot of cells will be cultured under Th9 cell conditions as control. We will determine the ability of Th9 cells to produce IFN-γ, IL-4, and IL-17

along with IL-9. If long term Th9 cells lose the ability to produce IL-9 and switch to other T helper subsets then it would also be informative to assess the expression of transcription factors associated with other T helper subsets including T-bet, GATA3, and RORγt. Furthermore when Th9 cells are switched to Th2 or Th17 phenotype it would be important to determine if Th9 cells switched to Th2 or Th17 can be reconverted to Th9 cells when they are activated under Th9-skewing conditions.

Epigenetic status of long term Th9 cultures

The epigenetic status of the *Il9* locus in Th9 cells which is a combination of enhanced positive histone marks and reduced repressive marks have been described previously (Chang et al., 2010). However, the chromatin modifications of the *Il17* gene which is expressed at a low level by Th9 cells has not been examined carefully. Repressive marks are abundant in Th17-associated genes in Th1 and Th2 cells (Wei et al., 2009). The *Tbx21* locus in Th2 and Th17 cells has both positive H3K4Me3 and negative H3K27Me3 marks. The instability of Th17 cells is correlated with remodeling of the *Ifng* locus when Th17 cells are treated with IL-12 (Mukasa et al., 2010). As recent studies demonstrate the presence of bivalent chromatin marks associated with a particular T helper subset, it will be informative to determine the epigenetic status of *Il9* gene in long term Th9 cultures. If the long term cultures do not maintain their phenotype it will also be important to define if the *Il9* gene is associated with more repressive chromatin marks and fewer positive chromatin marks. We will examine the common histone modifications including total and specific H3 and H4 acetylation marks and histone methylation marks. Furthermore, we could expand our studies by examining the epigenetic modification at the Th2 and Th17-

specific gene loci. It would be interesting to look at repressive marks at the *Il9* locus as well as the Th2 and Th17 associated genes when Th9 cells would be switched to Th2 and Th17 cells and then converted back to Th9 cells. Overall, these studies will elucidate epigenetic regulation associated with long term Th9 cells.

PART III: Defining the role of PU.1 in inflammatory disease models

Experimental autoimmune encephalomyelitis (EAE)

Th9 cells have been implicated in autoimmune diseases including EAE with distinct pathological phenotype (Jager et al., 2009). As PU.1-deficient Th9 cells have defective Th9 differentiation *in vitro* and reduced inflammation in an OVA-induced allergic airway disease model (Chang et al., 2010), it will be important to determine if PU.1-deficiency ameliorates EAE. To test this, we will immunize WT and PU.1-deficient mice with MOG peptide and assess the features of EAE. If the absence of PU.1 ameliorates EAE disease severity we will determine if PU.1-deficient animals treated with recombinant IL-9 after EAE immunization restores EAE to the level of wild-type animals treated with control antibody. As PU.1 deficiency in T cells does not affect IL-17 production (Chang et al., 2010) it will be relevant to assess if there is any change in MOG-specific IL-17 production in peripheral lymph nodes and in the CNS. Furthermore, PBS or Th9 cells from 2D2 mice (TCR transgenic mice specific to MOG peptide) will be transferred to *Sfpi1*$^{lck-/-}$ mice. These studies will determine if *in vitro* generated Th9 cells can restore EAE symptoms and demonstrate the importance of PU.1 for the development of Th9 cells *in vivo* and its role in EAE.

Define the role of PU.1 in worm infection

As IL-9 has been shown to be important for immune responses against worm infection (Faulkner et al., 1997; Faulkner et al., 1998; Richard et al., 2000), it will be informative to assess if PU.1-deficiency affects worm clearance. To test this, WT and *Sfpi1*$^{lck-/-}$ mice will be infected with *Trichuris muris*. As PU.1 is required for IL-9 production we speculate that the absence of PU.1 will adversely affect worm clearance compared to wild-type mice. Additionally we will assess cytokine production from colon, and from *in vitro* stimulated lymphocytes. Colon contractility will also be measured. As mucus production by goblet cells is a hallmark of helminthic infection we could further determine if PU.1-deficiency results in alteration of goblet cell numbers in the infection model. Whether Th9 cells regulate mast cells in inflammation has not been examined carefully. It will be interesting to determine the mast cell content of the colon during worm infection and whether PU.1 deficiency affects the recruitment of mast cells. Whether the absence of PU.1 results in defective recruitment or survival of mast cells in cecum will also be determined. Together these studies will determine the importance of PU.1 during worm immunity.

Define the effects of PU.1 during respiratory syncytial virus infection

Infants with respiratory syncytial virus (RSV) bronchiolitis have elevated levels of IL-9 in the bronchial sections. Following RSV challenge, mice vaccinated with vaccinia virus expressing respiratory syncytial virus G protein demonstrate exacerbated disease marked by increased IL-9 production (Dodd et al., 2009). Depletion of IL-9 during immunization alleviates the disease with reduced Th2 cytokines (Dodd et al., 2009). Therefore it will be

important to determine if the absence of PU.1 reduces the disease severity after wild-type and PU.1-deficient mice are vaccinated and then challenged with RSV. Weight of mice, total BAL leukocytes and differential BAL fluid leukocyte count will be determined along with the cytokine profile in the BAL fluid. To determine if the absence of PU.1 during the challenge or immunization phase affects the disease severity, we will mate mice having *Sfpi1* floxed allele with mice expressing tamoxifen-inducible estrogen receptor-Cre fusion. These mice will be treated with tamoxifen at different time points to remove PU.1 expression either during challenge or during immunization. To determine if PU.1 depletion results in altered RSV-specific serum antibody level, we could measure RSV-specific different IgG levels. As IL-9 elimination during immunization or challenge phase has different outcomes, it will be informative to transfer CD4+ T cells from *Sfpi1*$^{fl/fl}$ mice where PU.1 was deleted during immunization or during challenge to naïve recipient mice before infecting them with RSV 24 hours later. In addition to determining the cytokines produced in the BAL fluid it will also be relevant to assess the cytokines produced in the lung.

Together, these future studies would determine additional transcription factors downstream of STAT6 signal required for the development of Th9 cells. These studies will provide further insights into the role of PU.1 in *Il9* gene regulation and its role in inflammatory disease models. Transcription factors can be targeted for pharmacological interference because of their modular structure. Small molecule pharmaceutical targets include upstream activating pathways of transcription factors, DNA-binding activity, and the ligand-binding domains of transcription factors. As improvements in structure-based

techniques enhance the design for novel small molecule inhibitors targeting transcription factors have become more relevant as drug targets. Therefore if PU.1 is involved in multiple inflammatory diseases targeting the transcription factor will pave the way for future therapeutics.

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Peer Reviewed Publications

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2010	Chang, HC*., Sehra, S*., Goswami, R., Yao, W., Yu, Q., Stritesky, GL., Jabeen, R., Han, L., Nguyen, ET., McKinley, CD., Tepper, RS., Robertson., MJ., Perumal, N.B., Nutt, SL., and Kaplan, MH. 2010. *Nat Immunol*. 11(6): 527-534. * These authors contributed equally.
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