TRANSCRIPTION FACTORS AND CIS-ACTING ELEMENTS IN T HELPER CELL CYTOKINE EXPRESSION

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DEDICATION

To my parents,

whom I love and respect beyond expression.

Thank you for raising me up with endless love to be who I am today.

To my wife,

the best and forever companion in my life.

Thank you for supporting and loving me the way no one can.

I would never have reached this point without your love, support, encouragement, and trust.

Thank you.

Love you.

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TRANSCRIPTION FACTORS AND CIS-ACTING ELEMENTS IN T HELPER CELL CYTOKINE EXPRESSION

The immune system provides resistance to the myriad of pathogens in the environment, but can also respond inappropriately causing allergic inflammation and autoimmune disease. CD4+ T cells, which play a crucial role in adaptive immune system, can be divided into several subsets based on their effector functions. T helper 9 (Th9) cells, derived by the IL-4/STAT6 and TGF-β signaling pathways, produce IL-9 as a hallmark cytokine, as well as IL-10. Through IL-9 production, Th9 cells protect against parasite infection but are also involved in allergic inflammation and autoimmune diseases. Transcription factors that promote Th9 development include STATs, PU.1, BATF, and IRF4. In this study, we identify ETV5 as a factor that promotes IL-9 and IL-10 production by binding to cis-acting regulatory elements in the respective genes. At the *Il9* gene, ETV5 cooperates with PU.1 in regulating gene expression. At the *Il10* gene, ETV5 facilitates binding of other transcription factors to the locus. These studies and others suggested that there may be additional cis-acting regulatory elements in the *Il9* gene. We demonstrate that a conserved noncoding sequence (CNS) located 25 kb upstream of the *Il9* transcription start site, termed *Il9* CNS-25, is critical for regulating *Il9* expression in Th cell subsets. Th9 cells derived from *Il9* CNS-25 mutant (*Il9* $^{\Delta CNS-25}$) mice produce significantly less IL-9. *Il9* CNS-25 promoted chromatin modifications at the promoter and accessibility of the locus. *Il9* $^{\Delta CNS-25}$ mice showed attenuated airway inflammation compared to control mice. The *Il9* CNS-25 region in mice is conserved with an *IL9* CNS-18 region in the human genome. We deleted CNS-18 in primary human Th9 cells and observed diminished IL-9 production. Thus, we have identified transcription factors that regulate multiple cytokines

in Th cell lineages and have demonstrated that the *Il9* CNS-25/*IL9* CNS-18 elements are respectively critical for *Il9/IL9* gene expression.

Mark H. Kaplan, Ph.D. Chair

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3C	Chromosome conformation capture
Ab	Antibody
AICE	Activating protein 1-IRF composite element
APC	Antigen presenting cell
ASP	Aspergillus fumigatus
ATAC	Assay for transposase accessible chromatin
ATF	Activating transcription factor
BAL	Bronchoalveolar lavage
BATF	Basic leucine zipper transcription factor ATF-like
BCL6	B cell lymphoma 6
BMMC	Bone marrow derived mast cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation
CNS	Conserved noncoding sequence
CTCF	CCCTC-binding factor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DHS (HS)	DNase I hypersensitivity site
DLA	Dual luciferase assay
dLN	draining lymph node
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
E4BP4	E4 promoter-binding protein 4

EAE	Experimental autoimmune encephalomyelitis
ECR	Evolutionary conserved region
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ETS	E-twenty six
Etv5	ETS variant gene 5
FACS	Fluorescence activated cell sort
FBS	Fetal bovine serum
Foxo1	Forkhead box O 1
Foxp3	Forkhead box protein 3
FSC	Forward-scattered light
Galcer	Galactosylceramide
GATA3	GATA binding protein 3
GC	Germinal center
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR-related protein
GWAS	Genome wide association studies
HAT	Histone acetyltransferase
HDM	House dust mite
HMT	Histone methyltransferase
I.N.	Intranasal
IBD	Inflammatory bowel disease
ICOS	Inducible co-stimulator
ICS	Intracellular staining
IFN	Interferon

IL	Interleukin
ILC	Innate lymphoid cell
IRF	Interferon regulatory factor
JAK	Janus family tyrosine kinase
JunB	Jun B proto-oncogene
LCR	Locus control region
LSF	Lineage specific transcription factor
Med1	Mediator complex subunit 1
medLN	Mediastinal lymph node
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
NFAT	Nuclear factor of activated T cell
NF-κB	Nuclear factor Kappa-light-chain-enhancer of activated B cell
NIK	NF-κB inducing kinase
NKT	Natural killer T
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PAS	Periodic acid–Schiff
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositide 3-kinases
PMA	Phorbol 12-myristake 13-acetate
PMN	Polymorphonuclear leukocyte
PNPP	p-Nitrophenyl Phosphate
PRR	Pattern recognition receptor

qRT-PCR	Quantitative Reverse Transcription PCR
RA	Rheumatoid arthritis
RAG	Recombinant-activating gene
RHS	Rad50 hypersensitivity site
RNA	Ribonucleic acid
ROR	Retinoid-acid-related orphan receptor
SCF	Stem cell growth factor
Sfpi1	Spleen focus forming virus proviral integration site-1
SMC1A	Structural maintenance of chromosome protein A
SNP	Single nucleotide polymorphism
SSC	Side-scattered light
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
Tbx21	T-box transcription factor 21
TCR	T cell receptor
TF	Transcription factor
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TLSP	Thymic stromal lymphopoietin
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
Treg	T regulatory
TSS	Transcription start site

UC	Ulcerative colitis
WT	Wild type

INTRODUCTION

Innate and adaptive immunity

The immune system provides resistance to the myriad of pathogens in the environment, but can also respond inappropriately causing allergic and autoimmune diseases (1). The immune system can be largely classified into innate immunity and adaptive immunity. Innate immunity is a fast and non-specific first line of defense. The adaptive immunity is pathogen-specific, and provides long-lasting protection, contributing fast and effective protection against repeat infections. Therefore, innate and adaptive immunity cooperate to enhance protection against harmful foreign agents (1).

Innate immunity

Humans and other mammals are constantly exposed to various toxic substances such as pathogens or nontoxic allergens such as pollen. Physiologic barriers, part of innate immunity, provide a crucial first defense line against pathogens. The first barriers to the environment are the epithelial cell layers that are tightly connected between cells through cell-cell connections in the skin and mucosal layers. Epithelial cells also produce protective enzymes and antibacterial peptides in tears and saliva, and provide a low pH environment in stomach. Mucosal cells also contribute to the initial defense in the respiratory and gastrointestinal tracts by secreting mucus. Additionally, commensal microorganisms that reside at epithelial surfaces compete with harmful bacteria for space and nutrients to survive.

When pathogenic microorganisms cross the epithelial layer, various types of innate immune cells recognize and kill the pathogens by ingestion. The innate immune response is very fast upon pathogen exposure and innate immune cells immediately generate inflammatory mediators for protection. Innate immunity consists of cellular and humoral components. Innate immune cells are cellular components which include macrophages,

dendritic cells, mast cells, neutrophils, eosinophils, natural killer (NK) cells, and NK T cells. Humoral components including complement proteins, mannose binding lectin, lipopolysaccharide (LPS) binding protein and anti-microbial peptides, augment the activity of innate immune cells. Innate immune cells express invariant, germline encoded receptors that recognize pathogen associated molecular patterns (PAMPs) which are highly conserved microbial components or damage-associated molecular patterns (DAMPs), which are metabolic consequences of infection and inflammation. One of the families of pattern recognition receptors (PRRs) recognizing PAMPs are the Toll-like receptors (TLRs). There are 10 TLRs in humans and each TLR specifically recognizes microbial products. For example, TLR4 recognizes LPS, a component of the outer membrane of Gram-negative bacteria and TLR5 recognizes flagellin. TLR3 and TLR7 recognize double-stranded RNA and single stranded RNA respectively. Leucine-rich repeat containing receptor (NLR), one of the cytosolic PRRs, can recognize endogenous signals of cellular damage such as K^+ efflux and uric acid crystals. Neutrophils recognize and engulf microorganisms and digest them through intracellular vacuoles containing toxic effector molecules such as nitric oxide, superoxide and degradative enzymes. Neutrophils accumulate at the site of bacterial infections and produce large quantities of toxic reactive oxygen species, enzymes, cytokines such as TNF and IL-12, as well as chemokines. Macrophages and monocytes are recruited to the infection site and persist for long period. Macrophages also act as antigen presenting cells (APC). Eosinophils have prominent cytoplasmic granules containing toxic molecules which effectively eliminate helminthic parasites. Mast cells are a key player in the initiation of immediate hypersensitivity responses. Mast cells express FcεRI, high-affinity IgE receptor. After binding of antigen specific IgE, mast cells are sensitized. Upon exposure to the same antigen, mast cells rapidly release histamine and other inflammatory cytokines that stimulate tissue inflammation. NK cells, large granular lymphocytes, recognize virus-infected cells or tumor

cells and kill them by releasing cytotoxic granules that induce apoptosis of target cells. Through inhibitory receptors on their surface, NK cytotoxic activity is inhibited when their receptors bind to the self-major histocompatibility complex (MHC) molecules. Dendritic cells (DCs) are present in most tissues and express both MHC II and I molecules that are recognized by $CD4^+$ and $CD8^+$ T cells respectively. After ingestion of foreign antigens, DCs migrate to the draining lymph nodes (dLNs) via afferent lymphatics through chemokine receptors such as CCR7 and CCR8. In the lymph nodes, DCs process antigens and load peptides on the MHCII molecules which are recognized by T cell receptor (TCR) on naïve $CD4^+$ T cells (1, 2).

Adaptive immunity

During development in the thymus and the bone marrow, T and B lymphocytes express a randomly generated repertoire of T and B cell receptors through somatic gene recombination. Naïve T lymphocytes develop in the thymus, a primary lymph node, and circulate in the blood and enter the dLNs, secondary lymph nodes. In the dLNs, T cells are activated through interactions between TCR and MHC molecules. MHC I is constitutively expressed by all nucleated cells, while MHC II is expressed on APCs (1, 3).

$CD8^+$ T cells, also called cytotoxic T cells (CTL) when they are primed, express the CD8 coreceptor for MHC I. By recognizing cytosolic antigenic peptides bound to MHC I, $CD8^+$ T cells kill target cells infected with intracellular pathogens or tumor cells through cell-cell contact. Using CTL granules containing granzymes and perforin, $CD8^+$ T cells induce lysis or apoptosis of target cells. Additionally, TCR activation promotes Fas ligand (CD95L) expression on the $CD8^+$ T cells which bind to Fas (CD95) on the target cells leading to apoptosis of the target cells. Most $CD4^+$ and $CD8^+$ T cells have a TCR consisting of α and β chains. Unlike these αβ T cells, γδ T cells have a TCR consisting of γ and δ chains that recognize mycobacterial antigens presented by the CD1 family and through

other less well-defined mechanisms. Natural killer T cells (NKT), another subset of T cells, are characterized by the expression of the NK receptor and invariant TCR. NKT cells recognize glycolipid antigens presented by CD1d on APCs. Upon TCR activation, NKT cells rapidly release large amounts of cytokines such as IL-4, IFN-γ and IL-9, contributing to the regulation of immune responses. Adaptive humoral immunity is mediated by antibodies produced by plasma cells that develop from B cells. B cells produce five classes of immunoglobulins (Igs): IgA, IgD, IgE, IgG and IgM, which are classified based on their amino acid sequence in the constant region of antibody heavy chain. B cells develop in the bone marrow and express the B cell receptor (BCR) consisting of membrane bound Igs. In germinal centers (GCs), located inside of lymph nodes, B cells encounter follicular helper T (Tfh) cells. Tfh cells promote the development of memory B cells and long-lived plasma cells. IL-4, secreted by Tfh, mediate class switch of antibodies in B cells (1, 3).

$CD4^+$ T helper cells

$CD4^+$ T cells play an essential role in adaptive immune system. With the help of APCs, naïve $CD4^+$ T cells differentiate into Th subsets to regulate host defense and inflammatory responses (Figure 1). For optimal Th cell activation, APCs need to provide three signals to induce lineage specific transcription factors (LSFs) that promote the differentiation of naïve $CD4^+$ T cells. TCR signal is the first signal through the interaction between the TCR on the naïve $CD4^+$ T cells and the antigen peptide-MHC II complex on the APCs. Co-stimulatory signals are the second signals mediated by cell surface molecular interactions in a non-antigen dependent mechanism. For example, the co-stimulatory molecule CD28 on naïve $CD4^+$ T cells interacts with its corresponding ligands CD80 or CD86 on the APCs. The third signal is mediated by cytokines, which can activate the signal transducer and activator of transcription (STAT), Smads and NF-κB signaling pathways thereby inducing a number of pertinent TFs (4).

Each $CD4^+$ T helper cell subsets has its own specific master regulators that promote effector functions by inducing a subset specific cytokines. Th1 cells express T-bet and produce IFN-γ which stimulates macrophages to eliminate intracellular bacterial pathogens (5, 6). IL-12 binds to the IL-12 receptor which consists of IL-12Rβ1 and IL-12Rβ2, and activates STAT4 which collaborates with T-bet to promote IFN-γ production. IFN-γ further amplifies IFN-γ production by binding to the IFN-γ receptor which stimulates STAT1 signaling to promote T-bet expression (6-8).

Th2 cells express GATA3 and produce IL-4, IL-5 and IL-13 which further activate innate immune cells such as eosinophils. STAT6 activated through the IL-4 receptor, promotes the expression of GATA3 and Th2 cell specific cytokines (9-11).

Th17 cells express the master regulator RORγt and produce IL-17. Along with TGF-β signaling, a STAT3 signaling pathway activated by IL-6, IL-21 and/or IL-23 promotes Th17 cell development (12, 13). IL-17 is an important cytokine for protection against fungi and extracellular bacteria and is also known as a pro-inflammatory cytokine (14, 15). Th17 cells are involved in many autoimmune and allergic disease such as rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) (16).

Tfh cells express B-cell lymphoma 6 (Bcl6) which promotes Tfh cell differentiation while preventing differentiation of Th1, Th2 and Th17 cells. Tfh cells play an essential role in germinal center reactions which are important for antibody production. Tfh cells produce IL-4 which allows B cells to switch antibody class from IgM to IgE. The IL-6/STAT3 axis and the inducible costimulator (ICOS) signaling pathway induce Bcl6, basic leucine zipper transcription factor (BATF) and Interferon regulatory factor 4 (IRF4) which are important for Tfh cell development (17-19).

$Foxp3^+$ regulatory T (Treg) cells play a pivotal role in maintaining immune tolerance and controlling immune responses. Treg cells are also known as suppressor T cells because they repress Th cell effector functions (20, 21). Foxp3 promotes and maintains

Treg cell population by interacting directly or indirectly with numerous TFs. Foxp3 belongs to the forkhead-box/winged-helix transcription factor family, and the Foxp3 gene is located on the X chromosome (21, 22). Foxp3 mutations cause severe autoimmune and inflammatory diseases which are associated with a significant increase in Th1 cytokines. These diseases are called IPEX (immune dysregulation, poly-endocrinopathy, enteropathy, X-linked syndrome) in humans (23). Foxp3 mutant mice called “Scurfy”, show similar symptoms of IPEX, and these symptoms are relieved by introducing wild type (WT) Treg cells (21, 24). Treg cells use different suppressive mechanisms in lymphoid and non-lymphoid tissues. In lymphoid tissues, Treg cells suppress effector T cells by preventing dendritic cell maturation or directly inhibiting activation of effector T cells. In non-lymphoid tissues, Treg cells secrete inhibitory cytokines such as IL-10, IL-35 and TGF-β (25).

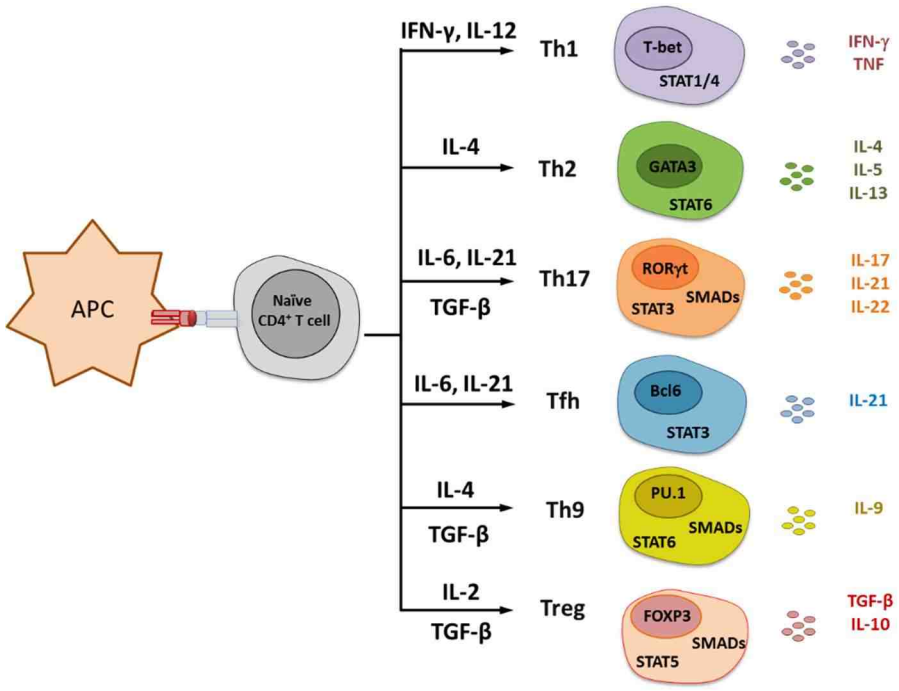

Figure 1. Model of differentiation and functions of Th cell subsets

Cytokines

Cytokines are soluble proteins secreted by various types of cells including immune cells. Cytokines have distinct roles in the immunoregulation and inflammation. Cytokines regulate the differentiation and proliferation of T cells through the Jak/STAT pathways. Cytokines induce lineage specific TFs that are important for the development of Th cell subsets. After differentiation, Th cells sense their cognate antigens and environmental cytokines to control immune responses by secreting Th cell specific effector cytokines. For optimal functions, the expression of cytokines needs to be strictly regulated (4).

Interleukin-10

IL-10, encoded by the *Il10* gene, is a critical regulatory cytokine produced by many Th subsets including Th2 and Th9 cells. As an anti-inflammatory cytokine, IL-10 suppresses inflammatory responses at the mucosal surfaces (26). In the absence of IL-10, mice develop spontaneous autoimmune inflammation (27). However, increased IL-10 production prevents clearance of pathogens resulting in chronic infection. Thus, transcriptional control of the *Il10* gene is essential in maintaining disease-free immune homeostasis (28).

Interleukin-9

IL-9, encoded by the *Il9* gene, is a 40kDa glycoprotein with 144 amino acids. Interleukin-9 was firstly discovered by stimulating murine T helper cell lines with concanavalin A (29). Naïve murine T cells cultured with IL-2, IL-4 and TGF-β express IL-9 (30). Secreted IL-9 binds to the IL-9 receptor (IL-9R), also known as CD129, which is a heterodimeric protein consisting of the IL-9Rα chain and the common γc chain (31, 32). IL-9R is expressed on various immune cells and mucosal layer cells such as smooth muscle cells in the lung or epithelial cells in the gut and lung (32-36). Upon binding of IL-

9 to its receptor, JAK1 and JAK3 are activated by phosphorylation leading to the activation of STAT1, STAT3 and STAT5 (37). As other cytokines, IL-9 functions pleiotropically, as a positive or negative regulator of immune responses. IL-9 contributes to allergic inflammation and autoimmune diseases. IL-9 is critical for protection against parasites such as helminths, for tumor immunity and for immunological tolerance during allograft skin transplantation (38). In the following section, the roles of IL-9 in immunity and diseases will be discussed in more details

Allergic asthma and atopic diseases

Allergic asthma is a chronic inflammatory disease of the lung airways characterized by coughing, wheezing and shortness of breath which is caused by airflow obstruction. In response to the allergens, the innate and adaptive immune systems are highly activated and mediate mucosal inflammation by recruiting eosinophils to the airways or by secreting proinflammatory mediators such as histamine (39).

Constitutive expression of IL-9 in the lung of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness (40). Genetic studies in asthmatic patients showed that genes related to asthma or atopy are located near on chromosome 5q31-q33, and the *Il9* gene is also located near on this chromosome (41). Asthmatic patients show higher serum IgE levels, *Il9* gene expression and a higher number of IL-9 producing cells (Th9) in the airways compared to healthy subjects (42-45). IL-9 receptor expression in airway epithelial cells, correlates with a high level of mucus production, and is also higher in asthma patients (46). IL-9 activates lung smooth muscle cells to produce cytokines and chemokines such as eotaxin which recruits eosinophils to the airways (47). IL-9 also stimulates airway epithelial cells resulting in goblet cell hyperplasia and a high amount of mucus production that leads to bronchial

hyperresponsiveness (48-50). IL-9 also acts as a growth factor for mast cells (51) and enhances IL-4 mediated IgE and IgG production by B cells (52, 53).

As a main source of IL-9, the Th9 cells are critical for the development of allergic airway disease. Increased numbers of Th9 cells are detected in asthmatic and atopy patients, and these patients express high levels of genes related to Th9 cell development and function (42-45). In experimental models of asthma, Th9 cells mediate allergic inflammation through their production of IL-9 (42, 54-56). Adoptive transfer of in vitro cultured Th9 cells into Rag2-deficient mice promotes allergic inflammation characterized by mast cell accumulation and mucus production, and these symptoms are ameliorated through administration of an IL-9 neutralizing antibody (55). IL-9, produced by Th9 cells, is required for accumulation of mast cells in the airways during asthma (57). These features of inflammation are attenuated by natural or engineered deficiencies in the IL-9-inducing TFs such as BATF, IRF4 and PU.1 (42, 44, 54-59).

Inflammatory bowel disease (IBD)

Inflammatory bowel diseases such as Crohn's disease (CD) and ulcerative colitis (UC) are characterized by chronic inflammation of the gastrointestinal tract that leads to chronic diarrhea and abdominal pain (60-62). IBD is caused by deregulated activation of several Th cell subsets. Th1 cells, activated by IL-18, IL-12 and IL-23, secrete IFN-γ which is essential for CD pathogenesis. Th2 cells produce Th2 specific cytokines inducing UC. Th17 cells, activated by IL-23 and IL-6, produce IL-17A/F, IL-21 and IL-22 contributing to both CD and UC (63-65). IBD patients show higher number of IL-9 producing cells and higher gene expression of *Il9* and *Il9r* compared with healthy people (66). In an oxazolone induced colitis model, Th9 cells promote UC by regulating intestinal epithelial cells. In that study, mice with PU.1 deficient T cells are resistant to colitis and IL-9 neutralizing antibody treatment attenuates inflammation (66). Moreover, IL-9 producing invariant NKT cells are

important for protection against autoimmune inflammation in the dextran sulfate sodium (DSS)-induced colitis model (67).

Multiple sclerosis

In addition to induction of allergic inflammation in the lung and gut, Th9 cells are also involved in multiple sclerosis, a T cell dependent demyelinating disease (68, 69). Th1 and Th17 cells are known as key players in an experimental animal model of multiple sclerosis (70). During an experimental autoimmune encephalomyelitis (EAE), both Th17 and Th9 cells produce IL-9, and IL-9 neutralization or IL-9Rα deficiency attenuate disease progression. Transfer of myelin oligodendrocyte glycoprotein (MOG) specific Th9 cells into RAG deficient recipient mice promotes EAE (69, 71, 72). However, other studies showed IL-9Rα deficient mice develop more severe EAE due to the fact that IL-9 can also have anti-inflammatory function in EAE by enhancing Treg cell activity (33, 73).

Rheumatoid arthritis (RA)

RA is a chronic autoimmune disease mediated by autoreactive Th1, Th17 cells and impaired function of Treg cells (74). In an experimental antigen induced arthritis model, IL-9 produced by type 2 innate lymphoid cells (ILC2) acts as an autocrine growth factor and upregulates ICOS ligand and glucocorticoid-induced TNFR-related protein (GITR) ligand on ILC2. Through interaction with these ligands, Treg cells enhance suppressive activity which inhibits Th17 cell mediated inflammation. Therefore IL-9 is critical for resolution of joint inflammation in RA (75).

Parasite infections

During nematode infections such as a *Nippostrongylus brasiliensis* infection, Th9 cells are essential for expulsion of the parasites from gastrointestinal tract (76). IL-9,

produced by Th9 cells, stimulates mast cells to secrete inflammatory mediators that stimulate epithelial cells to produce mucus, recruits eosinophils, increases intestinal permeability, and induces smooth muscle contraction (76).

Tumor immunity

Depending on the types of cancer, Th9 cells can function positively or negatively in tumor growth (77-81). In a melanoma model, adoptive transfer of Th9 cells into RAG deficient recipient mice following B16-F10 melanoma cancer cell injection reduces tumor growth compared to control (77, 78). Additionally, *Il9* deficient mice and mice treated with IL-9 neutralizing antibody show accelerated tumor masses. In this model, the roles of IL-9 in antitumor immunity depend on mast cell activation (77). Tumor specific Th9 cells promote $CD8^+$ CTL mediated anti-tumor immunity (78). IL-21 produced by Th9 cells also has an anti-cancer function by promoting $CD8^+$ CTL responses and IFN-γ production (81).

Cellular sources of IL-9

IL-9 can be produced by a number of immune cell populations. In this segment, we briefly discuss evidence for IL-9 production and function in these cells.

Type 2 innate lymphoid cells (ILC2)

Innate lymphoid cells (ILC), newly discovered immune cells, are groups of lineage negative lymphoid cells (82). These cells lack T, B cell receptors and other lineage markers, however their functions are similar to T helper cells. There are three types of ILCs: ILC1, ILC2 and ILC3. ILC2 secrete Th2 type cytokines such as IL-5, IL-6, IL-13 and IL-9 in response to helminth infection (76, 83). Moreover, ILC2 are the main source of IL-9 during papain induced lung inflammation (83).

Mast cells

Mast cells secrete both cytokines and pro-inflammatory mediators such as IL-6 and histamine. Toll-like receptor agonists and cytokines including IL-10 and IL-9 stimulate mast cells to produce IL-9 (84-86). As a growth factor, IL-9 is also important for survival and proliferation of mast cells (51). A recent study revealed that IL-9 producing mucosal mast cells (MMC9s) induce anaphylaxis in response to food antigens by producing IL-9 and inflammatory mediators (87).

Natural killer T (NKT) cells

NKT cells produce Th2 type cytokines and IL-9 in vitro (67). In allergic inflammation models, CD1d restricted NKT cell deficient mice are resistant to allergic inflammation caused by decreased IL-9 production from NKT cells and by reduced mast cell recruitment (88). Another study revealed that IL-9 producing NKT cells protect against DSS induced colitis. In this study, NKT cells cultured under Th9 condition produced IL-9. Additionally, α-galactosylceramide (α-GalCer) treated NKT cells produced more IL-9 compare to non-treated controls (67).

Th9 cells – IL-9 producing T cells

Th9 cells are the most recently described Th cell subset and are derived by the IL-4/STAT6 and TGF-β signaling pathways (32, 68, 89). Th9 cells are characterized by a high amount of IL-9 production and a high expression of PU.1, a master regulator for Th9 cell development (59). Th9 cells also express a high amount of IL-10, however Th9 cells do not co-express IL-4, IL-5, IL-13, IL-17 or IFN-γ. Like other Th cell subsets, Th9 cells can function as both positive and negative regulator of immune responses. Th9 cells protect against extracellular parasites such as helminths and important for anti-tumor immunity (76-78). However, Th9 cells are also involved in allergic inflammation or

autoimmune disease (42, 54-56). Therefore, Th9 cell development and IL-9 production need to be strictly regulated. To do this, many TFs cooperate with cis-regulatory elements near the *Il9* gene.

Regulation of Th9 cell development

Cytokines activate STAT signaling pathways which induce TFs involved in *Il9* expression (Figure 2). These TFs promote *Il9* expression by directly binding to the *Il9* promoter or by mediating the *Il9* locus chromatin remodeling to a poised status ready to be activated in response to TCR stimulation. TCR signaling activates NFAT and NF-κB, enhancing *Il9* expression by binding to the *Il9* promoter (38).

STAT6

IL-4 binds to the IL-4 receptor (IL-4R), consisting of the IL-4Rα and γc chains, and activates STAT6 through JAK activation (10). STAT6 regulates *Il9* expression in several ways (Figure 2). STAT6 directly binds to the *Il9* promoter and promotes IL-9 production (90). Previous report showed that STAT6 represses *Foxp3* and *Tbx21* which repress IL-9 production (90). Moreover, STAT6 induces IL-9 inducing TFs such as BATF and IRF4 (54). BATF is required for Th9 cell development as well as Th17, Tfh and Th2 cells. BATF deficiency impairs Th9 cell development and BATF deficient mice show attenuated airway inflammation in asthma model. Moreover, ectopic expression of BATF increases IL-9 production in Th9 cells. Like STAT6, BATF also directly binds to the *Il9* promoter and enhances *Il9* expression (54). IRF4 is another essential TF for Th9 cell development as in Th17 and Th2 cell development. IRF4 deficiency resulted in significantly less IL-9 production under Th9 cell condition and ectopic expression of IRF4 increases IL-9 production in Th9 cells. Moreover, mice with IRF4 deficient T cells show attenuated airway inflammation in the acute OVA/Alum asthma model (55).

BATF and IRF4 cooperate to regulate Th17 cell development (91, 92). BATF and IRF4 cooperatively bind to specific DNA elements called “activating protein 1-IRF composite elements (AICEs)” to enhance Th17 cell differentiation and gene expression (93, 94). In Th9 cells, BATF and IRF4 also cooperatively bind to the *Il9* promoter. Deficiency of either TF impairs the binding of the other TF to the *Il9* promoter, and mutation of AICE in the *Il9* promoter decrease *Il9* promoter reporter activity (54).

GATA3, a master regulator of Th2 cells, is also induced by IL-4/STAT6 signaling pathway in Th9 cells although expression is lower than in Th2 cells (32, 90). The function of GATA3 in Th9 cell development is controversial because GATA3 deficient T cells fail to produce IL-9 (68), whereas ectopic expression of GATA3 inhibits IL-9 production in Th9 cells (90). These data suggest that balancing GATA3 expression is important for optimal *Il9* expression.

STAT5

IL-2 binds to the IL-2 receptor, a heterotrimeric complex consisting of IL-2Rα called CD25, IL-2Rβ and γ chains, and activates STAT5 (89). The IL-2/STAT5 signaling pathway is also critical for Th9 cell development. Either IL-2 or STAT5 deficient T cells fail to produce IL-9 under Th9 cell condition (89, 95). STAT5 positively regulates *Il9* expression directly and indirectly. STAT5 directly binds to the *Il9* promoter and enhances *Il9* expression. STAT5 competes with Bcl6, a master regulator of Tfh cells and a repressor of *Il9* expression by binding to the *Il9* promoter, to bind to the *Il9* promoter and repress Bcl6 expression (71, 95)

Thymic stromal lymphopoietin (TSLP), an epithelial cell derived cytokine, is critical for Th2 mediated allergic inflammation (96). TSLP also induces *Il9* expression by activating STAT5 that binds to the *Il9* promoter. IL-2 neutralization does not affect the

ability of TSLP to induce *Il9* expression, suggesting that TSLP function is independent of IL-2 (42).

STAT3

STAT3 might function differently in mouse and human Th9 cells. Mouse Th9 cells produce a high amount of IL-10 which acts as a negative regulator of *Il9* expression (97). STAT3 deficient Th9 cells produce a relatively high amount of IL-9 at later differentiation stages meaning that activation of STAT3 by IL-10 results in instability of the Th9 cell phenotype. Additionally, IL-10R blockade using anti-IL-10R antibody contributes to the maintenance of IL-9 production (97). In contrast to murine Th9 cells, human Th9 cells produce more IL-9 in response to IL-10. In addition to IL-10, IL-6 and IL-21 also promote IL-9 production by activating STAT3 in human Th9 cells (98).

TGF-β signaling pathway

TGF-β can reprogram Th2 cells into Th9 cells (32, 68). TGF-β binds to the TGF-βR2 and activates Smad2, 3 and 4. The TGF-β signaling pathway induces active histone modifications at the *Il9* promoter (58, 99). By physical interaction with IRF4, Smad2/3 promote *Il9* expression by directly binding to the *Il9* promoter (58). Smad3 is also required for Notch signaling mediated *Il9* expression (73).

TGF-β signaling pathway also induces the expression of PU.1. PU.1 is a member of E-Twenty-Six (ETS) TF family and induced by a Smad independent TGF-β signaling pathway (59, 90, 100). PU.1 is the only TF that can induce *Il9* expression in cells other than Th9 cells. Ectopic expression of PU.1 in Th2 cells increases IL-9 production and decreases IL-4 expression. PU.1 deficient Th9 cells produce significantly less IL-9 and more IL-4, suggesting that PU.1 negatively regulates Th2 cell development (59). PU.1 directly binds to the *Il9* promoter and promotes *Il9* expression through permissive histone

acetylation at the *Il9* locus by recruiting GCN5, a histone acetyltransferase (HAT) (101). Mice with PU.1 deficient T cells are resistant to in vivo airway inflammation model (59).

Etv5

Etv5, or Ets variant gene 5, is an ETS family member that has largely been examined for its roles in tissue development. Etv5 coordinates limb development, controls spermatogonial gene expression, and regulates epithelial-mesenchymal gene expression (102-105). Etv5 was also identified as an IL-12-induced Th1 gene, though recent work suggests it only has a minimal role in IFN-γ production, contrary to the original report (106, 107). Etv5-deficient T cells have diminished Th17 but slightly increased Th2 development in vivo, and this correlates with decreased inflammation in the house dust mite model of allergic lung inflammation. The diminished pulmonary inflammation is due to the lack of IL-17-dependent immunity, because the addition of exogenous pulmonary IL-17 to Etv5 conditional mutant mice normalizes inflammation (107).

Foxo1

Forkhead box protein O1 (Foxo1) is required for IL-9 production in Th9, Th2, Th17 and Treg cells (108). The PI3K/AKT pathway negatively regulates Foxo1 activity. In the nucleus, Foxo1 is phosphorylated by AKT and moves out of the nucleus to the cytosol leading to inactivation of its transcriptional activity. Foxo1 directly binds to the *Il9* and *Irf4* promoters and promotes gene expression. Loss of Foxo1 through siRNA leads to less IL-9 production in Th9 and Th17 cells resulting in attenuated allergic inflammation (108).

Co-stimulation

Like other Th cell differentiation pathways, CD28 stimulation by CD80/CD86 on APCs is critical for Th9 cell development. The CD28/CD80 or CD86 stimulation transiently

activate NF-κB and NFAT which directly bind to the *Il9* promoter (109, 110). The ligation of OX40 on T cells by the OX40 ligand on APCs induces IL-9 in Th9 cells. OX40 activates the TRAF6, an ubiquitin ligase, which activates NF-κB-inducing kinase (NIK) and the noncanonical NF-κB signaling pathway to induce IL-9 (109). Notch and Smad3 cooperate to induce IL-9. The ligation of Notch on T cells by the Jagged2 on APCs promotes Th9 cell development. After ligation, the Notch1 intracellular domain (NICD1) moves to the nucleus and interacts with Smad3 to bind to the *Il9* promoter (73).

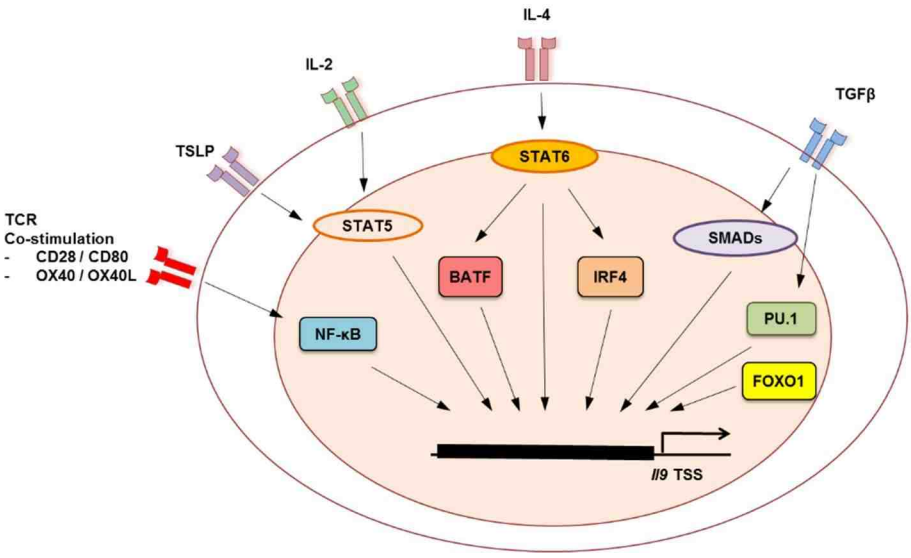

Figure 2. Transcriptional network for *Il9* expression in Th9 cells

Regulation of gene expression

For specific and precise gene regulation in different cell types, cooperation of TFs, trans- regulatory elements, and cis-regulatory elements is critical. TFs recognize and bind to specific DNA sequences in the genome through DNA binding domains, and these TFs recruit other TFs such as histone modifying enzymes through activation or other interaction domains (111).

Epigenetic pioneering TFs act to initiate gene expression by converting closed chromatin to open chromatin. DNase I hypersensitive sites (DHS) are the result of less tightly packaged nucleosomes or the absence of nucleosomes. The nucleosome is a basic structural unit of DNA packaging, and consists of 146 base pairs (bp) of DNA wrapped in histone octamer - two copies of core histones: H2A, H2B, H3 and H4. TFs can't bind to highly nucleosome packed regions, called heterochromatin. However, nucleosome free regulatory elements, called euchromatin, are accessible to lineage specific TFs which dictate lineage specific chromatin landscape by recruiting chromatin remodeling complexes such as HATs and histone methyltransferases (HMTs). These epigenetic modifications on cis-regulatory elements allow the binding of other TFs to regulate gene expression (112-115).

Recent various genome wide association studies (GWAS) have revealed that almost 90% of disease related single nucleotide polymorphisms (SNPs) are enriched in the intronic and intergenic regions. Many SNPs in cis-regulatory elements are closely link to diseases and these mutations affect the binding of TFs and chromatin conformation which result in abnormal gene expression (112, 116-118).

Cis-regulatory elements

There are several types of cis-regulatory elements: promoters, enhancers, silencers, insulators and locus control regions (LCRs). These cis-elements are located in different regions of the gene (119) These regions can be evolutionarily highly conserved between species and provide binding sites for multiple TFs to control gene expression (120). Accessibility of TFs to these regions is regulated by epigenetic mechanisms such as DNA methylation, histone modifications, chromatin remodeling and intra/ inter-chromosome interactions (111).

Enhancers

The location of enhancers varies among genes. Distal enhancers can be located far from a gene transcription start site (TSS), and a DNA loop mediates enhancer-promoter interactions (Figure 3)(121, 122). Active enhancers are characterized by high DNase1 sensitivity, strong binding of p300, co-ocupation of TFs, and active histone modification marks such as H3K4me1, H3K4me2 and H3K27ac, but low H3K4me3, an active promoter marker (123).

Histone modifications are one of the key post transcriptional gene regulation mechanisms. Histone tails can be modified with methylation, acetylation, phosphorylation, ubiquitylation and sumoylation (111). Chromatin status is defined as “poised”, “active” or “silent” depending on modifications of histone tails which affect gene expression patterns (124-127).

Mono or di-methylation of lysine (K) 4 residue in Histone 3 (H3) tail is a poised or primed enhancer marker which is strictly regulated by HMTs. H3K4me1 modified enhancers without H3K27ac are termed as “poised” -ready to be activated by subsequent stimulation. Enhancers marked by both H3K4me1 and H3K27ac are termed as “active”-opened chromatin status which allows the binding of TFs (128). Acetylation of tails of H3 such as H3K27ac is a critical mechanism for active gene expression and it is strictly regulated by histone deacetylases (HDACs) or HAT such as p300 (129, 130). Active promoters are marked by H3K4me3 and active transcription elongation is marked by H3K36me3 which is spread throughout the gene body (131). H3K27me3, catalyzed by histone methyltransferase Ezh1 or Ezh2, a subunit of polycomb repressive complex2 (PRC2), is associated with “silent” enhancer- repressive and closed chromatin status. The H3K4me3 and H3K27me3 bi-functional pattern is distinct among Th cell subsets (132).

TFs shaping the epigenetic landscape in differentiating Th cells

In naïve $CD4^+$ T cells, most gene loci of lineage specific TFs and cytokines are inactive or have a repressed status marked by low DNase1 sensitivity and repressive histone modifications. After TCR and cytokine stimulation, these gene loci are converted to permissive chromatin with high DNase1 sensitivity and active histone modifications which allow the binding of TFs. TCR signaling induces epigenetic pioneering TFs such as NFAT and AP-1 that regulate chromatin landscape and other general TFs. In combination with TCR stimulation, the STAT signaling pathway is essential for establishing lineage specific epigenetic landscapes required for Th cell development. STATs sense environmental cytokines secreted by APCs. After binding of a cytokine to a cytokine receptor, JAKs activate STATs, and dimerized STATs translocate to the nucleus and initiate Th cell specific gene expression (111).

Recent genome wide studies showed that about 20% of the binding sites for STATs are located in promoters with the remainder located in regulatory regions or within genes. These data suggest that STATs are multifunctional, not only directly regulating gene expression but also initiating the chromatin landscape by inducing histone modification at regulatory elements through recruitment of p300 (8, 116, 133, 134). For example, STAT6 and STAT4 bind to enhancers and initiate lineage specific chromatin landscape through epigenetic modifications in Th2 and Th1 cell respectively (8).

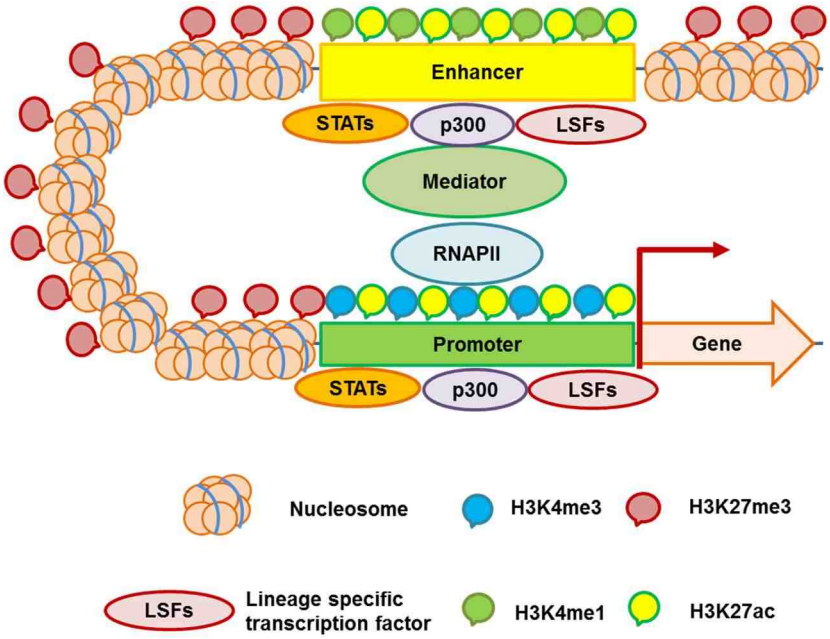

Figure 3. Cooperation of TFs and enhancer for gene expression. STATs bind to the enhancer and mediate active histone modifications by recruiting p300, contributing to induce LSFs through DNA looping. Expressed LSFs bind to the enhancer with STATs and p300, leading to DNA looping which promote recruitment of RNA polymerase II (RNAPII) to the gene promoter.

Enhancers for cytokine expression in Th cell subsets

In Th cell subsets, cytokine loci are regulated by sophisticated enhancer structures which contribute to tightly regulated gene expression in response to various stimulations. In this section, the roles of enhancers in cytokine expression will be discussed in more details.

Il10 regulatory elements

The *Il10* and *IL10* genes are located on chromosome 1 in both mouse and human genomes. Both the *Il10/IL10* genes include 5 exons (Figure 4) (135). The *Il10* locus contains a promoter, intronic regions and several CNSs. CNS1 is located 5 kb upstream

of *Il0* TSS. CNS2, CNS3 (+6.4) and CNS4 are located behind of 3' of *Il10* gene (136). However, not only these CNSs, conserved regions within *Il10* gene provide binding sites for TFs. GATA3 regulates *Il10* expression independently of IL-4 by binding to the *Il10* promoter, *Il10* intronic region +3.7 and CNS3. By doing so, GATA3 induces permissive chromatin structure at the *Il10* locus (137). E4 promoter-binding protein 4 (E4BP4) is a key player for *Il10* expression in most Th subsets, and its function is independent on GATA3. In Th2 cells, E4BP4 binds to most intronic regions, CNS2 and CNS3, but not *Il10* promoter. E4BP4 deficient Th2 cells show repressive chromatin structure at the *Il10* locus suggesting that E4BP4 epigenetically regulates the *Il10* locus in Th2 cells (138). CNS3 region contains AP-1 binding sites which allow the binding of c-Jun and JunB. In Th2 cells, these proteins bind to the CNS3 and promote *Il10* expression. But these proteins do not activate *Il10* promoter activity in reporter assays, suggesting that they might not bind to the *Il10* promoter (136). In Th2 cells, IRF4 binds to both *Il10* promoter and CNS3 contributing to *Il10* expression. In the absence of PU.1, IRF4 binds more strongly to the *Il10* locus and enhances *Il10* expression, suggesting that PU.1 acts as an *Il10* repressor by inhibiting IRF4 activity (139). In Th1 cells, Ets-1, another ETS family member, negatively regulates *Il10* expression by recruiting HDAC1 to the *Il10* promoter and intronic regions (140). Therefore, *Il10* regulatory elements and *Il10*-inducing or -repressing TFs cooperate to control *Il10* expression.

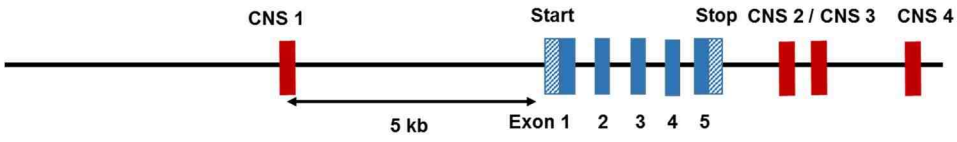

Figure 4. Schematic of the *Il10* locus in mice

Il4-Il5-Il13 regulatory elements

Th2 cells expressing cytokines such as IL-4, IL-5 and IL-13 are activated by the IL-4/STAT6 signaling pathway (141). IL-4 activates STAT6 which induce GATA3, an essential TF for Th2 cell development (9). STAT6 cooperates with GATA3 to mediate chromatin remodeling of Th2 cytokine locus (142).

The Th2 cytokine locus, encoding IL-4, IL-5 and IL-13, contains more than 15 DHSs. The *Il13* locus located 12.5 kb behind of 3' of the *Il4* locus and the *Il5* locus is located 86 kb upstream of the *Il13* promoter. The *Il4, Il13* and *Il5* genes are coordinately regulated by Th2 LCR. Th2 LCR located between *Il5* and *Il13* promoters and contains Rad50 hypersensitivity site (RHS) 4-6. RHS6 and RHS7 sites provide binding sites for STAT6 and GATA3. GATA3 binds to these sites and recruits p300 to make these regions transcriptionally permissive. During differentiation, the Th2 LCR region is positioned adjacent to the *Il4, Il5* and *Il13* promoters by looping to promote gene expression (143). HS2, located in second intron of *Il4* gene, is critical for IL-4 expression but dispensable for IL-5 and IL-13 expression (144). Deletion of HS V, another DHS located downstream of *Il4* gene, does not affect Th2 cytokines under Th2 cell condition, however deletion of this region impairs IL-4 and IL-13 expression in Tfh cells (145). GATA3 represses *Tbx21* expression by recruiting HDAC to the *Tbx21* enhancer region during Th2 cell development suggesting that GATA3 acts as both positive and negative regulator in same Th cell lineage (146).

Ifng regulatory elements

Th1 cells producing IFN-γ are activated by the IL-12/STAT4 and STAT1 signaling pathway. Transcriptional regulation of IFN-γ is controlled by the Th1 specific TFs: T-bet, STAT1, STAT4 and STAT5. These TFs combinatorially regulate *Ifng* gene expression by binding to 12 enhancers in the *Ifng* locus (123, 147). In contrast to the Th2 cytokine locus,

the *Ifng* locus is dispersed in naïve CD4$^+$ T cell and begin to close to the *Ifng* promoter during Th1 cell development (6, 148, 149). T-bet is important for recruiting CTCF, CCCTC binding factor, to enhancer regions which result in loops between enhancers and the *Ifng* promoter (148).

Il17 regulatory elements

Th17 cells produce proinflammatory cytokines such as IL-17A and IL-17F, two highly homologous cytokines. Th17 cells are differentiated from naïve CD4$^+$ T cell by the TGF-β and STAT3 signaling pathways, the latter activated by IL-6, IL-21 and IL-23 (15). STAT3 directly binds to the *Il17* gene promoter and induces *Rorc* which encodes RORγt, a master regulator of Th17 cell development (150, 151). In addition to RORγt, another orphan nuclear receptor RORα is also important for IL-17A and IL-17F expression (152). STAT3 mediates permissive histone modifications at the *Il17* gene locus that contains the *Il17a*, *Il17f* promoters and eight CNSs (153, 154). CNS2, located upstream of *Il17a,* interacts with the *Il17a* and *Il17f* promoters leading to gene expression. *Il17a* and *Il17f* promoters do not contain ROR response element (RORE), a binding sequence for ROR, however CNS2 does have ROREs (152). Therefore, RORα and RORγt bind to the CNS2 and mediate chromatin remodeling resulting in *Il17* gene expression.

Il9 regulatory elements

The Th9 cells are main producer of IL-9. The *Il9* gene is located on chromosome 13 in the mouse genome and the *IL9* gene is located on chromosome 5 in the human genome. Both *Il9/IL9* genes consist of 5 exons. The *Il9* locus contains 3 CNSs which are highly conserved between mice and human. CNS0 (-6) is located 6.3 kb upstream of the *Il9* TSS, CNS1 is the promoter region of *Il9*, and CNS2 (+5.5) is located 5 kb behind of the *Il9* TSS (Figure 5) (155). Many IL-9-inducing factors such as IRF4, BATF, STAT6 and

PU.1 directly bind to the *Il9* promoter and promote *Il9* expression (54, 55, 59, 90). STAT6 also binds to the CNS-6 region in Th9 cells but binding strength is lower than in the *Il9* promoter (90). However, detailed functions of CNSs at the *Il9* locus and existence of other enhancers for *Il9* expression are not fully understood.

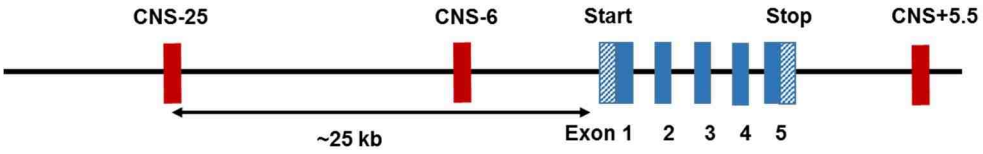

Figure 5. Schematic of the *Il9* locus in mice

Research goals

Transcription factors and cis-regulatory elements cooperately regulate gene expression. Many studies revealed TFs and cis-regulatory elements which regulate cytokines expression such as *Il4-Il5-Il13, Il17a-Il17f, Ifng* and *Il10* loci. However, identification and functions of trans- and cis-regulatory elements in *Il9* and *Il10* loci are still not completely defined. The goals of this research are to identify transcription factors binding to cis-regulatory elements of *Il9* and *Il10* loci and to define new enhancers that promote *Il9* gene expression.

MATERIALS AND METHODS

Mice

C57BL/6 and BALB/C mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). *Etv5*$^{fl/fl}$ mice were provided by Dr. Xin Sun (105). *Etv5*$^{fl/fl}$ mice were crossed with CD4-Cre transgenic mice to generate CD4 conditional Etv5 deficient mice (*Etv5*$^{fl/fl}$ CD4-Cre^{+}). *Etv5*$^{fl/fl}$ CD4-Cre^{-} littermates were used as wild-type (WT) control mice (107). *Stat6* deficient mice and *Irf4*$^{fl/fl}$ Lck Cre+ mice were described previously (156, 157). CNS-25 deleted mice (*Il9* $^{\Delta CNS-25}$) were generated by CRISPR/Cas9 mediated gene editing (Taconic) (Figure 6 and Table 1). Mice were maintained under specific pathogen-free conditions. All experiments were performed with the approval of the Indiana University Institutional Animal Care and Use Committee.

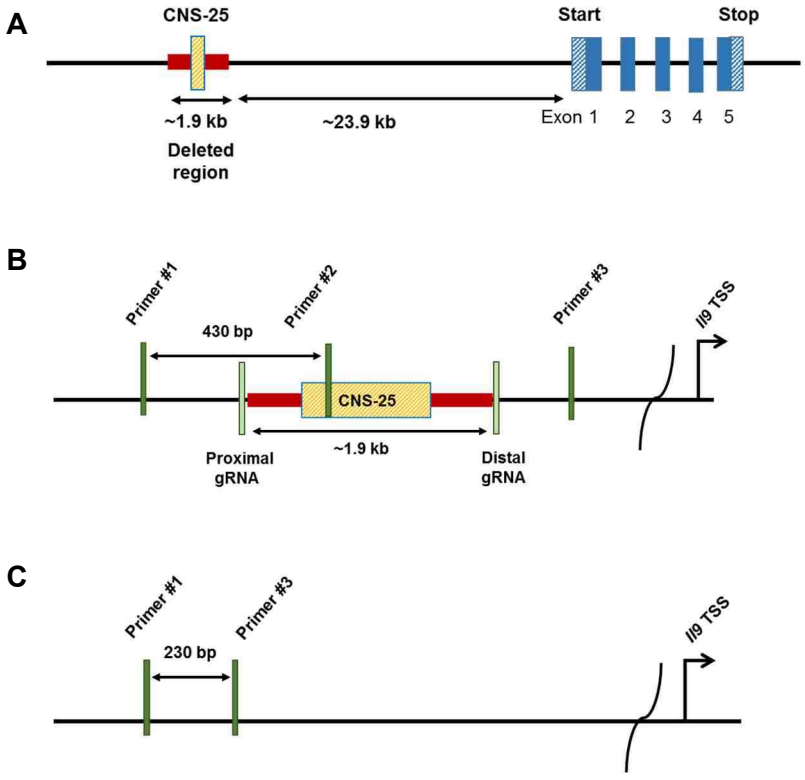

Figure 6. Strategy for generating *Il9* $^{\Delta CNS-25}$ mice

D

Proximal gRNA

aggtaattccaagtgtaacattgcacagtgaacttgtcagggttagttacaatcacctagctaactcggaggcaggtgcatttctgagaag
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tcaacctgccatgtttgtttccatttggggactcgaatgcaaggctaactgcctagcaaattgtttttcatttcctggcgttta

Distal gRNA

Figure 6. Strategy for generating *Il9*$^{\Delta CNS\text{-}25}$ mice. (A) Mouse *Il9* genomic locus (B) *Il9* CNS-25 targeting gRNAs and genotyping primer set for detecting WT allele: Oligo #1 and #2 (C) Mouse *Il9* locus after CRISPR/Cas9 mediated gene editing and genotyping primer set for detecting CNS-25 deletion allele: Oligo #1 and #3 (D) Sequence of mouse *Il9* CNS-25 region and sequences of CNS-25 targeting gRNAs. CNS-25 region is marked with yellow highlight and deleted region is marked with red line.

Table 1. Sequences of mouse *Il9* CNS-25 targeting gRNAs and *Il9*$^{\Delta CNS\text{-}25}$ mice genotyping primers

CNS-25 mice	Protospacer sequence (5'-3')		
Il9 CNS-25 KO 5' (Proximal gRNA)	CAATCACCTAGCTAACTCGG		
Il9 CNS-25 KO 3' (Distal gRNA)	TGCATTCGAGTCCCCAAATG		
Genotyping primers	primer sequence (5'-3')	Program	
CNS-25 Primer #1	AAACCATTGTGTGATGTACCTGG	95°C 5 mins	
		95°C 30 sec	X 34 cycle
CNS-25 Primer #2	AAGCCTCATGACATCCTTTGA	58 °C 30 sec	
		72 °C 1 min	
CNS-25 Primer #3	AACTGACCAGATTTACTAGGTCCC	72 °C 10 mins	
		4 °C overnight	

In vitro mouse T cell differentiation

Naïve $CD4^+CD62L^+$ T cells from mice were positively selected from the enriched $CD4^+$ T cells from spleen and lymph nodes using MACS beads and columns (Miltenyi Biotec). Naive $CD4^+CD62L^+$ T cells were activated with plate-bound anti-CD3 (2 µg/ml 145-2C11 ; BioXCell) and soluble anti-CD28 (0.5 µg/ml ; BD Pharmingen) in complete culture media, Roswell Park Memorial Institute 1640 (RPMI 1640, ThermoFisher Scientific) containing 10% Fetal bovine serum (FBS, Atlanta Biologicals), 1% antibiotics (penicillin and streptomycin / stock; Pen 5000 µ/ml, Strep 5000 µg/ml), 1 mM sodium pyruvate, 1 mM L-Glutamine, 2.5 ml of Non-essential amino acids (Stock; 100 X), 5mM HEPES (all from LONZA) and 57.2 µM 2-Mercapoethanol (Sigma-Aldrich), to generate Th0 cells (10 U hIL-2) or with additional cytokines (all from PeproTech) and antibodies (all from BioXCell) to generate Th2 cells (10 ng/ml IL-4; and 10 µg/ml anti-IFNγ XMG), Th9 cells (20 ng/ml

IL-4, 2 ng/ml hTGF-β1, 10 μg/ml anti-IFNγ XMG, 10 μg/ml anti-IL-10 receptor antibody), Treg cells (5 ng/ml hTGF-β1, 10U hIL-2, 10 μg/ml anti-IFNγ,10 μg/ml anti-IL-4), Th17 cells (100 ng/ml IL-6, 2 ng/ml hTGF-β1,10 μg/ml anti-IFNγ,10 μg/ml anti-IL-4) and Th1 cells (20 ng/ml IL-12, 10 U hIL-2, 10 μg/ml anti-IL-4 11B11) culture conditions. Cells were grown at 37°C under 5% CO_2 and were expanded after 3 days with original concentration of cytokines in fresh medium. Cells were harvested on day 4 or 5 for analysis.

Isolation of human PBMCs from buffy coat

Peripheral blood mononuclear cells (PBMCs) were isolated from human buffy coat (Indiana Blood Center, IN) by density gradient centrifugation using Ficoll-Paque (GE Healthcare). 15 ml of the buffy coat was diluted with 35 ml of ice cold buffer (PBS with 2 mM EDTA). 25 ml of this diluted suspension was gently added to 15 ml of Ficoll-paque in a 50 ml conical tube. After centrifuging at 400 x g for 40 mins at 20°C, the upper layer was gently removed by aspiration. Mononuclear cell layer was transferred to a new 50 ml conical tube and filled with buffer up to 50 ml. After mixing, cells were centrifuged at 300 x g for 10 mins at 20°C. After removing supernatant, cells were resuspended again with 50 ml of the buffer and centrifuged at 200 x g for 15 mins at 20°C. After repeating this washing step, the number of cells were counted and used for the isolation of human naïve $CD4^+$ T cells.

In vitro human T cell differentiation

Human naïve $CD4^+$ T cells were isolated from human PBMCs using magnetic separation (Miltenyi Biotec), and these cells were activated with a receptor crosslinking bead, Dynabead human T-activator CD3/CD28 (ThermoFisher Scientific) in complete media to generate Th0 cells (10 U hIL-2) and Th9 cells (20 ng/ml hIL-4, 2 ng/ml hTGF-β1,

10 μg/ml anti-IFNγ). 3 x 10^5 of naïve $CD4^+$ T cells and an equal ratio of beads in 300 μl of culture media were added to 48 well plate. Cells were grown at 37°C under 5% CO_2 and were expanded after 3 days with original concentration of cytokines in fresh medium. Cells were harvested on day 4 or 5 for analysis.

Retrovirus production

Platinum E cells were grown in 10 ml of Dulbecco's modified eagle medium 1640 (DMEM 1640) with 10% FBS and 1% antibiotics in a 100 mm tissue culture dish. When confluency reached 80~90%, cells were transfected with control vector or retroviral vector containing Etv5, GATA3, E4BP4, BATF, IRF4 open reading frame (ORF) using lipofectamine 3000 (ThermoFisher Scientific). For transfection, 18 μg of vector, 6 μg of pCL-Eco and 25 μl of P3000 were mixed in 500 μl of Opti-MEM®I reduced-serum medium (ThermoFisher Scientific), and 25 μl of lipofectamine 3000 was mixed in another 500 μl of Opti-MEM®I. After combining, this mixture was incubated for 10-15 mins at room temperature (RT). The mixture was gently pipetted into culture dish. After 16 hours, the media containing retrovirus was collected and changed with new fresh media. After 24 hours, the media was collected and centrifuged at 1500 rpm for 5 mins to remove cell debris. Supernatant containing retrovirus was used for retroviral transduction or stored at -80 °C for subsequent use.

Retroviral transduction

Activated mouse $CD4^+$ T cells were infected on day 1 with retrovirus containing control or expressing the interested gene by centrifugation at 2300 rpm at 32°C for 90 mins in the presence of 8 μg/ml polybrene (Sigma-Aldrich). After spin infection, the supernatant was replaced with the fresh Th cell condition media. Cells were expanded on day 3 and analyzed on day 4 or 5.

CRISPR /Cas9 plasmid construct

PX330A_D10A-1X2 (Addgene #58772) was modified by adding ClaI site in front of hU6 promoter, termed as 'new pX330A_D10A-1X2'. gRNAs were designed using Feng Zhang lab's online tool (http://crispr.mit.edu/). After annealing of gRNA oligos, the gRNAs duplexes were cloned to new pX330A_D10A-1X2 and pX330S-2 (Addgene #58778) using BbsI (BpiI) (Table 2). Through Golden gate assembly using Eco31I, new pX330A_D10A-1X2 which contains gRNA cassette containing two hU6 promoter and two gRNAs was made. By using Cla1 and Kpn1, the gRNA cassette from this vector was inserted to new lentiCRISPR v2 (modified from lentiCRISPR v2, Addgene #52961, by adding new cloning site containing Cla1 and Kpn1) using Cla1 and Kpn1. Finally, DNA element containing gRNA cassette of this new lentiCRISPR v2 was replaced with lentiCas9-EGFP (Addgene #63592) using Not1 and Nhe1 (Figure 7). Plasmids and gRNA sequences are listed in Table 2 and 3.

Table 2. CRISPR/Cas9 plasmids

Construct	Oligo	Overhang	Protospacer	Overhang
New PX330A_D10A 1X2	sgRNA-1 sense	5'-CACCG	(N)20	
	sgRNA-1 antisense	3'-C	(N)20 complement	CAAA-5'
PX330S_2	sgRNA-1 sense	5'-CACCG	(N)20	
	sgRNA-1 antisense	3'-C	(N)20 complement	CAAA-5'

Table 3. Sequences of gRNAs targeting *hIL9* CNS-18 and GM38602

gRNAs	Target location	Protospacer sequence (5'-3')	Genome
hIL9 CNS-18 5A	CNS-18	CAGAGTAGATCTTCCATTGG	Human
hIL9 CNS-18 3A	CNS-18	AGAGATGGGGTCTCCCTATG	Human
mGM38602 5A	GM38602	GCCCGGGCTCGATCTATTAA	Mice
mGM38602 3A	GM38602	CCTCTCCAGGTGTTAACTAC	Mice

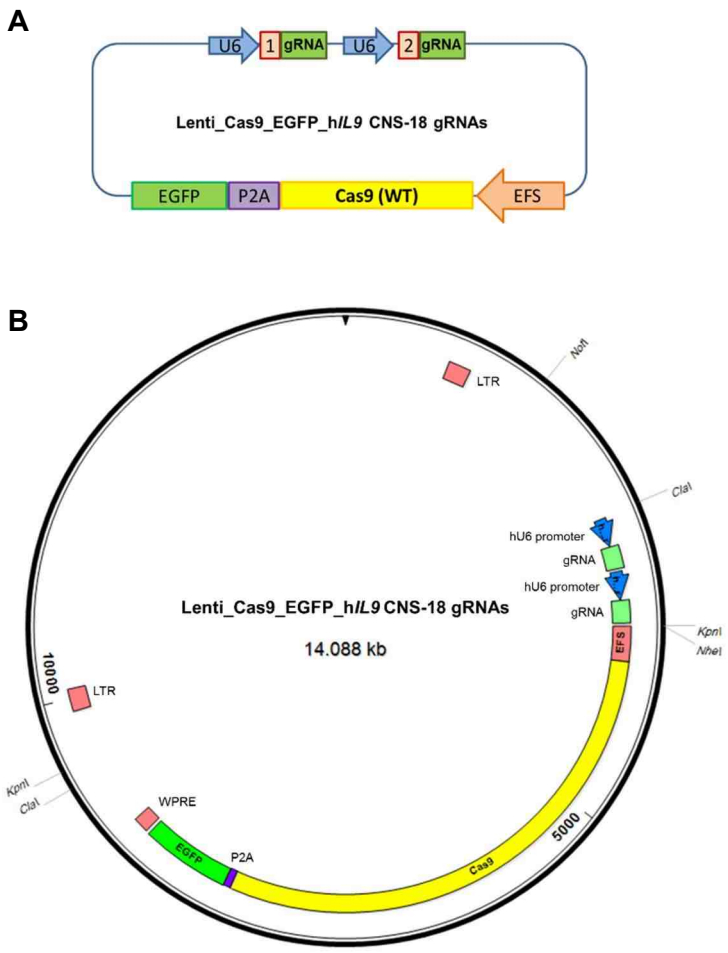

Figure 7. Single lentiviral vector expressing Cas9 and gRNAs. (A) Simplified map and (B) full map for vector

Lentivirus production

Human embryonic kidney (HEK) 293T cells were grown with 10 ml of DMEM 1640 with 10% FBS and 1% antibiotics in a 100 mm tissue culture dish. When confluency reached 95~99%, cells were transfected with lentiviral vectors expressing Cas9 and gRNAs targeting h*IL9* CNS-18 or GM38602 as a negative control using lipofectamine 3000. For transfection, 10 µg of lentiviral vector, 8 µg of PAX2, 5 µg of PMDG.2 and 40 µl of

P3000 were mixed in 1,500 µl of Opti-MEM®I, and 40 µl of lipofectamine 3000 was mixed in another 1,500 µl of Opti-MEM®I. After combining, these mixtures were incubated for 10-15 mins at RT, and gently pipetted into culture dish. After 6 hours, media was changed to the lentivirus packaging media; Opti-MEM®I-GlutaMAX (ThermoFisher Scientific) with 5% FBS and 0.2 mM sodium pyruvate, and incubated another 16 hours. The first batch of virus was harvested, and fresh lentivirus packaging media was added. After 24 hours, the second batch of virus was collected and combined with the first batch of virus. After filtering media containing lentivirus using a 0.45 µm filter, Lenti-X-concentrator (Takara) was added 1:3 volume and incubated at 4°C overnight with rotation. The following day, the mixture was centrifuged at 1,500 x g for 45 mins at 4°C. The pellet was resuspended in 500 µl of PBS, aliquoted to 100 µl, and stored at -80 °C for future use.

Lentiviral infection

Sterile non-tissue-culture treated 24 well plates were coated with 50 µg/ml of Retronectin (Takara) and incubate at 4°C overnight. The next day, after washing the plate with PBS, blocking buffer (PBS with 2% bovine serum albumin (BSA)) was added and incubated at RT for 30 mins. After removing the buffer, lentivirus stock (100µl) and 500 µl of culture media was added to the plate. After loading lentivirus to the plate using centrifugation with 2000 x g at 32°C for 2 hours, day 1 cultured human T cells were transferred to the plate and incubated for 2~3 days. Cells were harvested on day 4 or 5 for analysis using flow cytometry or sorted by fluorescence activated cell sorter (FACS; BD FACSAria) based on EGFP for gene expression analysis.

***Aspergillus fumigatus* extract-induced allergic airway inflammation**

Mice were challenged intranasally with *Aspergillus fumigatus* (*A.fumigatus*; Greer Laboratories) extract every other day for 21 days. *A. fumigatus* (25-100µg) extract was

diluted with PBS (20-50 µl) and administered into the nose. Mice were sacrificed 1 day after final intranasal challenge. Bronchoalveolar lavage (BAL) cells were collected by lavaging the lungs with 1 ml PBS. To prepare single cell suspension from lungs, lung tissues were chopped and incubated with 0.5 mg/ml of collagenase A (Roche) in DMEM at 37°C for 45 mins. After grinding tissues with mesh stainless steel strainer, red blood cells were removed by Ammonium-Chloride-Potassium (ACK) lysing buffer (LONZA). After stopping the reaction by adding buffer (PBS with 0.5 % BSA), cells were washed with the buffer followed by filtering through 70 µm nylon mesh to remove debris. Single cell suspensions were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 6 hours to assess cytokine production using intracellular staining. Cytokines in BALF were measured by enzyme linked immunosorbent assay (ELISA).

House dust mite extract-induced allergic airway inflammation

Mice were challenged intranasally with house dust mite (HDM; Greer Laboratories) extract every other day for 16 days. HDM extract (25 µg) was diluted with 20 µl PBS and administered into the nose. Mice were sacrificed 1 day after final intranasal challenge. The subsequent procedures for sample harvesting are the same as for the *A. fumigatus* extract-induced allergic airway inflammation experiment.

Reverse transcription (RT) and quantitative real-time PCR (qPCR)

Total RNA was extracted using TRIzol reagent (ThermoFisher Scientific) and reverse transcribed using cloned Avian Myeloblastosis Virus (AMV) reverse transcriptase (ThermoFisher Scientific). For qPCR, Taqman real time PCR assay (ThermoFisher Scientific) was used for gene expression analysis. Taqman probes used for qPCR are listed in Table 4. Gene expression was normalized to housekeeping gene expression (β2-microglobulin). The relative gene expression was calculated by the change-in-threshold

($2^{-\Delta C_T}$) method. All experiments were performed in duplicate in two independent experiments and results are presented as standard error of means (SEM) of biological replicates.

Table 4. Taqman probes for qPCR

Mouse	
Gene	Cat. No.
β2m	Mm00437762_m1
Batf	Mm00479410_m1
Bcl6	Mm00477633_m1
Jun	Mm00495062_s1
Foxp3	Mm00475165_m1
Gata3	Mm00484683_m1
Id3	Mm00492575_m1
Ifng	Mm01168134_m1
Il9	Mm00434305_m1
Il10	Mm00439614_m1
Il17a	Mm00439618_m1
Il4	Mm00445259_m1
Irf1	Mm00515191_m1
Irf4	Mm00516431_m1
Junb	Mm04243546_s1
Nfil3	Mm00600292_s1
Rorc	Mm00441139_m1
Runx3	Mm00490666_m1
Spi1	Mm00488142_m1
Stat6	Mm01160477_m1
Tbx21	Mm00450960_m1
Human	
Gene	Cat. No.
B2M	Hs99999907_m1
IL10	Hs99999035_m1
IL21	Hs00222327_m1
IL9	Hs00914237_m1
STAT6	Hs00598625_m1

Flow cytometric analysis

For cytokine staining, in vitro cultured $CD4^+$ T cells were stimulated with PMA and ionomycin for 2 hours followed by monensin for a total of 6 hours. After fixation with 1% formaldehyde for 10 mins at RT, cells were washed two times with FACS buffer (PBS with 0.5 % BSA). For transcription factor staining, cells were fixed with Foxp3/Transcription factor fixation buffer (eBioscience) at 4°C in dark for 30 mins or overnight. Fixed cells were permeabilized with permeabilization buffer (eBioscience), and stained for cytokines and TFs with fluorochrome conjugated antibodies at 4°C in dark for one hour. Stained cells were washed two times with FACS buffer and resuspended with 500 µl of FACS buffer for flow analysis. Fluorescent antibodies for flow cytometric analysis are listed in Table 5.

Table 5. Fluorescent antibodies for flow cytometric analysis

Antigen/Name	Clone	Fluorochrome	Company	Cat. No.
CD11b	M1/70	PerCP-Cy5.5	eBioscience	45-0112-82
CD11c	N418	PE-Cy7	eBioscience	25-0114-82
CD3	145-2C11	PerCP-Cy5.5	BD Biosciences	551163
CD4	GK1.5	FITC	BD Biosciences	553729
	GK1.5	PE	BioLegend	100408
	GK1.5	PerCP-Cy5.5	BioLegend	100434
	GK1.5	PE-Cy7	BioLegend	100422
	GK1.5	APC	BioLegend	100412
	GK1.5	APC-Cy7	BD Biosciences	552051
F4/80	BM8	FITC	BioLegend	123108
Foxp3	MF23	FITC	BD Biosciences	560403
IFN-γ	XMG 1.2	PerCP-Cy5.5	eBioscience	45-7311-82
IL-10	JES5-16E3	FITC	BioLegend	505006
	JES5-16E3	PE	eBioscience	12-7101-82
IL-17A	eBio17B7	PE-Cy7	eBioscience	25-7177-82
IL-4	11B11	AF647	BioLegend	504110
IL-9	RM9A4	PE	BioLegend	514104
Ly6G	1A8	APC	BioLegend	127613
SiglecF	E50-2440	PE	BD Biosciences	552126
Fixable Viability dye		eFluor 780	eBioscience	65-0865-14

Enzyme linked immunosorbent assay (ELISA)

Cytokine capturing antibodies were coated on 96 well plate (NUNC) with 50 µl of coating buffer (dH_2O with 0.1 M $NaHCO_3$ pH 9) and incubated at 4°C overnight. After washing the plate three times with washing buffer (PBS with 0.05% Tween-20), 250 µl of ELISA buffer (PBS with 2% BSA) was added to well and incubated at RT for one hour to prevent non-specific binding. After washing the plate three times with washing buffer, 100 µl of diluted samples (10-100 fold diluted with ELISA buffer) and standard cytokines were added and incubated at 4°C overnight. The next day, after washing the plate three times with washing buffer, 100 µl of the biotinylated secondary antibody was added and incubated at RT for one hour. After washing the plate three times with washing buffer, 100 µl of washing buffer with 0.05% of avidin-alkaline phosphatase (Sigma-Aldrich) was added and incubated at RT for one hour. After washing the plate three times with washing buffer, 100 µl of the substrate buffer with p-Nitrophenyl Phosphate (PNPP) (Sigma-Aldrich), a phosphatase substrate, was added to each well. Reaction was stopped by adding stop solution (1N NaOH) and measured with the Biorad Microplate 680 ELISA reader. Primary and secondary antibodies for ELISA are listed in Table 6.

Table 6. ELISA capture and biotinylated secondary antibodies

Capture Abs	Clone	Stock	Final conc.	Company	Cat. No.
IL-17A	TC11-18H10	0.5mg/ml	2ug/ml	BD Biosciences	555068
IL-10	JES5-2A5	1mg/ml	4ug/ml	BD Biosciences	551215
IL-4	11B11	0.5mg/ml	2ug/ml	BD Biosciences	554434
IL-9	D8402E8	1mg/ml	1ug/ml	BD Biosciences	551218
IFN-γ	R4-6A2	1mg/ml	2ug/ml	BD Biosciences	551216
Secondary Abs	Clone	Stock	Final conc.	Company	Cat. No.
IL-17A	TC11-18H10	0.5mg/ml	1ug/ml	BD Biosciences	555067
IL-10	SXC-1	0.5mg/ml	1ug/ml	BD Biosciences	554423
IL-4	BVD6-24G2	0.5mg/ml	1ug/ml	BD Biosciences	554390
IL-9	D9302C12	0.5mg/ml	1ug/ml	BioLegend	504804
IFN-γ	XMG1.2	0.5mg/ml	1ug/ml	BD Biosciences	554410

Chromatin Immunoprecipitation (ChIP)

In vitro differentiated Th cells (1 x 10^7) were cross-linked for 15 mins with 1% formaldehyde at RT with rotation. The reaction was quenched by adding 0.125 M glycine and incubated at RT for 5 mins. Cells were washed with ice cold PBS two times. After the second wash, cell pellets were processed for next step or stored at -80 °C for subsequent use. Fixed cells were lysed by 400 µl of cell lysis buffer and incubated on ice for 10 mins. After centrifugation, supernatant was discarded and lysates were incubated with 400 µl of nuclear lysis buffer on ice for 10 mins. Nuclei were degraded and chromosomal DNA were fragmented by sonication using sonicator. After sonication, debris were removed by centrifugation at 13000 rpm for 10 mins at 4°C and supernatant was transferred to new

tube. 40 µl of supernatant containing lysates of 1 x 10^6 cells was used for one ChIP experiment. The supernatant was diluted 10 fold with ChIP dilution buffer. After pre-clearing with salmon sperm DNA, bovine serum albumin, and a protein agarose A bead slurry (50%, Millipore), the supernatant was incubated with the ChIP antibodies for either rabbit polyclonal Etv5, p300, E4BP4, IRF4, STAT6, STAT5 (Santa Cruz Biotechnology), GATA3, IRF4 D9P5H, BATF D7C5, PU.1 (Cell Signaling Technology), SMC1 or CRSP1/TRAP220 (Bethyl Laboratory), H3K14ac or normal rabbit IgG (Millipore), H3K4me1, H3K27ac, H3K4me3 or H3K27me3 (Abcam) at 4°C overnight with rotation. ChIP antibodies are listed in Table 7. The following day, immunocomplexes containing antibody/protein/DNA were incubated with Protein Agarose A or G beads at 4°C for 2~4 hours. After centrifuging at 2000 rpm for 2 mins at 4°C, the supernatant of the IgG control sample was kept for input samples. Immunocomplexes were washed one time with low salt, high salt, LiCl and two times with TE buffer. After the last wash of TE buffer, complexes were incubated with 250 µl of elution buffer at RT for 15 mins with rotation. After centrifuging at 2000 rpm for 2 mins at RT, the supernatant was transferred to new fresh tube. After repeating the elution step, 25 µl of 4M NaCl was added to 500 µl of supernatant to reverse cross-links at 65°C overnight. The next day, DNA was purified using phenol-chloroform extraction, and resuspended in 200 µl of nuclease free water and analyzed by qPCR. SYBR green master mix (Applied Biosystems) was used to measure amplification of DNA using 7500 Fast Real-Time PCR system (Applied Biosystems). ChIP primer sequences are listed in Table 8. After normalization to the Input DNA, the amount of output DNA of each target protein was calculated by subtracting that of the IgG control.

Table 7. Antibodies for ChIP assay

Transcription factor				
Antigen/Name	Clone	Host	Company	Cat. No.
BATF	D7C5	Rabbit	Cell Signaling Technology	8638
C-Jun	H-79	Rabbit	Santa Cruz Biotechnology	sc-1694
CRSP1/TRAP220	polyclonal	Rabbit	Bethyl Laboratories	A300-793A
CTCF	D31H2	Rabbit	Cell Signaling Technology	3418
E4BP4	C-18	Goat	Santa Cruz Biotechnology	Sc-9550
	V-19	Goat	Santa Cruz Biotechnology	Sc-9549
ETV5	H-100	Rabbit	Santa Cruz Biotechnology	Sc-22807
GATA3	D13C9	Rabbit	Cell Signaling Technology	Sc-268
GCN5	H-75	Rabbit	Santa Cruz Biotechnology	Sc-20698
IRF4	D9P5H	Rabbit	Cell Signaling Technology	15106
	H-140	Rabbit	Santa Cruz Biotechnology	Sc-28696
Jun-B	210	Rabbit	Santa Cruz Biotechnology	Sc-73
P300	N-15	Rabbit	Santa Cruz Biotechnology	Sc-584
PU.1	9G7	Rabbit	Cell Signaling Technology	2258
SMC1	polyclonal	Rabbit	Bethyl Laboratories	A300-055A
STAT5	C-17	Rabbit	Santa Cruz Biotechnology	Sc-835
STAT6	M-20	Rabbit	Santa Cruz Biotechnology	Sc-981
Histone modification				
Antigen/Name	Clone	Host	Company	Cat. No.
H3ac	polyclonal	Rabbit	Millipore	06-755
H3K27ac	polyclonal	Rabbit	Abcam	Ab4729
H3K27me3	mAbcam6002	mouse	Abcam	Ab6002
H3K4me1	polyclonal	Rabbit	Abcam	Ab8895
H3K4me3	polyclonal	Rabbit	Abcam	Ab8580
H4K16ac	polyclonal	Rabbit	Millipore	07-329
H4K5ac	monoclonal	Rabbit	Millipore	04-118
H4K5ac	monoclonal	Rabbit	Millipore	04-118
H4K8ac	polyclonal	Rabbit	Millipore	07-328

Normal Rabbit IgG	polyclonal	Rabbit	Millipore	12-370

Table 8. Sequences of ChIP primers

Mouse		
Primers	Forward (5'-3')	Reverse (5'-3')
Il9 CNS-6 -6 kb	GAGCTGAACGCAGGCCAAGAACGA	CTTGGAACTAGTTATCTCTCCACTG
Il9 promoter -5bp ~ -67bp	GTGGGCACTGGGTATCAGTTTGATGT	CAGTCTACCAGCATCTTCCAGTCTAG
Il9 CNS -25_1	ATGTCATGAGGCTTGTCTGC	ACTCCTAATCTTCAAGCCCCT
Il9 CNS -25_2	AGCAGGCGACCACTTTAAAA	GCCAACTCTCAGCATGTGTT
Il9 -35 kb	GAGGGAGAGGGGAAAACACA	TACCGCTCCGCAGTCTAAAT
Il9 -12 kb	GTTGCCTTGGTTATGGTGCT	AGAATGGCCCATGAAGACCA
Human		
Primers	Forward (5'-3')	Reverse (5'-3')
hIL9 CNS-4.5	GTCACCTCACCTGTCTCCTT	ACATTGGTGCAGGGTTTGAG
hIL9 promoter	AAGTGGCCCCAACTTACAGA	CGCTTGCAGACACCTTCAAA
hIL9 CNS -18_1	ACCTAGCCCACTGTGCAACT	CATGATGACCCTGTGGTCTG
hIL9 CNS -18_2	TTTCAGAGTCAGAAGAAAAGATGG	CATTTAGGGTGTTGCCTTTCA
hIL9 -30 kb	AGACCAAGGACGTTAGAGCA	GTTGCCATTTTAGCTAGCTTTGG
hIL9 -12 kb	CTGGGCTCTTTGGAGAAATG	CAATGTGGCTTTTGGGATTT

Luciferase reporter assay

HEK 293T cells were grown in DMEM 1640 with 10% FBS and 1% antibiotics in a 12 well plate. When confluency reached 80~90%, cells were co-transfected with 1 μg of the *Il10* promoter or CNS3 luciferase reporter vector (pGL3 basic) and 1 μg control or Etv5 expressing vector (pcDNA3.1) and 0.5 μg of pRL-TK for endogenous control using lipofectamine 2000 (ThermoFisher Scientific).

EL4 cells, mouse lymphoma cell line, were grown in RPMI 1640 with 10% FBS and 1% antibiotics in a 12 well plate. 2 x 10^6 cells were co-tranfected with 5 μg of the *Il9*

gene locus containing pGL3 basic vector and 5 μg control or interested gene expressing pcDNA3.1 vector and 0.5 μg of pRL-TK for endogenous control using Amaxa Nucleofection Kit L (Lonza).

After 24 hours of transfection, cells were stimulated with PMA/ionomycin (only for EL4 cells) for 6 hours and washed with PBS two times. After the second wash, cells were lysed through 250 μl of passive lysis buffer followed by sonication. After centrifugation at 14000 rpm for 1 min at 4°C, supernatant was transferred to new tube to assess luciferase activities. Luciferase activities were measured using the dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. Firefly values were normalized to renilla values; Firefly values from luciferase reporter vectors, renilla values from pRL-TK

Statistical analysis

Two tailed Student's *t*-test or one-way analysis of variance (ANOVA) were used to generate *p*-value data for all data. Post hoc Tukey test was used for multiple comparisons. $p \leq 0.05$ was considered statistically significant.

RESULTS

Part I. The ETS family transcription factors Etv5 and PU.1 function in parallel to promote Th9 cell development

Etv5 promotes IL-9 production

A recent report demonstrated that Etv5 promotes IL-17 production and is induced in response to STAT-activating cytokines (107). However, Etv5 had limited functions in Th1 cells and seemed to repress IL-4 production. The repression of IL-4 was reminiscent of the phenotype observed in T cells that lack PU.1 expression (158). Thus, we were interested in determining the effects of Etv5 on Th9 development. To test this, we isolated naive T cells from *Etv5*$^{fl/fl}$ mice that were either negative or positive for CD4-Cre (referred to in the figures as Etv5 $^{\Delta CD4}$) and cultured them under Th2 or Th9 skewing conditions. By intracellular cytokine staining, we observed a diminished percentage of IL-9–producing cells in Th2 and Th9 cultures lacking the expression of Etv5 (Figure 8A). We also observed an increased percentage of IL-4^{+} cells in Th2 and Th9 cultures lacking Etv5 (Figure 8A). This observation was consistent with decreased IL-9 and increased IL-4 in the supernatants of Etv5-deficient Th2 and Th9 cultures, and with mRNA expression of these cytokines (Figure 8B, 8C). We further observed that Th2 cytokines, including IL-5 and IL-13, were increased in the absence of Etv5 (Figure 8B). To directly demonstrate the function of Etv5, we transduced developing Th9 cells with retroviruses containing either no cDNA or expressing Etv5. Ectopic expression of Etv5 in Th9 cells increased the production of IL-9 and further decreased the minimal production of IL-4 (Figure 8D). Similarly, ectopic expression of Etv5 in Th2 cells repressed IL-4 production and increased the percentage of cells positive for IL-9 (Figure 8D). Thus, Etv5 regulates production of IL-9.

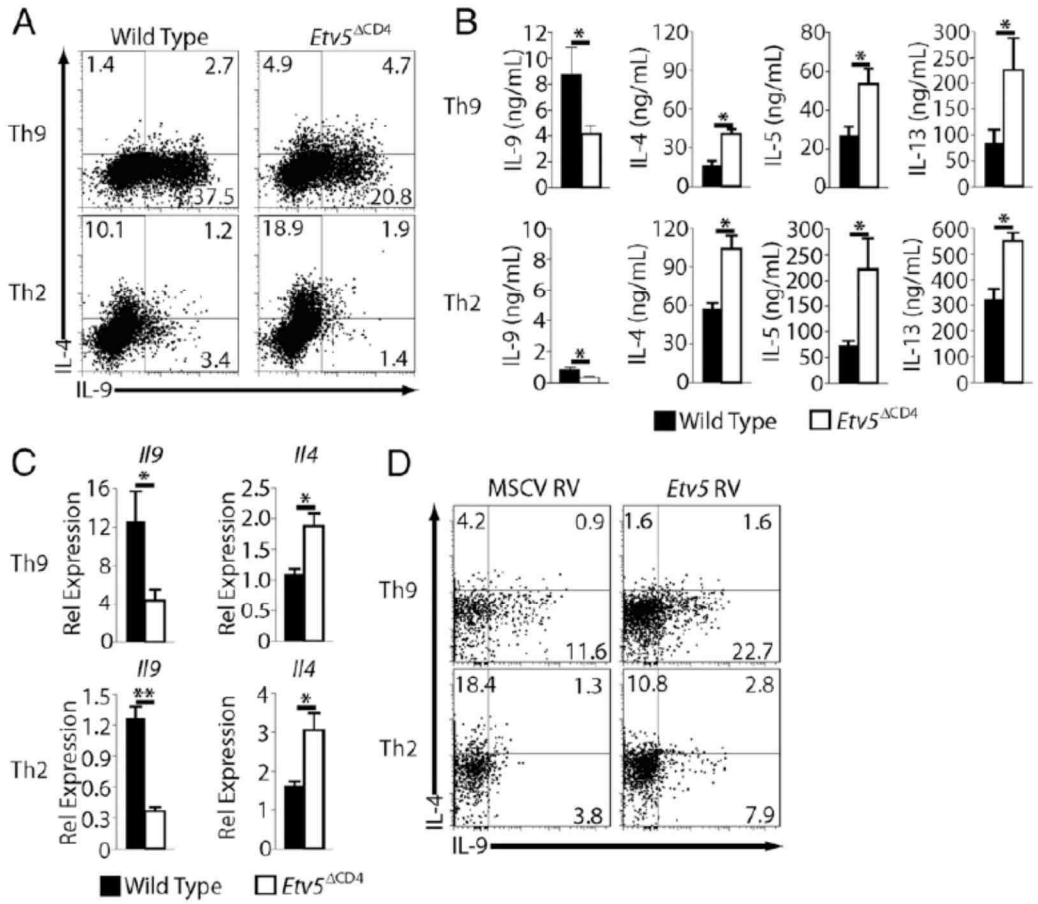

Figure 8. Etv5 promotes IL-9 and inhibitsTh2-associated cytokine production. (A–D) Wild type or Etv5 $^{\Delta CD4}$ naive CD4^{+}T cells were cultured in vitro under Th2 or Th9 conditions for 5 days. (A) Representative FACS profiles of IL-9–and IL-4–producing cells after stimulation with PMA/ionomycin for 6 hours. Depicted cells were gated as FSC^{lo}SSC^{lo}CD4^{+}. (B) Cytokines secreted after stimulation with anti-CD3 for 24 hours. (C) *Il9* and *Il4* expression after stimulation with anti-CD3 for 6 hours. Data were normalized to *β2m* expression. *Il9* expression was relative to wild type Th2, and *Il4* was relative to wild type Th9. (D) Wild type naive CD4^{+}T cells were cultured for 5 days in Th9 condition and were transduced with control or Etv5-expressing retrovirus on day 2 of culture. Representative FACS profiles of IL-9–and IL-4–producing transduced cells. Cells were stimulated with PMA/ionomycin. Depicted cells were gated as FSC^{lo}SSC^{lo}CD4^{+}Thy1.1^{+}. Data are represented as mean ± SEM. (A–C) Data depicted are from two independent experiments (four to five mice per experiment) (D) Data depicted are from three independent experiments (two mice per experiment). A two-tailed Student *t* test was used for pairwise comparisons. *p< 0.05, **p<0.01.

Etv5 gene expression is induced after T cell activation. We compared the expression of Etv5 during the development of Th2, Th9, and inducible Treg cells, and observed that although there is no induction of Etv5 during Treg development, Etv5 is dynamically regulated in Th2 and Th9 cells (Figure 9A). We observed a rapid increase in Th2 cells and a slower increase in Th9 cells that ultimately fell to similar levels after 5 days in culture (Figure 9A, 9B). After stimulation of Th9 cells after 5 days of culture, *Etv5* mRNA is rapidly induced by 4 hours (Figure 9C). Consistent with regulation dissimilar from *Sfpi1*, we observed *Etv5* mRNA was dependent on STAT6 (Figure 9D). *Etv5* mRNA was also decreased in Th9 cultures by the absence of IRF4, suggesting Etv5 expression is downstream of a STAT6/IRF4 network. In contrast, *Etv5* expression was increased in Th9 cultures that lacked the expression of BATF or PU.1, suggesting that IL-4–dependent expression does not require BATF (Figure 9D).

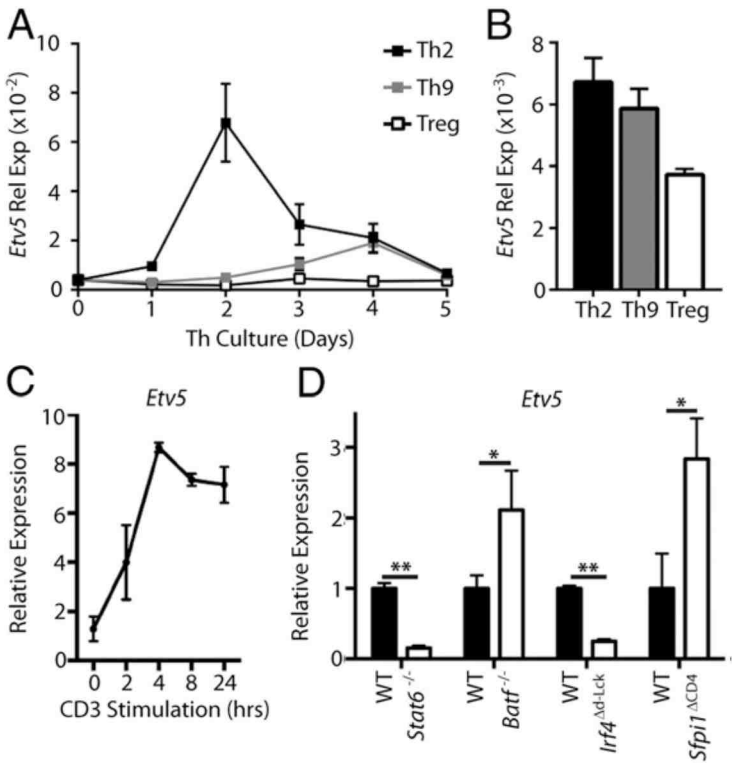

Figure 9. Etv5 expression in Th cell subsets. (A–C) Wild type naive CD4^{+}T cells were cultured in vitro under Treg, Th2, or Th9 conditions for 5 days. (A) Every 24 hours during the culture period, RNA was isolated to assay for *Etv5* expression by qRT-PCR in each culture condition. (B) Comparison of *Etv5* expression among the subsets at day 5 of culture. (C) *Etv5* mRNA levels in Th9 cells activated with anti-CD3 for the indicated time points. (D) T cells from mice of the indicated genotypes were cultured in vitro under Th9 condition over 5 days. *Etv5* expression was measured using qRT-PCR at day 5 of culture. A two-tailed Student *t* test was used for pairwise comparisons. **p*< 0.05, ***p*<0.01. (A–D) Data are represented as mean ± SEM from at least two independent experiments.

Chromatin remodeling at the *Il9* locus by Etv5

Previous reports indicated that PU.1 bound to the *Il9* promoter and recruited Gcn5-containing complexes to alter the histone modifications at the *Il9* locus (59, 101). We first tested whether Etv5 also binds to the *Il9* locus at three CNS regions: CNS-6, CNS1 (*Il9* promoter), and CNS+5.5 (155). Etv5 binding was significantly enriched at the CNS-6 and CNS+5.5 regions (Figure 10A). This was in contrast with PU.1 binding that is enriched primarily at the *Il9* promoter (59, 90). Moreover, Etv5 binding at CNS+5.5 was significantly greater in Th9 cells than in either Th2 or Th17 cells, and binding at CNS-6 was significantly greater in Th9 than Th17, with a trend toward increased binding compared with Th2 cells (Figure 10B). The binding of Etv5 to a nonconserved sequence in the *Il9* locus was barely detectable (Figure 10B). PU.1 and Etv5 also had differential effects on the recruitment of HAT proteins to the locus. In contrast with PU.1-deficient Th9 cells that had increased p300 association and decreased Gcn5 association with the *Il9* locus (101), Etv5-deficient cells had normal Gcn5 recruitment but significantly decreased p300 recruitment, correlating with significant decreases in the overall histone H3 acetylation and H4K16 acetylation at the *Il9* promoter (Figure 10E, 10F). The histone acetylation events were specific because there was no difference in the acetylation of H4K5 or H4K8 between control and Etv5- deficient Th9 cells (Figure 10G, 10H). These results suggest that although PU.1 and Etv5 have overlapping biological functions, they promote the expression of *Il9* by acting on the *Il9* locus through distinct binding sites.

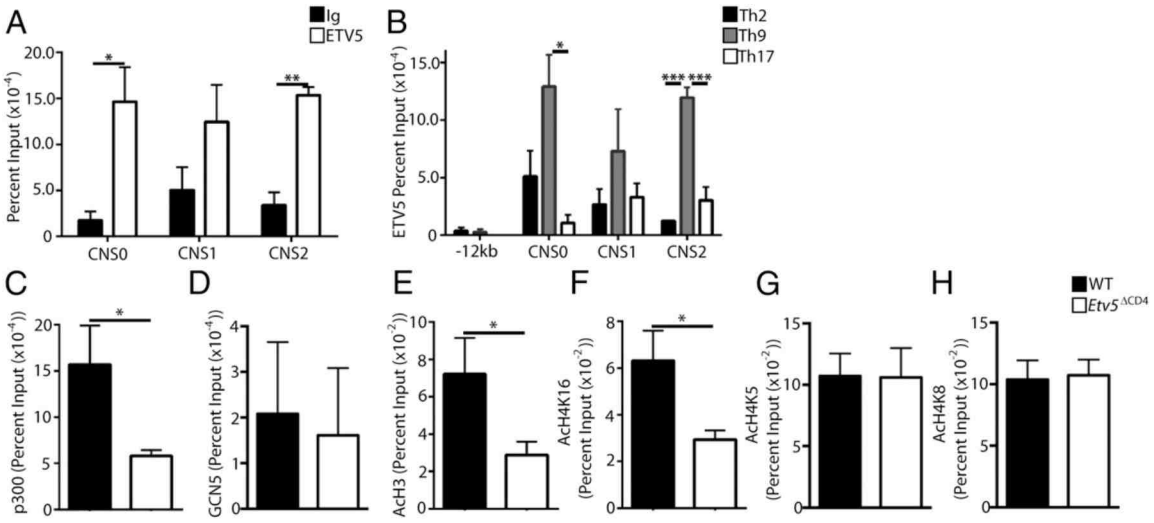

Figure 10. Etv5 binds and enhances histone acetylation at the *Il9* locus (A–H) Wild type or Etv5 $^{\Delta CD4}$ naive CD4^{+}T cells were cultured in vitro under Th9 condition, unless depicted otherwise, for 5 days. (A) Etv5 binding, with respective isotype controls, at the *Il9* locus. (B) Etv5 binding within indicated Th cultured cells at the *Il9* locus and a -12 kb region of the *Il9* locus (negative control). (C and D) p300 or GCN5 binding to the *Il9* promoter. (E–H) Depicted histone acetylation levels at the *Il9* promoter. (B–H) Percent input depicted are the Etv5 ChIP values after subtraction of the control Ig ChIP values. Data are represented as mean ± SEM from two to three independent experiments (three mice per experiment). A two-tailed Student *t* test was used for pairwise comparisons. One-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. **p*<0.05, ***p*<0.01, ****p*<0.001.

Parallel effects of PU.1 and Etv5 in vitro

These results suggested that Etv5 has distinct and overlapping functions with PU.1 in the development of Th9 cells. To test this directly, we mated mice with a conditional PU.1-expressing allele (*Sfpi1* $^{fl/fl}$) with the *Etv5*$^{fl/fl}$ mice, in the context of the CD4-Cre transgene. Naive CD4$^+$ T cells were isolated from control, *Etv5*$^{fl/fl}$ CD4-Cre, *Sfpi1*$^{fl/fl}$ CD4-Cre, and *Sfpi1* $^{fl/fl}$/*Etv5*$^{fl/fl}$ CD4-Cre mice, and cultured under Th9 conditions. Consistent with our previous reports, Th9 cultures that were deficient in PU.1 had diminished IL-9 production (Figure 11). Similarly decreased levels of IL-9 were observed in Etv5-deficient Th9 cultures. Importantly, Th9 cultures that were doubly deficient in PU.1 and Etv5 showed an additive effect of the deletion of each gene and had the lowest percentages of IL-9 producing Th cells. Interestingly, although deficiency in either PU.1 or Etv5 increases the production of IL-4, there was not an additive effect of deficiency in both factors, with double-deficient Th9 cultures having intermediate percentages of IL-4 producing Th cells.

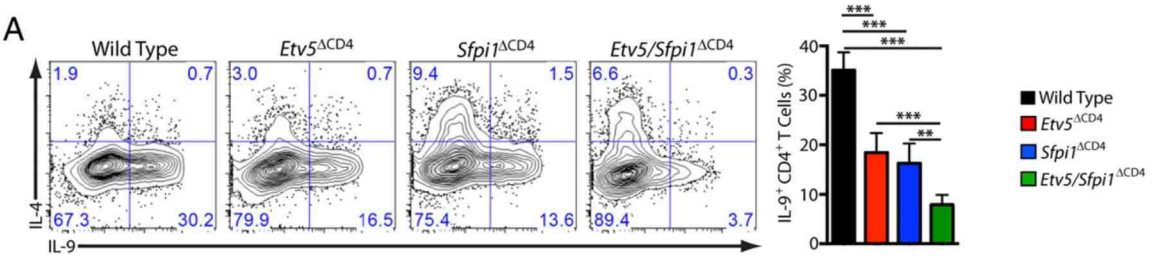

Figure 11. Effect of deficiency in *Etv5* and *Sfpi1* on Th9 differentiation. Wild type, *Etv5* $^{\Delta CD4}$, *Sfpi1* $^{\Delta CD4}$, or *Etv5/Sfpi1* $^{\Delta CD4}$ naive CD4$^+$ T cells were cultured in vitro under Th9 condition for 5 days. Representative FACS profiles of IL-9– and IL-4–producing cells. Cells were stimulated with PMA/ionomycin. Depicted cells were gated as FSC^{lo}SSC^{lo}CD4$^+$. Data are represented as mean ± SEM from three independent experiments (two to three mice per experiment). One-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. **p*<0.05, ***p*<0.01, ****p*<0.001. Experiments performed in collaboration with Dr. Matthew Hufford.

Parallel effects of PU.1 and Etv5 in vivo

We then tested whether combined deficiency in PU.1 and Etv5 had distinct effects on IL-9 production when tested in vivo. We used the OVA/Alum allergic airway inflammation model that has previously been shown to be partially dependent on IL-9 and PU.1 expression in Th cells (59). Mice were sensitized and challenged as portrayed in Figure 12A. Overall, we observed that there were parallel effects of deficiency in both PU.1 and Etv5 in Th cells, with each affecting distinct aspects of inflammation. We observed that PU.1 alone had a greater effect on total inflammation as assessed by histology and eosinophils in the lung (Figure 12B, 12D, 12H). Etv5 deficiency resulted in significant decreases in mucus production, polymorphonuclear leukocytes (PMNs) infiltration, and mast cell accumulation (Figure 12C, 12F, 12H, 12I). Among these parameters, the double-deficient mice were not significantly different from the respective single-deficient mice, suggesting that PU.1 and Etv5 were controlling separate aspects of inflammation. However, only the double-deficient mice were significantly lower than wild type mice in total lung cell accumulation, *Muc5ac* expression, and *Mcpt2* expression (Figure 12E, 12G, 12J). Moreover, double-deficient mice demonstrated *Mcpt1* expression that was significantly lower than wild type and either of the single-deficient mice (Figure 12J).

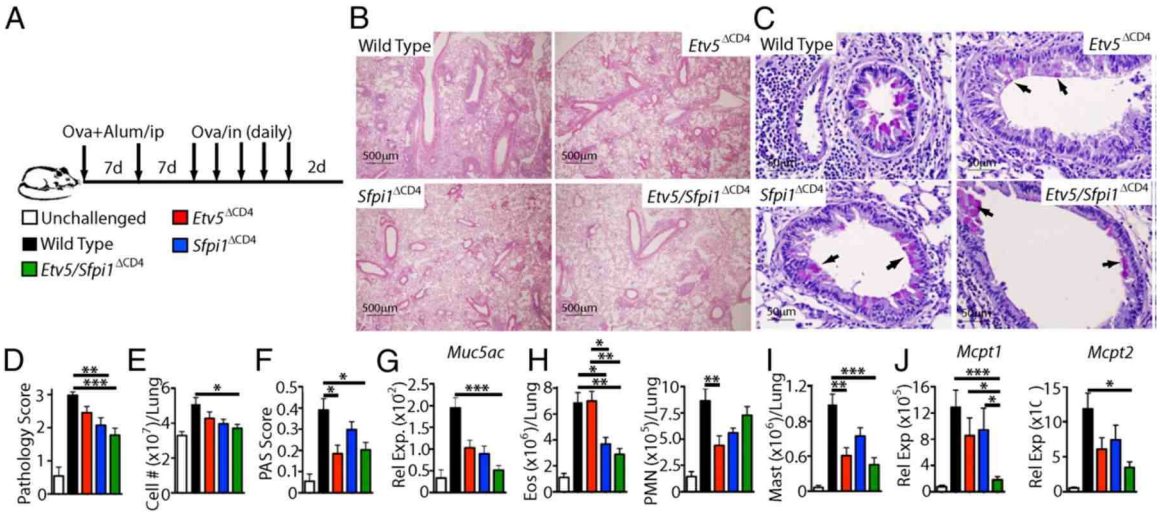

Figure 12. Pathology of mice deficient in both *Etv5* and *Sfpi1* in an allergic disease model. (A) Schematic of OVA/Alum model used to promote allergic inflammation. Unchallenged mice were sensitized with OVA/Alum but did not receive OVA challenge intranasally. Representative images of H&E (B) and PAS staining (C) of inflamed lung tissue. (D) Tabulated pathology scores from H&E-stained tissues. (E) Absolute cell numbers of the lungs. (F) Tabulated PAS scores. (G) *Muc5ac* expression in the lung. Data were normalized to *β2m* expression. (H–I) Eosinophil ($FSC^{lo}SSC^{int/hi}SigLecF^{+}CD11c^{-}$), neutrophil ($FSC^{lo}SSC^{int}Ly6G^{+}CD11b^{hi}$), and mast cell ($FSC^{int}SSC^{int}Fc\varepsilon R1^{+}cKit^{+}$) absolute numbers in the lung. (J) *Mcpt1* and *Mcpt2* expression in the lung. Data were normalized to *β2m* expression. (D–J) Data represented as mean ± SEM from two independent experiments (five to six mice per treatment per experiment). One-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. **p*<0.05, ***p*<0.01, ****p*<0.001. Experiments performed in collaboration with Dr. Matthew Hufford.

We then tested whether this corresponded to decreased IL-9 production in vivo. Although there was a similar reduction in total $CD4^+$ T cells in the lung in each single- or double-deficient mouse (Figure 13A), there was an overall decrease in the number and percentage of IL-9 producing Th cells in the lung, with T cells from double-deficient mice demonstrating IL-9 reduced to background amounts (Figure 13B, 13C), although these values were not significantly decreased compared to mice with PU.1-deficient Th cells. This is in contrast with the numbers and percentages of lung Th2 cytokine–producing Th cells that were, respectively, modestly decreased or unaffected (Figure 13B, 13C). The number of IL-13– producing Th cells in the lung was decreased in double-deficient mice, although the percentages in the lung were not decreased, suggesting a link to overall inflammation (Figure 12). Moreover, in the periphery, Th2 responses developed normally. OVA stimulation of draining lymph node cells yielded normal or slightly increased production of Th2 cytokines (Figure 13D). This was in contrast with OVA-stimulated IL-9 concentrations that were significantly decreased in cultures from mice with PU.1-deficient or double deficient T cells (Figure 13D). Together, these data demonstrate a role for Etv5 in regulating IL-9 production and Th9 development in vitro and regulating allergic inflammation in parallel with PU.1.

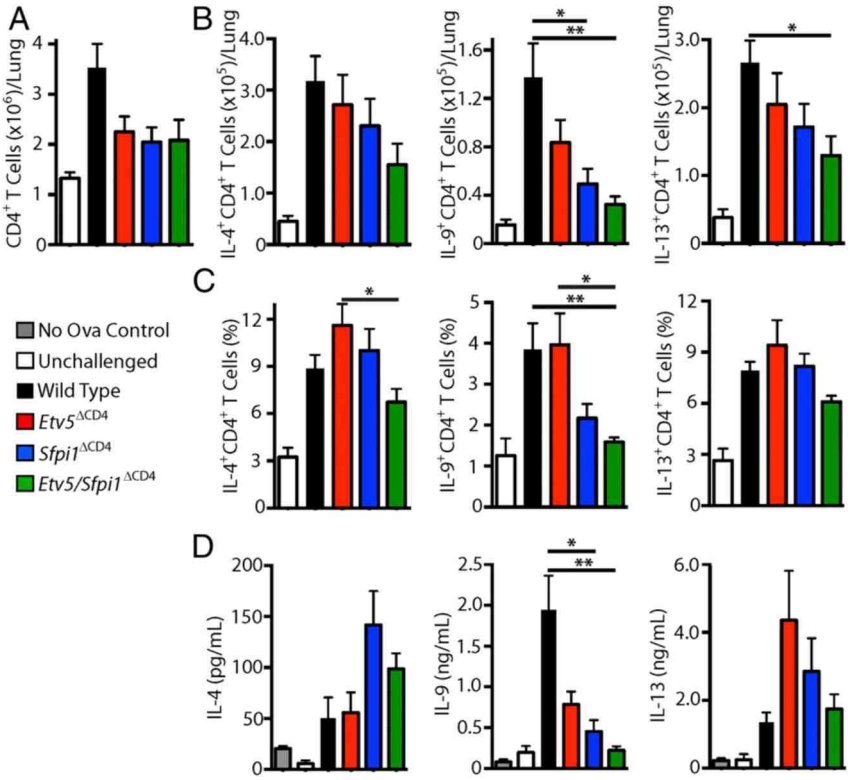

Figure 13. CD4⁺ T cell responses in *Etv5*- and *Sfpi1*-deficient mice during allergic inflammation. Pulmonary allergic inflammation was generated as in Figure 12. (A) Absolute number of CD4⁺ T cells ($FSC^{lo}SSC^{lo}CD3\varepsilon^{+}CD4^{+}CD8^{-}$) in the lung. Numbers (B) and percentage (C) of cytokine producing CD4⁺ T cells in the lung. (D) Cells were collected from the lung draining lymph nodes in allergic mice. Equivalent numbers of cells were cultured with exogenous Ova for 36 hours. Supernatant was analyzed for depicted cytokines. Data are represented as mean ± SEM from two independent experiments (five to six mice per treatment per experiment). One-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. **p*<0.05, ***p*<0.01, ****p*<0.001. Experiments performed in collaboration with Dr. Matthew Hufford.

Part II. Etv5 regulates IL-10 production in Th cells

Etv5 promotes IL-10 production in Th1 and Th2 cells

Our previous studies have demonstrated that Etv5 promotes the production of IL-17A, IL-17F, and IL-21 in Th17 cells, while it had a modest repressive effect on Th2 cytokines (107). Interestingly, a recent study showed that Etv5 is highly expressed in IL-10-producing Th1 cells (159). To determine if Etv5 had a role in regulating IL-10 production, we isolated naïve $CD4^+$ T cells from *Etv5*$^{fl/fl}$ CD4-Cre mice and Cre-negative littermate control and cultured them under Th1 and Th2 cell polarizing conditions before measuring IFN-γ, IL-4 and IL-10 production using intracellular cytokine staining. Although total IFN-γ-producing Th1 cells or IL-4-producing Th2 cell populations were not affected by Etv5 deficiency, we observed a significant decrease in IL-10-producing Th1 and Th2 cells in the absence of Etv5 (Figure 14A-D). Consistent with these results, *Il10* gene expression and secreted IL-10 concentrations were significantly decreased in Etv5 deficient Th1 and Th2 cells (Figure 14C &D). To further test the function of Etv5, we introduced Etv5 into Th1 and Th2 cells using retroviral transduction. Ectopic expression of Etv5 strongly enhanced IL-10 production in both Th1 and Th2 cells (Figure 14E). However, the total number of Th1 or Th2 cells were not affected (data not shown). Taken together, these data suggested that Etv5 positively regulates IL-10 production in both Th1 and Th2 cells without affecting lineage specific cytokine production.

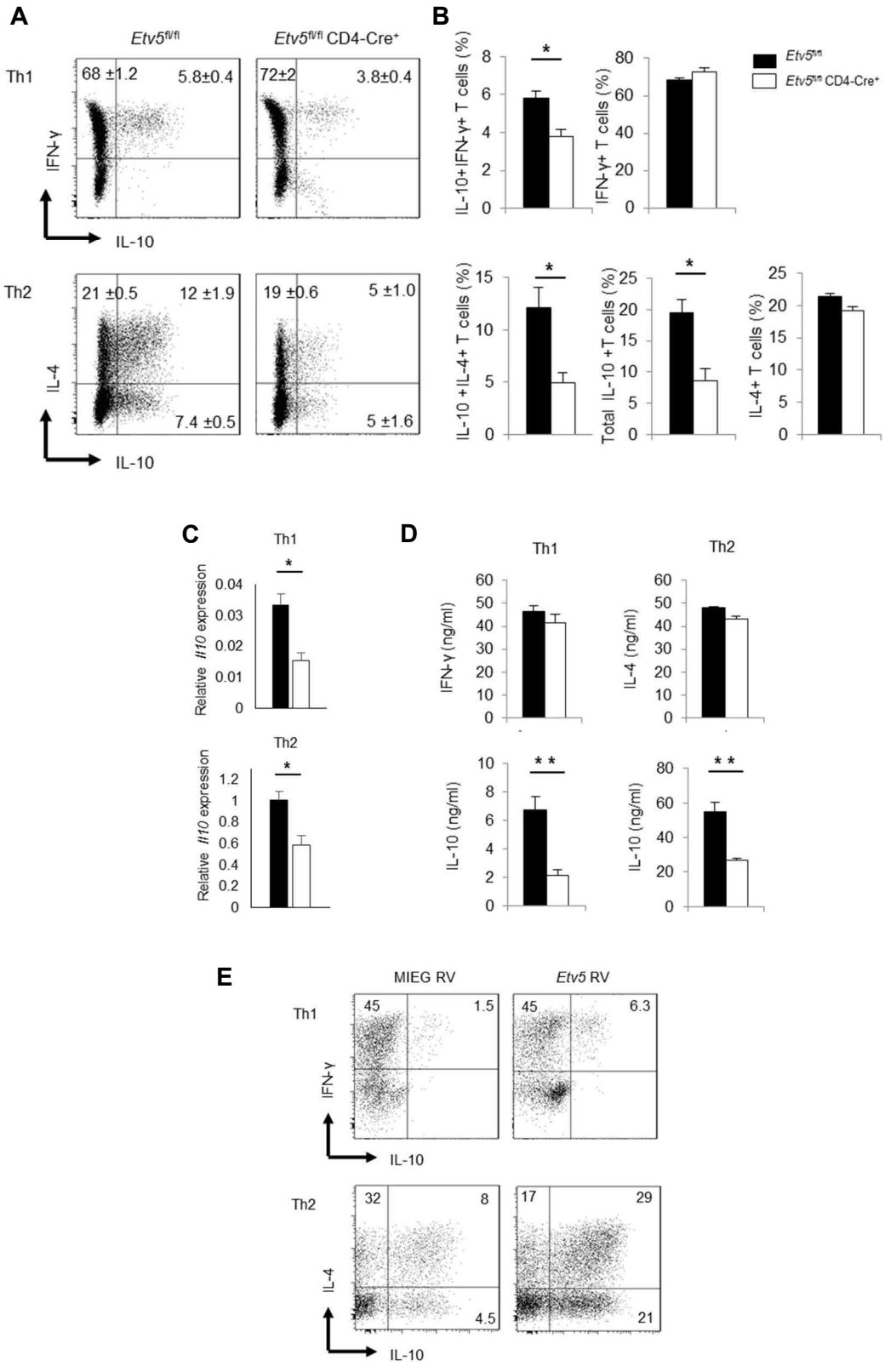

Figure 14. Etv5 promotes IL-10 but does not affect IL-4 and IFN-γ production.

Figure 14. Etv5 promotes IL-10 but does not affect IL-4 and IFN-γ production. (A-D) Naïve $CD4^+$ T cells from control and *Etv5*$^{fl/fl}$ CD4 Cre^+ mice were cultured under Th1 or Th2 cell conditions for 5 days. (A) On day 5, Th1 and Th2 cells were stimulated with PMA/ionomycin for 6 hours to measure cytokine production using intracellular staining. (B) Average percentage of IL-10-producing Th1 and Th2 cells. (C-D) Th1 and Th2 cells were restimulated with anti-CD3 for 6 hours or overnight to measure *Il10* gene expression by using qRT-PCR (C) or to assess cytokine production by means of ELISA (D). (E) Naïve $CD4^+$ T cells from wild type mice were differentiated under Th1 and Th2 cell conditions. Twenty four hours after initiation of culture, cells were transduced with control or Etv5 expressing retrovirus. On day 5, cells were stimulated with PMA/ionomycin for 6 hours to measure cytokine production using intracellular staining. Data are mean ± SEM of 4 mice per group and representative of three independent experiments. A two-tailed Student *t* test was used for pairwise comparisons. *$p < 0.05$, **$p < 0.005$.

Etv5 regulates IL-10 production in vivo

A. fumigatus-challenged *Il10*-/- mice showed exaggerated airway inflammation with significant increased production of Th2 cytokines (160). To define the effect of Etv5 on Th2 cells in vivo, we sensitized mice with *A. fumigatus* extract every other day for 21 days to induce type 2 inflammation (Figure 15A). One day following the final challenge, we collected and counted BAL cells. There was no difference in cell numbers between control mice and mice that had Etv5-deficient T cells (Figure 15B). However, the IL-10-producing $CD4^+$ T cell population was significantly decreased in mice that had Etv5-deficient T cells in both BAL and lung, compared to control mice (Figure 15C). This result was consistent with decreased IL-10 present in the BAL fluid in mice with Etv5-deficient T cells (Figure 15E). However, Etv5 deficiency did not affect the production of IL-4 in vivo (Figure 15C-D). These data demonstrated that Etv5 plays a crucial role in regulating IL-10 production in vivo.

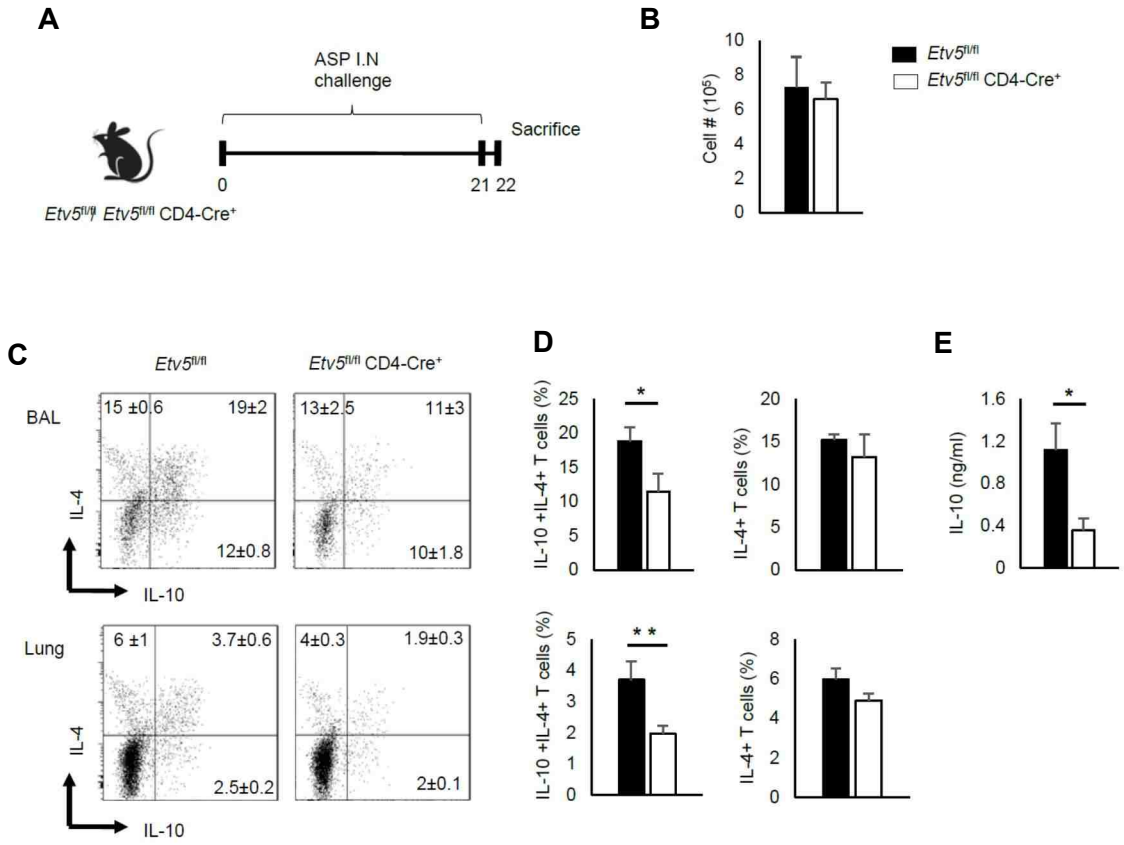

Figure 15. Etv5 deficient Th2 cells produce less IL-10 in *A. fumigatus* extract-induced airway inflammation. (A) Control and *Etv5*$^{fl/fl}$ CD4 Cre^{+} mice were intranasally challenged with *A.fumigatus* extract every other day for 21 days. (B) Total cell count in the BAL of *A.fumigatus* extract-challenged control and *Etv5*$^{fl/fl}$ CD4 Cre^{+} mice. (C-D) Lung and BAL cells were stimulated with PMA/ionomycin for 6 hours to measure cytokine production using intracellular staining. Representative dot plots (C) and average percentage of IL-10 producing Th2 cells in BAL and lung (D) are indicated. Cells for flow cytometric analysis were gated on lymphocyte size and granularity, and the expression of CD4 and TCRβ. (E) IL-10 production of BAL cells was measured using ELISA. Data are mean ± SEM of 6 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pairwise comparisons. *$p < 0.05$, **$p < 0.005$.

Etv5 directly binds to the *Il10* locus and promotes IL-10 production

The *Il10* gene locus consists of a promoter and several CNS regions (28). Previous studies reported that several IL-10 inducing transcription factors bind to the *Il10* locus and promote *Il10* expression (136-139). Specifically, CNS3 is a primary target region of IL-10-inducing transcription factors and critical for IL-10 regulation (Figure 16A). To test the ability of Etv5 to directly activate gene expression from *Il10* regulatory elements, we co-transfected 293T cells with Etv5 expressing vector and reporter vector containing the *Il10* promoter or CNS3 region. Etv5 significantly increased CNS3 reporter activity but did not activate the *Il10* promoter reporter activity (Figure 16B). We then tested whether Etv5 directly binds to the the *Il10* promoter and CNS3 region using ChIP assay. Etv5 strongly bound to CNS3 region in Th2 cells but not in Th0 cells that have the minimal expression of IL-10 (Figure 16C and data not shown). As a control, Etv5 did not bind to several regulatory elements at the *Il4* locus, consistent with a lack of altered IL-4 production in Th2 cells lacking Etv5 (Figure 16C). These data suggest that Etv5 directly binds to the *Il10* CNS3 region and promotes gene expression from binding this element.

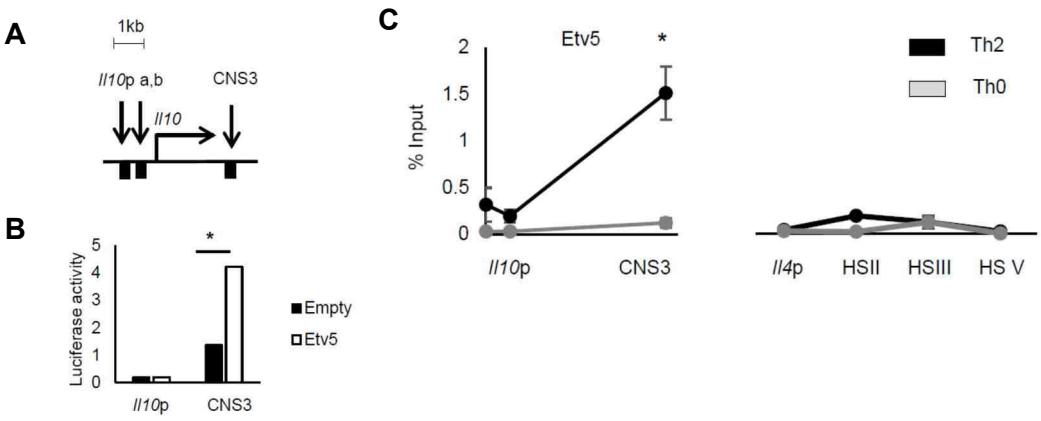

Figure 16. Etv5 binds to the *Il10* locus in Th2 cells. (A) Schematic of the *Il10* locus. (B) Luciferase activity of 293T cells transfected with Etv5 expressing vector or control vector along with *Il10* locus reporter vectors. (C) ChIP analysis of Etv5 binding to the *Il10* and *Il4* loci in Th2 or Th0 cells. Data are mean ± SEM of two independent experiments or 4 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pairwise comparisons. *$p < 0.05$.

Etv5 recruits IL-10-inducing transcription factors onto the *Il10* locus

Although the reporter assay would suggest that Etv5 can directly activate the *Il10* gene, it is also possible that Etv5 regulates the expression of other transcription factors that are required for IL-10 expression. To determine whether Etv5 regulates the expression of those transcription factors, we measured gene expression of the transcription factors in control or Etv5-deficient Th2 cells. The expression of *Nfil3*, a global IL-10 regulator that encodes E4BP4, was not affected by Etv5 deficiency (Figure 17A). Similarly, gene expression of other transcription factors was not affected by Etv5 deficiency (Figure 17A).

Despite normal expression of other IL-10-inducing transcription factors, it was still possible that they were not able to bind to the *Il10* locus in the absence of Etv5. To test whether these factors were still binding the *Il10* locus in the absence of Etv5, we used ChIP assays to assess binding in control and Etv5-deficient Th2 cells. E4BP4 bound to the *Il10* promoter, intronic region (+2.5 kb) and more strongly CNS3 region in wild type Th2 cells (Figure 17B). However, in absence of Etv5, E4BP4 failed to bind to these regions, suggesting that Etv5 deficiency affected the binding of E4BP4 across the entire *Il10* locus, especially at the CNS3 region. In the CNS3 region, recruitment of all transcription factors was significantly decreased in Etv5 deficient Th2 cells (Figure 17C). Etv5-deficient Th2 cells also showed decreased H3K4me3. (Figure 17D). p300 strongly bound to the CNS3 region, but binding was greatly decreased in Etv5-deficient Th2 cells (Figure 17D). Histone 3 lysine 14 acetylation, linked to p300 activity, was also significantly decreased in Etv5 deficient Th2 cells (Figure 17D). Thus, Etv5 is required for the maximal binding of IL-10-inducing transcription factors to the CNS3 region in Th2 cells.

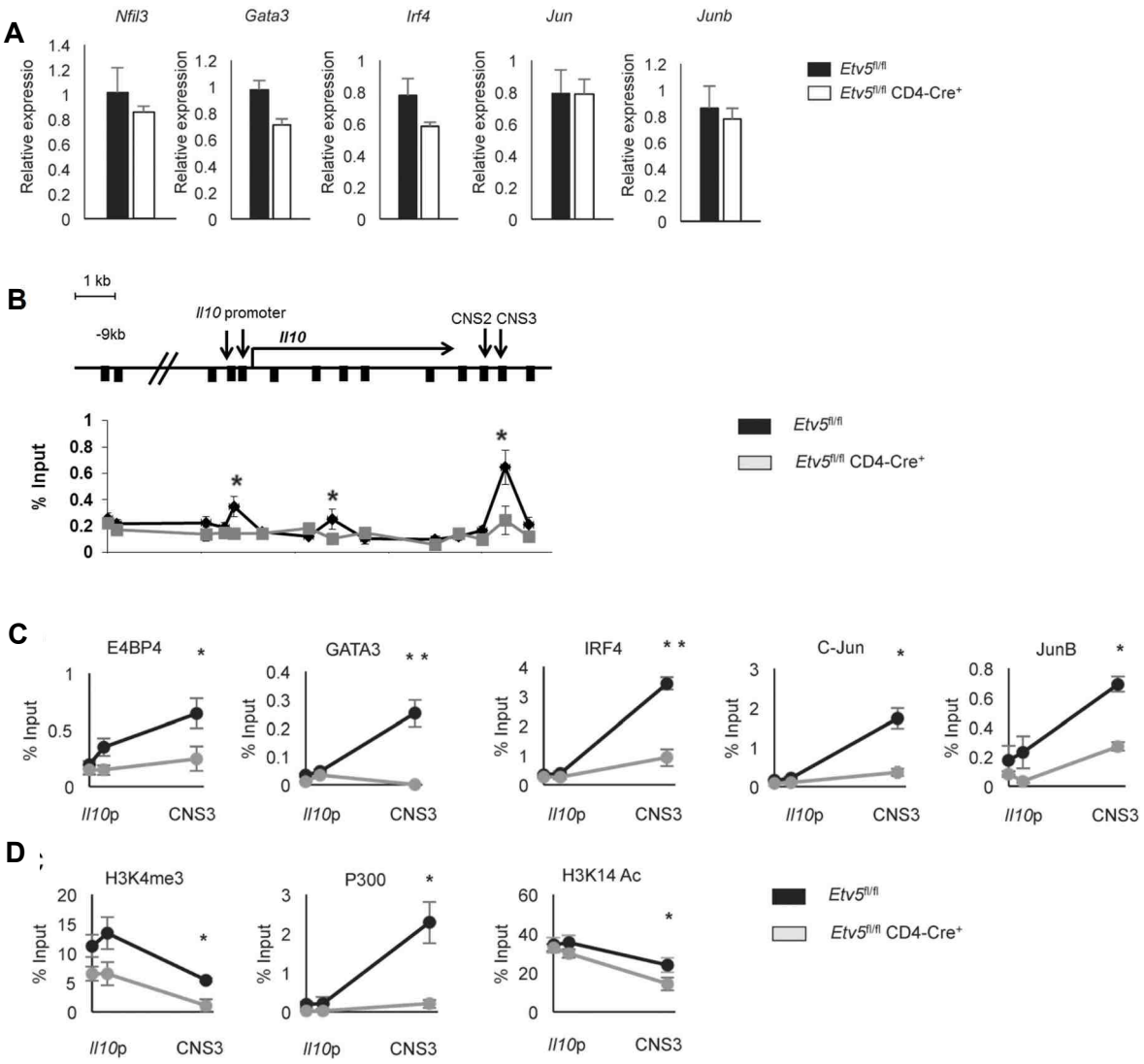

Figure 17. Etv5 enhances the binding of IL-10-inducing transcription factors. (A) Expression of IL-10-inducing transcription factors in control and *Etv5*$^{fl/fl}$ CD4 Cre^{+} Th2 cells were measured using qRT-PCR. (B-D) ChIP analysis of E4BP4 (B), IL-10 inducing transcription factors (C), or histone modifications and p300 (D) at the *Il10* locus in WT or Etv5-deficient Th2 cells. Data are mean ± SEM of 4 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pairwise comparisons. *$p < 0.05$, **$p < 0.005$.

Etv5 functionally cooperates with GATA3 and E4BP4

The diminished binding of multiple transcription factors to the *Il10* locus suggests that transduction of those factors into Etv5-deficient T cells would also have diminished effects. To test this directly, we ectopically introduced Etv5, E4BP4, or GATA3 into wild type or Etv5-deficient Th2 cells using retroviral transduction and measured IL-10 production using intracellular staining. In wild type Th2 cells, ectopic expression of each factor significantly promoted IL-10 production (Figure 18A-B). In Etv5-deficient Th2 cells, ectopic Etv5 expression rescued IL-10 production to control levels (Figure 18A-B). Ectopic expression of GATA3 and E4BP4 increased IL-10-producing cells, but effects were still decreased in the absence of Etv5, compared to wild type Th2 cells. These data further support an important role for Etv5 in the appropriate functions of GATA3 and E4BP4 in *Il10* regulation.

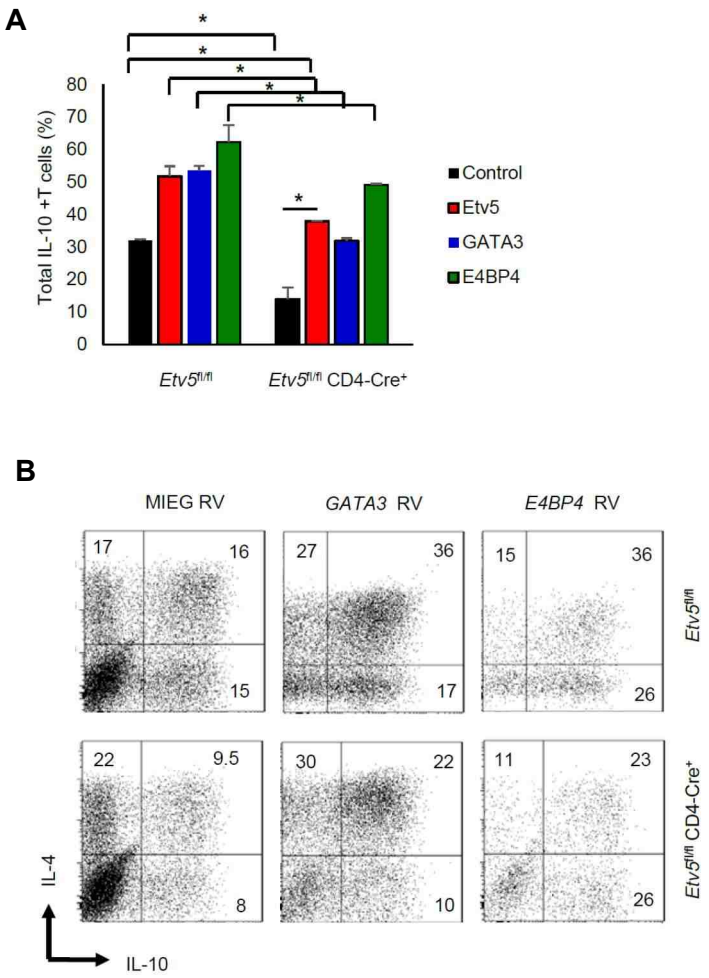

Figure 18. Etv5 deficiency decreases the ectopic expression effects of IL-10 inducing transcription factors. WT and Etv5-deficient naïve $CD4^+$ T cells were cultured under Th2 cell condition for 5 days. After one day of culture, cells were transduced with control or transcription factor-expressing retrovirus. On day 5, cells were restimulated with PMA/ionomycin for 6 hours to assess IL-10 production using intracellular staining analysis. (A) Average percentage of IL-10 producing cells and (B) representative of flow cytometry image of data in (A). Data are mean ± SEM of 3 mice per group and representative of three independent experiments. A two-tailed Student *t* test was used for pairwise comparisons. *$p < 0.05$, **$p < 0.005$.

Ectopic Etv5 expression restores its function in Etv5 deficient Th2 cells

Our data suggests that IL-10-inducing transcription factors require Etv5 for optimal binding to the *Il10* locus and IL-10 induction. To test whether re-introduction of Etv5 rescues the binding of other transcription factors to the *Il10* locus, we introduced Etv5 into Etv5 deficient Th2 cells using retroviral transduction followed by ChIP assay. Etv5 deficient Th2 cells infected by empty virus showed decreased binding of IRF4, GATA3 and E4BP4 at the CNS3 region, compared to wild type Th2 cells infected by the same virus (Figure 19), consistent with data in Figure 17C using non-transduced cells. Importantly, ectopic Etv5 expression in Etv5-deficient Th2 cells restored the binding of all three transcription factors at the CNS3 region indicating that Etv5 plays a pivotal role in regulating the binding of transcription factors to the *Il10* locus.

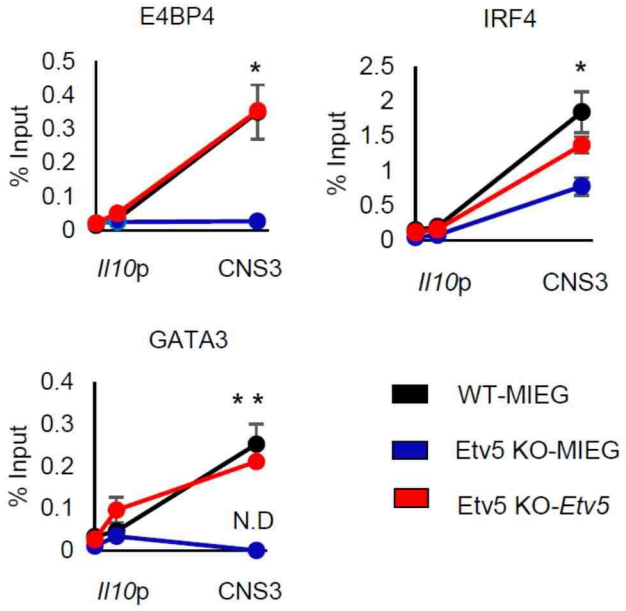

Figure 19. Ectopically introduced Etv5 in Etv5-deficient Th2 cells increases transcription factor binding to the *Il10* locus. After one day of culture, Etv5-deficient Th2 cells were transduced with control or Etv5 expressing retrovirus. On day 5 of culture, ChIP assay was performed to examine transcription factor binding at the *Il10* locus. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pairwise comparisons. *$p < 0.05$, **$p < 0.005$.

Part III. Identification of novel enhancers for *Il9* gene expression in T cells

p300 ChIP and ChIP-seq

Enhancers function independent of their location which makes identification of enhancers more challenging. To narrow down enhancer candidates for *Il9* gene expression, we used several strategies. Because enhancers are highly conserved between species, we screened CNSs around the *Il9* locus using evolutionary conservation of region (ECR) browser. In addition to CNS+5.5 and CNS-6, a region 25 kb upstream of the *Il9* TSS in mice is highly conserved with a -18 kb region of the human *Il9* gene (Figure 20A). p300 binding is an indicator of active enhancers (111). To examine the binding of p300 across the *Il9* locus, p300 ChIP assay was performed in T cell subsets. In Th9 cells, p300 strongly bound to CNS-25, CNS-6 and the *Il9* promoter (*Il9p*) (Figure 20B). Consistent with p300 ChIP analysis, p300 ChIP-seq data showed strong signals at the CNS-25, CNS-6 and *Il9* promoter (Figure 20C). We examined this region in Th2 cells using publically available data (GSE 22104 (8)/ GSE 40463 (123)). We observed signals for p300 and H3K4me1 at the CNS-25 and CNS-6 but not at the *Il9* promoter, consistent with low or absent *Il9* expression in Th2 cells. STAT6 binding was also observed at the CNS-25 and CNS-6.

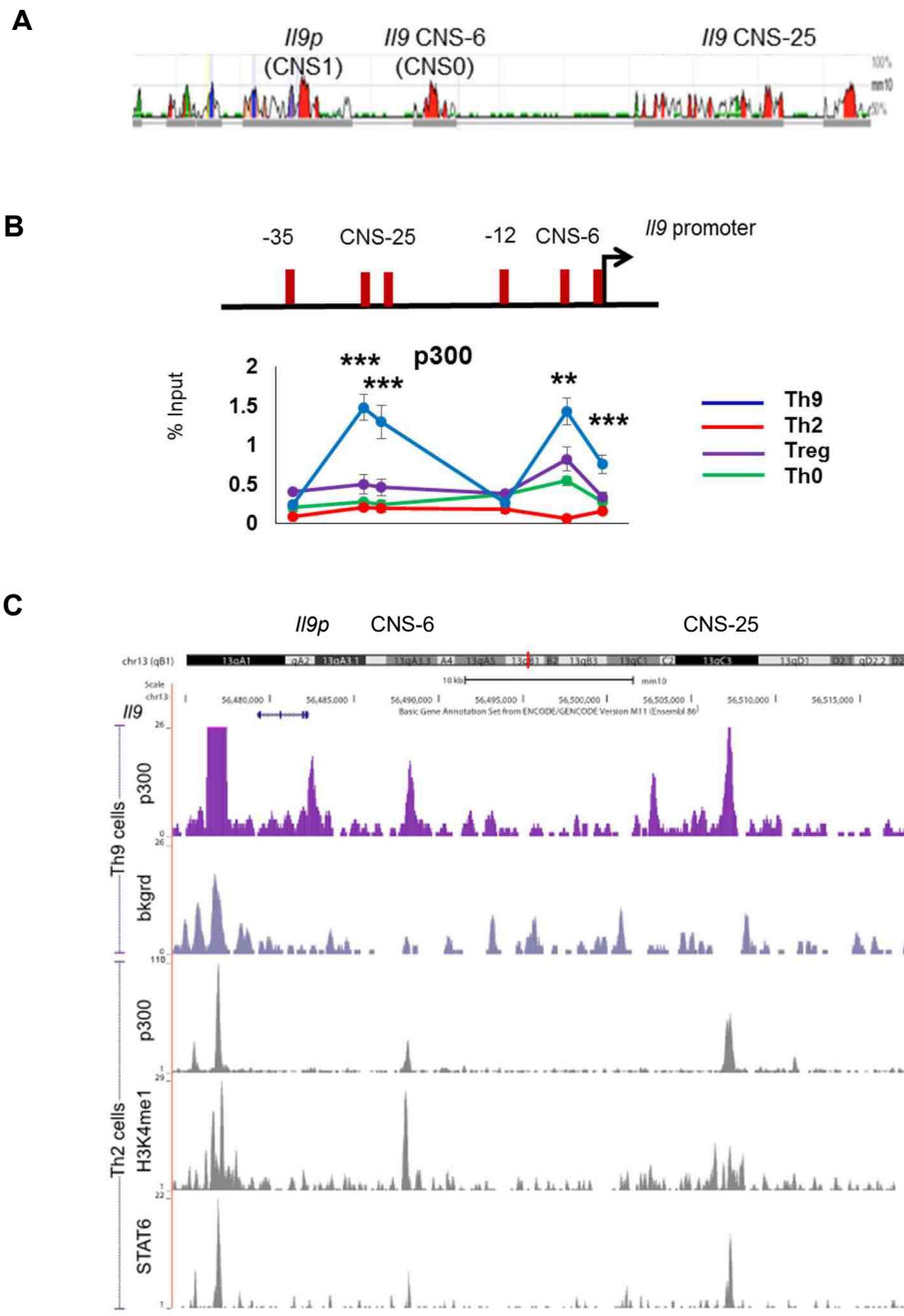

Figure 20. p300 binds to the CNS-25, CNS-6 and the *Il9* promoter. (A) Schematic of the mouse *Il9* locus and conserved regions with the human *IL9* locus. (B) Naïve CD4$^+$ T cells were isolated from WT mice and cultured under different Th cell polarizing conditions. On day 5, cells were harvested for ChIP analysis using p300 antibody. ChIP analysis of p300 at the CNS regions of the *Il9* locus and at -12 kb, -35 kb regions for negative control. Percent input depicted are the histone modification ChIP values after subtraction of the control IgG ChIP values. (C) ChIP seq analysis of p300 in Th9 cells and p300, STAT6 and H3K4me1 in Th2 cells. Data are mean ± SEM (n=4/experiment). One-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. ***p* <0.01, ****p* <0.001

Analysis of CNS-25 enhancer activity

Based on p300 ChIP and ChIP-seq analysis, we hypothesized that the CNS-25 region might be an enhancer for *Il9* gene. To test whether CNS-25 has an enhancer activity, dual luciferase reporter assays were performed. We co-transfected EL4 cells with the *Il9p* reporter vectors with or without CNS-25, and an IRF4 expression vector. Consistent with previous reports, IRF4 significantly increased *Il9p* activity (Figure 21A). In the absence of IRF4, CNS-25 did not enhance *Il9p* activity. However, when co-transfected with IRF4, CNS-25 significantly enhanced *Il9p* activity. Because enhancers function independently of their direction and location, *Il9p* reporter vector was modified by adding reverse direction of CNS-25 into upstream of *Il9* promoter or 3' of the luciferase gene. Regardless of direction and location, CNS-25 significantly enhanced IRF4-induced *Il9p* activity. These data suggest that CNS-25 has an enhancer activity. BATF and IRF4 cooperate to bind to an AICE element in the *Il9* promoter and promote *Il9* gene expression (54). After screening AICE sites in CNS-25 region, the IRF4 consensus sequence in these sites were point mutated in a CNS-25-*Il9p* reporter vector (Figure 21B). After co-transfection of EL4 cells with these vectors and IRF4 expression vector, *Il9p* reporter activities were measured. AICE#1~#3 mutations caused modest decrease in *Il9p* reporter activities. Point mutations in AICE#4~#7 did not affect *Il9p* activity. Next, we deleted sequences between AICE#1 and AICE#2 (25bp), AICE#3 and AICE#5 (40bp), AICE#6 and AICE#7 (85bp) in the CNS-25-*Il9p* reporter vector (Figure 21C). After co-transfection of EL4 cells with these vectors and IRF4 expression vector, *Il9p* reporter activities were measured. Similar to the point mutations in AICEs, deletion of AICEs showed only modest and insignificant decreases in *Il9p* reporter activity. These data suggest that these sites might not be actual AICEs. Another possibility is that point mutations or deletions of AICEs in CNS-25 might not be sufficient for a significant effect on the *Il9* reporter activity because of redundancy of AICEs.

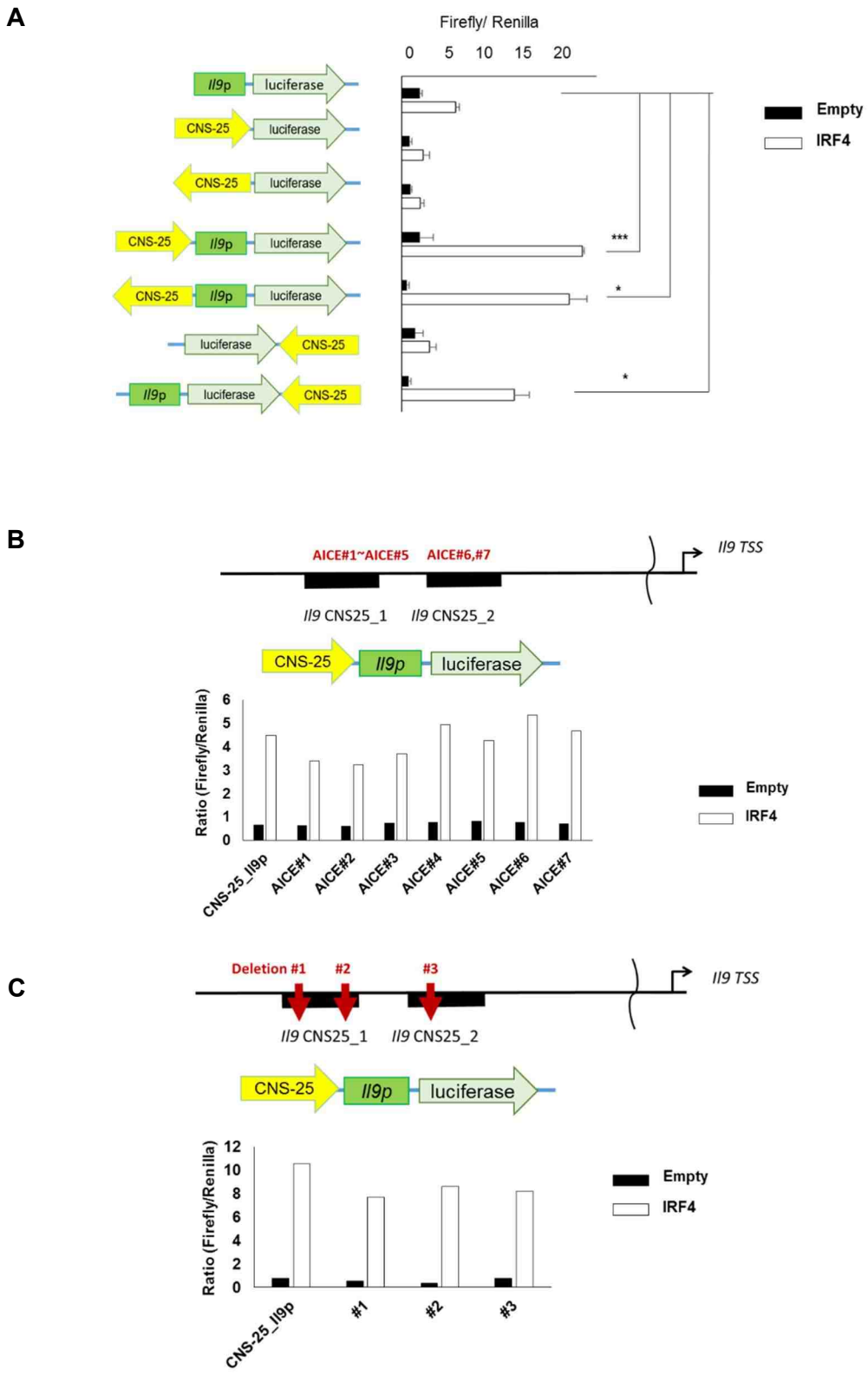

Figure 21. CNS-25 enhances *Il9* promoter activity.

Figure 21. CNS-25 enhances *Il9* promoter activity. (A) EL4 T cells were transfected with IRF4 expressing vector or control vector along with the *Il9* locus reporter vectors following activation with PMA/ionomycin for 6 hours to assess reporter activities. (B) Schematic of the CNS-25-*Il9p* reporter vector with point mutations in AICE candidates and their effects on *Il9p* activity (C) Schematic of the CNS-25-*Il9p* reporter vector with deletions of AICE candidates and their effects on *Il9p* activity. Data are mean ± SEM of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. **p* < 0.05, ***p* <0.01, ****p* <0.001

Histone modifications and the binding of transcription factors at the *Il9* locus

Previous studies showed that active enhancers have opened chromatin status with active histone modifications such as H3K4me1 and H3K27ac (127). These histone modifications change closed chromatin, called heterochromatin, to opened chromatin, called euchromatin, which allows TFs to bind to accessible DNA (111).

To determine the chromatin status of CNS-25 in T cell subsets, ChIP assays were performed using antibodies against active enhancer marks H3K4me1 and H3K27ac, and repressive mark H3K27me3. Both H3K4me1 and H3K27ac modifications at the CNS-25 were significantly higher in Th9 cells than other T cell subsets (Figure 22B). In contrast, H3K27me3 was significantly lower at both CNS-25 and *Il9* promoter in Th9 cells compared to other T cell subsets. These results indicate that the CNS-25 has an activated and opened chromatin status in Th9 cells which allow TFs easily get access to this region. Next, we measured H3K4me3, an active promoter marker, to determine whether CNS-25 has a promoter activity. H3K4me3 was the highest at the *Il9* promoter in Th9 cells among T cell subsets. However, H3K4me3 was much lower at the CNS-25 than at the *Il9* promoter in Th9 cells suggesting that CNS-25 does not have a promoter activity. The histone modification analyses further support that CNS-25 is an *Il9* enhancer.

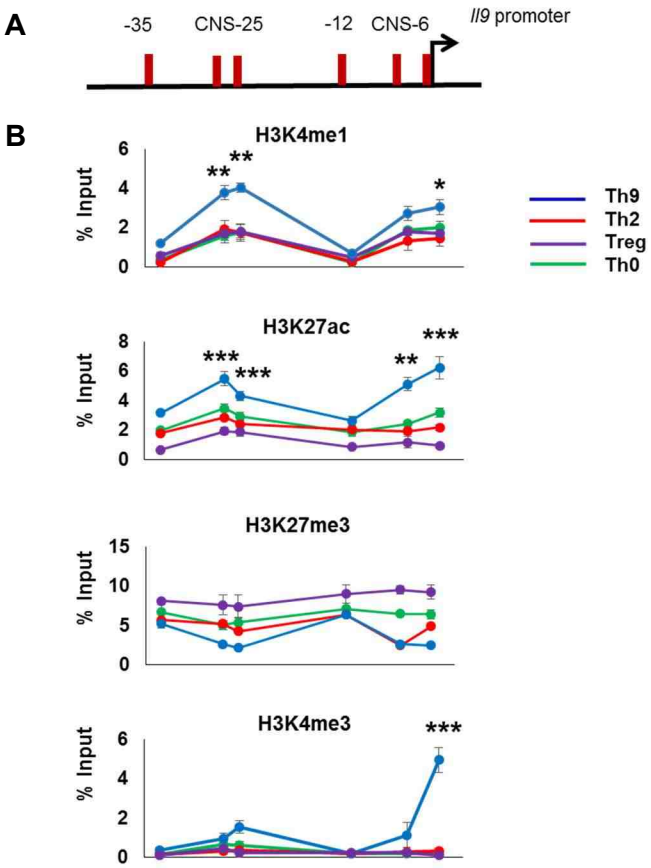

Figure 22. Active histone modifications at the *Il9* locus in Th cell subsets. Naïve CD4$^+$ T cells were isolated from WT mice and cultured under different Th cell polarizing conditions. On day 5, cells were harvested for ChIP analysis using H3K4me1, H3K27ac, H3K27me3 and H3K4me3 antibodies. (A) Schematic of the *Il9* locus in mice. (B) ChIP analysis of histone modifications at the *Il9* locus. Data are mean ± SEM (n=4/experiment). One-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. **p* < 0.05, ***p* <0.01, ****p* <0.001

In addition to specific histone modifications, active enhancers are co-occupied with many TFs (111). The results of histone modifications at the CNS-25 suggest that *Il9* inducing TFs might bind to the CNS-25. To examine this, further ChIP assays were performed to measure the binding of TFs such as BATF, IRF4, STAT6, STAT5 and PU.1. Consistent with previous reports (54, 55, 59, 90), BATF, IRF4, STAT6, STAT5 and PU.1

strongly bound to the *Il9* promoter in Th9 cells (Figure 23B). Interestingly, BATF, IRF4, STAT6 and STAT5 also strongly bound to the CNS-25 in Th9 cells. However, PU.1 binding was not detected at CNS-25.

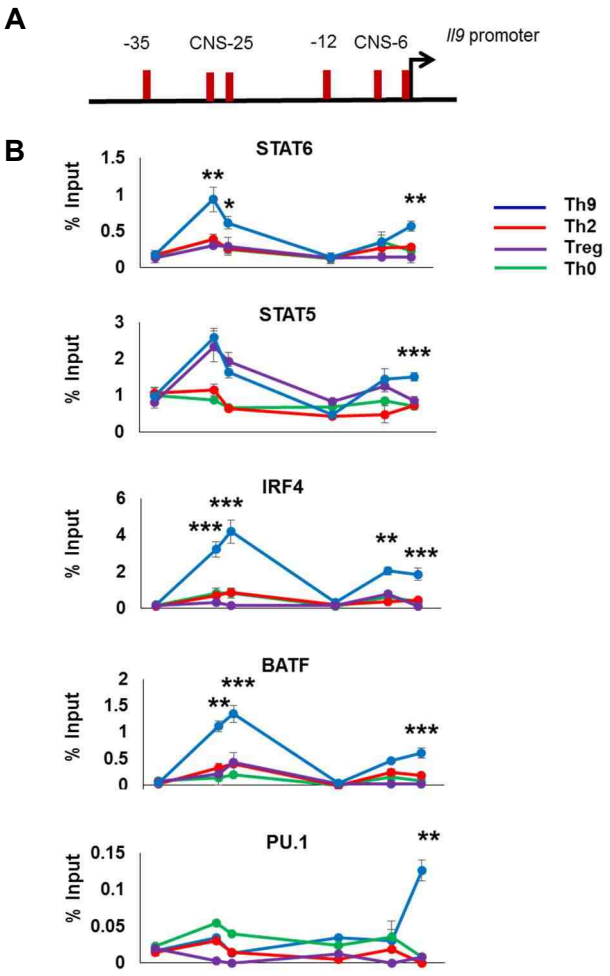

Figure 23. IL-9-inducing transcription factors co-occupy the *Il9* locus in Th9 cells. Naïve $CD4^+$ T cells were isolated from WT mice and cultured under different Th cell polarizing conditions. On day 5, cells were harvested for ChIP analysis using STAT6, STAT5, IRF4, BATF and PU.1 antibodies. (A) Schematic of the *Il9* locus in mice. (B) ChIP analysis of the binding of transcription factors at the *Il9* locus. Data are mean ± SEM (n=4/experiment). One-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Effects of STAT6 and IRF4 deficiency on CNS-25 chromatin modifications

Previous studies revealed that STAT6 and IRF4 are critical IL-9-inducing factors. Both STAT6 and IRF4 deficient CD4$^+$ T cells cultured under Th9 condition showed dramatically reduced IL-9 production (55, 90). To examine the effects of STAT6 and IRF4 deficiency on CNS-25 chromatin modifications, ChIP assays were performed. Either STAT6 or IRF4 deficient Th9 cells failed to recruit p300 and BATF to the CNS-25 (Figure 24A & B). Moreover, H3K4me1 level was also significantly decreased in these cells which might result in failure of the activation of CNS-25.

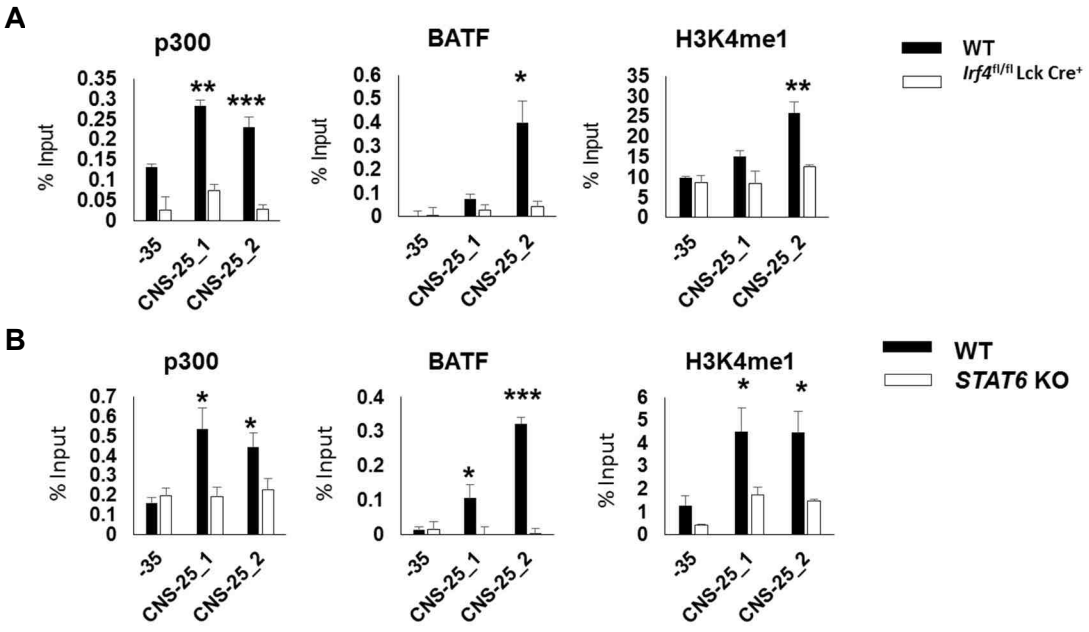

Figure 24. IRF4 and STAT6 are important for mediating chromatin modifications at the CNS-25. Naïve CD4$^+$ T cells were isolated from *Irf4*$^{fl/fl}$ Lck Cre$^+$, *STAT6* KO and WT mice and cultured under Th9 cell polarizing condition. On day 5, *Irf4*$^{fl/fl}$ Lck Cre$^+$ (A) or *STAT6* KO (B) Th9 cells cells were harvested for ChIP analysis using p300, BATF and H3K4me1 antibodies. Data are mean ± SEM of 4 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. **p* < 0.05, ***p* <0.01, ****p* <0.001

CNS-25 is critical for regulating *Il9* gene expression in vitro

To define the function and specificity of CNS-25 for *Il9* gene expression, CNS-25 germline deficient (*Il9* $^{\Delta CNS-25}$) mice were generated by Taconic using a CRISPR/Cas9 approach. (Figure 6). Mutant mice were bred to homozygosity and appeared normal. Lymphoid organ cellularity and composition appeared normal. Proliferation rate and viability of T cells were normal (data not shown). First, we compared IL-9 production in Th cell subsets between *Il9* $^{\Delta CNS-25}$ mice and their littermates as a wild type control. After isolation of naïve $CD4^+$ T cells from these mice, cells were cultured under the indicated Th cell conditions for 4 or 5 days. On day 4 or day 5, IL-9 production of Th cell subsets in wild type and *Il9* $^{\Delta CNS-25}$ mice was analyzed using intracellular staining. IL-9 production was significantly decreased by about 75% in *Il9* $^{\Delta CNS-25}$ Th9 cells compared to wild type Th9 cells on day 4 (Figure 25A, 25B). This defect in IL-9 production by *Il9* $^{\Delta CNS-25}$ Th9 cells on day 4 was similar in the presence or absence of anti-IL-10R neutralizing antibody on day 5 (Figure 25A, 25B).

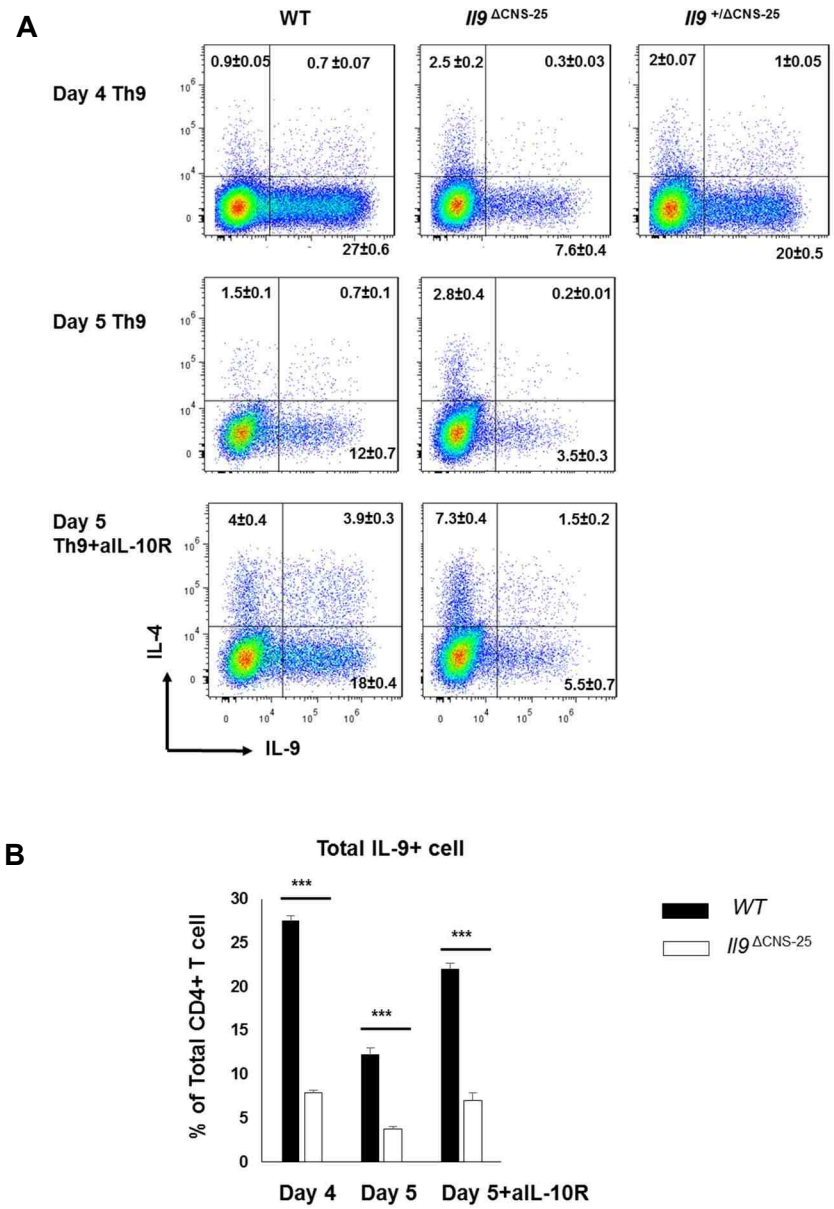

Figure 25. CNS-25 deletion impairs IL-9 production in Th9 cells. Naïve CD4$^+$ T cells were isolated from WT or *Il9* $^{\Delta CNS-25}$ mice and cultured under Th9 cell polarizing condition with or without anti-IL-10R antibody. (A) On day 4 or day 5, cells were restimulated with PMA/ionomycin for 6 hours to measure cytokine production using intracellular staining. (B) Average percentage of IL-9 producing T cells in total CD4$^+$ T cells. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. **p* < 0.05, ***p* <0.01, ****p* <0.001

To confirm flow cytometry data, the amount of IL-9 in the supernatant and *Il9* gene expression in Th9 cells were measured using ELISA and qRT-PCR, respectively. Consistent with flow cytometry data, the amount of IL-9 and *Il9* gene expression level were significantly decreased in *Il9* $^{\Delta CNS-25}$ Th9 cells on day 4 and day 5 (Figure 26 and 27). However, IL-4 and IL-10 production and their gene expression were not affected by CNS-25 deletion suggesting that CNS-25 specifically regulates *Il9* expression (Figure 26 and 27).

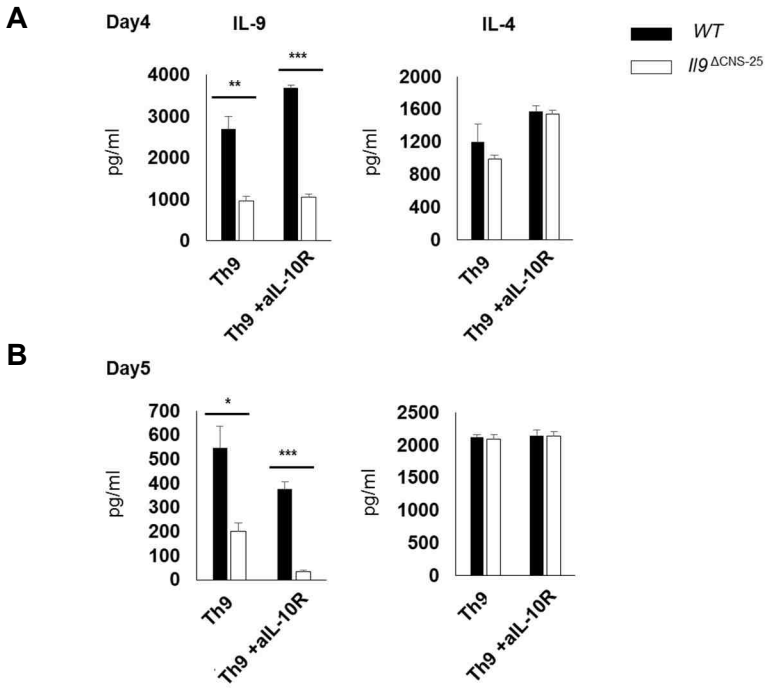

Figure 26. IL-9 production was impaired by CNS-25 deletion in Th9 cells. Day 4 (A) or day 5 (B) cultured Th9 cells were restimulated with anti-CD3 overnight to assess cytokine production by means of ELISA. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. **p* < 0.05, ***p* <0.01, ****p* <0.001

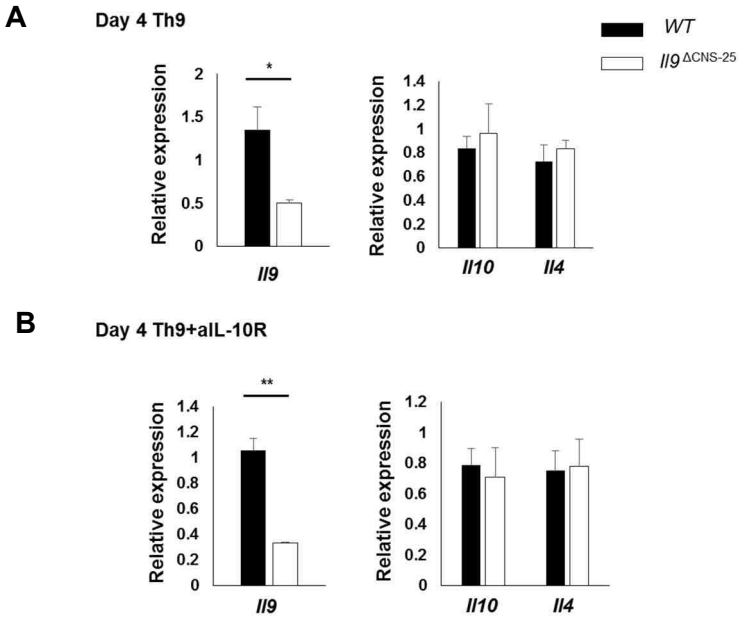

Figure 27. *Il9* gene expression was impaired by CNS-25 deletion in Th9 cells. Day 4 cultured Th9 cells with (A) or without anti-IL10R antibody (B) were restimulated with anti-CD3 for 6 hours to measure cytokine gene expression using qRT-PCR. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. **p* < 0.05, ***p* <0.01

To exclude the possibility that CNS-25 deletion affects *Il9* expression by altering TF expression, TF gene expression was measured using qRT-PCR. *Batf, Irf4, Stat6* and *Sfpi1* gene expression were similar between wild type and *Il9*$^{\Delta CNS\text{-}25}$ Th9 cells (Figure 28A). Additionally, gene expression of IL-9 repressing TFs such as T-bet, Foxp3, RORc, Bcl6, Runx3, Id3 and IRF1, were also not affected by CSN-25 deletion (Figure 28B). These data strongly indicate that CNS-25 specifically regulates *Il9* expression without affecting other cytokine or TF expression which might affect Th9 cell development.

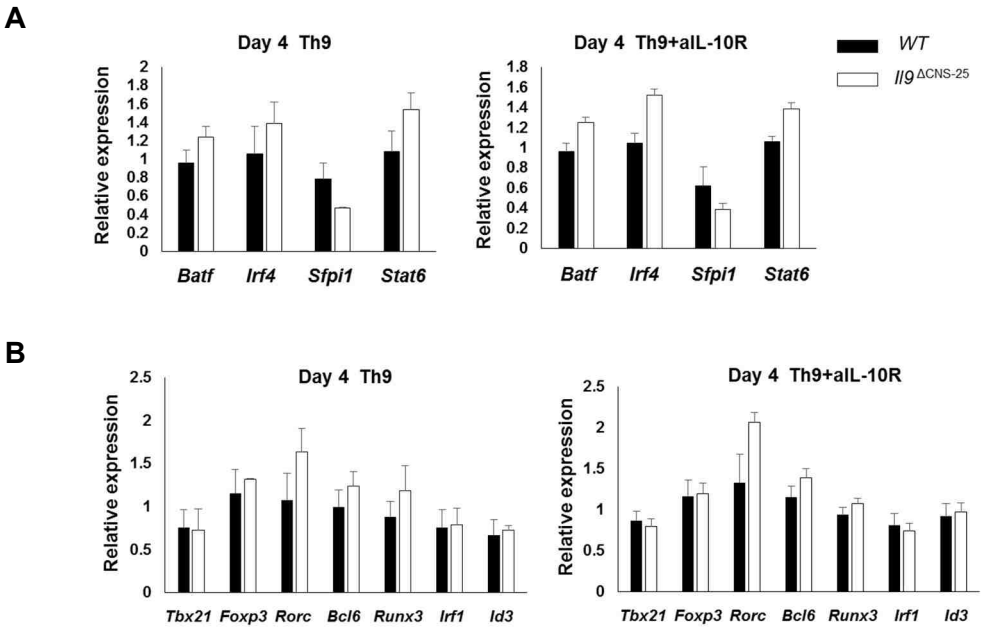

Figure 28. CNS-25 deletion does not affect gene expression of TFs regulating *Il9* expression in Th9 cells. On day 4, Th9 cells were restimulated with anti-CD3 for 6 to measure cytokine gene expression using qRT-PCR. The gene expression of IL-9-inducing (A) or repressing (B) TFs in WT and *Il9* $^{\Delta CNS\text{-}25}$ were measured using qRT-PCR. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. **p* < 0.05, ***p* <0.01, ****p* <0.001

Next, we studied the effect of CNS-25 deletion on *Il9* expression in other Th cell subsets. Human Th17 cells produce IL-9 in response to IL-10 and pro-inflammatory cytokines, and murine Th17 cells also produce IL-9 (98, 161). As a previous report showed, Th17 cells produced IL-9 although the expression was much less than in Th9 cells (compare Figure 29 to Figure 25 and 26). However, *Il9* $^{\Delta CNS-25}$ Th17 cells barely produced IL-9. And CNS-25 deletion did not affect IL-17 production. To confirm this cytometry data, the amounts of IL-9 and IL-17A in the supernatant of Th17 cells were measured by ELISA. IL-9 production was significantly decreased in *Il9* $^{\Delta CNS-25}$ Th17 cells, however IL-17A production was not changed (Figure 29C, 29D). Additionally, the gene expression of *Il17a* and *Rorc* were also not altered in *Il9* $^{\Delta CNS-25}$ Th17 cells (Figure 30).

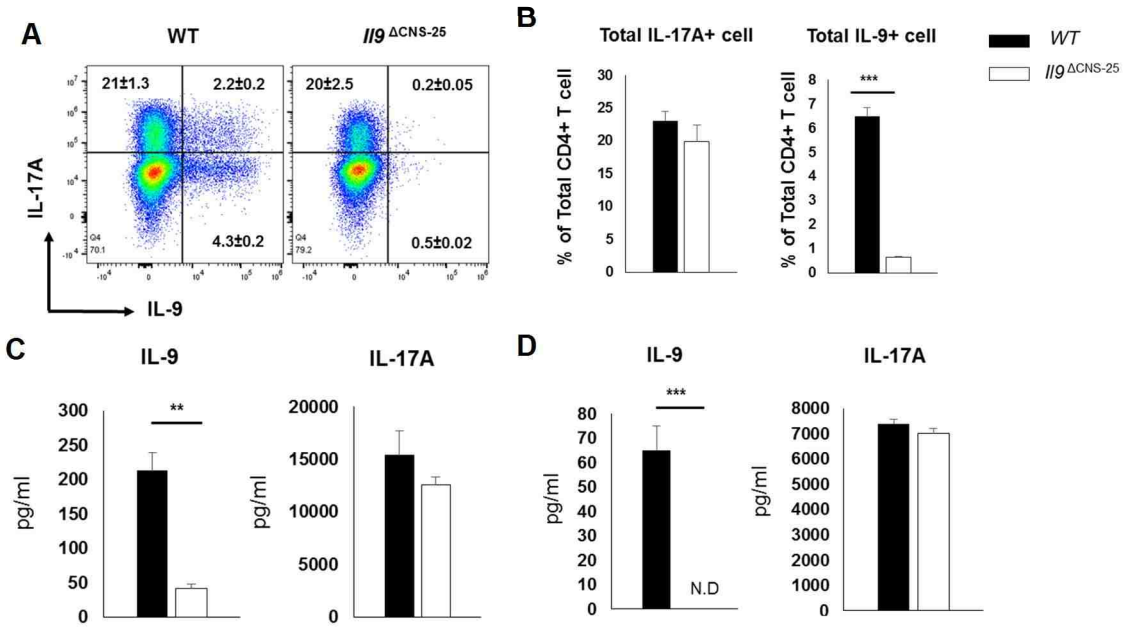

Figure 29. CNS-25 deletion impairs IL-9 production in Th17 cells. Naïve CD4$^+$ T cells were isolated from WT or *Il9* $^{\Delta CNS-25}$ mice and cultured under Th17 cell polarizing condition. On day 4, cells were harvested for analysis. (A) Cells were restimulated with PMA/ionomycin for 6 hours to measure cytokine production using intracellular staining. (B) Average percentage of IL-9 producing T cells in total CD4$^+$ T cells. Day 4 (C) or day 5 (D) cultured Th17 cells were restimulated with anti-CD3 overnight to assess cytokine production by means of ELISA. Data are mean ± SEM of 3 mice per group and representative of two

independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. N.D., not detected.

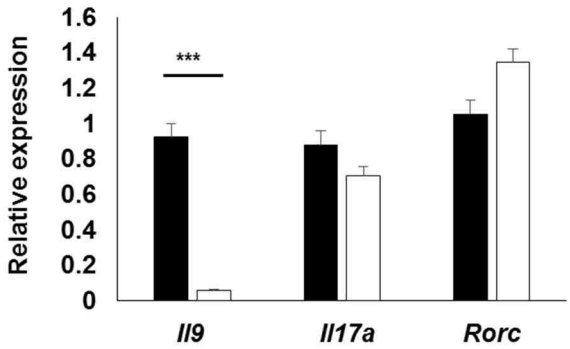

Figure 30. CNS-25 deletion does not affect the gene expression of *Il17a* and *Rorc* in Th17 cells. Day 4 cultured Th17 cells were restimulated with anti-CD3 for 6 hours to measure cytokine gene expression using qRT-PCR. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. **$p < 0.01$, ***$p < 0.001$

In contrast to the effects of CNS-25 deletion in Th9 and Th17 cells, *Il9* $^{\Delta CNS-25}$ Th2 cells produced more IL-9 than wild type Th2 cells although still much less than Th9 cells (compare Figure 31 to Figure 25 and 26). However, *Il9* $^{\Delta CNS-25}$ Th2 cells produced normal amount of IL-4. To confirm this cytometry data, the amounts of IL-9 and IL-4 in the supernatant of Th2 cells were measured by ELISA (Figure 31C, 31D). IL-9 production was significantly increased in *Il9* $^{\Delta CNS-25}$ Th2 cells, however IL-4 production was not changed. Additionally, the gene expression of *Il4* and *Gata3* were not altered in *Il9* $^{\Delta CNS-25}$ Th2 cells (Figure 32). These data suggest that CNS-25 might act as a repressor of *Il9* gene expression in Th2 cells.

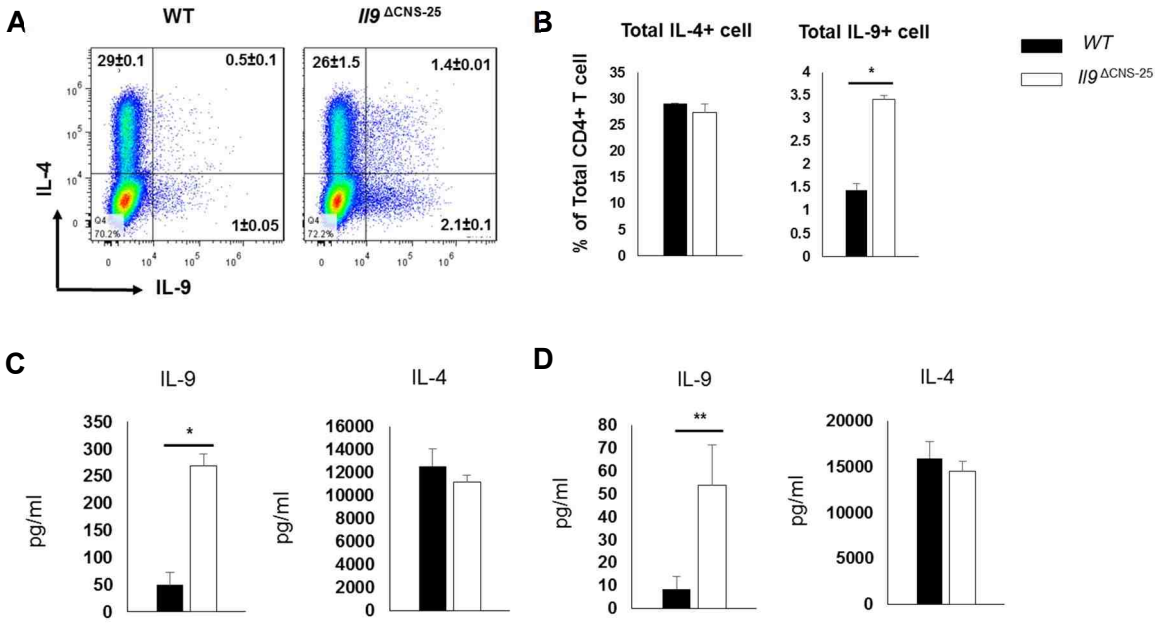

Figure 31. Deletion of CNS-25 promotes IL-9 production in Th2 cells. Naïve CD4$^+$ T cells were isolated from WT or *Il9* $^{\Delta CNS-25}$ mice and cultured under Th2 cell polarizing condition. On day 4, cells were harvested for analysis. (A) Cells were restimulated with PMA/ionomycin for 6 hours to measure cytokine production using intracellular staining. (B) Average percentage of IL-9 producing T cells in total CD4$^+$ T cells. Day 4 (C) or day 5 (D) cultured Th2 cells were restimulated with anti-CD3 overnight to assess cytokine production by means of ELISA. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. **p* < 0.05, ***p* <0.01, ****p* <0.001

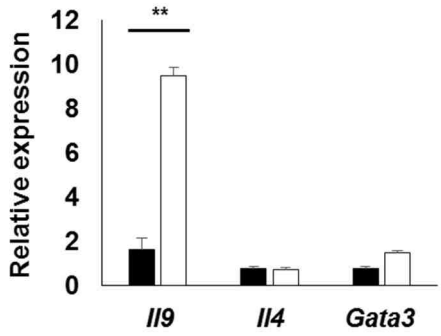

Figure 32. CNS-25 deletion does not affect the gene expression of *Il4* and *Gata3* in Th2 cells. Day 4 cultured Th2 cells were restimulated with anti-CD3 for 6 hours to measure cytokine gene expression using qRT-PCR. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. ***p* <0.01, ****p* <0.001

We also examined whether CNS-25 deletion affects IL-9 production and the development of Treg and Th1 cells. To do this, *Foxp3* and *Ifng* expression were measured, as hallmarks of Treg and Th1 cells respectively. Both *Il9* $^{\Delta CNS-25}$ Treg and Th1 cells expressed normal *Foxp3* and *Tbx21*, which suggest that the CNS-25 deletion does not affect Treg and Th1 cell development (Figure 33, 34). In Treg cells, *Il9* gene expression was significantly decreased in *Il9* $^{\Delta CNS-25}$ Treg cells, however *Il10* expression was not altered (Figure 33). In Th1 cells, IL-9 was barely detected and there were no differences between wild type and *Il9* $^{\Delta CNS-25}$ Th1 cells in either ICS or qRT-PCR (Figure 34). We compared *Il9* expression in Th cell subsets and it was much higher in Th9 cells than other Th cell subsets (Figure 35). These results strongly indicate that CNS-25 is a critical enhancer for regulating *Il9* expression in T cell subsets, especially in Th9 cells, without affecting other cytokines or TF expression which might affect Th9 cell development.

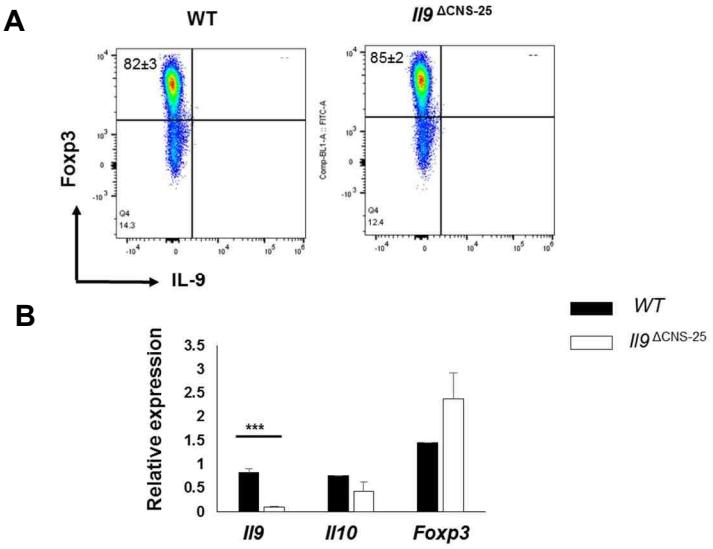

Figure 33. Deletion of CNS-25 impairs IL-9 production in Treg cells. Naïve CD4^{+} T cells were isolated from WT or *Il9* $^{\Delta CNS-25}$ mice and cultured under Treg cell polarizing condition. On day 4, cells were harvested for analysis. (A) Cells were stained with Foxp3. (B) Treg cells were restimulated with anti-CD3 for 6 hours to measure cytokine gene expression using qRT-PCR. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. ***p <0.001

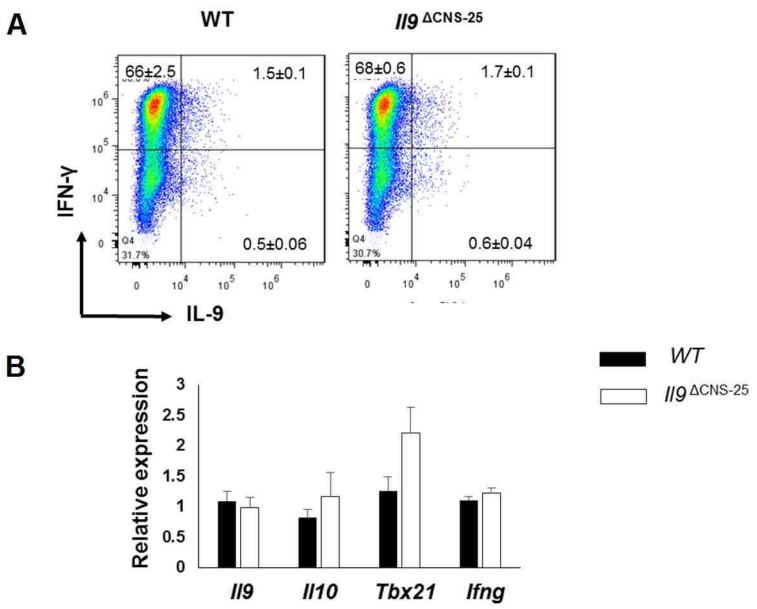

Figure 34. Deletion of CNS-25 does not impair IL-9 production in Th1 cells. Naïve $CD4^+$ T cells were isolated from WT or *Il9* $^{\Delta CNS-25}$ mice and cultured under Th1 cell polarizing condition. On day 4, cells were harvested for analysis. (A) Cells were restimulated with PMA/ionomycin for 6 hours to measure cytokine production using intracellular staining. (B) Th1 cells were restimulated with anti-CD3 for 6 hours to measure cytokine gene expression using qRT-PCR. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons.

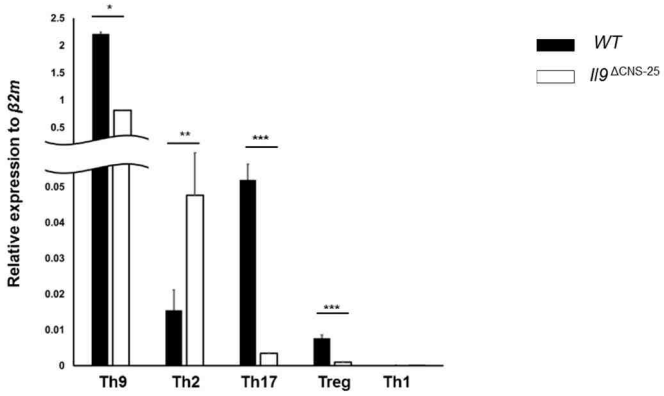

Figure 35. Relative *Il9* expression among Th cell subsets. *Il9* expression in Th cell subsets was measured by qRT-PCR and normalized to *β2m* expression.

CNS-25 deletion attenuates allergic inflammation in asthma models

To define the function of CNS-25 in vivo, the HDM and chronic *A. fumigatus* airway inflammation models were used.

In the HDM extract-induced airway inflammation model, we intranasally challenged wild type, *Il9* $^{\Delta CNS-25}$ and *Il9* $^{+/\Delta CNS-25}$ mice with HDM extract 8 times, every other day for 16 days (Figure 36A). The day following the final challenge, we collected and counted BAL cells (Figure 36B). The population of IL-9-producing $CD4^+$ T cells in the lung was measured using intracellular cytokine staining, and it was significantly decreased in the lung of *Il9* $^{\Delta CNS-25}$ mice, compared to wild type and *Il9* $^{+/\Delta CNS-25}$ mice.

In the *A. fumigatus* extract-induced airway inflammation model, we intranasally challenged wild type and *Il9* $^{\Delta CNS-25}$ mice with *A. fumigatus* extract, 3 times a week for 6 weeks (Figure 37A). The day after the final challenge, we collected and counted BAL cells (Figure 37B). The number of BAL cells was significantly decreased in *Il9* $^{\Delta CNS-25}$ mice. The IL-9-producing $CD4^+$ T cell population in BAL and lung was significantly decreased in *Il9* $^{\Delta CNS-25}$ mice (Figure 37C, 37D). These results strongly indicate that CNS-25 is a critical enhancer for promoting *Il9* expression in $CD4^+$ T cells in vivo.

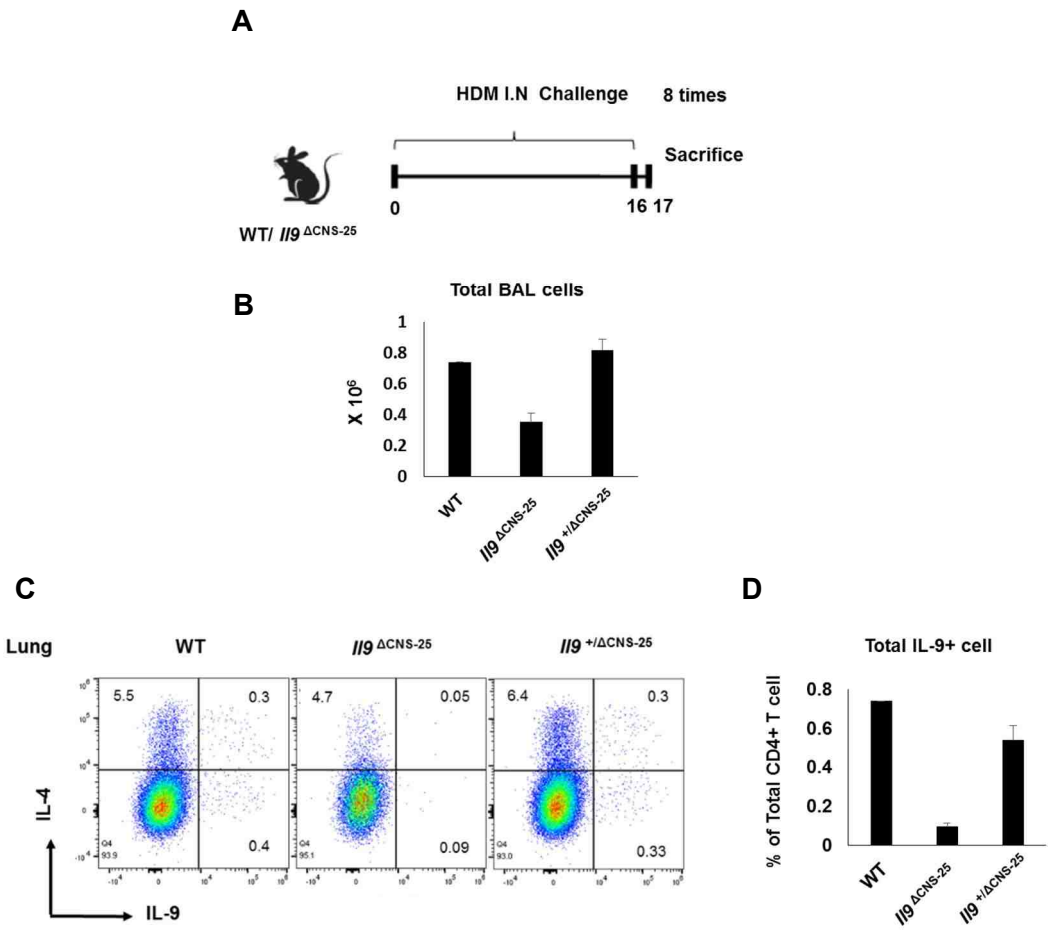

Figure 36. CNS-25 deficient Th9 cells produce less IL-9 in HDM extract-induced airway inflammation. (A) WT, *Il9* $^{\Delta CNS-25}$ and *Il9* $^{+/\Delta CNS-25}$ mice were intranasally challenged with HDM extract every other day for 16 days. (B) Total cell count in the BAL. (C) Lung cells were restimulated with PMA/ionomycin for 6 hours to measure cytokine production using intracellular staining. Representative dot plots (C) and average percentage (D) of IL-9 producing $CD4^+$T cells in the lung. Cells for flow cytometric analysis were gated on lymphocyte size and granularity, and the expression of CD4 and TCRβ. Data are mean ± SEM of 3 mice per group. A two-tailed Student *t* test was used for pair wise comparisons. **p* < 0.05, ***p* <0.01

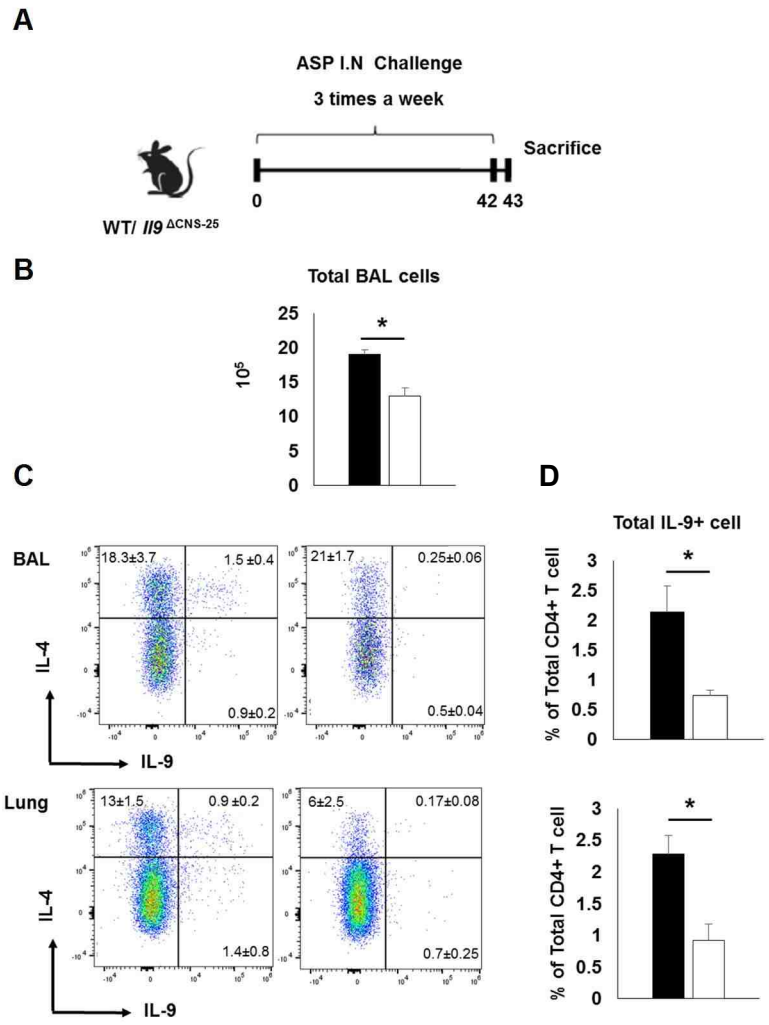

Figure 37. CNS-25 deficient Th9 cells produce less IL-9 in *A. fumigatus* extract-induced airway inflammation. (A) WT and *Il9* $^{\Delta CNS-25}$ mice were intranasally challenged with *A. fumigatus* extract every other day for 42 days. (B) Total cell count in the BAL. (C-D) BAL and lung cells were restimulated with PMA/ionomycin for 6 hours to measure cytokine production using intracellular staining. Representative dot plots (C) and average percentage of IL-9 producing $CD4^+$ T cells in BAL and lung (D). Cells for flow cytometric analysis were gated on lymphocyte size and granularity, and the expression of CD4 and TCRβ. Data are mean ± SEM of 5 mice per group. A two-tailed Student *t* test was used for pair wise comparisons. *$p < 0.05$

Effects of CNS-25 deletion on histone modifications and TF binding at the *Il9* promoter

Both in vitro and in vivo data showed that CNS-25 is a critical enhancer for *Il9* expression. To define the molecular functions of CNS-25 on the *Il9* locus in Th9 cells, histone modifications and the binding of IL-9-inducing TFs to the *Il9* promoter were measured using ChIP assay. The active promoter markers H3K4me3 and H3K27ac were significantly higher in wild type Th9 cells than in *Il9*$^{\Delta CNS\text{-}25}$ Th9 cells (Figure 38B). However, H3K27me3 was similar between groups. Consistent with Figure 23, IL-9-inducing TFs strongly bound to both CNS-25 and *Il9* promoter in Th9 cells (Figure 39B). However, CNS-25 deletion caused significantly less binding of the TFs in the *Il9* promoter (Figure 39B). Based on the data, we conclude that CNS-25 is critical for the binding of TFs and histone modifications at the *Il9* promoter.

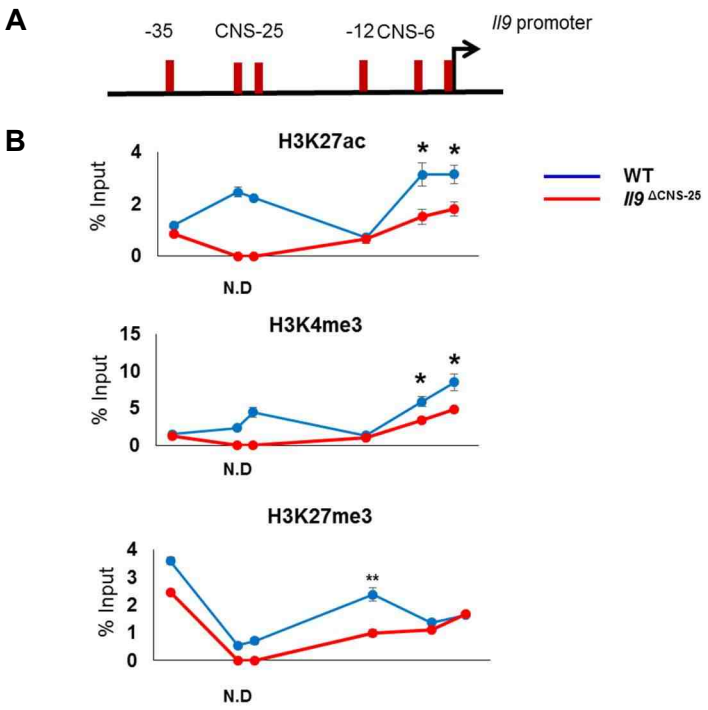

Figure 38. CNS-25 deletion impairs active histone modification at the *Il9* promoter. Naïve $CD4^+$ T cells were isolated from WT or *Il9* $^{\Delta CNS-25}$ mice and cultured under Th9 cell polarizing condition. On day 5, cells were harvested for ChIP analysis using H3K27ac, H3K27me3 and H3K4me3 antibodies. (A) Schematic of the *Il9* locus in mice. (B) ChIP analysis of histone modifications at the *Il9* locus. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. **p* < 0.05, ***p* <0.01. N.D., not detected.

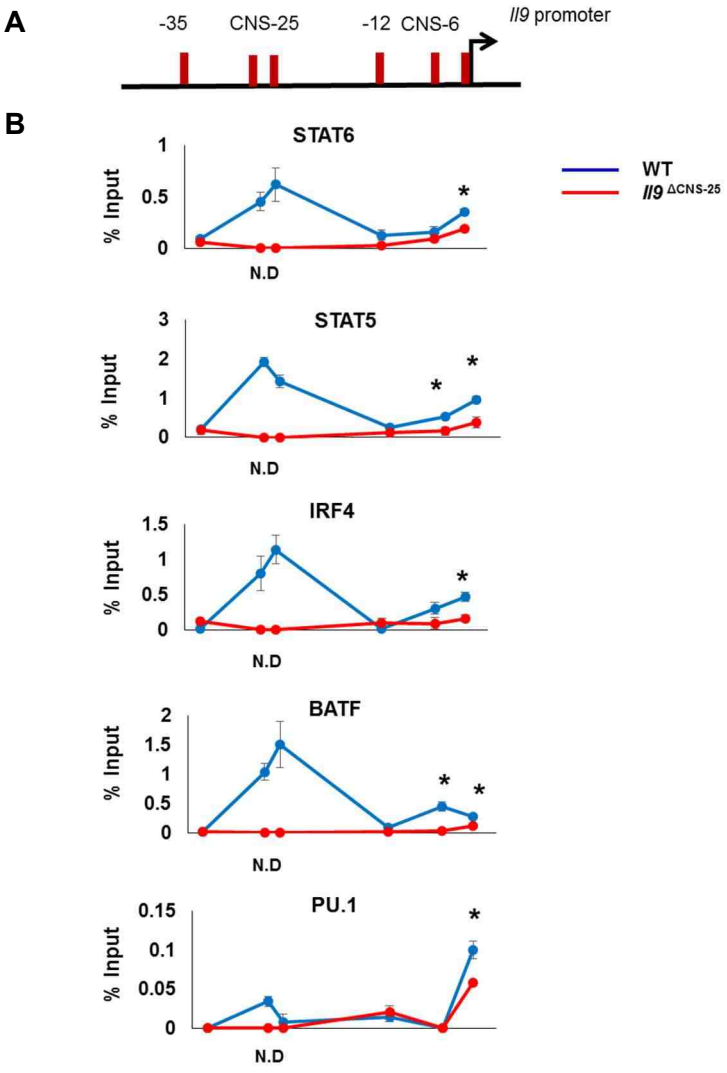

Figure 39. CNS-25 deletion impairs the binding of IL-9-inducing TFs at the *Il9* promoter. Naïve CD4^{+} T cells were isolated from WT or *Il9* $^{\Delta CNS-25}$ mice and cultured under Th9 cell polarizing condition. On day 5, cells were harvested for ChIP analysis using STAT6, STAT5, IRF4, BATF and PU.1 antibodies. (A) Schematic of the *Il9* locus in mice. (B) ChIP analysis of the binding of TFs at the *Il9* locus. Data are mean ± SEM of 3 mice per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. *$p < 0.05$. N.D., not detected.

DNA Looping

To function properly, enhancers need to be in proximity to a promoter and they accomplish this by making DNA loops (111). Med1, a mediator complex subunit, Smc1a, structural maintenance of chromosomes 1a, and CTCF, CCCTC binding factor, are all involved in making 3D DNA loop chromatin structure (148, 162). To provide evidence for DNA looping between CNS-25 and the *Il9* promoter, ChIP assays were performed using Smc1a, Med1 and CTCF antibodies. These proteins strongly bound to both CNS-25 and the *Il9* promoter in Th9 cells, suggesting that CNS-25 functions through a DNA loop (Figure 40).

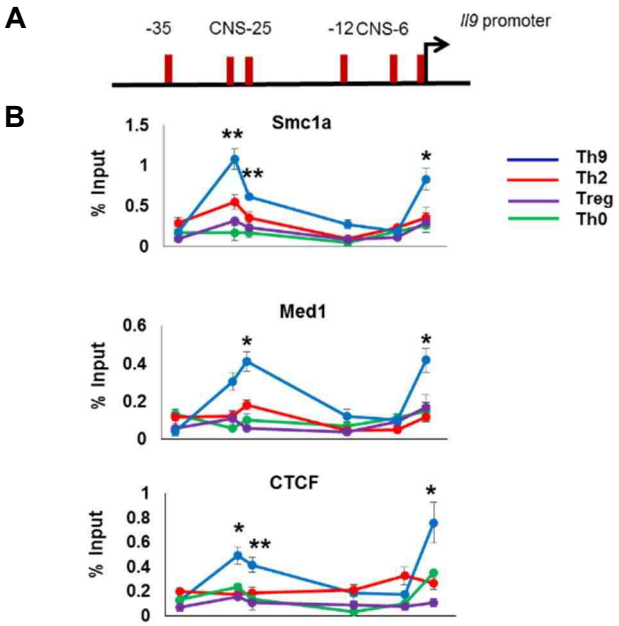

Figure 40. DNA loop mediating TFs bind to both CNS-25 and the *Il9* promoter in Th9 cells. Naïve $CD4^+$ T cells were isolated from WT mice and cultured under different Th cell polarizing conditions. On day 5, cells were harvested for ChIP analysis using Smc1a, Med1 and CTCF antibodies. (A) Schematic of the *Il9* locus in mice. (B) ChIP analysis of the binding of at the *Il9* locus. Data are mean ± SEM (n=4/experiment). One-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. **p* < 0.05, ***p* <0.01

Conserved Functions of *IL9* CNS-18 in Human

Cis-regulatory elements can be highly conserved between species (111). As shown in Figure 41A, the *Il9* CNS-25 region in mice shows high homology with an *IL9* CNS-18 region in the human genome, suggesting that *IL9* CNS-18 might have similar function of *Il9* CNS-25. To measure chromatin status of *IL9* CNS-18 in human T cell subsets, ChIP assays were performed using antibodies against H3K4me1 and H3K27ac. Both H3K4me1 and H3K27ac levels at the CNS-18, and H3K4me3 level at the *IL9* promoter were significantly higher in Th9 cells than in Th0 cells (Figure 41C).

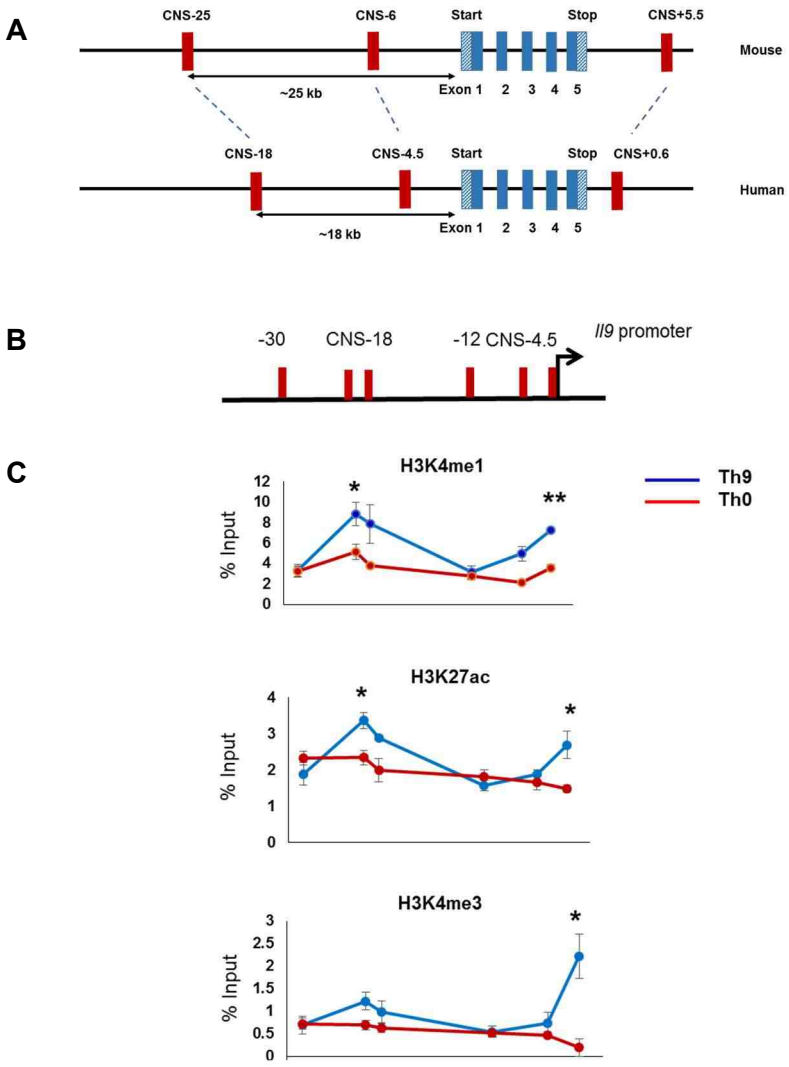

Figure 41. Active histone modifications at the *IL9* CNS-18 in human T cells. Naïve CD4$^+$ T cells were isolated from human PBMCs and cultured under Th0 and Th9 cell polarizing conditions. On day 4, cells were harvested for ChIP analysis using H3K4me1, H3K27ac and H3K4me3 antibodies. (A) Schematic of CNSs in *Il9/IL9* locus in mouse and human genomes (B) Schematic of the *IL9* locus in human. (B) ChIP analysis of histone modifications at the *IL9* locus. Data are mean ± SEM of 3 donors per group and representative of two independent experiments. A two-tailed Student *t* test was used for pair wise comparisons. *p < 0.05, **p <0.01

To prove whether *IL9* CNS-18 is also critical for *IL9* gene expression in human Th9 cells, we applied the CRISPR/Cas9 system to delete CNS-18 region in primary human T cells. To do this, we generated a single lentiviral vector expressing Cas9 and gRNAs targeting CNS-18. Cas9 in this vector is fused with EGFP with a connecting sequence encoding the self-cleaving 2A peptide, so EGFP can be used as a marker for Cas9 expression. Lentiviruses expressing both Cas9 and gRNAs targeting CNS-18 or targeting an irrelevant mouse DNA sequence for a negative control, were produced through transfection of HEK 293T cells. After infection of human Th9 cells with lentivirus, IL-9 production between control and CNS-18 deleted Th9 cells were measured using ICS on day 4. Consistent with mouse experiments, CNS-18 deleted Th9 cells produced significantly less IL-9 compared to control Th9 cells (Figure 42A). Even though IL-9 production in Th9 cells was different among donors, the pattern of IL-9 reduction after CNS-18 deletion was similar. The deletion efficiency of CNS-18 region was examined using ChIP primers for CNS-18 region and normalized to -12 kb sequence. The deletion efficiency was around 40 to 50% (Figure 42C).

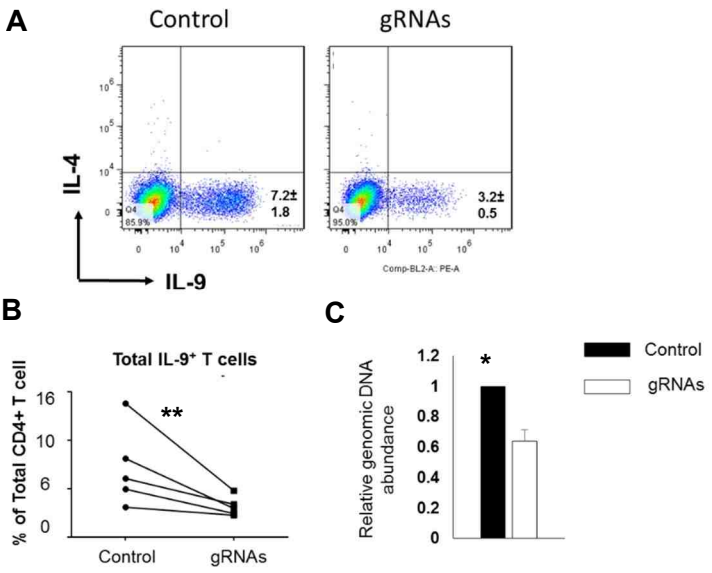

Figure 42. Deletion of *IL9* CNS-18 impairs IL-9 production in human Th9 cells. Naïve $CD4^+$ T cells were isolated from human PBMCs and cultured under Th9 cell polarizing condition. After one day of culture, cells were transduced with lentivirus expressing Cas9 and gRNAs targeting CNS-18. On day 4, cells were restimulated with PMA/ionomycin for 6 hours to assess IL-9 production using intracellular staining analysis. (A) Representative of flow cytometry image of data (B). (B) Percentage of IL-9 producing $CD4^+$ T cells from individual donors after *IL9* CNS-18 deletion. (C) On day 4, Cas9 and gRNAs expressing Th9 cells were sorted based on EGFP expression. Deletion efficiency of *IL9* CNS-18 was measured using qPCR with ChIP primer for CNS-18 and normalized to -12 kb sequence. Data are mean ± SEM of 5 donors per group. A ratio paired *t* test was used for pair wise comparisons. $^{**}p < 0.01$

To confirm the ICS results, Cas9 and gRNAs expressing human Th9 cells were sorted based on EGFP expression on day 4, and mRNA was isolated for assessing cytokine expression. Consistent with the cytometry data, *IL9* gene expression was significantly decreased in CNS-18 deleted Th9 cells (Figure 43), However, *IL10* and *IL21* expression was not altered by CNS-18 deletion. Overall, these data strongly indicate that CNS-18 in human, highly conserved in mice, plays a key role for enhancing *IL9* gene expression.

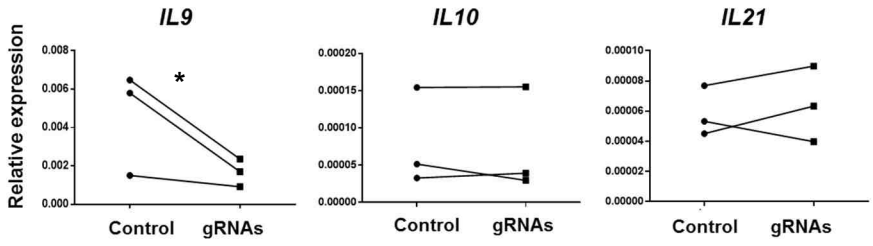

Figure 43. *Il9* gene expression was impaired by CNS-18 deletion in Th9 cells. On day 4, Cas9 and gRNAs expressing Th9 cells were sorted based on EGFP expression. Gene expression of cytokines was measured using qRT-PCR. Data are normalized to *β2m* expression. Data are mean ± SEM of 3 donors per group. A ratio paired *t* test was used for pair wise comparisons. **p* < 0.05

DISCUSSION

For optimal differentiation and function of $CD4^+$ T cells, lineage specific TFs and cis-regulatory elements cooperate to regulate gene expression (111). Here, we identified Etv5 as an IL-9- and IL-10-inducing TF in Th9 and Th2 cells, respectively. Etv5 binds to these gene promoters and CNS regions, suggesting that Etv5 not only directly promotes gene expression but also mediates active chromatin structure at the *Il9* and *Il10* gene loci.

The *Il9* locus consists of 3 CNSs: CNS-6, CNS+5.5 and the *Il9* promoter. IL-9-inducing TFs including PU.1 directly bind to the *Il9* promoter and promote *Il9* expression (155). Interferon regulatory factors such as IRF1 and IRF4 also bind to the CNS-6 and CNS+5.5 (163). We revealed that Etv5 binds to the CNS-6 and CNS+5.5, and recruits p300 to these regions which might contribute to active histone modifications. And Etv5 cooperates with PU.1 to regulate *Il9* expression in vitro and in vivo.

Th9 cells may produce IL-10 though the amount varies with culture conditions. The *Il10* locus consists of 4 CNSs and the *Il10* promoter (136). The binding sites for *Il10* regulating TFs are spread across these sites. For example, GATA3 binds to the *Il10* promoter, intronic regions and CNS3 region (137). E4BP4 binds to the CNS2, CNS3 and intronic regions, but does not bind to the *Il10* promoter (138). We revealed that Etv5 promotes IL-10 production in Th1 and Th2 cells. In Th2 cells, Etv5 mainly binds to the CNS3 region and recruits p300 contributing to a permissive chromatin structure in CNS3. In the absence of Etv5, active histone modifications at the CNS3 are decreased, which impairs the binding of IL-10-inducing TFs.

To further resolve *Il9* regulation, we identified CNS-25, a novel enhancer for *Il9* expression in mice. CNS-25 specifically regulates *Il9* expression without affecting the expression of other cytokines and TFs. CNS-25 promotes active chromatin modifications at the *Il9* promoter, potentially through DNA looping. *IL9* CNS-18 in the human genome, conserved with *Il9* CNS-25, is also critical for *IL9* expression in human Th9 cells. Overall,

we have identified that Etv5 regulates expression of multiple cytokines, and demonstrated that the *Il9* CNS-25/*IL9* CNS-18 elements are critical for *Il9/ IL9* expression respectively.

Part I. The ETS family transcription factors Etv5 and PU.1 function in parallel to promote Th9 cell development

Transcription factors play an obligate role in defining the phenotype and function of Th cells. In this part, we have defined the contribution of the ETS family transcription factor Etv5 to the Th9 phenotype. Etv5 shares many functions with the ETS family factor PU.1 that we previously demonstrated was required for Th9 development (59, 101). Both Etv5 and PU.1 regulate IL-9 production, although they appear to work through different regulatory elements in the *Il9* gene (Figure 44). Apart from IL-9, PU.1 and Etv5 regulate some common and some distinct genes associated with the Th9 phenotype. Moreover, T cells that lacked both Etv5 and PU.1 had decreased IL-9 production in vitro.

The concept of transcription factors from the same family having overlapping functions is observed in other T cells. In Th17 cells, the loss of IL-17 production is more severe in mice that lack both RORγt and RORα than either factor individually (152). Similarly, Tc17 cells develop from CD8 T cells that are doubly deficient in the T-box factors T-bet and Eomesodermin (164). This paradigm is also seen among ETS family factors that are divided into four classes based on binding sequence and nine subfamilies based on homology (165). The partial redundancy between PU.1 and Spi-B, two factors in the same subfamily, has been examined in several models including myeloid and lymphoid cell development, B cell function, and the development of leukemia (166-169). Other examples of factors from the same subfamily having overlapping function include Etv1 and Etv4 that cooperate in prostate cancer, ELK1 and ELK4 in thymocyte development, and FLI-1 and ERG in hematopoiesis (170-172). However, PU.1 and Etv5 are from different subfamilies. This is not without precedent and ETS2 and ELF5, factors from

distinct subfamilies, cooperate in development (173). Some of this redundancy is clearly dependent on overlapping binding specificities of the family-defining DNA binding domain (174). Yet, the protein sequences outside of the DNA binding domain are divergent and suggest that the partial redundancies observed are limited by the ability of each ETS family member to interact with other transcription factors at gene-regulatory elements (165).

Although Etv5 and PU.1 were clearly cooperating on IL-9 production within in vitro–derived cells, we observed a trend but not a significant effect of Etv5 deficiency on IL-9 production in vivo. This could be caused by several reasons including compensation by increased the expression of PU.1 (Figure 11) or other signals in vivo that might bypass the requirement for Etv5 in the OVA/ alum model. Despite these data, we observed distinct effects of PU.1 and Etv5 on the overall allergic inflammation, suggesting that PU.1 and Etv5 are not exerting effects in vivo strictly through IL-9. This is consistent with the effects of deficiency of either factor or both in T cells during the development of allergic inflammation. Although deficiency in either factor results in diminished inflammation, PU.1 deficiency had a slightly greater effect on eosinophil accumulation during allergic inflammation than did deficiency in Etv5. Conversely, Etv5 deficiency had a greater effect on neutrophil recruitment, consistent with our previous studies (107). Importantly, although we and others have shown that the HDM model of airway inflammation is dependent on IL-17 (107, 175), the OVA-alum model is largely IL-17 independent (176), so that the effects of Etv5 deficiency on IL-17 should not impact the total cellular inflammation in our studies. Both Etv5 and PU.1 contribute to the ability of Th9 cells to promote accumulation and gene expression of mast cells in lung tissue, again consistent with our previous results on the role of Th9 cells in mast cell accumulation in multiple models of allergic airway disease (57). We did observe that IL-13–producing T cells in the lung were also decreased in mice with T cells lacking both Etv5 and PU.1, although the proportion of these cells was not different, suggesting the decrease in number was linked to decreased overall

inflammation. IL-13 production from draining lymph node cells in mice with T cells lacking Etv5 and PU.1 was normal, leading to the conclusion that there is normal development of the Th2 response in these mice. Still, it is possible that the observed decrease in IL-13–producing T cells might contribute some aspect of the diminished inflammation observed in vivo.

Although we have focused on conventional $CD4^+$ T cells in this part it is possible that Etv5 might be functioning in other types of cells. Other sources of IL-9 in the immune system include NKT cells, γδ T cells, and innate lymphoid cells (38). Among these, the CD4-Cre should only affect expression in NKT cells. We previously suggested that PU.1 was not required for IL-9 production by NKT cells (57), but it is not clear whether Etv5 might play a role within these cells. The role of NKT cells in asthma models is still debated, and what controls IL-9 within these cells is not yet clear.

As noted earlier, the ETS transcription factor family is divided based on binding specificity and homology (165). As we have shown that PU.1 and Etv5 preferentially bind to different sites in the *Il9* locus and recruit different HATs to the locus, they appear to have distinct functions at a convergent target gene. It is not clear whether this is a common theme among cooperating ETS factors. The ability of PU.1 and Etv5 to cooperate across subfamilies suggests that redundancy of function is not restricted to family members that have similar binding site specificities or similar structures outside of the common ETS DNA binding domain. PU.1 interacts with GATA3 and IRF4 in Th2 cells (139). PU.1-IRF4 and PU.1-GATA3 complexes are different because IRF4 does not interact with GATA3. These data suggest that PU.1 might interact with IRF4 at the *Il9* promoter. BATF, another essential factor for *Il9* expression, also binds to the AICE site in *Il9* promoter suggesting that IRF4 is required for BATF binding at the *Il9* promoter (54). IRF4 strongly binds to the CNS-6, CNS+5.5 and *Il9* promoter (55, 163). We further revealed that BATF only binds to the *Il9* promoter, not CNS-6 and CNS+5.5. PU.1 might interact with BATF or be required

for those TFs binding at the *Il9* promoter. Interactions of Etv5 with IRF4 or BATF have not been studied yet. Etv5 might interact with IRF4 to regulate *Il9* expression in CNS-6 and CNS+5.5. The functions of CNS-6 and CNS+5.5 are still not fully understood. These regions might be essential for inducing *Il9* expression by mediating the *Il9* locus chromatin conformation. Moreover, Etv5 might be important for the mechanism recruiting p300 to the CNS-6 and CNS+5.5 elements. Further work will help to elucidate the rules governing the development of evolutionary cooperation among this family of transcription factors.

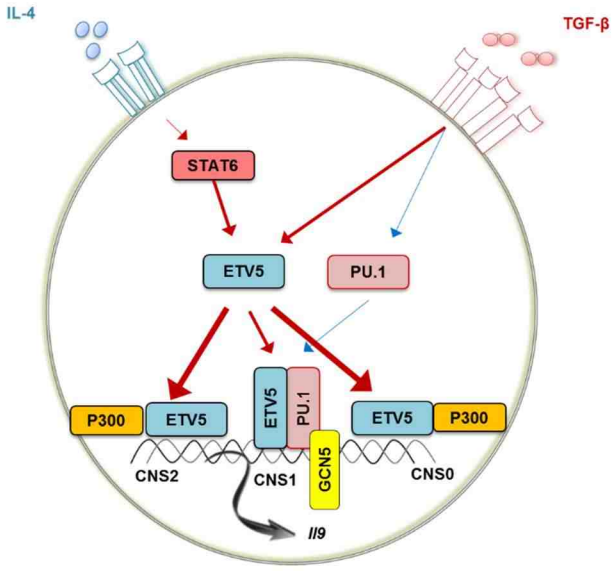

Figure 44. Etv5 and PU.1 regulate *Il9* expression in Th9 cells through different regulatory elements in the *Il9* locus

Part II. Etv5 regulates IL-10 production in Th cells

IL-10 is a critical regulatory cytokine. In the absence of IL-10, mice develop spontaneous autoimmune inflammation (27). Thus, transcriptional control of the *Il10* gene is essential in maintaining disease-free immune homeostasis (28). In this part, we identify Etv5 as a regulator of IL-10 in Th1 and Th2 cells, in vivo and in vitro. Etv5 binds directly to the *Il10 locus* and facilitates the binding of a number of other transcription factors that regulate IL-10 production (Figure 45).

The regulation of IL-10 production may be distinct among subsets of Th cells. Previous reports identified several transcription factors that are involved in *Il10* regulation in Th2 cells. GATA3 remodels the *Il10* locus independently of IL-4 production in Th2 cells (137). JunB and c-Jun proteins bind to the CNS3 region in the *Il10* locus and induce IL-10 production in Th2 cells (136). In Th2 cells, IRF4 directly binds to the *Il10* locus and promotes IL-10 production. E4BP4 is a reported universal IL-10 regulator in all Th cell subsets (138, 139). E4BP4 deficiency causes impairment of IL-10 production, but does not affect IL-13 production in Th2 cells. In this part, we add Etv5 to the list of factors that promote IL-10 production, and show that it works in concert with other factors by facilitating their binding to the *Il10* locus. It is not clear if Etv5 functions by directly interacting with other transcription factors bound to the locus, or if it remodels chromatin allowing other transcription factors to bind more efficiently. Nevertheless, Etv5 is required for the other factors to optimally induce IL-10 production.

Etv5 adds to a subset of ETS family transcription factors that regulate *Il10*. Ets-1 represses IL-10 expression in Th1 cells by recruiting HDACs to the *Il10* promoter and intronic regions (140). Polymorphisms in the human *Il10* gene increase binding of the ETS factor Elk1 (177). PU.1 also negatively regulates IL-10 in Th2 and Th9 cells (100, 139). At least some of the mechanism of PU.1 function in Th2 cells is by interfering with IRF4 binding to the *Il10* locus regulatory elements (139). As each of these factors seems to

function through different mechanisms, and having different effects on IL-10, it seems unlikely that there would competition among them. This could be formally tested in future studies.

Etv5 does not work in a subset specific manner, regulating IL-10 production in both Th1 and Th2 cells. Etv5 did not dramatically affect the lineage identifying cytokines for these Th subsets. This is in contrast to Etv5 function in Th17 where it is required for optimal expression of the lineage associated cytokines (107). We detected little IL-10 production from Th17 or Th9 cells in our culture conditions (data not shown), making it impossible to determine if Etv5 is contributing to *Il10* expression in these subsets. This function might best be addressed in vivo in disease models where there are more IL-10-producing Th9 and Th17 cells.

Mice that have Etv5-deficient T cells clearly do not behave as IL-10-deficient mice. The $Etv5^{fl/fl}$ CD4 Cre^{+} mice do not develop spontaneous autoimmune inflammation. Moreover, *A. fumigatus* challenged *Il10*-/- mice showed exaggerated airway inflammation and excessive Th2 cytokines in BAL (160). In our studies, *A. fumigatus* extract-challenged $Etv5^{fl/fl}$ CD4 Cre^{+} mice demonstrated no difference in the overall inflammation, but had a reduced number of IL-10-producing T cells in the BAL and lung. This is consistent with the partial role for Etv5 in IL-10 production that we observe in vitro and in vivo. The approximate 50% decrease in IL-10 production is not sufficient to result in the more dramatic effects of IL-10-deficiency in vivo. Additionally, in non-T cells, where Etv5 is not deleted, IL-10 production is likely normal, further attenuating the effects of the T cell-specific deficiency of Etv5 in vivo.

Our studies identify Etv5 as a promoter of IL-10 regulation in Th subsets. It binds to the *Il10* CNS3 element, and facilitates the binding of several other IL-10-inducing factors that together control IL-10 production. This latest component of the *Il10* enhancer complex

provides new insight into regulation of immunoregulatory cytokine production in Th subsets and could provide another potential target for modulating IL-10 production in vivo.

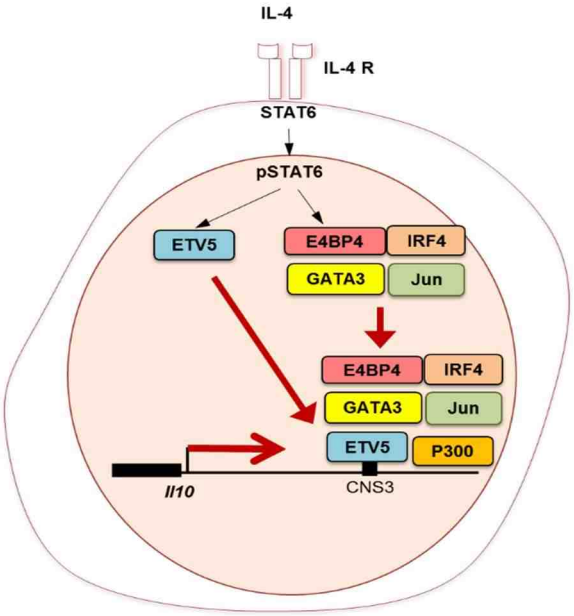

Figure 45. Etv5 regulates *Il10* expression in Th2 cells by recruiting IL-10-inducing factors to the *Il10* CNS3

Part III. Identification of novel enhancers for *Il9* gene expression in T cells

TFs and cis-regulatory elements cooperate to regulate gene expression, In $CD4^+$ Th cell subsets, cis-regulatory elements including enhancers and LCRs for cytokine expression were identified (111). Even though many *Il9* regulating TFs have been identified, enhancers for *Il9* gene expression had not been studied. Previous studies reported three CNS regions around the *Il9* locus: *Il9* promoter, CNS+5.5 and CNS-6 (155). Many studies focused on the *Il9* promoter, and revealed that a few TFs can bind to the CNS-6 or CNS+5.5. These CNSs are marked by active histone modifications and co-occupied with several TFs, suggesting that these regions might be enhancers for *Il9* expression. However detailed functions of these regions were not studied yet. In this part, we identified *Il9* CNS-25/ *IL9* CNS-18 as novel enhancers for *Il9/ IL9* expression in mouse and human Th9 cells.

Enhancers function independent of their direction and location which make it challenging to identify them. Previous studies proved that active histone modifications such as H3K4me1, H3K27ac and p300 binding are active enhancer marks (8, 127, 132). Moreover, enhancers are evolutionally conserved between different species.

After screening CNSs in the *Il9* locus between the mouse and human genomes using the ECR browser (online tool for visualizing whole genome alignments of multiple vertebrates), we detected CNS located 25 kb upstream of the *Il9* TSS, termed as CNS-25. To define whether the region has enhancer features, we examined p300 binding in Th subsets using ChIP assay and ChIP seq. We could see strong binding of p300 at the CNS-25, CNS-6 and *Il9* promoter in Th9 cells. H3K4me1 and H3K27ac modifications at the CNS-25 were significantly higher in Th9 cells. H3K27me3, a repressive mark, was significantly lower at the CNS-25 and *Il9* promoter in Th9 cells. Moreover, IL-9-inducing TFs also strongly bound to the CNS-25 and *Il9* promoter in Th9 cells. These ChIP analyses suggest that CNS-25 has active enhancer features in Th9 cells.

To identify the function of CNS-25, we generated CNS-25 deletion mice, termed as *Il9* $^{\Delta CNS-25}$, using the CRISPR/Cas9 system. We examined the effects of CNS-25 deletion on *Il9* expression in Th cell subsets through ICS, ELISA and qRT-PCR. IL-9 production and gene expression were significantly decreased in Th9, Th17 and Treg cells from *Il9* $^{\Delta CNS-25}$ mice. Effects of CNS-25 deletion on IL-9 production in Th9 cells were independent of the time point analyzed or culture conditions with or without αIL-10R antibody. The CNS-25 deletion did not affect the expression of other cytokines and TFs in all Th cell subsets, suggesting that CNS-25 specifically regulates *Il9* expression. An opposite effects of the CNS-25 deletion was measured in Th2 cells. *Il9* $^{\Delta CNS-25}$ Th2 cells produced more IL-9 compared to wild type Th2 cells while expressing normal level of IL-4, suggesting that CNS-25 might act as a repressor of *Il9* gene expression in Th2 cells.

In this study, we showed that the function of CNS-25 is regulated by the binding of IL-9-inducing TFs. However, the function of CNS-25 also might be regulated by IL-9 repressing TFs, because enhancer functions are regulated by both activating TFs and repressing TFs. One of known IL-9 repressing TFs is GATA3, however, the function of GATA3 in *Il9* expression is still controversial. Even though ectopic expression of GATA3 represses IL-9 production in Th9 cells, GATA3 deficient T cells failed to differentiate into Th9 cells (68, 90). Additionally, GATA3 binds to the *Il9* promoter to promote *Il9* expression (178). These data suggesting that the amount of GATA3 might be important for regulating *Il9* expression. Existence of GATA3 binding candidate sites in CNS-25 and high *Gata3* expression in Th2 and Th9 cells suggest that GATA3 might bind to the CNS-25 and regulate *Il9* expression in Th2 and Th9 cells. The DNA binding inhibitor Id3 negatively regulates *Il9* expression by inhibiting the binding of GATA3 at the *Il9* promoter in Th9 cells (178). As a repressor, Id3 might bind to the CNS-25 and repress *Il9* expression in Th2 cells. STAT3 is a negative regulator of IL-9 in Th9 cells and impairs the stability of Th9 cells (97). In Th2 cells, however, STAT3 is required for the development and cytokine

expression of Th2 cells (179). We showed that STAT5 and STAT6 bound to the CNS-25 in Th9 cells. Based on these data, we hypothesized that STAT3 might compete with STAT5 and STAT6 to bind to the CNS-25 and repress *Il9* expression in Th2 cells.

Next, we examined the function of CNS-25 in vivo airway inflammation models. We used HDM and *A. fumigatus* extract to induce airway inflammation. In both models, total number of BAL cells was significantly decreased in *Il9* $^{\Delta CNS-25}$ mice. And, IL-9 production was also significantly decreased in lung $CD4^+$ T cells of *Il9* $^{\Delta CNS-25}$ mice. In *A. fumigatus*-induced airway inflammation model, IL-9 producing $CD4^+$ T cells in BAL was also decreased in *Il9* $^{\Delta CNS-25}$ mice. These data showed that CNS-25 is essential for IL-9 production in $CD4^+$ T cells in vivo. In addition to Th9 cells, ILC2, NKT and mast cells produce IL-9 (67, 83-87). CNS-25 deletion might impair IL-9 production by these cells which might attenuate airway inflammation in these models.

Next, we studied the molecular mechanisms by which CNS-25 may function in regulation *Il9* expression. To do this, we compared the levels of H3K27ac, H3K4me3, and the binding of TFs at the *Il9* promoter between wild type and *Il9* $^{\Delta CNS-25}$ Th9 cells. As expected, active histone marks and the binding of TFs were significantly decreased in *Il9* $^{\Delta CNS-25}$ Th9 cells. These data indicate that CNS-25 mediates active histone modifications at the *Il9* promoter which promote the binding of IL-9-inducing TFs to the *Il9* promoter. H3K27me3 modification at the *Il9* promoter in both wild type and *Il9* $^{\Delta CNS-25}$ Th9 cells was much less compared to negative control regions (-35 and -12 kb regions). This indicates that H3K27me3 function was not altered by CNS-25 deletion. IL-9-inducing TFs still bound to the *Il9* promoter in *Il9* $^{\Delta CNS-25}$ Th9 cells, but much lower level than in wild type Th9 cells. This might contribute to maintain basal level of H3K27me3 which leads to produce more IL-9 in *Il9* $^{\Delta CNS-25}$ Th9 cells than in other Th cell subsets.

Enhancers function by looping to the promoter and many proteins are involved in this process. To establish whether CNS-25 is a part of a DNA loop, we examined the

binding of CTCF, Smc1a and Med1 at the CNS-25 and *Il9* promoter in Th subsets. These proteins strongly bound to both CNS-25 and *Il9* promoter in Th9 cells, suggesting that CNS-25 might interact with the *Il9* promoter by making a DNA loop, which mediates active histone modifications at the *Il9* promoter. However, these TFs did not bind to CNS-6, suggesting that CNS-6 might not be part of a DNA loop although active histone modification marks and the binding of IL-9-inducing TFs were measured. Further study will be required to identify functions of CNS-6. To get more clear evidence for a DNA loop at the *Il9* locus, chromatin conformation capture assay (3C assay) will be performed in the future.

The CNS-25 region in mice is highly conserved with CNS-18 in human. Because CNS-25 is critical for *Il9* expression in mice Th9 cells, we hypothesized that CNS-18 has identical function in human Th9 cells. To test that, we deleted CNS-18 in primary human Th9 cells using the CRSIPR/Cas9 system. The efficiency of CNS-18 deletion was around 40%, which might make it hard to detect deletion effects. However, CNS-18 deleted Th9 cells produced significantly less IL-9 than control Th9 cells while expressing normal levels of *IL10* and *IL21*. This data suggest that *IL9* CNS-18 might be more critical for *IL9* induction not just for enhancement, or other CNSs in *IL9* locus in human might not be involved in *IL9* expression.

In mouse Th9 cells, pro-inflammatory cytokines and IL-10 act as negative regulators of *Il9* expression. Th9 cells cultured with IL-10R blocking antibody and STAT3 deficient Th9 cells produce higher IL-9 and maintain its expression long term in culture compare to normal Th9 cells (97). However, human Th9 cells produce more IL-9 in response to the pro-inflammatory cytokines such as IL-6, IL21 and IL-23, and IL-10 (98). Similar to this study, another report revealed that human Th17 cells produce more IL-9 after adding pro-inflammatory cytokines (161). Based on these reports, we hypothesized

that STAT3 might bind to the CNS-18 and interact with other TFs to regulate *IL9* expression in human Th9 cells.

Our studies demonstrated that the *Il9* CNS-25 and *IL9* CNS-18 are critical for *Il9/IL9* expression in Th9 cells. *Il9* CNS-25 mediates active histone modifications at the *Il9* promoter potentially through a DNA loop (Figure 46). Additionally, we revealed that CNS-25 is critical for *Il9* expression in airway inflammation models.

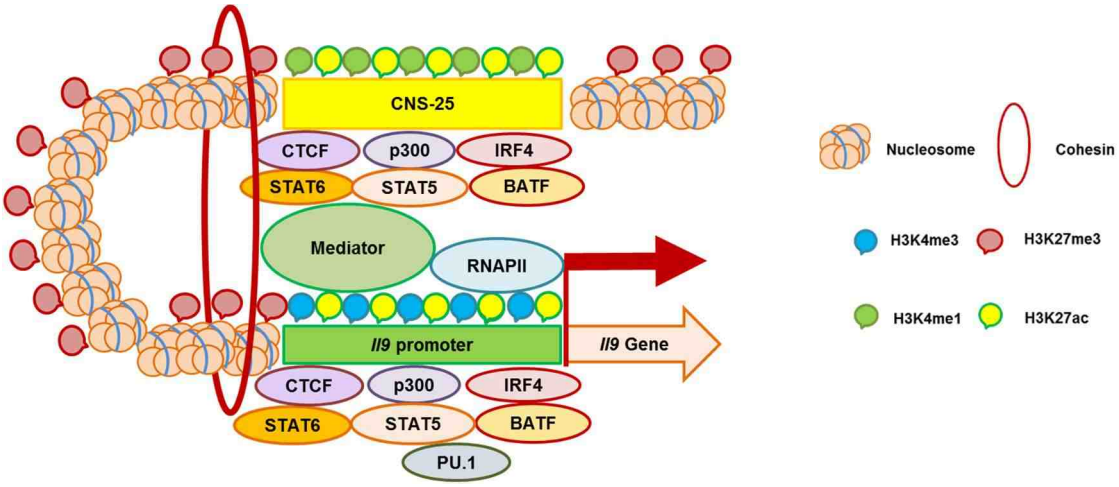

Figure 46. CNS-25 regulates *Il9* expression in Th9 cells through DNA loop

FUTURE DIRECTIONS

The functions of cis-regulatory elements in the *Il10* locus

Th9 cells produce a high amount of IL-9 and IL-10 (38). Many studies have revealed TFs regulating the *Il10* gene in Th cell subsets. However, the functions of cis-regulatory elements in the *Il10* locus were not fully understood. To define functions of *Il10* CNSs, mutant mice lacking these regions will be generated. After isolation of naïve $CD4^+$ T cells from these mice, these cells will be cultured under Th cell conditions. IL-10 production and gene expression between wild type and mutant Th cells will be compared using ICS, ELISA and qRT-PCR. To prove whether CNSs are clinically relevant, SNPs in *Il10* CNSs of patients with ulcerative colitis or Crohn's disease will be analyzed. If SNPs are located in binding sites for TFs at the *Il10* CNSs, this could provide information about potential cooperation of the cis-regulatory elements at the *Il10* locus and TFs in these diseases. Even in the absence of mice lacking *Il10* CNSs, we can still examine the possibility of interactions between these regions with the *Il10* promoter through 3C assay. Overall, these studies will provide more detailed molecular mechanisms of *Il10* gene regulation.

The functions of STAT3 and GATA3 in Th9 cells

In this study, we identified *Il9* CNS-25 as a novel enhancer for *Il9* expression. We revealed that CNS-25 were co-occupied by many IL-9-inducing TFs in Th9 cells. And there is a high possibility that other *Il9* regulating TFs can bind to the CNS-25. As discussed in discussion section, GATA3 might be linked to the function of the CNS-25. To prove whether GATA3 binds to the CNS-25 and mediates the *Il9* locus conformation change in Th9 cells, GATA3 ChIP assay will be performed. If GATA3 binds to the CNS-25 region, the effects of knockdown of GATA3 on the 3D structure at the *Il9* locus in Th9 cells will be measured using 3C assay. GATA3 promotes the *Il9* promoter activity in a reporter assay

(178). To define whether GATA3 affects CNS-25 enhancer activity, reporter assay will be performed using GATA3 expressing vector and *Il9* promoter reporter vectors with or without CNS-25. These studies will help us define additional roles of GATA3 for *Il9* expression.

The functions of STAT3 might be different between mouse and human Th9 cells. To define functions of STAT3 in mouse and human Th9 cells, the binding of STAT3 at the *Il9* CNS-25 and *IL9* CNS-18 will be measured using ChIP assay. To measure STAT3 activity, reporter assay will be performed using constitutively active STAT3 expressing vector and *Il9* CNS-25-*Il9* promoter or *IL9* CNS-18-*IL9* promoter vector. Through these studies, we will be able to define the different functions of STAT3 in mouse and human Th9 cells.

Other cis-regulatory elements in the *Il9* locus

We have shown that CNS-6 also has enhancer features with strong binding of p300 and IRF4, high H3K27ac and low H3K27me3. Additionally, Etv5 also binds to the CNS-6. However, detailed functions of CNS-6 have not been studied yet. To prove whether CNS-6 has enhancer functions for *Il9* expression, CNS-6 mutant mice will be generated. To define functions of CNS-6, similar experiments used to analyze function of CNS-25 will be performed. To study potential possibility of the interactions between CNS-6 and *Il9* promoter or CNS-6 and CNS-25, 3C assay will also be performed.

The functions of CNS-25 in IL-9 producing non-T cells

In addition to Th9 cells, many immune cells produce IL-9 (38). *Il9* $^{\Delta CNS\text{-}25}$ mice are CNS-25 germline mutant mice, meaning that all IL-9 producing cells in the mice might be affected by the CNS-25 deficiency. One of IL-9 producing cells is mast cells. IL-9 is a growth factor for mast cells and it is also produced by mast cells. Bone marrow derived

mast cells (BMMCs) produce IL-9 (84). And multifunctional MMC9s were identified in IgE mediated food allergy experiment (87). To define the function of CNS-25 in mast cells, bone marrow cells from wild type and *Il9*$^{\Delta CNS-25}$ mice will be isolated and cultured with stem cell growth factor (SCF) and IL-3 to selectively differentiate BMMCs. After 4 weeks culture, BMMCs will be stimulated with IL-33 to measure IL-9 production using ICS and ELISA. The expression of other cytokines such as IL-6 will be measured using qRT-PCR or ELISA to prove that CNS-25 specifically regulates *Il9* expression.

The functions of GATA1 are still controversial in mast cell. In human mast cells, GATA1 is essential for of *FCER1A* expression encoding FcεRIα (180). However, another report revealed that GATA1 is dispensable for mast cell differentiation in adult mice (181). In mouse mast cells, p38 kinase activates GATA1 contributing to IL-9 production (85). We confirmed GATA1 binding candidate sites in CNS-25 region. To examine whether GATA1 is an essential TF for IL-9 production by binding to the CNS-25, GATA1 ChIP assay will be performed. To study whether GATA1 enhances *Il9* promoter activity, reporter assays will be performed by co-transfecting mast cells with GATA1 expression vector and *Il9* promoter vectors with or without CNS-25. In addition, the functions of GATA1 will be examined by overexpression or knockdown of GATA1 through lentiviral infection.

ILC2s produce IL-9 in response to IL-33, IL-25 or papain (83). To study the functions of CNS-25 in ILC2, wild type and *Il9*$^{\Delta CNS-25}$ mice will be intranasally challenged with IL-33 for 3 days. Following the last day of challenge, lung will be harvested for ICS and magnetic separation of ILCs. After separation, mRNA will be isolated to measure *Il9* gene expression using qRT-PCR.

NKT cells, another lineage of IL-9 producing cells, will be studied by intranasal challenge of wild type and *Il9*$^{\Delta CNS-25}$ mice with α-GalCer. 48 hours after challenge, BAL and lung cells will be isolated to measure IL-9 production using ICS. In addition to ICS, NKT cells will be isolated using CD1d tetramer specific fluorescent antibody and its micro

beads for magnetic separation. Then mRNA will be isolated to measure *Il9* gene expression using qRT-PCR.

Together these studies will further define IL-9 regulation in multiple lymphoid cell types and provide a basis for better understanding the mechanisms that control *Il9* gene expression. Furthermore, these study will help us to develop treatments for IL-9 mediated allergic or autoimmune disease.

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2016	American Association of Immunologists Travel award	
2017	Erica Daniel Kepner Award	
	Indiana University	Indianapolis, IN
2018	American Association of Immunologists Travel award	

Abstracts

2016	Koh BH, Hufford MM, Sun X and Kaplan MH. Etv5 Regulates IL-10 Production in Th Cells. The American Association of Immunologists (AAI) Annual Meeting. 2016, Seattle, WA
2018	Koh BH, Qayum AA, Fu Y, Ulrich B, Janga SC and Kaplan MH. Identification of a conserved multi-lineage *Il9* enhancer. The American Association of Immunologists (AAI) Annual Meeting. 2018, Austin, TX

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Deak PE, Kim B, Koh BH, Qayum AA, Kiziltepe T, Kaplan MH and Bilgicer B. Inhibition of drug allergy immunoglobulin-E with covalent heterobivalent inhibitors. (Submitted)