TWIST1 AND ETV5 ARE PART OF A TRANSCRIPTION FACTOR NETWORK DEFINING T HELPER CELL IDENTITY

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DEDICATION

I would like to dedicate this thesis to my family.

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ABSTRACT

Duy Pham

TWIST1 AND ETV5 ARE PART OF A TRANSCRIPTION FACTOR NETWORK DEFINING T HELPER CELL IDENTITY

CD4 T helper cells control immunity to pathogens and the development of inflammatory disease by acquiring the ability to secrete effector cytokines. Cytokine responsiveness is a critical component of the ability of cells to respond to the extracellular milieu by activating Signal Transducer and Activator of Transcription factors that induce the expression of other transcription factors important for cytokine production. STAT4 is a critical regulator of Th1 differentiation and inflammatory disease that attenuates the gene-repressing activity of Dnmt3a. In the absence of STAT4, genetic loss of Dnmt3a results in de-repression of a subset of Th1 genes, and a partial increase in expression that is sufficient to observe a modest recovery of STAT4-dependent inflammatory disease. STAT4 also induces expression of the transcription factors Twist1 and Etv5. We demonstrate that Twist1 negatively regulates Th1 cell differentiation through several mechanisms including physical interaction with Runx3 and impairing STAT4 activation. Following induction by STAT3-activating cytokines including IL-6, Twist1 represses Th17 and Tfh differentiation by directly binding to, and suppressing expression of, the *Il6ra* locus, subsequently reducing STAT3 activation. In contrast, Etv5 contributes only modestly to Th1 development but

promotes Th differentiation by directly activating cytokine production in Th9 and Th17 cells, and Bcl6 expression in Tfh cells. Thus, the transcription factors Twist1 and Etv5 provide unique regulation of T helper cell identity, ultimately impacting the development of cell-mediated and humoral immunity.

Mark H. Kaplan, Ph.D.-Chair

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LIST OF ABBREVIATIONS

3C	Chromosome conformation capture assay
AHR	Aryl hydrocarbon receptor
AP-1	Activator protein 1
APC	Antigen presenting cells
ATF	Activating transcription factor
BAF	BRG1/BRM-associated factor
BAL	Bronchioalveolar lavage
BATF	Basic leucine zipper transcription factor ATF like
Bcl-6	B-cell lymphoma 6 protein
BCR	B cell receptor
bHLH	basic helix-loop-helix
BSA	Bovine serum albumin
BTLA	B and T lymphocyte attenuator
CBF-β	Core binding factor, beta subunit
CBP	CREB-binding protein
CCL	C-C chemokine ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CFA	Complete Freund’s adjuvant
ChIP	Chromatin immunoprecipitation
CNS	Conserved non-coding sequence
CREB	cAMP response element-binding

CTLA	Cytotoxic T lymphocyte antigen
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C chemokine receptor
DAPA	DNA affinity precipitation assay
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DTT	Dithiothreitol
BAC	Bacterial artificial chromosome
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
Eomes	Eomesodermin
ERK	Extracellular signal-regulated kinase
ETS	E-twenty six
ETV5	ETS variant gene 5
FBS	Fetal bovine serum
Foxp3	Forkhead box protein 3
GATA3	GATA binding protein 3
GC	Germinal center
Gcn5	General control non-derepressible 5
Gfi-1	Growth factor independence 1

GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR-related protein
GL-7	T and B cell activation marker
GM-CSF	Granulocyte macrophage colony-stimulating factor
H&E	hematoxylin and eosin
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	House dust mite
HIES	Hyper-IgE syndrome
Hlx	H2.0-like homeobox-1
ICOS	Inducible co-stimulator
ICS	Intracellular staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, X-linked
IRF	Interferon regulatory factor
IκB	Inhibitor of κB
JAK	Janus family tyrosine kinase
JMJD	JmjC domain-containing protein
LCMV	Lymphocytic Choriomeningitis
LCR	Locus control region
LPP	Lipoma-preferred partner

Maf	Musculoaponeurotic fibrosarcoma
MBD2	Methyl-CpG-binding domain protein 2
MBL	Mannose-binding lectin
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
P300	E1A binding protein p300
PAMP	Pathogen-associated molecular pattern
PAS	periodic acid-Schiff
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PD-1	Programmed death-1
Phox2b	Paired-like homeobox 2b
PI3	Phosphatidylinositol 3
PIAS	Protein inhibitor of activated STAT
PMA	Phorbol 12-myristake 13-acetate
PNA	Peanut agglutinin

PRR	Pattern recognition receptor
PTP-BL	protein tyrosine phosphatase-Basophil like
qRT-PCR	quantitative real-time polymerase chain reaction
ROR	Retinoid-acid-related orphan receptor
Runx	Runt-related transcription factor
SAP	SLAM-associating protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sfpi1	Spleen focus forming virus proviral integration site-1
SH2	Src-homology domain
Shh	Sonic hedgehog
shRNA	short hairpin RNA
siRNA	small interfering RNA
SLAM	Signaling lymphocytic activation molecule
SOCS	Suppressor of cytokine signaling
SRBC	Sheep Red Blood Cells
SSC	Spermatogonial stem cells
STAT	Signal transducer and activator of transcription
SWI/SNF	SWItch/Sucrose NonFermentable
T-bet	T-box expressed in T cells
TBP	TATA-binding protein
Tbx21	T-box transcription factor 21
TCF	T cell factor 1
Tcfe2a	Transcription factor E2-alpha

TCR	T cell receptor
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptors
TLSP	Thymic stromal lymphopoietin
TMPRSS2	Transmembrane protease serine 2
TNF	Tumor necrosis factor
Treg	T regulatory
WT	Wild type

INTRODUCTION

Innate and adaptive immunity

Immune responses that include innate and adaptive immunity are vital biological functions protecting the host from pathogens and toxic substances. Innate immunity including macrophages, neutrophils, dendritic cells, natural killer cells, mast cells, eosinophils, and basophils, acts as the first line of defense against microorganisms and infections. Adaptive immunity, requiring lymphocytes provides a more versatile and long-term protection. Both innate and adaptive immunity works in concert to provide effective and sufficient protective mechanisms against harmful agents in the environment.

The host body is constantly exposed to microorganisms present in the environment. The common routes of entry are through mucosal surfaces (airway, gastrointestinal tract, and reproductive tract) and external epithelia (skin, wound, and abrasions). The host body utilizes different methods to protect against the pathogens. Epithelial surfaces act as the first line of defense providing mechanical (structure), chemical (pH and enzymes), or microbiological (normal flora) barriers against infection. When pathogens cross epithelial barriers, they are recognized and ingested by dendritic cells or ingested and killed by other innate cells such as macrophages and neutrophils. Dendritic cells and macrophages reside in the submucosal tissues. They are helped in pathogen clearance by the recruitment of neutrophils to the site of infection. These cells express receptors that recognize repeating patterns on the surface of the

pathogens, thus being able to pathogen-specific molecular patterns. These receptors, known as pattern-recognition receptors, include mannose-binding lectin (MBL), macrophage mannose receptor, and scavenger receptors that trigger phagocytosis, and Toll-like receptors (TLRs) that activate innate immune function and cytokine production. Upon ingestion of the pathogens, macrophages and neutrophils utilize several mechanisms for killing such as vesicular containing acidification, toxic oxygen-derived products, toxic nitrogen oxides, antimicrobial peptides, and enzymes. In addition, recognition of the pathogens by macrophages results in the initiation of an inflammatory response that helps the clearance of microorganisms, prevents the spread of the infection in the bloodstream, and promotes tissue repair. TLR activation in macrophages and other innate cells results in the production of pro-inflammatory cytokines (IL-1, IL-6, IL-12, and TNFα), chemokines (CXCL8), and co-stimulatory molecules (CD80 and CD86) that amplify innate and adaptive immune responses. Another component of the innate immune response is the complement system that is made up of many distinct plasma proteins. The complement system works by a series of proteolytic reactions mediated by proteases that result in recruitment of inflammatory cells, opsonization of pathogens, and killing of pathogens.

Other innate immune cells such as natural killer (NK) cells, NKT cells, $\gamma\delta$ T cells, B-1 cells, mast cells, basophils, and eosinophils are also important in innate immune responses. NK cells are activated by interferons (IFNs) and macrophage-derived cytokines to protect against intracellular infections. NK cell

activation results in the release of cytotoxic granules that induce apoptosis of target cell. NK cells express receptors for self antigens that prevent their activation by uninfected cells. Natural killer T (NKT) cells, γδ T cells, and B-1 cells belong to the group of innate-like lymphocytes. NKT cells are characterized by the expression of invariant T-cell receptor α chain that recognizes glycolipid antigens presented by CD1 molecules. NKT cells exist in the thymus and in peripheral lymphoid organs that secrete IL-4, IL-10, and IFNγ and have regulatory function. γδ T cells are a subset of the T cells with antigen receptors composed of γ and δ chains. γδ T cells are found in the lymphoid and epithelial tissues, respond to antigens through mechanism that are still not entirely defined. B-1 cells are subset of B cells that express CD5 receptor. B-1 cells produce antibodies in response to carbohydrate antigens in a T-cell independent mechanism. Mast cells, basophils, and eosinophils arise from myeloid progenitors and have various distinct roles in innate immune responses. While mast cells and basophils defend against pathogens and wound healing by releasing granules containing histamine and active agents, eosinophils release toxic proteins, and free radicals to kill antibody-coated parasites. In addition, these cell types play important roles in mediating allergic reactions.

Although innate immunity is often sufficient to protect against foreign pathogens, it fails to provide a specific and long-term protection against recurrent invaders. Thus, adaptive immunity, requiring T and B lymphocytes primed by antigen presenting cells (B cells, dendritic cells, and macrophages) play a crucial role in

eliminating pathogens when the innate immune system is insufficient to limit infection. The main functions of the adaptive immunity are to discriminate between self- and non-self- antigens, to regulate inflammatory responses and eliminate specific pathogens, and to develop immunological memory. T and B cells express T- and B-cell receptors (TCR and BCR, respectively) to recognize antigen. B-cell receptors are composed of membrane-bound and secreted immunoglobulin (Ig). While membrane-bound immunoglobulin acts as receptor for antigen, the secreted Ig binds antigens and elicits humoral effector functions. One unique function of TCR and immunoglobulins is their ability to recognize variable molecules with high specificity and affinity through the variable (V) region that binds to antigens. However, TCR (composed of TCRα and TCRβ chains) on T cells differs from BCR on B cells in that it recognizes peptide fragments of foreign proteins bound to the major histocompatibility complex (MHC) molecules class I and class II. The two classes of MHC molecule are expressed differentially on cells that present antigens to CD4 (MHC class II) and CD8 (MHC class I) T cells. CD8 T cell function is to eliminate intracellular pathogens such as viruses through T cell-mediated cytotoxicity of infected cells. CD8 T cells induce infected cells to undergo programmed cell death, secrete proteins (perforin, granzymes, and granulysin) to trigger apoptosis, and express Fas ligand to activate apoptosis. In contrast, CD4 T cells provide helps to other cells to carry out effector functions, and are known as T helper (Th) cells (Figure 1). For example, Th1 cells activate macrophages to become more efficiently in clearing intracellular microorganisms. Th2 cells play a role in controlling defense

against extracellular parasites. Th9 cell function is to provide protection against helminth infection, although IL-9 can be involved in autoimmune diseases and allergic inflammation. Th17 cells produce IL-17 cytokine that plays important role in inflammation and anti-microbial immunity. Regulatory T (Treg) cells are important in maintaining tolerance to self-antigens and play a crucial role in autoimmune diseases. A recently described subset of T helper cells, known as T follicular helper (Tfh) cells, specializes in helping B cells to differentiate into plasma cells that secrete high affinity and specificity antibodies to mediate humoral immune responses against extracellular pathogens. The role of other T helper cell subsets will be discussed in the subsequent sections.

Altogether, the innate and the adaptive immune responses create an effective barrier to pathogens and environmental toxins.

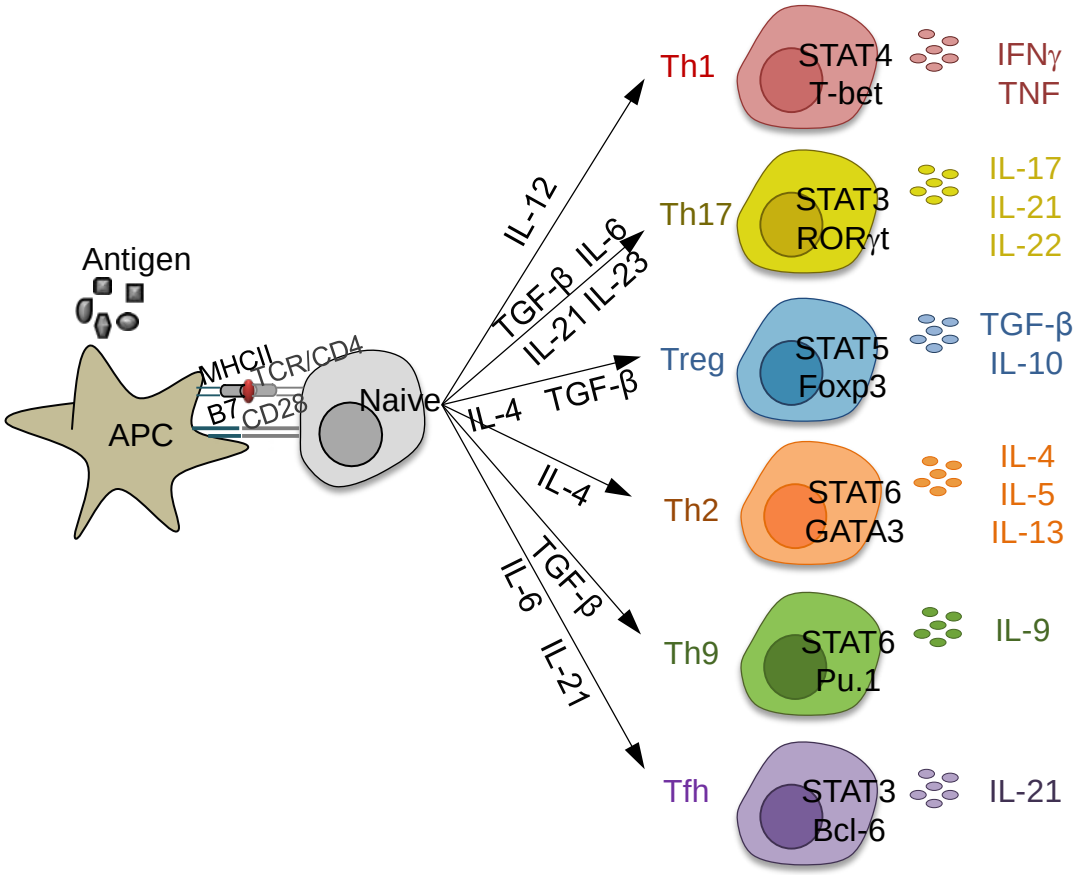


Figure 1. Model of T helper cells

JAK-STAT pathway

Responsiveness to the extracellular milieu is a core component of the adaptability of the immune system. Cytokines mediate intracellular communication and can promote the differentiation and proliferation of responsive cells. One of the signaling pathways activated by cytokines is the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway that was discovered during studies of gene induction by interferons (IFNs) (Darnell et al., 1994). There are four members of the JAK family including JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2) with conserved kinase domain (Stark et al., 1998). There are seven members of the STAT family including STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (Darnell, 1997). The role of STAT proteins in T helper cell development will be discussed in the subsequent sections.

The structure of STAT proteins includes a DNA-binding domain, a transactivation domain, and a SRC homology 2 (SH2) domain. In the JAK/STAT signaling pathway, receptor dimerization and subsequent activation of JAK tyrosine kinases occurs upon the binding of a cytokine to the cell surface receptor. Activated JAKs phosphorylate specific residues on the receptor to create a docking site for STAT binding. STATs are phosphorylated by JAKs, then dimerize, leave the receptor, translocate to the nucleus, and activate gene transcription (Figure 2) (Shuai and Liu, 2003). Because of the important role of the JAK/STAT pathway in controlling immune responses, it is tightly regulated at

several levels. JAKs are regulated at the post-translational level by suppressor of cytokine signaling (SOCS) proteins, protein tyrosine phosphotases (PTP), and by ubiquitylation and ISGylation (Shuai and Liu, 2003). SOCS regulates JAK by inhibiting JAK kinase activity or by binding directly to the activated receptor (Croker et al., 2008). PTPs dephosphorylate JAKs thus inhibiting signaling (Xu and Qu, 2008). Ubiquitylation of JAKs results in degradation (Ungureanu et al., 2002), but it is not known how JAKs are regulated by ISGylation. STATs are regulated at the post-translational level by modifications including phosphorylation, methylation, acetylation, ubiquitylation, ISGylation, and sumoylation that each alter STAT function in dimerization, nuclear translocation, DNA binding, and transactivation (Shuai and Liu, 2003). In addition, STATs are regulated by the Protein Inhibitor of Activated STAT (PIAS) family that has been shown to inhibit STAT-mediated gene activation (Chung et al., 1997; Liu et al., 1998).

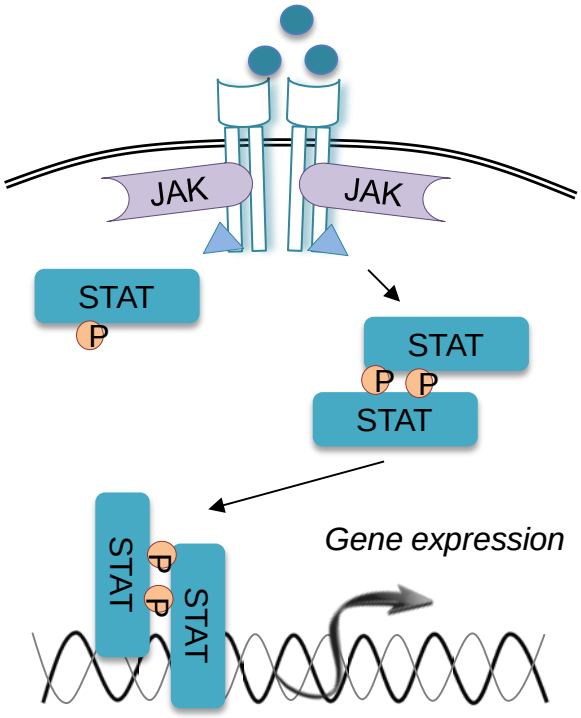


Figure 2. Activation of the JAK-STAT pathway following cytokine stimulation

Dysregulation of JAK/STAT signaling pathway results in defects in immune responses. For example, STAT1 mutations have been documented in patients with susceptibility to viral and mycobacterial infections (O'Shea et al., 2011). Patients with hypermorphic mutations in STAT1 are susceptible to autoimmunity, cerebral aneurysms, and squamous-cell carcinoma (O'Shea et al., 2013). Dominant-negative mutations of STAT3 are linked to hyper-IgE syndrome (HIES) (O'Shea et al., 2011). Constitutively tyrosine phosphorylated STAT3 in intestinal T cells is correlated with Crohn's disease (Lovato et al., 2003). *Stat4*-deficient mice have been linked with increased nephritis and mortality in a model of lupus (Watford et al., 2004). Because *Stat4*-deficient mice lack Th1 responses, they are more susceptible to infectious pathogens such as *L. monocytogenes*,

Mycobacterium tuberculosis, *Babesia*, *Leishmania major*, *L. mexicana*, *T. gondii*, and *Trypanosoma cruzi* (Watford et al., 2004). STAT4 mutation with a defect in nuclear translocation is associated with recurrent *M. avium* infection in patients (Toyoda et al., 2004). In addition, polymorphisms of STAT4 have been described in multiple studies and are associated with diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjogren's syndrome, and Crohn's disease (O'Shea et al., 2011). Given that STAT5B plays important roles in regulating IL-2 and growth-hormone signaling and Treg cell differentiation, dysregulation of STAT5B has been linked with immunodeficiency and autoimmune diseases (Cohen et al., 2006; Imada et al., 1998; Udy et al., 1997; Yao et al., 2007). Patients that lack STAT5B have severe opportunistic infections and elevated levels immunoglobulins (O'Shea et al., 2013). STAT6 is required for the development of allergic inflammation and immunity to helminth parasites. As the result, *Stat6*-mutant mice have decreased pulmonary inflammation and increased inflammatory immunity. In addition, polymorphisms of STAT6 are associated with allergic disease (Goenka and Kaplan, 2011).

In summary, JAK/STAT pathway plays a crucial role in controlling the immune responses and is highly regulated to prevent immune disorders.

Transcriptional regulation of Th1 cells

Th1 cells are critical regulators of inflammation and play obligate roles in immunity to intracellular pathogens and in the development of autoimmune inflammation (Kaplan, 2005; Watford et al., 2004). Th1 development initiates when a T cell, activated by antigen in the context of MHC class II molecules, is stimulated by IL-12 and IFNγ (Afkarian et al., 2002; Hsieh et al., 1993; Wenner et al., 1996). IL-12 and IFNγ stimulation result in the phosphorylation of STAT4 and STAT1 respectively, both transcription factors that are required for optimal Th1 differentiation (Afkarian et al., 2002; Kaplan et al., 1996a; Thierfelder et al., 1996). The *Tbx21* gene, encoding the T-box transcription factor T-bet, is a critical target for both factors, with STAT1 binding early in differentiation, and STAT4 binding later (Lighvani et al., 2001; Schulz et al., 2009; Yang et al., 2007). STAT4 and T-bet are required for the expression of many genes expressed in Th1 cells, although both factors activate the expression of a subset of Th1 genes in the absence of the other factor (Thieu et al., 2008). IFNγ production is the hallmark of Th1 cells, and both STAT4 and T-bet, in cooperation with other transcription factors including Hlx1 and Runx3, activate expression from the *Ifng* locus (Figure 3) (Djuretic et al., 2007; Mullen et al., 2002).

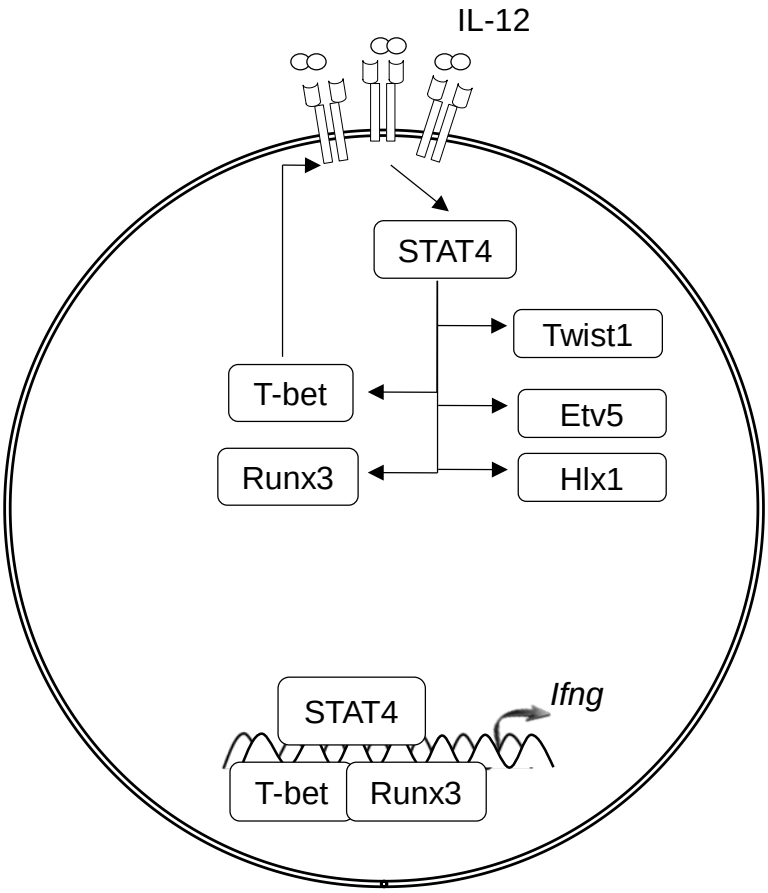


Figure 3. The transcriptional network in Th1 cells

STAT4 binds a multitude of DNA sequences throughout the genome, and genome-wide studies have begun to define targets in Th1 cells. Using a chromatin immunoprecipitation (ChIP)-on-chip approach, STAT4 was found to bind at least 1500 sites in the 10 kb span around murine promoters in response to acute IL-12 stimulation in differentiating T cells (Good et al., 2009). Using a ChIP-seq approach in differentiated Th1 cells, STAT4 bound almost 4500 sites, with slightly over one-third located in the promoter regions, providing a good concordance of the two studies (Good et al., 2009; Wei et al., 2010). Both studies found the STAT consensus sequence TTCN3GAA in the majority of bound sites.

Despite this, numerous genes that bound STAT4 were not induced by IL-12 stimulation, and for genes that were induced, there was no correlation between the peak intensity of STAT4 binding to a site and the fold induction of the associated gene (Good et al., 2009; Wei et al., 2010). Thus, although we now have a detailed appreciation for STAT4 target genes in Th1 cells, it is still not clear how STAT4 activates long-lasting changes in Th1 gene expression.

Epigenetic events that include DNA methylation and histone modification play a key role in T cell differentiation. DNA methyltransferase (DNMT) enzymes catalyze DNA methylation at CpG dinucleotides, resulting in gene repression in T helper cells (Allis et al., 2007; Kouzarides, 2007b; Pekowska et al., 2011; Yu et al., 2007; Yu et al., 2012). Histone modifications that occur at the tails of the core histones include methylation, acetylation, phosphorylation, sumoylation, and ubiquitination. The addition and removal of histone marks alter chromatin into either an active or repressed state correlating with the amount of transcription at the locus. Tri-methylation of histone H3 lysine 4 (H3K4) correlates with active gene transcription (Kouzarides, 2007a). Acetylation of histones at specific lysine residues (H3K9, K18, K27 and K36) result in decreased association with DNA and greater access for trans-acting factors (Kouzarides, 2007a; Shahbazian and Grunstein, 2007). In contrast, tri-methylation of histone H3 lysine 27 (H3K27) is associated with gene repression (Kouzarides, 2007a; Shahbazian and Grunstein, 2007). Histone modifying enzymes are recruited to DNA by transcription factors and are able to recognize histone marks thus allowing the extension of histone

modification across adjacent regions of the target locus (Kouzarides, 2007a). For example, Jumonji C domain protein (Jmjd3) is a histone demethylase that specifically removes the methyl group of H3K27 methylation. The result is gene activation involving in developmental processes and the inflammatory responses (Swigut and Wysocka, 2007).

Once STAT4 is bound to a locus, it can recruit other transcription factors and chromatin modifying enzymes. At the *Il2ra* locus, STAT4 is responsible for the recruitment of c-Jun-containing complexes and the histone acetyltransferase CBP (O'Sullivan et al., 2004). In the absence of STAT4, IL-12 does not induce histone acetylation at the *Il2ra* locus (O'Sullivan et al., 2004). At the *Il18r1* locus, STAT4 is required for global histone acetylation, H3K9 acetylation, H3K4 di- and tri-methylation, and for limiting H3K27 tri-methylation (Yu et al., 2008; Yu et al., 2007). Genome wide analysis of STAT4 binding sites also found a requirement for STAT4 in H3K4me3 (Wei et al., 2010). At the *Ifng* and IL12Rβ2 loci, STAT4 recruits BAF-containing SWI/SNF complexes that are required for nucleosome remodeling (Letimier et al., 2007; Zhang and Boothby, 2006). The *Ifng* locus contains multiple regulatory elements and conserved non-coding sequence (CNS) within 60-70 kb upstream and downstream of its promoter. STAT4 and other STATs (including STAT1 and STAT5) have been shown to facilitate histone acetylation and to recruit chromatin-remodeling complexes to the *Ifng* locus resulting to the induction of permissive epigenetic modification and the activation of *Ifng* gene expression (Wilson et al., 2009a).

STAT4 also limits DNA methylation of the *Il18r1* locus by reducing the association of Dnmt3a, one of the two de novo DNA methyltransferases, with the *Il18r1* promoter, and the promoters of several additional Th1 genes (Yu et al., 2007). T cells deficient in Dnmt3a, but not Dnmt3b, the other de novo DNA methyltransferase, have increased IFNγ production and increased flexibility in their ability to switch from Th2, Th17 or Treg cultures to an IFNγ-secreting phenotype. Dnmt3a also represses alternative lineage gene expression in Th1 cells. The *Il13* locus is particularly sensitive to Dnmt3a-deficiency, and GATA3 can more effectively induce *Il13* expression in Dnmt3a-deficient Th1 cells than in wild type Th1 cells (Yu et al., 2012). Thus, Dnmt3a is required for appropriate gene repression in T helper subsets. Collectively, epigenetic modification mediated by multiple factors is essential for Th1 cell differentiation (Figure 4).

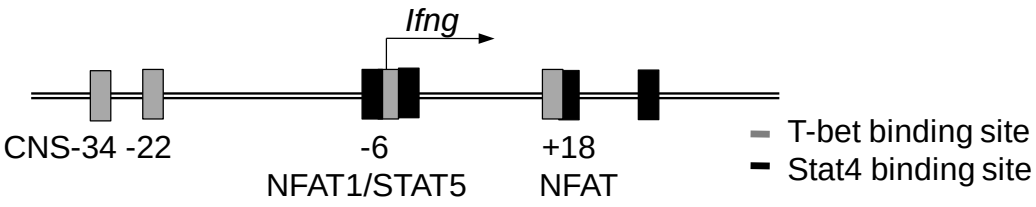


Figure 4. Schematic diagram of the *Ifng* gene

Transcriptional regulation of Th2 cells

T helper type 2 (Th2) cells are important for host immunity to extracellular parasites. Th2 cells mediate function through the production of IL-4, IL-5, IL-13, and IL-25. Defects in Th2 cell development result in asthma and other allergic inflammatory diseases. Th2 cell differentiation occurs under the presence of IL-4 and anti-IFNγ. IL-4 activates STAT6 that directly binds to gene encoding transcription factors including GATA3, Gfi-1, BATF, and Runx1. While GATA3 is a Th2 master transcription factor and Gfi-1 and BATF are required for its optimal cell expansion, Runx1 represses GATA3 expression thus inhibiting Th2 cell differentiation (O'Shea et al., 2011; Zhu et al., 2002). Other signaling pathways such as β-catenin and T cell factor 1 (TCF1) have been reported to contribute to Th2 cell differentiation through the induction of GATA3 expression in an IL-4-independent manner (Paul and Zhu, 2010). Other STAT proteins also promote the differentiation of Th2 cells. IL-2-STAT5 signaling pathway has been shown to be important in regulating Th2 cell differentiation, and that both GATA3 and STAT5 bind to regulatory elements in the Th2 cytokine loci (Zhu et al., 2003). In addition, STAT3 activation has been detected in Th2 cell development, and that STAT3 is required for STAT6 bound to target genes in Th2 cells (Stritesky et al., 2011). Mice with *Stat3*-deficient T cells display reduced inflammation in a mouse model of allergic airway inflammation (Stritesky et al., 2011). Thus, the integration of multiple STAT proteins and transcription factors is required for optimal Th2 cell development (Figure 5).

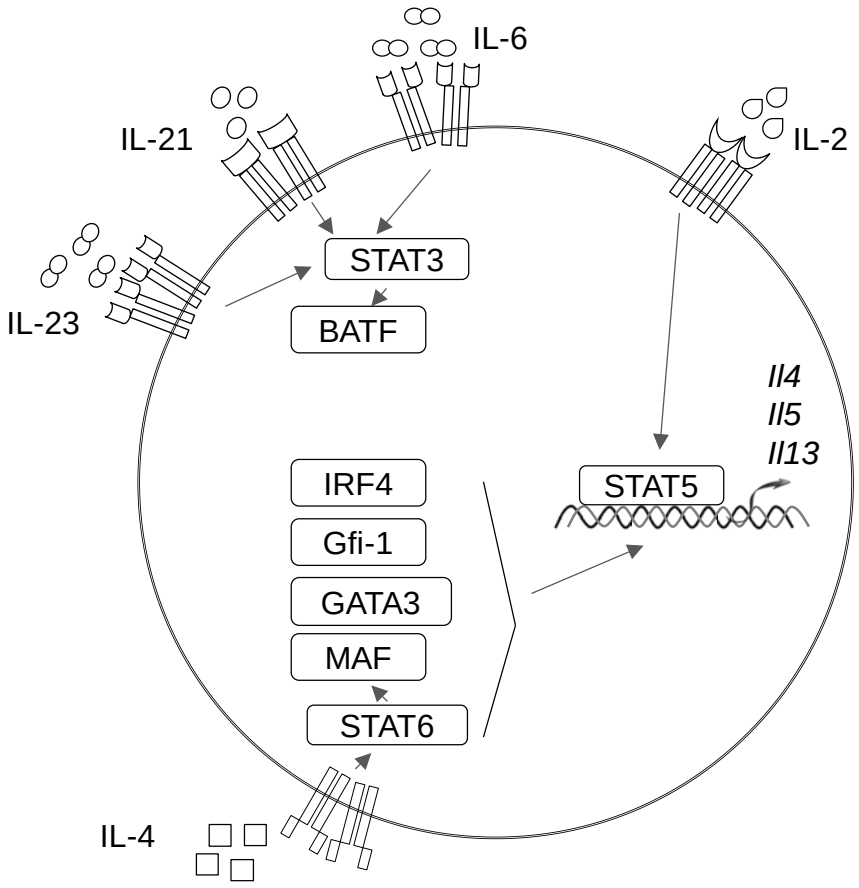


Figure 5. The transcriptional network in Th2 cells

Originally, IL-4 was viewed as a central cytokine that acts on STAT6 in Th2 cell differentiation. However, other cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 have been demonstrated to be important for Th2 cell development. TSLP produced from epithelial cells, mast cells, and basophils signals through TSLPR expressed on $CD4^+$ T cells and activates STAT5 (Al-Shami et al., 2005; Yao et al., 2013). In contrast, IL-25 is produced by Th2 cells, and it is suggested to be required for the balance between Th1 and Th2 cells during *T.muris* infection model (Owyang et al., 2006). IL-33 together with TSLP could induce the production of IL-13 without TCR signaling (Guo et al., 2009)

Epigenetic modifications also play an important part in regulating Th2 cell differentiation. The *Il4, Il5,* and *Il13* loci contain DNase I hypersensitive sites located in CNS regions that are accessible by regulatory transcription factors. For example, STAT6 and GATA3 have been shown to bind to hypersensitive site V (HSV) of the *Il4* and *Il13* loci (Wilson et al., 2009a). GATA3 not only induces Th2 gene transcription but also recruits histone acetyltransferases (HATs) and histone H3K4 methyltransferases, displacing MBD2 and associated HDCA-containing complexes, diminishing DNMT1 association and inhibiting DNA methylation, and recruiting chromatin-remodeling complexes to the Th2 cytokine loci (Wilson et al., 2009a). Similarly, STAT3 and STAT6 are required for some histone modifications, accessibility of chromatin (STAT3), and recruiting HATs and other chromatin modifying enzymes to Th2 gene loci (Stritesky et al., 2011; Wilson et al., 2009a). The regulation of Th2 cytokine gene expression is represented below (Figure 6).

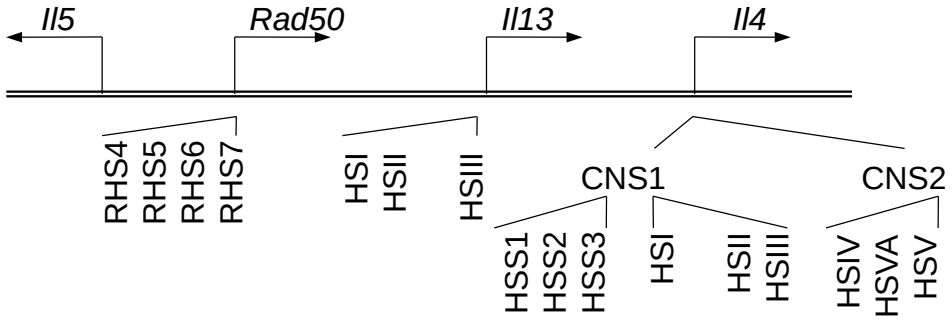


Figure 6. Schematic diagram of the Th2 genes

Transcriptional regulation of Th9 cells

T helper type 9 (Th9) cells have emerged as a new T helper cells in recent years. IL-9 is the hallmark cytokine of Th9 cells that is required for allergic inflammation and immunity to intestinal parasites. IL-9 has effects on various cell types such as smooth muscle cells, stem cells, lymphocytes, mast cells, and epithelial cells that are important for the development of immunity and inflammation (Goswami and Kaplan, 2011).

Th9 cell differentiation requires both IL-4 and TGF-β signaling (Dardalhon et al., 2008; Veldhoen et al., 2008b). IL-4 activates STAT6 that induces the expression of transcription factors that are important for Th9 differentiation such as GATA3, IRF4, and BATF (Goswami et al., 2012; Jabeen and Kaplan, 2012). STAT6 can promote *Il9* expression, though it binds the *Il9* gene poorly compared to other genes (Jabeen and Kaplan, 2012). In addition, STAT6 represses T-bet expression that together with Runx3 negatively regulates IL-9 production in Th1 cells (Goswami et al., 2012). While IRF4 promotes *Il9* expression by directly binding to its locus (Staudt et al., 2010), one potential GATA3 function in Th9 cells is to repress the expression Foxp3 (Mantel et al., 2007). Th9 cell differentiation also requires TGF-β signaling pathway that induces PU.1 expression (Goswami et al., 2012). PU.1 has been shown to repress Th2 cell development and promotes *Il9* expression by directly binding to its locus suggesting that PU.1 is the master transcription factor that drives Th9 cell differentiation (Chang et al., 2010). Other cytokines and STAT protein have been

shown to regulate Th9 cells. TSLP activates STAT5 that directly binds and promotes *Il9* gene expression in Th9 cells (Yao et al., 2013). Infants with atopic dermatitis have increased IL-9 and TLSP in the serum compared to non-atopic infants (Yao et al., 2013). IL-2, IL-25, and IL-1 cytokines can enhance IL-9 production from $CD4^+$ T cells by activating transcription factors including NF-κB that bind to the *Il9* promoter (Jabeen and Kaplan, 2012). A transcription factor network of Th9 cells is summarized in Figure 7.

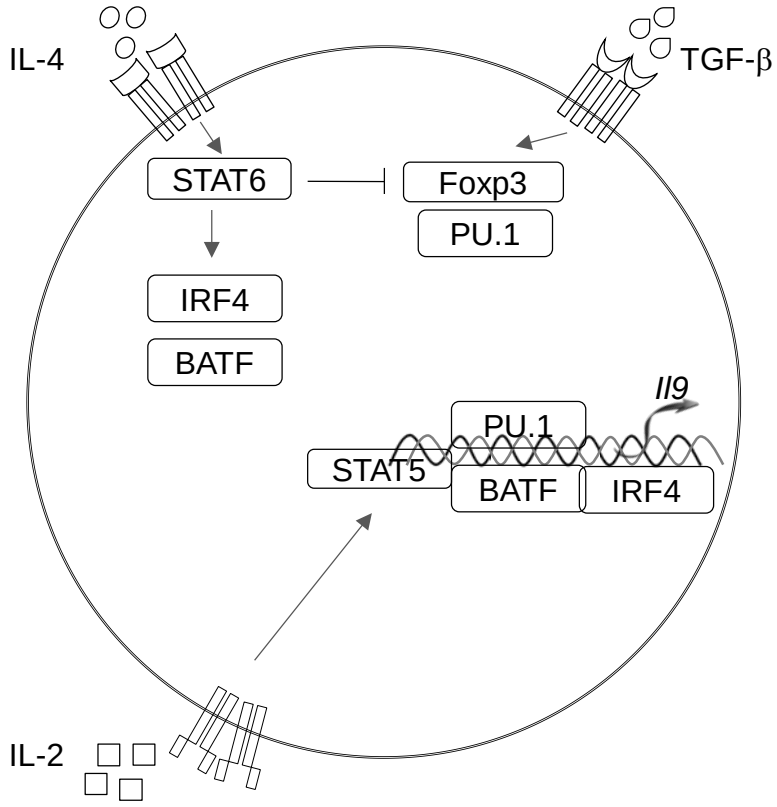


Figure 7. The transcriptional network in Th9 cells

Epigenetic programming also plays an important role in regulating IL-9 production in $CD4^+$ T cells. The *Il9* gene contains at least three CNS 6 kb upstream and downstream of the *Il9* promoter that contains binding sites for PU.1, IRF4, GATA3, NFAT, and STAT4 proteins (Perumal and Kaplan, 2011) suggesting a complex regulation of *Il9* that requires multiple *cis*-regulatory elements and specific *trans*-activating factors. In addition, PU.1 recruits and forms a complex with Gcn5 (a histone acetyltransferase (HAT)) resulting in an increased histone acetylation at the *Il9* locus (Goswami and Kaplan, 2012). There are decreased association of p300/CBP associated factor (PCAF) and increased association of histone deacetylases at the *Il9* locus in Th9 cells suggesting histone modification plays an important role in the regulation of *Il9* in Th9 cells (Figure 8) (Goswami and Kaplan, 2012).

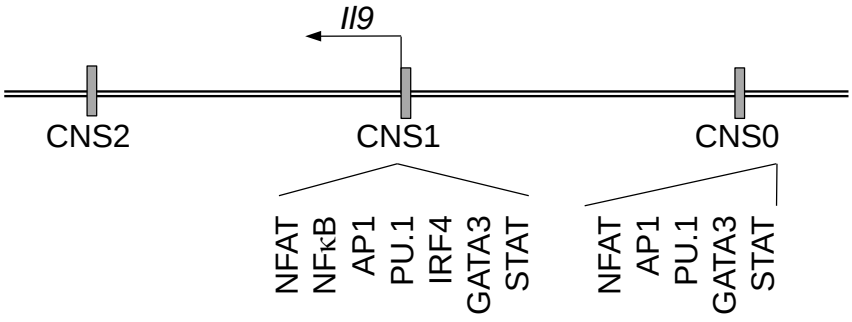


Figure 8. Schematic diagram of the *Il9* gene

Transcriptional regulation of Th17 cells

T helper type 17 (Th17) cells secrete IL-17A, IL-17F, IL-21, and IL-22, and are primary mediators in controlling infection and promoting autoimmune diseases (Korn et al., 2009). Naïve $CD4^+$ T cells differentiate into Th17 cells in the presence of IL-1β, TGF-β, and IL-6 while IL-23 is required for the maintenance of IL-17-producing cells (Harrington et al., 2005; Mangan et al., 2006; Stritesky et al., 2008). Cytokine stimulation results in the induction of a network of transcription factors including RORγt, BATF, IRF4, and other factors (Figure 9)(Ciofani et al., 2012; Wu et al., 2013; Yosef et al., 2013). Some of the factors in this network are required for the development of additional Th subsets, and cooperate with specialized factors to promote acquisition of distinct phenotypes.

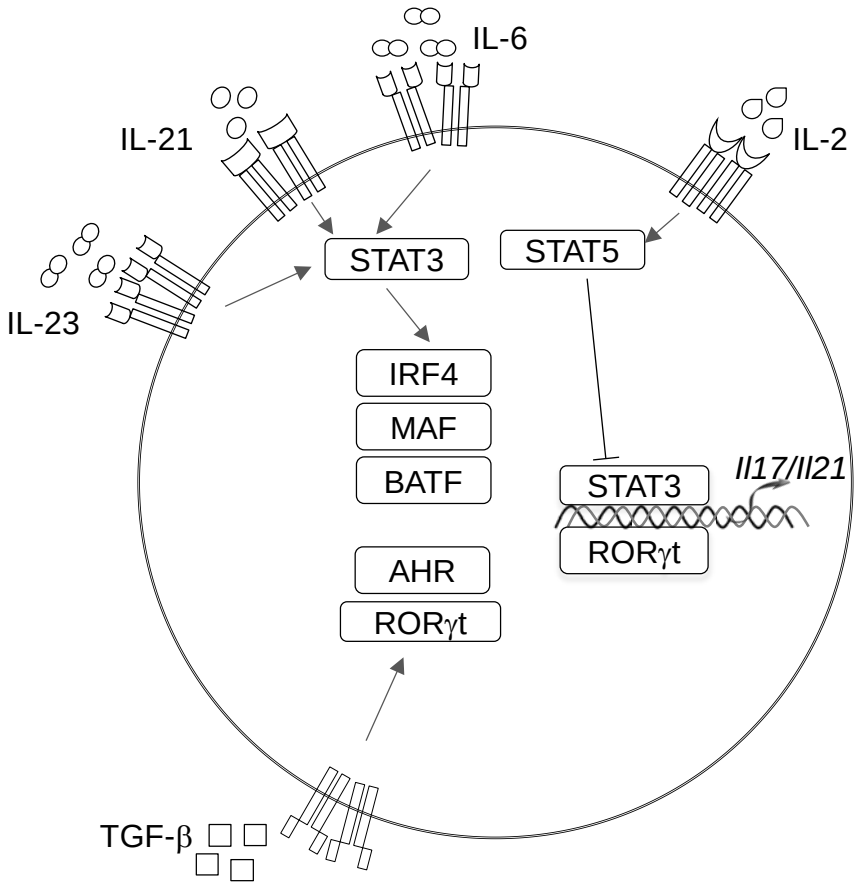


Figure 9. The transcriptional network in Th17 cells

IL-6 and IL-23 activate STAT3 that directly binds to genes encoding transcription factors including *Rorc, Maf, Batf, Irf4,* and *Ahr* that promote *Il17* expression and Th17 development (Brustle et al., 2007; Durant et al., 2010; Ivanov et al., 2006; Manel et al., 2008; Schraml et al., 2009; Veldhoen et al., 2008a; Xu et al., 2009). Bcl-6 is another STAT3 target gene that has been shown to indirectly control Th17 cell differentiation by suppressing Th2 cell differentiation (Durant et al., 2010; Mondal et al., 2010). STAT3 promotes *Il23r* expression that is required for the development of inflammatory Th17 cells (Zhou et al., 2007). Conversely, decreased receptor expression interferes with the ability of a cell to respond to

the cytokine environment. STAT5 inhibits expression of *Il6ra* and *Il6st*, limiting Th17 differentiation (Liao et al., 2011). STAT3 is also required for multiple T helper cell lineages including Th2, Th17, and Tfh (Ma et al., 2012a; Mathur et al., 2007; Nurieva et al., 2008; Stritesky et al., 2011; Yang et al., 2008b). As part of its function STAT3 activates genes that are common among these lineages (*Maf*, *Batf,* and *Irf4*) and genes that are lineage-specific, such as *Rorc* for Th17 and *Bcl6* for Tfh (Bauquet et al., 2009; Brustle et al., 2007; Durant et al., 2010; Ivanov et al., 2006; Schraml et al., 2009; Yu et al., 2009). However, a balance between positive and negative regulatory factors controls the differentiation of each of these subsets. The IL-2-STAT5 signaling pathway limits IL-17 production, and the balance between STAT3 and STAT5 activation determines the ability of cells to produce inflammatory cytokines (Durant et al., 2010; Yang et al., 2011).

Il17 gene regulation is controlled by epigenetic events at multiple CNS regions (Figure 10). Upon Th17 cell differentiation, eight CNS in the *Il17a-Il17f* locus becomes more accessible because of the increased histone H3 acetylation and K4 tri-methylation at the locus (Akimzhanov et al., 2007). CNS2 has been reported to interact with both *Il17a-Il17f* promoters and is required for their transcription in Th17 cells (Wang et al., 2012). Moreover, Wang et al. showed that CNS2 is required for the recruitment of histone-modifying enzymes p300 and Jmjd3 to the *Il17a-Il17f* locus.

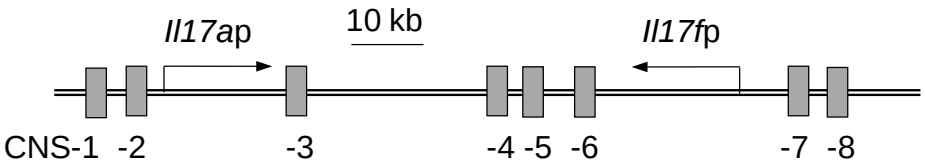


Figure 10. Schematic diagram of the *Il17* gene

Transcriptional regulation of T regulatory cells

Regulatory T (Treg) cells are unique in the ability to maintain immunological self-tolerance and homeostasis by suppressing various effector lymphocytes. There are two types of Treg cells: natural Treg (nTreg) cells that arise in the thymus early in the development and inducible Treg (iTreg) cells that develop in the peripheral lymphoid organs upon TCR engagement or antigen presented in an inappropriate mileu. Treg cells are characterized by the expression of *Ctla4, Il2ra* and transcription factor Foxp3, a master regulator of its functions (Hori et al., 2003; Ohkura et al., 2013). Mutation or deletion of *Foxp3* results in immunodysregulation polyendocrinopathy enteropathy X-linked syndrome with autoimmune diseases in human and fatal systemic autoimmunity in mice (Ohkura et al., 2013).

In addition to Foxp3 that controls Treg cell development, Bcl-6 has been shown to indirectly regulate Treg cell function by potently repressing *Gata3* transcriptional transactivation (Sawant et al., 2012). Although the suppression function of *Bcl6*$^{-/-}$ Treg cells are normal compared to wild type cells in the Th1-type colitogenic T cell responses *in vivo, Bcl6*$^{-/-}$ Treg cells fail to control Th2 responses in a model of allergic airway disease resulting in increased lung inflammation (Sawant et al., 2012). Runx1 belongs to the Runx family and controls IL-2 production in Treg cells through the association with Foxp3 (Ono et al., 2007). In addition, Runx1 is required for Foxp3 expression and suppressive function of Treg cells (Kitoh et al., 2009; Rudra et al., 2009). Eos is a member of

Ikaros family and has been shown to play an important role in iTreg function by controlling the repressive activity of Foxp3 (Pan et al., 2009b). Another member of Ikaros family, Helios, is important for nTreg cell development, but how Helios functions is unknown (Wei et al., 2009). DNA-binding inhibitor Id3 controls Treg cell differentiation by diminishing GATA3 binding at the *Foxp3* promoter (Maruyama et al., 2011).

Furthermore, STAT proteins have an indispensible role in Treg cell differentiation. IL-2 activates STAT5 that binds to the *Foxp3* promoter and the first intron of *Foxp3* and initiates gene transcription (O'Shea et al., 2011). In addition, STAT5 induces *Il2ra* expression, which is upregulated during Treg cell development (O'Shea et al., 2011). In contrast, STAT4 has been shown to limit the development of iTreg by increasing the repressive chromatin modifications at the *Foxp3* locus thus impairing STAT5 binding to *Foxp3* (O'Malley et al., 2009*)* . A transcription factor network of Treg cells and the regulation of the *Foxp3* gene expression are summarized in Figure 11 and 12.

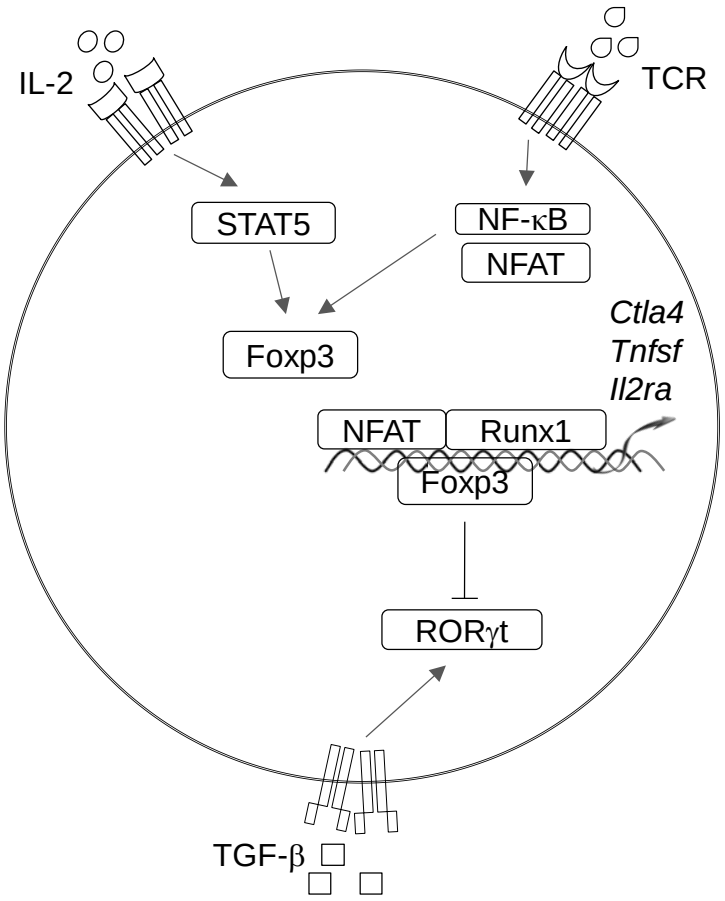


Figure 11. The transcriptional network in Treg cells

Epigenetic modifications such as histone modifications and DNA methylation play an essential role in Treg cell development. DNAs of Treg-associated genes such as *Foxp3, Ctla4,* and *Eos* have been shown to be hypomethylated that associates with an open chromatin structure leading to gene transcription (Ohkura et al., 2012) . Moreover, Ets-1, a member of the ETS family of transcription factors, binds and demethylates *Foxp3* CNS2 region resulting to enhanced gene expression at the *Foxp3* locus (Polansky et al., 2010).

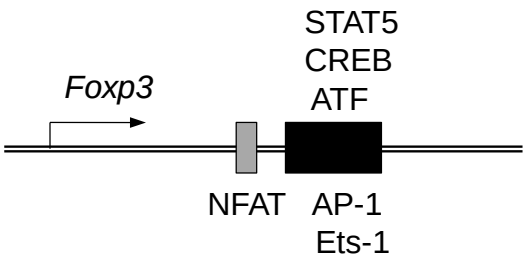


Figure 12. Schematic diagram of the *Foxp3* gene

Transcriptional regulation of T follicular helper cells

One crucial function of T helper cells is to help B cells. T follicular helper (Tfh) cells are specialized to provide help to B cells. These Tfh cells help germinal center B cells to differentiate into plasma cells and memory B cells that produce high affinity antibodies for long-lasting protection through somatic hypermutation and affinity maturation. A unique feature of Tfh cells is their location. Tfh cells express high levels of chemokine receptor CXCR5 that facilitates the relocation of Tfh cells from the T cell zone of the lymphoid tissue into the B cell follicle (Deenick and Ma, 2011; Ma et al., 2012b). The result is a T cell-B cell interaction that provides B cell help. In addition to CXCR5 expression, Tfh cells also express programmed death-1 (PD-1) and inducible co-stimulator (ICOS) that provide an inhibitory signal to the T cells, (Deenick and Ma, 2011). Furthermore, Tfh cells coexpress other surface markers such as CD40 ligand, OX40, CXCR5, CD200, B and T lymphocyte attenuator (BTLA), members of the SLAM family, and SLAM-associated protein (SAP) (Deenick and Ma, 2011). Tfh cells produce IL-4 and IL-21 that are required for optimal B cell help and plasma cell differentiation, respectively (Crotty, 2011).

Tfh cell differentiation is controlled by the master regulator transcriptional repressor Bcl-6 (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Bcl-6 controls Tfh cell differentiation by antagonizing transcription factors important for Th1 (T-bet), Th2 (GATA3), or Th17 (Rorγt) differentiation (Crotty, 2011). Bcl-6 also represses Blimp-1 expression, an antagonist of Bcl-6 (Martins and Calame,

2008). Bcl-6 negatively regulates miRNA cluster miR-17-92 that inhibits CXCR5 in Tfh cells (Yu et al., 2009). In addition to Bcl-6, other transcription factors such as MAF, BATF, and IRF4 are also involved in Tfh differentiation (Crotty, 2011). Transcription factor MAF together with NFAT and JunB control *Il4* expression (Zhu et al., 2010), and directly binds to the *Il21* promoter (Hiramatsu et al., 2010). *Batf*-deficient mice have defect in Tfh cells, B cell CSR, and GC development (Betz et al., 2010). In addition, *Irf4*-mutant mice have defects in Tfh cell development (Crotty, 2011). Both BATF and IRF4 control Tfh cell differentiation by regulating *Bcl6* expression (Bollig et al., 2012; Ise et al., 2011).

STAT proteins have been suggested to be involved in Tfh cell development. IL-6 and IL-21 activate STAT3 that is required for the development of Tfh cells (Nurieva et al., 2008). *Stat3*-deficient T cells failed to differentiate into Tfh cells resulting in a poor B cell responses (Nurieva et al., 2008). However, it has been observed the redundancy in the role of IL-6 and IL-21 in Tfh cell development (Eto et al., 2011) suggesting a complex role of STAT3 in Tfh cell differentiation. STAT4 also plays an important role in the early Tfh cell development. IL-12 activates STAT4 signaling that promotes both *Il21* and *Bcl6* gene expression (Nakayamada et al., 2011). Since STAT4 induces T-bet expression that represses *Bcl6* and other markers of Tfh cells, STAT4 is a positive regulator of both Th1 and Tfh cells (Nakayamada et al., 2011). In contrast, STAT5 signaling has been reported to repress the development of Tfh cells (Johnston et al., 2012;

Nurieva et al., 2012). Therefore, multiple STAT proteins and transcription factors play an essential role in controlling Tfh cell development (Figure 13).

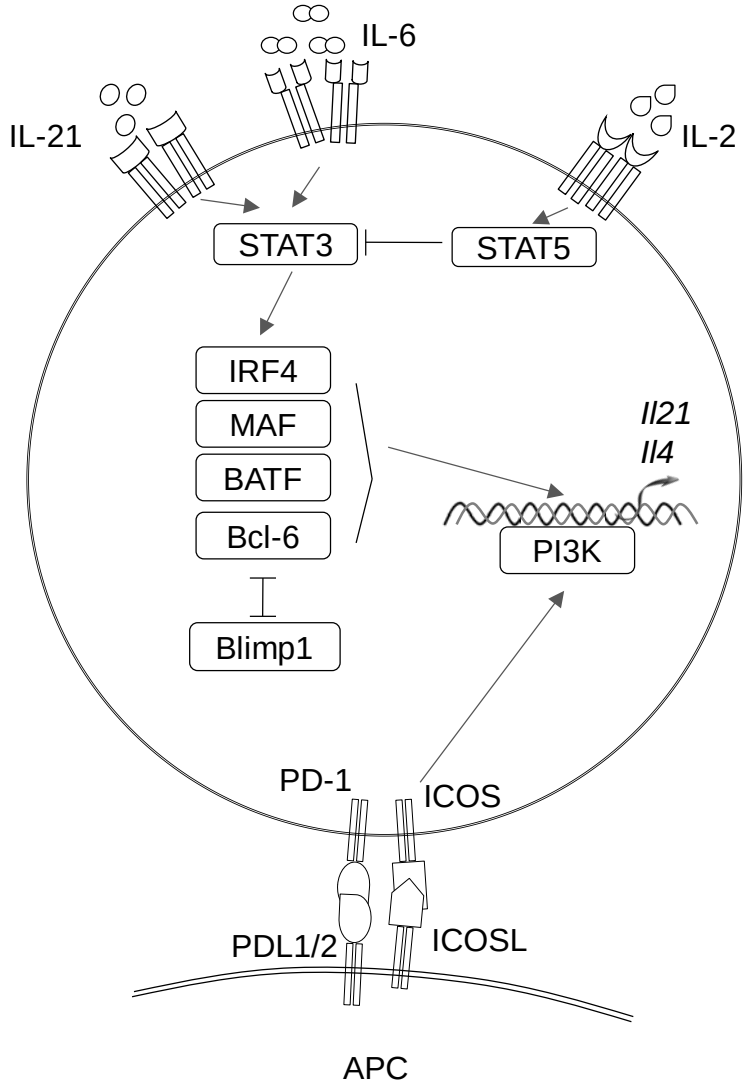


Figure 13. The transcriptional network in Tfh cells

T helper cell plasticity and overall functions

Naïve $CD4^+$ T cells can differentiate into T helper subsets with distinct effector functions that require in specific cytokine signaling, STAT proteins, and transcription factors. However, $CD4^+$ T cells also have the ability to redirect their functional programs under certain conditions into other types of effectors cells. $CD4^+$ T cells co-express transcription factors of different lineages that result in multifunctional effector cells. In an LCMV infection model, Th2 cells can acquire a Th1 phenotype through antigen-specific TCR stimulation and an inflammatory environment by converting into $GATA3^+$T-bet^+ cells that coproduce both IL-4 and IFNγ (Hegazy et al., 2010). Similarly, the existence of $Foxp3^+Ror\gamma t^+$ (Zhou et al., 2008) and $Foxp3^+$T-bet^+ cells (Koch et al., 2009) have been described in specific *in vivo* conditions. $CD4^+$ T cells also acquire the ability to switch to another T helper cells through receptor expression. Stimulating Th2 cells with type 1 interferons that activate both STAT1 and STAT4 induces IL-12Rβ2 expression on those cells (Hegazy et al., 2010). As a result, type 1 interferon stimulated Th2 cells are able to respond to IL-12. Thus, type 1 interferons together with IL-12 and IFNγ are able to promote IFNγ-producing Th2 cells (Hegazy et al., 2010). Th17 cells express receptors for both IL-12 and IL-23 thus could be converted to Th1 cells in the presence of IL-12 (Bluestone et al., 2009). Epigenetic modification also plays an essential role in T helper cell plasticity. While trimethylation of histone H3 lysine 4 (H3K4me3) is associated with active gene transcription, trimethylation of histone H3 lysine 27 (H3K4me27) indicates inactive gene transcription. For example, H3K4me3 at the *Ifng* locus and

H3K27me3 at the *Il4* and *Il17* loci are found with Th1 cells. In contrast, H3K4me3 at the *Il4* locus and H3K27me3 at the *Ifng* and *Il17* loci are found in Th2 cells. However bivalent epigenetic status at the *Tbx21* and *Gata3* loci also exist in Th17 and Treg cells suggesting a preconditioned to be converted toward Th1 or Th2 cell fates (Zhou et al., 2009).

Transcriptional repressor Twist1

Twist1 is a transcriptional repressor and a member of the basic helix-loop-helix (bHLH) family of proteins that plays a positive role in dorso-ventral patterning in *Drosophila* embryos (Barnes and Firulli, 2009). Twist1 functions as either a homodimer or heterodimer with other bHLH factors, where the dimerization partners dictate the function (Castanon et al., 2001). Altering the balance between Twist1 and Hand2 association has a significant impact on limb and craniofacial defects in humans with Saethre-Chotzen syndrome (Firulli et al., 2005). Twist1 has been shown to determine the onset of osteoblast differentiation (Bialek et al., 2004), and functionally represses muscle development (Hamamori et al., 1997; Hebrok et al., 1994; Spicer et al., 1996). Twist1 also plays a role in mediating outflow track cushion formation within the developing heart (Vincentz et al., 2008). In addition, Twist1 maintains energy homeostasis by altering mitochondria metabolism in the brown fat (Pan et al., 2009a). Twist1 not only plays a central role in embryonic development, it is an essential factor in tumor development and progression. Twist1 has been associated with epithelial-mesenchymal transition (EMT) in tumorigenesis (Vernon and LaBonne, 2004). Twist1 is an oncogene that plays important role in breast cancer, prostate cancer, hepatocellular carcinoma, and other (Kwok et al., 2005; Matsuo et al., 2009; Vesuna et al., 2012). Although the role of Twist1 in developmental and cancer biology has been explored (Yang et al., 2004), the function of Twist1 in the immune response is only beginning to be understood. Twist1 regulates cytokine production in macrophages through a negative

feedback loop by targeting NF-κB activation that results in decreased TNFα and IL-1β (Sharif et al., 2006; Sosic et al., 2003). The repressive mechanism may involve binding of Twist1 to E-boxes in cytokine promoters to inhibit the transcriptional activity of NF-κB (Sharif et al., 2006; Sosic et al., 2003). In Th1 cells, NF-κB, NFAT, and IL-12-STAT4 signaling can induce *Twist1* expression, and Twist1 limits inflammation by suppressing IFNγ and TNFα production (Niesner et al., 2008). However, a detailed mechanism of how Twist1 regulates cytokine production in Th1 cells and other T helper subsets has not been described.

Transcription factor Etv5

Etv5 belongs to the PEA3 subfamily of ETS transcription factors that regulates gene expression by binding to a conserved motif GGAA/T (Oh et al., 2012). Etv5 has been shown to play important role in coordinating limb development (Zhang et al., 2010b; Zhang et al., 2009), controlling gene expression in the spermatogonial stem cell (Wu et al., 2011), and regulating EMT in many type of cancers (Oh et al., 2012). Etv5 inhibits sonic hedgehog (*Shh)* expression in the anterior limb bud that is essential for the anterior-posterior (A-P) patterning of the vertebrate limb (Zhang et al., 2009). In addition, Etv5 physically interacts with Twist1 to inhibit *Shh* expression in the limb bud (Zhang et al., 2010b). Etv5 positively regulates microRNA-21 (mir21), *Bcl6b,* and LIM homeobox 1 (*Lhx1)* that are known to control spermatogonial stem cells (SSC) self-renewal (Niu et al., 2011). Chromosomal translocations resulting from the fusion between Etv5 and transmembrane protease serine 2 (TMPRSS2) correlate with prostate cancer (Oh et al., 2012). In endometrial cancer, Etv5 cooperates with lipoma-preferred partner (LPP) and promotes zinc finger E-box-binding transcription factor Zeb1 expression leading to the suppression of E-Cadherin, an important factor that is associated with EMT (Colas et al., 2012).

Although the role of Etv5 in developmental and cancer biology has been defined, the function of Etv5 in the immune response is still poorly understood. The IL-12-STAT4 signaling pathway induces Etv5 expression in Th1 cells to augment IFNγ production in Th1 cells through an unknown mechanism (Ouyang et al., 1999;

Thieu et al., 2008). Thus, it remains to elucidate the role of Etv5 in the development of Th1 and other T helper cells.

Goal of the thesis

Intensive research has been conducted to describe lineage-specific transcription factors regulating the development of different T helper cell subsets. However, how different positive and negative factors integrate together to control the plasticity of T cell subsets at the transcriptional level is not fully understood. Thus, the main goal of this thesis is to define the functional interaction of transcription factors that have a positive or negative regulatory role in the development of T helper cells.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). *Stat4*$^{-/-}$ and *Stat3*-mutant mice were previously described (Kaplan et al., 1996b; Stritesky et al., 2011). *Twist1*$^{fl/fl}$ (Chen et al., 2007) and *Etv5*$^{fl/fl}$ mice (Zhang et al., 2009) were crossed with CD4-Cre transgene mice to generate *Twist1*$^{fl/fl}$ CD4-Cre^{+} and *Etv5*$^{fl/fl}$ CD4-Cre^{+} mice with Cre-negative littermates as WT mice. *Twist1*$^{fl/fl}$ CD4-Cre mice were backcrossed to C57BL/6 mice for six generations with Cre-negative littermates as wild type mice for *in vivo* experiments. *Twist1*$^{cc/wt}$ mice (Bialek et al., 2004) were crossed with *Twist1*$^{fl/fl}$ CD4-Cre^{+} mice to generate *Twist1*$^{fl/cc}$ CD4-Cre^{+} and *Twist1*$^{fl/wt}$ CD4-Cre^{+} as WT control. *Dnmt3a*$^{fl/fl}$ CD4-Cre positive C57BL/6 mice (Yu et al., 2012) were mated with *Stat4*$^{-/-}$ to generate *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ CD4-Cre positive with Cre-negative littermates as control mice. The following primers and PCR cycling conditions were used for genotyping: for *Cre*, forward 5'-GTGAAACAGCATTGCTGTCACTT-3' and reverse 5'-GCGGTCTGGCAGTAAAAACTATC-3', initial denaturation at 94^{o}C for 2 min, 30 cycles of 94^{o}C for 45 sec, 60^{o}C for 45 sec, and 72^{o}C for 45 sec, and a final extension for 10 min at 72^{o}C; for *Twist1*, forward 5'-AGATTGGG CACCGTAGCAG-3' and reverse 5'-TGACAGCAGTAGTGGCAA GC-3', wild type allele 320 bp and mutant allele 370 bp ,initial denaturation at 94^{o}C for 3 min, 35 cycles of 94^{o}C for 30 sec, 60^{o}C for 1 min, and 72^{o}C for 1 min, and a final extension for 2 min at 72^{o}C; for *Twist1cc,* forward 5'-

ACGAGCTGGACTCCAAGATG-3' and reverse 5'-GGAGCTCCGCTGCTAGTG-3', 40 cycles of 94^{o}C for 30 sec, 59^{o}C for 15 sec, and 72^{o}C for 12 sec, and a final extension for 5 min at 72^{o}C, purify the PCR product and send for sequencing; for *Etv5* forward 5'-CTCGCAGAGGACAAGGTAGTGAC-3', reverse 1 5'-GTGTGCACGACATGTTCAAGG-3' and reverse 2 5'-CTCTTGCACAGGACCCATGTTAG-3', wild type allele 270 bp and mutant allele 368 bp, initial denaturation at 94^{o}C for 3 min, 35 cycles of 94^{o}C for 30 sec, 58^{o}C for 45 sec, and 72^{o}C for 1 min, and a final extension for 10 min at 72^{o}C; for *Dnmt3a,* forward 5'-CTGTGGCATCTCAGGGTGATGAGCA-3' and reverse 5'-AAGCCTCAGGCCCTCTAGGCAAGA-3', wild type allele 100 bp and mutant allele 200 bp, initial denaturation at 94^{o}C for 2 min, 35 cycles of 94^{o}C for 45 sec, 65^{o}C for 45 sec, and 72^{o}C for 45 sec, and a final extension for 7 min at 72^{o}C. 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.

In vitro T cell differentiation

Naïve CD4^{+}CD62L^{+} T cells were isolated from spleen and lymph nodes using a MACS isolation system (Miltenyi Biotec). CD4^{+} T cells were activated with plate-bound anti-CD3 (2 ug/ml 145-2C11 BD Pharmingen) and soluble anti-CD28 (37.51, 0.5 ug/ml BD Pharmingen for Th0, Th1, Th2, and Th17 or 1 ug/ml for Th9 and Treg) with additional cytokines (all from PeproTech) and antibodies to generate Th1 (5 ng/ml IL-12; and 10 ug/ml anti-IL-4 11B11), Th2 (10 ng/ml IL-4;

and 10 ug/ml anti-IFNγ XMG), Th9 (20 ng/ml IL-4; 2 ng/ml TGF-β; and 10 ug/ml anti-IFNγ XMG) or Th17 (100 ng/ml IL-6; 10 ng/ml IL-23,; 10 ng/ml IL-1β; 2 ng/ml TGFβ;10 ug/ml anti-IL-4, 11B11; and 10 ug/ml anti-IFNγ, XMG) or Treg (2 ng/ml TGF-β, and 10 ug/ml anti-IL-4, 11B11) culture conditions. In some culture conditions, Th17 cells were generated without TGF-β or with IL-21 (10 ng/ml) instead of IL-6. Cells were expanded after 3 days without additional cytokines (Th0, Th1 and Th2), with 50 U/ml human-IL-2 (Treg), full concentration (Th9) or half concentration of IL-6 (Th17) of the original cytokines in fresh medium. Cells were harvested on day 5 for analysis. In some experiments, non-polarized T cells and Th1 cells (expanded after 3 days in fresh medium) were harvested on days 3 and 5 respectively, and restimulated with 5 ng/ml IL-12 for 1, 4, and 6 h for further analyses.

Retroviral expression vectors and retroviral transduction

Bicistronic retrovirus expressing EGFP only (MIEG) and T-bet and EGFP (T-bet) were previously described (Chang et al., 2005; Mathur et al., 2006) PBMN-IRES-GFP-IL-12Rb2c was a kind gift from Dr. Takashi Usui (Kyoto University). *Etv5, Il12rb2c* or *Twist1* (Open Biosystems) cDNAs were digested and sub-cloned into MIEG3-EGFP, MIEG3-hCD4, or MSCV-YFP, respectively. Flag-tagged Twist1 (Firulli et al., 2005) and Flag-tagged Twist1cc pCDNA3.1 that was made using QuikChange Site-Directed Mutagenesis Kit (Stratagene) with primer pair forward 5'-TCAGCTACGCCTTCCCCGTCTGGAGGATG-3' and reverse 5'-CATCCTCCAGACGGGGAAGGCGTAGCTGA-3', were digested, and subcloned

into MIEG3-EGFP. *Runx3* cDNA (Open Biosystems) was amplified 5'-BglII-forward 5'- AGATCTATGCGTATTCCCGTAGACCC -3' 3'- SalI and reverse 5'-GTCGACTCAGTAGGGCCGCCA-3'), digested and sub-cloned into MSCV-Thy1.1. *HLX1* (Open Biosystems) cDNA was amplified forward Hlx1-EcorI-F5'-ATCAGAATTCATGTTCGCAGCCG-3' and reverse Hlx1-XhoI-R 5'-ACTACTCGAGCTATAAGCAGCCAAGCG-3'), sub-cloned into the TOPO vector (Invitrogen), digested and sub-cloned into either MIEG-EGFP or MSCV-Thy1.1. *Twist1*-targeting shRNA oligo was designed as described (Niesner et al., 2008) and introduced into RNAi-Ready pSIREN-RetroQ-ZsGreen according to the manufacture manual (Clonetech). Retroviral stocks were prepared by calcium phosphate transfection of 70% confluent Phoenix GP cells using 15 ug of DNA, 3 ug of env DNA and 10 ug of gag pol DNA per transfection. The medium (DMEM, 10% FBS, 5% Penicillin/streptomycin) was replaced after 12 h, and viral supernatants were collected after 24 h and 48 h later. Purified $CD4^+$ T cells were cultured under Th1 cell differentiation condition. On day 2, cells were transduced with retrovirus expressing vector control or gene of interest centrifugation at 2000 rpm at 25^{o}C for 1 h in the presence of 8 ug/ml polybrene. Viral supernatant was replaced with the former culture supernatant supplemented with 50 U/ml human IL-2. After spin infection, cells were expanded on day 3 and analyzed on day 5.

Gene expression analysis (qRT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies) and reversed transcribed to make cDNA according to the manufacturer's instructions (Invitrogen Life Technologies). Quantitative Real Time (qRT)-PCR reaction was set up including cDNA, TaqMan Fast Universal Master Mix, commercially available primers, and DEPC H_2O to 10 ul. Gene expression was normalized to housekeeping gene expression (β2-microglobulin). The relative gene expression was calculated by the change-in-threshold ($-\Delta\Delta C_T$) method.

Table1-Taqman RT-PCR primers

Species	Gene	Product catalog number
Mouse	*Ahr*	Mm00478932_m1
	Ahrr	Mm00477443_m1
	B2m	Mm00437762_m1
	Batf	Mm00479410_m1
	Ccr4	Mm00438271_m1
	Ccr8	Mm01351703_m1
	Crem	Mm00516347_m1
	Cxcl1	Mm04207460_m1
	Cxcl10	Mm00445235_m1
	Cxcl2	Mm00436450_m1
	Cxcl3	Mm01701838_m1
	Erg	Mm01214248_m1

Mouse	*Etv5*	Mm00465816_m1
	Fasl	Mm00438864_m1
	Furin	Mm00440646_m1
	Gata3	Mm00484683_m1
	Hlx1	Mm00468656_m1
	Id3	Mm00492575_m1
	Ifng	Mm01168134_m1
	Il12rb2	Mm00434200_m1
	Il17a	Mm00439618_m1
	Il17rb	Mm00444709_m1
	Il18r1	Mm00515178_m1
	Il1rn	Mm01337566_m1
	Il4	Mm00445259_m1
	Il6ra	Mm00439653_m1
	Il6st	Mm00439665_m1
	Il9	Mm00434305_m1
	Irf1	Mm01288580_m1
	Itgae	Mm00434443_m1
	Jmjd3	Mm01332680_m1
	Maf	Mm01546091_s1
	Ptpn13	Mm00478426_m1
	Rorc	Mm01261022_m1
	Runx3	Mm00490666_m1

Mouse	*Sfpi1*	Mm00488142_m1
	Socs1	Mm00782550_s1
	Socs3	Mm00545913_s1
	Stat3	Mm01219775_m1
	Stat4	Mm00448890_m1
	Tbx21	Mm00450960_m1
	Tcfe2a	Mm01175588_m1
	Tnfsf13b	Mm00446347_m1
	Twist1	Mm00442036_m1
Human	*B2M*	Hs00984230_m1
	BATF	Hs00232390_m1
	IFNG	Hs00989291_m1
	IL17A	Hs00174383_m1
	IL17F	Hs00369400_m1
	MAF	Hs00193519_m1
	RORC	Hs01076122_m1
	TWIST1	Hs00361186_m1

Enzyme-linked immunosorbent assay (ELISA)

To assess cytokine production, 96-well NUNC MaxiSorp plates were coated with capture antibodies in 0.1 M $NaHCO_3$ (pH 9) buffer overnight at 4^oC. Plates were washed three times with 0.05% Tween20/PBS buffer and blocked with 2% BSA/PBS for 2 h at room temperature. Cell-free supernatant collected after 24 h

of anti-CD3 (2 ug/ml) stimulation or standards were diluted in 2% BSA/PBS, added to plates, incubated overnight at 4^{o}C or 2 h at room temperature. Plates were washed three times with 0.05% Tween20/PBS buffer and biotinylated antibodies (final concentration at 1 ug/ml) in 2% BSA/PBS, Avidin-alkaline phosphatase (1:2000 dilution) in 2% BSA/PBS were added sequentially at room temperature for 2 h and 1 h, respectively. Washes were performed between each step. For plate development, 5 mg/ml phosphatase substrate (Sigma 104) in substrate buffer (10% diethanolamine, 0.05 mM $MgCl_2$, 0.02% NaN_3, pH 9.8) was added. The absorbance was measured at 405 nm (Bio-Rad microplate reader model 680). Similar protocol was used to assess antigen-specific antibodies titer except 96-well NUNC MaxiSorp plates were coated with 10 ug/ml Myelin Oligodendrocyte Glycoprotein Peptide (MOGp35-55) or Sheep Red Blood Cell membrane protein in 0.1 M $NaHCO_3$ (pH 9) buffer overnight at 4^{o}C. Plates were washed three times with 0.05% Tween20/PBS buffered blocked with 2% BSA/PBS for 2 h at room temperature. Serum was diluted in 2% BSA/PBS, added to plates, and incubated overnight at 4^{o}C or 2 h at room temperature.

Table 2-ELISA capture antibodies

Capture antibodies	**Clone**	**Company**	**Final Concentration**
GM-CSF	MP1-22E9	BD Pharmingen	2 ug/ml
IFNγ	R4-6A2	BD Pharmingen	2 ug/ml
IL-10	JES5-2A5	BD Pharmingen	4 ug/ml

IL-13	eBio13A	eBioscience	2 ug/ml
IL-17A	TC11-18H10	BD Pharmingen	2 ug/ml
IL-17F	Cat#AF2057	R&D	2 ug/ml
IL-2	JES6-1A12	BD Pharmingen	4 ug/ml
IL-4	11B11	eBioscience	2 ug/ml
IL-9	D8402E8	Biolegend	1 ug/ml
TNFα	1F3F3D4	eBioscience	2 ug/ml

Table 3-ELISA biotinylated antibodies

Biotinylated antibodies	**Clone**	**Company**	**Final Concentration**
GM-CSF	MP1-31G6	BD Pharmingen	1 ug/ml
IFNγ	XMG1.2	BD Pharmingen	1 ug/ml
IgE	R35-72	BD Pharmingen	1 ug/ml
IgG	Poly4053	Biolegend	1 ug/ml
IgG1	A85-1	BD Pharmingen	1 ug/ml
IgG2a/c	R19-15	BD Pharmingen	1 ug/ml
IL-10	SXC-1	BD Pharmingen	1 ug/ml
IL-13	eBio1316H	eBioscience	1 ug/ml
IL-17A	TC11-8H4	BD Pharmingen	1 ug/ml
IL-2	JES6-5H4	BD Pharmingen	1 ug/ml
IL-4	BVD6-24G2	BD Pharmingen	1 ug/ml

IL-9	D9302C12	Biolegend	1 ug/ml
IL-17F	RD BAF2057	R&D	1 ug/ml
TNFα	XT3/XT22	eBioscience	1 ug/ml

Cell sorting and flow cytometry

Transduced cells were collected on day 5, stained with fluorochrome conjugated surface antibodies and sorted for single or doubly positive cells using a Reflection cell sorter (iCyt). Sorted cells were rested or re-stimulated with 2 ug/ml anti-CD3 for qRT-PCR (6 h) and ELISA (24 h) to assess gene expression and cytokine production, respectively. For surface staining, resting T cells were stained with with fluorochrome conjugated surface antibodies for 30 min at 4^{o}C, washed with 2% BSA/PBS and fixed with 2% paraformaldehyde for 10 min at room temperature before analysis. For phosphorylated STAT and transcription factor (T-bet and Runx3) analyses, cells were fixed with 1.5% formaldehyde for 10 min at room temperature, permeabilized using 100% ice cold methanol for 10 min at 4^{o}C, and stained for fluorochrome conjugated antibodies for 30 min at room temperature before analysis. Foxp3/Transcription factor staining kit (ebioscience) was used for other transcription factor staining (Foxp3, Twist1, Bcl-6, and Rorγt). For cytokine staining, cells were stimulated with either 2 ug/ml anti-CD3 with Golgi Plug inhibitor (1:1000 dilution, BD Pharmingen) or 50 ng/ml PMA and 500 ng/ml Ionomycin and 3 uM monensin (Sigma Aldrich) added for the last 3 h of a 5 h activation, fixed with 2% paraformaldehyde for 10 min at room temperature, permeabilized using 0.1% saponin for 10 min at 4^{o}C, and stained

for fluorochrome conjugated antibodies for 30 min at 4^{o}C before analysis. For viability staining, cells were washed twice with PBS and stained for fixable viability dye eFluor 780 (ebioscience) for 30 min at 4^{o}C. For biotinylated antibodies intracellular staining, fixed with 2% paraformaldehyde for 10 min at room temperature, permeabilized using 0.1% saponin for 10 min at 4^{o}C, and stained for biotinylated antibodies for 30 min at 4^{o}C follows by fluorochrome conjugated streptavidin for an additional 30 min at 4^{o}C. Cells were washed in between steps using 2% BSA/PBS.

Table 4-Flow cytometry antibodies

Antibody	**Clone**	**Company**
B220	RA3-6B2	BD Pharmingen
Bcl-6	K112-91	BD Pharmingen
CD19	6D5	BD Pharmingen
CD3	G4.18	BD Pharmingen
CD4	RPA-T4	BD Pharmingen
CD8	HIT8a	BD Pharmingen
CD90.1	OX-7	Biolegend
CXCR5	SPRCL5	eBioscience
Etv5	C-20	Santa Cruz
Fas	Jo2	BD Pharmingen
Foxp3	236A/E7	eBioscience
GATA3	L50-823	BD Pharmingen

GL-7	GL7	BD Pharmingen
hCD4	11830	R&D
ICOS	C398.4A	Biolegend
IFNγ	XMG1.2	Biolegend
IL-12Rβ2	305719	R&D
IL-17A	TC11-18H10	BD Pharmingen
IL-17F	O79-289	BD Pharmingen
Il-18Rα	BG/IL18RA	Biolegend
IL-4	11B11	BD Pharmingen
IL-9	RM9A4	Biolegend
Jmjd3	Cat#NBP1-06640B	Novus Biologicals
NK1.1	PK136	BD Pharmingen
PD-1	29F.1A12	Biolegend
PNA	Cat#B-1075	Vector Laboratories
pSTAT3	4/P-STAT3	BD Pharmingen
pSTAT4	38/P-STAT4	BD Pharmingen
pSTAT5	47	BD Pharmingen
Rorγt	AFKJS-9	eBioscience
Runx3	527327	R&D
Streptavidin	Cat#12-4317	eBioscience
T-bet	4B10	Biolegend
TNFα	MP6-XT22	Biolegend

Twist1	Cat#IC6230A	R&D

Immunoblot and Immunoprecipitation

Sorted cells or differentiated T cells were pelleted by centrifugation at 2000 rpm at 4^{o}C for 5 min, incubated in lysis buffer (10% glycerol, 1% IGEPAL, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 1 mM EDTA (pH 8) for 20 min on ice, and supernatant was collected by spinning for 15 min max speed at 4^{o}C. Whole-cell protein lysates diluted in 4X loading buffer (250 mM Tris-HCl, 2% SDS, 20% βME, 40% glycerol and 0.04 mg/ml bromophenol blue) were boiled at 100^{o}C for 5 min, spun at max speed at 4^{o}C for 5 min, loaded, run on 4-12% Bis-Tris gel at 100 V for 2 h, and transferred onto nitrocellulose membrane (Whatman GmbH) at 40 mA overnight at 4^{o}C. The membrane was blocked using 5% non-fat dry milk in 0.05%Tween20/PBS for 1 h at room temperature, immunoblotted using primary antibodies and secondary antibodies diluted in 0.05% Tween20/PBS overnight at 4^{o}C and 1 h at room temperature, respectively; and washed three times with 0.05% Tween20/PBS between steps. The membrane was developed using Western Lighting Chemiluminescence reagent (Perkin elmer Life Sciences, Wellesley, MA) and classic blue autoradiography film BX (Molecular Technologies). The membrane was stripped using stripping buffer (10% SDS, 62.5 mM Tris-HCl and 0.7% βME) and re-probed with other primary antibodies. For immunoprecipitation, whole-cell protein lysates were generated from differentiated T cells or 293T cells transfected with constructs expressing genes of interest using calcium phosphate. Whole-cell protein lysates were pre-cleared

with Protein A agarose (Thermo Scientific) for 1 h at 4^{o}C, before immunoprecipitation of equal amounts of protein with primary antibodies overnight at 4^{o}C. Immunocomplexes were captured by Protein A agarose for 1 h at 4^{o}C and eluted at 100^{o}C for 5 min in Laemmli's sample buffer. Immunoprecipitates were separated by 10% SDS-PAGE and immunoblotted with primary antibodies.

DNA affinity precipitation assay

Differentiated T cells were pelleted by centrifugation at 2000 rpm at 4^{o}C for 5 min, washed with cold PBS, and the following buffers were added sequentially and incubated on ice: lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl and 0.3% $MgCl_2$) for 10 min, 20% NP-40 for 10 min, cells were pelleted, nuclear lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40 with proteinase inhibitor) for 20 min. Cells were pelleted, and supernatant was stored at -20^{o}C as nuclear extract. Oligonucleotides containing a Twist1 binding site were described previously (Firulli et al., 2007). Additional oligonucleotides (biotinylated at 5') containing wild type and mutated (**bold**) Runx3 and T-bet binding sites (underline) are: wild type Runx3 and T-bet 5'-ACCTATGTGGTCTGCCTTTTCTTCTTTCTGGGCACGTTGA-3', mutant Runx3 5'-ACCTA**A**G**A**GG**A**CTGCCTTTTCTTCTTTCTGGGCACGTTGA-3', mutant T-bet 5'-ACCTATGTGGTCTGCCTTTTCTTCTTTCTGGGC**T**CGTTGA-3', and mutant Runx3-T-bet 5'-ACCTA**A**G**A**GG**A**CTGCCTTTTCTTCTTTCTGGGC**T**CGTTGA-3'. Biotinylated

oligonucleotides were incubated with streptavidin-agarose beads for 30 min at 4^{o}C. The complex was washed three times with pull-down buffer (25 mM HEPES, 15 mM NaCl, 0.5 mM DTT, 0.5% NP-40, 0.1 mM EDTA pH 7.5, and 10% glycerol). Nuclear extracts were added and incubated at 4^{o}C for 2 h. The complex was washed three times with pull-down buffer, eluted at 100^{o}C for 5 min in Laemmli's sample buffer, and separated using 10% SDS-PAGE gel.

Chromatin Immunoprecipitation (ChIP)

Cultured T cells (5-10 $\times$ 10^{6}) were cross-linked for 10 min with 1% formaldehyde, quenched with 0.125 M Glycine for 5 min and washed with cold PBS. Cell pelleted were resuspended in lysis buffer (5 mM Pipes, 85 mM KCl, 0.5% NP-40 with protease inhibitor) for 10 min on ice, spun down and aspirated the supernatant. Nuclei were resuspended in nuclear lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 1% SDS and protease inhibitor) for 10 min on ice. An ultrasonic processor (Vibra-cell) was used to shear genomic DNA to a size range of 200-300 bps at 30% amplitude for 8 sets of 10 second bursts. Cells were pelleted at max speed for 15 min at 4^{o}C, and supernatant was kept at -80^{o}C for the subsequent steps. Supernatant was diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1% v/v TritonX-100, 1.25 mM EDTA pH 8, 16.7 mM Tris-HCl pH 8, 166 mM NaCl, 20 ug/ml salmon sperm DNA, 10 ug/ml BSA and protease inhibitor) and pre-cleared with Protein A (or G) agarose bead slurry (50%) for 1.5 h at 4^{o}C. Primary antibodies were added, mixed, and incubated overnight at 4^{o}C. The immunocomplexes were precipitated with protein A (or G) agarose beads for

2 h at 4^{o}C, washed with low salt/high salt/LiCl salt buffers and two times with TE buffer, and eluted with elution buffer (2 mM EDTA, 20 mM Tris-HCl pH 8, and 20% SDS) after mixing the bead/immunocomplexes for 10 min at room temperature following by 10 min at 37^{o}C and cross-links reversed at 65^{o}C overnight supplemented with 10 mg/ml protease K. DNA was purified by phenol-chloroform extraction and ethanol precipitation, resuspended in H_2O, and analyzed by qPCR with Taqman or SYBR primers. DNA was quantified from the standard curve generating from serial dilutions of input DNA. The percentage input was calculated by subtracting the amount of immunoprecipitated DNA from the IgG control from the amount of immunoprecipated DNA from the specific antibody and normalized against the amount of input DNA.

Table 5-Immunoblots and ChIP antibodies

Antibody	**Clone**	**Company**
β-Actin	C4	Santa Cruz
Dnmt3a	64B1446	IMGENEX
E47	G127-32	BD Pharmingen
Etv5	H-100	Santa Cruz
Flag	M2	Sigma Aldrich
H3K27ac	Cat#07-360	Millipore
H3K27me3	Cat#07-449	Millipore
H3K36ac	Cat#ab9050	Abcam
H3K4me3	Cat#ab8580	Abcam

H3K9/18ac	Cat#07-593	Millipore
IgG	12-370	Millipore
Jmjd3	RB10082	Abgent
PEBP2β	FL-182	Santa Cruz
Runx3	6821C3a	Santa Cruz
STAT3	C-20	Santa Cruz
STAT4	C-40	Santa Cruz
T-bet	4B10	Santa Cruz
Twist1	Twist2C1a	Santa Cruz

Chromosome conformation capture assay (3C)

Chromosome conformation capture assay (3C) was performed as described (Hadjur et al., 2009; Hagege et al., 2007; Sekimata et al., 2009) with some modifications. 10^7 cells were cross-linked with 2% formaldehyde for 10 min at room temperature and quenched with 0.125 M Glycine for 5 min. Cells were lysed with ice-cold lysis buffer (10 mM Tris-HCl pH 8, 10 mM NaCl, 0.2% NP-40, Protease inhibitor cocktail) for 30 min. Nuclei were resuspended in 0.5 ml of restriction enzyme buffer (NEB3) containing 0.3% SDS and shaken for 1 h at 37^{o}C. Triton X-100 (final concentration 1.8%) was added and shaken for 1 h at 37^{o}C to sequester the SDS. Crosslinked DNA was digested overnight with 400-800 U BglII containing 1 mM ATP that has been shown to enhance digestion efficiency (Court et al., 2011). Enzyme was inactivated by addition of SDS (final concentration 1.6%) and samples were shaken for 20 min at 65^{o}C. The reaction

was diluted with 7 ml of ligation buffer (50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 1 mg/ml BSA), and Triton X-100 (final concentration 1%) was added and shaken for 1 h at 37^{o}C. DNA fragments were ligated with 4000 U T4 ligase (NEB) for 4 h at 16^{o}C followed by 40 min at room temperature. Crosslinks were reversed by incubation with 300 ug proteinase K overnight at 65^{o}C. The samples were further incubated with 300 ug RNase for 30-45 min at 37^{o}C, and the DNA were purified by phenol-chloroform extraction and ethanol precipitation. Ligation products were quantified by qPCR using primers as listed below. To generate control templates for the positive controls and to correct for differences in ligation and PCR efficiency between different templates, BAC clones were used to generate control template containing all possible ligation products. Equimolar amounts of three BAC clones spanning the mouse *Ifng* locus (RP23-353P23, RP23-138P22, and RP23-55O21) and BAC spanning the mouse *Gapdh* locus (RP23-410F11) from CHORI were mixed, digested, phenol chloroform extracted and ethanol precipitated, and ligated at a DNA concentration of 300 ng/ul. This sample was used as the DNA reference standard. Relative crosslinking frequencies between the analyzed pairs were calculated as described (Hagege et al., 2007) and were normalized to control interaction frequencies using primer pairs within the *Gapdh* locus.

Table 6: SYBR and Taqman primers for ChIP assay

Assay		**Gene**	**Sequence (5'→3')**
SYBR	Stat3	*Rorc*	F-AGCTTTGCTGTGGAAGATGTTTC

	binding		R-GAAGGGCTGGTAGGGAAGTCA
SYBR	STAT3 binding	*Il17a*	F-GGATTAAGGGCACACGTGTTG R-TTTCCCCACTCTGTCTTTCCA
		Maf	F-AACAGTGTTGGCTTTGTCTACTATGGGAT R-GTAGGCGGTGCTCTGATATATTGCTGTT
		Batf	F-CATTGCTGTGATGATGATGGTGACGAT R-AAGGCTGGCAAGATGACTCAGAGAATA
		Etv5 proximal	F-GGTGCTGATTGGTCAATGG R-CTGCCCATCACCTCTTGTTT
		Etv5 distal	F-CGCTACCCACGTTCTAGTCA R-CCTGCCTGTGTCATGATAGG
		Twist1 proximal	F-GCCAGGTCGGTTTTGAATGG R-CGTGCGGGCGGAAAGTTTGG
		Twist1 distal	F-AGCATGCAGGGCTTAATTTG R-ACTGTGCTTCCAAAGGTGCT

SYBR	Tbet/Runx3 binding	*Ifng* CNS-34	F-AGCTCCCATTAATGACACACC R-CTGAGACTGTGGTTGACACTC
		Ifng CNS-22	F- AAAGGTAAGGAAGCCAAAGAGAGTA R-GGCGCTGACATCATGCTTC
		Ifng CNS-6	F-CCCAGTGAGTGCTTTAAAATTTCT R-CTGGATGGTTTTGAAGGATAATGT
		Ifng CNS+46	F-GCAGCCCGGAGCAAGAGT R-CCCAATATTACTTCAGTGACAGCA
SYBR	Twist1 binding	*Bcl6*	F-CCCAACATAATTGTCCCAAA R-GCGAGAGAGTTGAGCCGTTA
		Il6ra	F-CGTGGCTCAGATCGGTGT R-GCCATCCTACTGGGCTTTC
		Icos	F-ACACCA CATCAACCTCCACA R-GAAGACAAAGACACGGCAGA
		Runx3	F-TCCTGTAGCCCCACTTTC R-TCGTCTATTCTGCCCTCG
		Tbx21	F-TGGGCATACAGGAGGCAGCA R-TCGCTTTTGGTGAGGACTGAAG
SYBR	Etv5 binding	*Bcl6*p	F-TCTTCGCTGTAGCAAAGCTC R-GGAAGAATTAGCCCCAGACC
		Il17 CNS1	F- GGAAGGTGCATGTGGCTGACT

			T R-AATGTGCCAGTCCCTTGGATGA
		Il17 CNS2	F-GCCTCCCATGTGGTCATTAT R-AGGCTCCTTCTCCATTGGTT
		Il17 CNS3	F-TTTGCTCATGCCCATATGTC R-TGAACCAACTTTCCCCACTC
		Il17 CNS4	F-CTCAAATCCGTGTGCCTTCT R-CATCTTGAAGCTGAGGCTGA
		Il17 CNS5	F-ACAGCCACACAACTCCCCACTC R-GGGCCTCCAAAATGACCTTCAC
		Il17 CNS6	F-ACAGGTGACCGCCAATAAAC R-GACATACCACAGCCCTTCGT
		Il17 CNS7	F-TGGCAAGGGGTAGGGTCAATCT R-GCAGACAGTGAGCAGGGCTTTC
		Il17 CNS8	F-ATGGTGGCCCTTAAAGCAGGAAR- CATGGGATCCTGGCTCATTCTC
SYBR	Etv5 binding	*Il17a* promoter	F-GCCTTTGTGATTGTTTCTTGCAG R-CCTTGCCCAAAGAAACCCTCTC
		Il17f promoter	F-GGGAATCAAAGGGGGACCCTAA R-AAAGCAGAACCCACACGCAGAG
SYBR	Other	*Cxcl10*	F- TCATCAGGACAGGGTCCACTGAGACT R-

			CTTTCAGAGATTTAGAGACTCAATGGCC
		Irf1	F-ACAGGAAAGCACACCCGCATCTTGT R-TGTCAAGAAGGGAGAGATTATGTCCCTGT
		Socs3	F-CCTTCGCAAACTTGCTTTGCATATTCT R-AAGTGACTTGAGCCTTTCTTTCCCTGG
Taqman	Promoter	*Etv5*	F-AGCTGGATTTGCCTGAAGACA R-CACACACGTCCCCATTGGT Probe: TAAACAAGAGGTGATGGGC
		Furin	F-GAAAGGCTGGCAGGAGAAGA R-TAGCCAGACCCCCTGAAGGC Probe: TGTGCCTGGGTTGC
		Hlx1	F-GGAATGACAGCTCCGAATAAAACT R-TCACCAGAGCTGCTCCAAGA Probe: ATTTGTGGTCTGCCCTCCTCCTGGC
		Ifng	F-ACCCCAAATGGTGTGAAGTAAAA

		promoter	R-CCCACCTGTGCCATTCTTGT Probe: TGCTTTCAGAGAATCC
Taqman	Promoter	*Il8r1*	F-TGAGATCCAGGCAGGAGAACTC R-AGAGCTCCACAGTTCCCAGAAC Probe: CTCTATCGCCTCAGCG

Table 7-3C primers

Assay	**Gene**	***Location**	**Primer Sequence (5'→3')**
	Ifng	-71	F-AGGACCTATACGCTGGCAGT
		-34	R-GCTTCTCTAATGAACTGTAATGTGC
		-6	R-TACACTCATTCCTGCCTGGT
		Promoter	R-CAGATGTAAGATGGGATCTC
		Promoter	F-GTGTATGCTCCGTGGCTAGT
		+46	F-GACCCATAGCTCTTGCCTCT
	Gapdh	Anchor1	R-ACACAGGCAAAATACCAATG
		Anchor2	R-GAATGCTTGGATGTACAACC
Taqman probe	*Ifng*	Promoter	R-6FAM-CCATAGTGAAAAGTCACATGGCTGAGAAACACTTC
		Promoter	F-6FAM-AGATCTCTCAGACACTGACTGAGCC
		-34	R-6FAM-CCTCTGCAAGCCTCACAGAGCA
		+46	6FAM-CCATCTACTGCAAAAAGAAGCT

	Gapdh	anchor	R-6FAM-AGATCTTAATTCCTGGTCCCTT
Internal control	*Ifng*		F-CCCAGTGAGTGCTTTAAAATTTCT R-CTGGATGGTTTTGAAGGATAATGT
	Gapdh		F-ACAGTCCATGCCATCACTGCC R-GCCTGCTTCACCACCTTCTTG

*Location corresponds to the distance in kilobases from *Ifng* transcription start site (chr 10: 177844040 UCSC mm8, UCSC Genome Browser: http://www.genome.ucsc.edu/). F or R designate forward or reverse primers as compared to direction of *Ifng* gene or *Gapdh* gene transcription.

Induction of EAE and analysis

Induction and scoring of EAE disease has been described previously (Mo et al., 2008a). In brief, a cohort of 8-12 week old female WT and *Twist1*-mutant mice (7 mice/group) were immunized (s.c.) with 100 ug of myelin oligodendrocyte glycoprotein (MOGp35-55) peptide antigen (Genemed Synthesis) in 150 ul emulsion of complete Freund's adjuvant (CFA) (Sigma Aldrich) on days 0 and 7. Mice were injected (i.p.) with 100 ng of pertussis toxin (Sigma Aldrich) on days 0 and 2. The clinical signs were scored daily for 30 days. On day 12 following induction of EAE, splenocytes were isolated and stimulated with MOG peptide for 48 h and cytokine production was measured by ELISA. Mononuclear cells were isolated from brain using a 30%/70% Percoll gradient and stimulated with PMA and ionomycin for 2 h followed by monensin for a total of 6 h before staining for intracellular cytokine production.

Sheep Red Blood Cells (SRBCs) immunization

SRBCs (Rockland, cat#R406-0050) were washed three times with PBS. Mice were injected with 1×10^9 cells (i.p.) and sacrificed after 9 days for the analysis.

House Dust Mice (HDM)-induced allergic airway inflammation

Wild type and *Etv5*-mutant mice were sensitized by intranasal injection of 40 ug HDM (GREER allergy immunotherapy) in PBS each day for three consecutive days over five weeks. Mice were sacrificed 24 h after the final intranasal challenge of week 5. Serum was collected by cardiac puncture, and HDM-specific antibodies were measured by ELISA. The trachea was cannulated, and lungs were lavaged three times with 1 ml PBS to collects BAL cells and cell-free BAL fluid. Cell-free BAL fluid was used to measure cytokine production by ELISA. BAL cells, and the single-cell suspension from lungs generated using the lung dissociation kit from Miltenyi Biotec, were stimulated with PMA and ionomycin for 2 h followed by monensin for a total of 5 h for cytokine analysis by ICS. Single-cell suspension from the lungs was used for gene expression analysis by qRT-PCR. Total splenocytes and cells from mediastinal lymph nodes were stimulated with HDM for 5 days, and cytokine production was analyzed by ELISA. Lung tissue was analyzed following for paraffin-embedding and staining with hematoxylin and eosin (H&E) for evaluation of the infiltration of inflammatory cells, and periodic acid-Schiff (PAS) for mucus production. Eosinophils, neutrophils, T cells, B cells and mononuclear cells in the BAL fluid and the lungs

were characterized by cell size and expression of CD3, B220, CCR3, CD11c, and major histocompatibility complex class II, analysis by flow cytometry.

Human helper T cell differentiation

The use of human cells was approved by the Institutional review Board of Indiana University. Naïve $CD4^+$ T cells were isolated from PBMCs using magnetic beads (Miltenyi Biotec). For Th17 cell differentiation, naïve $CD4^+$ cells were activated with anti-CD3 (2 ug/ml; HIT3a; BD Pharmingen) and soluble anti-CD28 (0.5 ug/ml; CD28.2; Biolegend) with additional cytokines and antibodies 10 ng/ml human IL-1β (R&D), 25 ng/ml human IL-21 (Cell Sciences), 25 ng/ml human IL-6 (R&D), 25 ng/ml human IL-23 (R&D), 5 ng/ml human TGF-β (R&D), 10 ug/ml anti-IFNγ (R&D) and 10 ug/ml anti-IL-4 (R&D). On day 3, cells were expanded with additional media and half concentration of cytokines. Cells were harvested for analysis on day 5.

Transfection of siRNA

siRNAs targeting *Twist1, Etv5, Tbx21,* and *TWIST1* were purchased from Santa Cruz. For mouse T cell transfection, $CD4^+$ T cells were transfected with siRNA on day 2 using Amaxa Nucleofector kit (Ionza), rested overnight with 50 U/ml hIL-2, and restimulated with anti-CD3 for 24 h for gene expression and cytokine production analyses. For human Th17 cell transfection, day 5 differentiated Th17 cells were transfected with siRNA using Human T Cell Nucleofector kit (Lonza),

rested overnight with 50 U/ml hIL-2, and restimulated with anti-CD3 for 24 h for gene expression analysis.

Luciferase reporter assay

The *IL6RA, IL17A,* and *IL-17F* promoter reporters were purchased from SwitchGear Genomics. For analyzing the effect of Twist1 on *IL6RA* promoter activity or Etv5 on *IL17A* and *IL17F* promoter activity, Jurkat T cells were grown in RPMI 1640 with 10% FBS and transfected with 2-5 ug of the *IL6RA, IL17A,* and *IL-17F* luciferase reporter plasmids and control or increasing concentration of plasmid expressing Twist1 or Etv5 via Fugene6 reagent (Roche). After 24 h, transfected cells were stimulated with PMA and ionomycin for 6 h before analyzing with the dual luciferase system (Promega).

RESULTS

Part I- Opposing roles of STAT4 and Dnmt3a in Th1 gene expression

IL-12 induces distinct patterns of gene expression

T-bet is a master transcription factor of Th1 cells that has been shown to be induced by IFNγ signaling during early differentiation and by IL-12 later during Th1 cell differentiation (Schulz et al., 2009). Similarly, our data showed that adding anti-IFNγ to wild type Th1 cultures resulted in decreased T-bet expression for the first two days of differentiation, while *Stat4*$^{-/-}$ Th1 cells had reduced T-bet expression the last three days of differentiation (Figure 14). *Ifngr*$^{-/-}$ Th1 cells had decreased T-bet expression during five days of differentiation and that might due to a decrease in *Il12rb2* expression and pSTAT4 level (Figure 14). The data indicated that T-bet expression is controlled sequentially and cooperatively by IFNγ-STAT1 and IL-12-STAT4 signaling.

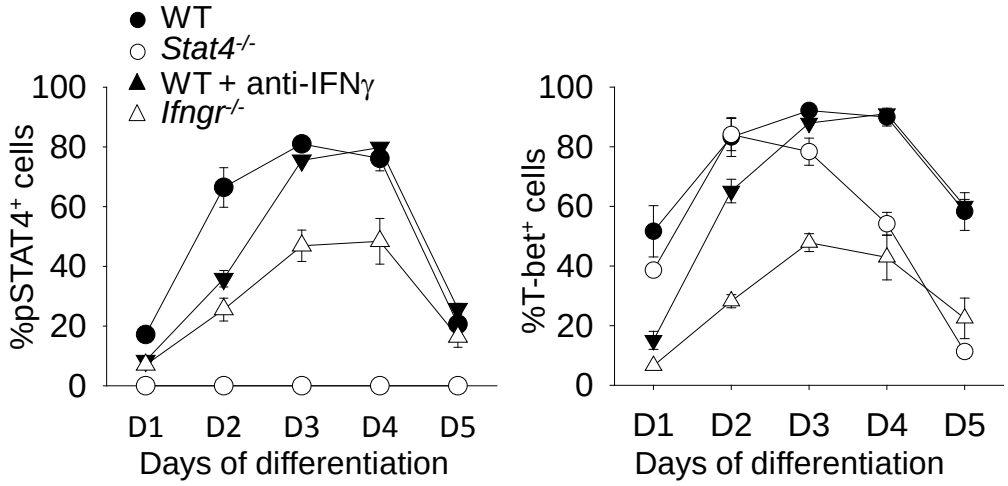


Figure 14. Gene expression pattern in WT, *Stat4*$^{-/-}$, and *Ifngr*$^{-/-}$ Th1 cells. Naïve CD4^{+}CD62L^{+} T cells were isolated from WT, *Stat4*$^{-/-}$, and *Ifngr*$^{-/-}$ mice and differentiated under Th1-polarizing conditions with anti-IFNγ was added to WT cell culture. The levels of phospho-STAT4 (pSTAT4) and T-bet expression were measured by ICS each day. Data are mean of two mice ± S.D. and representative of three independent experiments with similar results.

Since STAT4 activated the IFN-induced T-bet expression at distinct time points, we wanted to examine this in other genes. In previous work we defined STAT4 target genes in activated T cells (Good et al., 2009), and observed that a subset of STAT4 bound genes (*Socs3*, *Cxcl10*, *Irf1*, and others) is classically defined as being interferon-induced genes (Ramana et al., 2002). Thus, we next wanted to compare the IL-12-induced gene expression patterns between Th1-associated genes and interferon-inducible genes. We examined purified naïve $CD4^+$ T cells activated with anti-CD3 for 72 h, a time point when the cells begin to express IL-12Rβ2 and become IL-12-responsive. We first examined mRNA for Th1-associated genes and interferon-inducible genes, placing *Tbx21* (encoding T-bet) in the interferon-inducible category because previous work demonstrated it to be primarily IFNγ-inducible early during Th1 differentiation (Schulz et al., 2009). We observed that in contrast to the Th1-associated genes that displayed an increase in relative expression over the course of 6 h, the IFN-inducible genes displayed a rapid increase in expression at 1 h and decreased thereafter (Figure 15). This was paralleled by the pattern of STAT4 binding to the genes, increasing over the time period at the Th1 gene loci, but initially increasing and then stabilizing or decreasing in the IFN-inducible genes (Figure 16A).

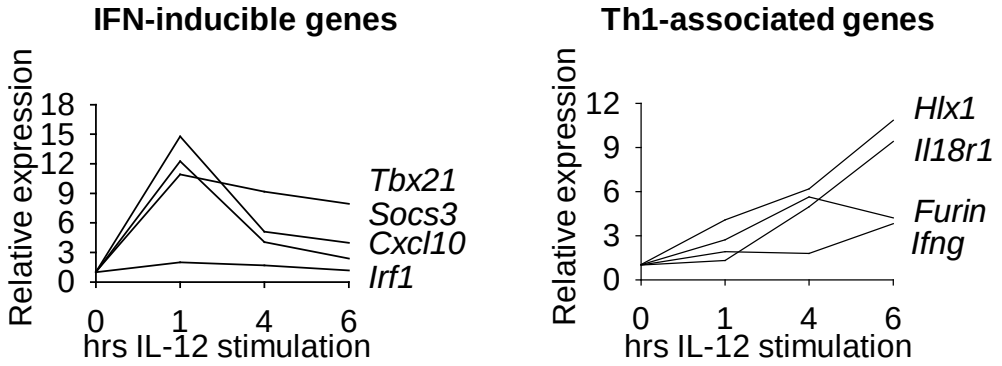


Figure 15. IL-12-induced gene expression in activated T cells. $CD4^{+}CD62L^{+}$ T cells were isolated from WT mice and activated with anti-CD3 and anti-CD28. After 3 days, cells were harvested, stimulated with IL-12 for the indicated times and used for analysis of gene expression analysis by qRT-PCR. Data are representative of three independent experiments with similar results.

We then examined chromatin modifications at each set of genes to define events that would correlate with distinct patterns of gene expression. Acetylation of H3K9/18 showed a similar pattern in both sets of genes over 6 h of IL-12 stimulation (Figure 16B). Acetylation and methylation of H3K27 over the 6 hours of IL-12 stimulation displayed varied patterns among the genes that did not obviously segregate between IFN-inducible and Th1-associated genes, although 2 of the 3 Th1-associated genes showed increased H3K27 acetylation (Figure 16B). Acetylation of H3K36 followed patterns that paralleled STAT4 binding, steadily rising over time in Th1-associated genes, and displaying modest or transient changes in acetylation at three of the four IFN-inducible genes (Figure 16B).

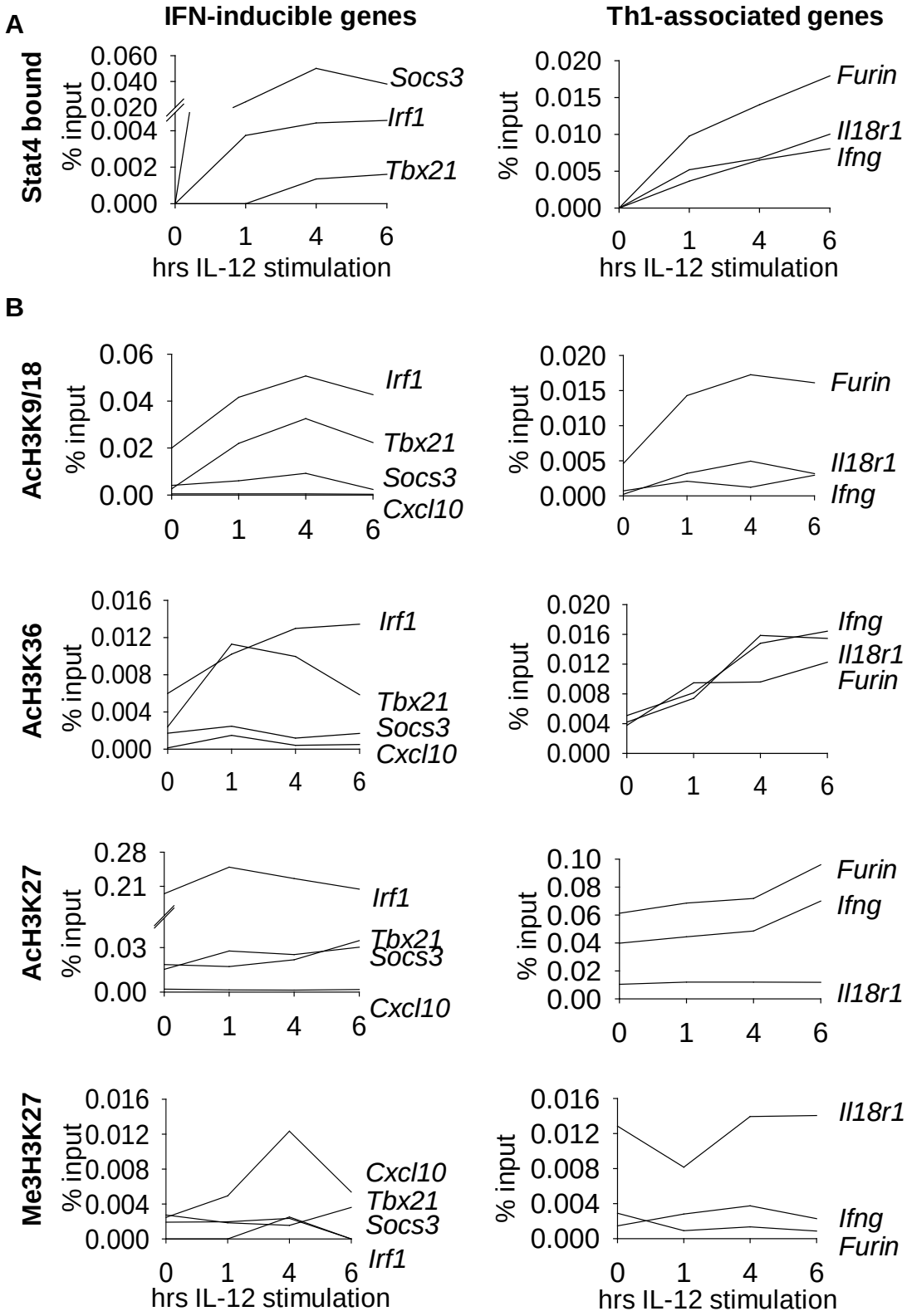

A
IFN-inducible genes
Th1-associated genes
Stat4 bound
% input
0.060
0.040
0.020
0.004
0.002
0.000
Socs3
Irf1
Tbx21
0.020
0.015
0.010
0.005
0.000
Furin
Il18r1
Ifng
0 1 4 6
hrs IL-12 stimulation
B
AcH3K9/18
0.06
0.04
0.02
0.00
Irf1
Tbx21
Socs3
Cxcl10
0.020
0.015
0.010
0.005
0.000
Furin
Il18r1
Ifng
AcH3K36
0.016
0.012
0.008
0.004
0.000
Irf1
Tbx21
Socs3
Cxcl10
0.020
0.016
0.012
0.008
0.004
0.000
Ifng
Il18r1
Furin
AcH3K27
0.28
0.21
0.03
0.00
Irf1
Tbx21
Socs3
Cxcl10
0.10
0.08
0.06
0.04
0.02
0.00
Furin
Ifng
Il18r1
Me3H3K27
0.016
0.012
0.008
0.004
0.000
Cxcl10
Tbx21
Socs3
Irf1
0.016
0.012
0.008
0.004
0.000
Il18r1
Ifng
Furin
0 1 4 6
hrs IL-12 stimulation

Figure 16. STAT4 binding and histone modification pattern in IL-12-stimulated activated WT T cells. (A-B) $CD4^+CD62L^+$ T cells were isolated from WT mice and activated with anti-CD3 and anti-CD28. After 3 days, cells were harvested, stimulated with IL-12 for the indicated times and used for analysis of STAT4 binding (A) and histone modification (B) analyses by ChIP assay. Data are representative of three independent experiments with similar results.

We then performed similar experiments in differentiated Th1 cells. Naïve $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 and polarized towards the Th1 phenotype in the presence of IL-12 and anti-IL-4. Relative expression of the genes was similar to the patterns observed in activated T cells with IFN-inducible genes displaying transient induction, and Th1-associated genes displaying more sustained induction (Figure 17).

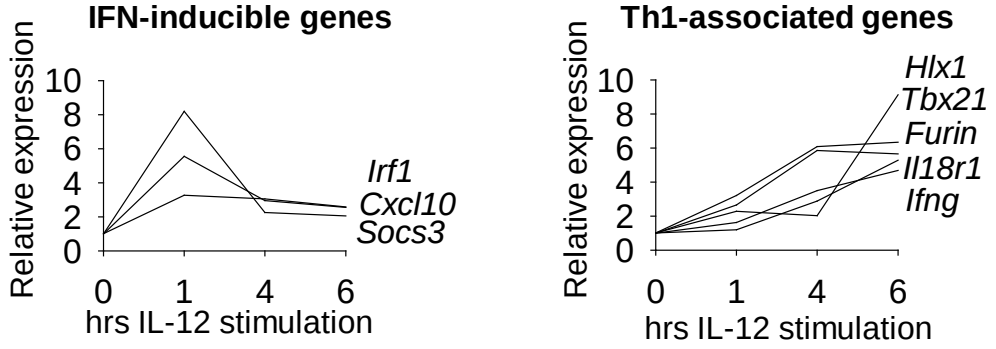


Figure 17. IL-12-induced gene expression in WT Th1 cells. $CD4^+CD62L^+$ T cells were isolated from WT mice and cultured under Th1-polarizing conditions. After 5 days, cells were harvested, stimulated with IL-12 for the indicated times and used for analysis of gene expression of Th1 genes or IFN-inducible target genes by qRT-PCR. Data are representative of three independent experiments with similar results.

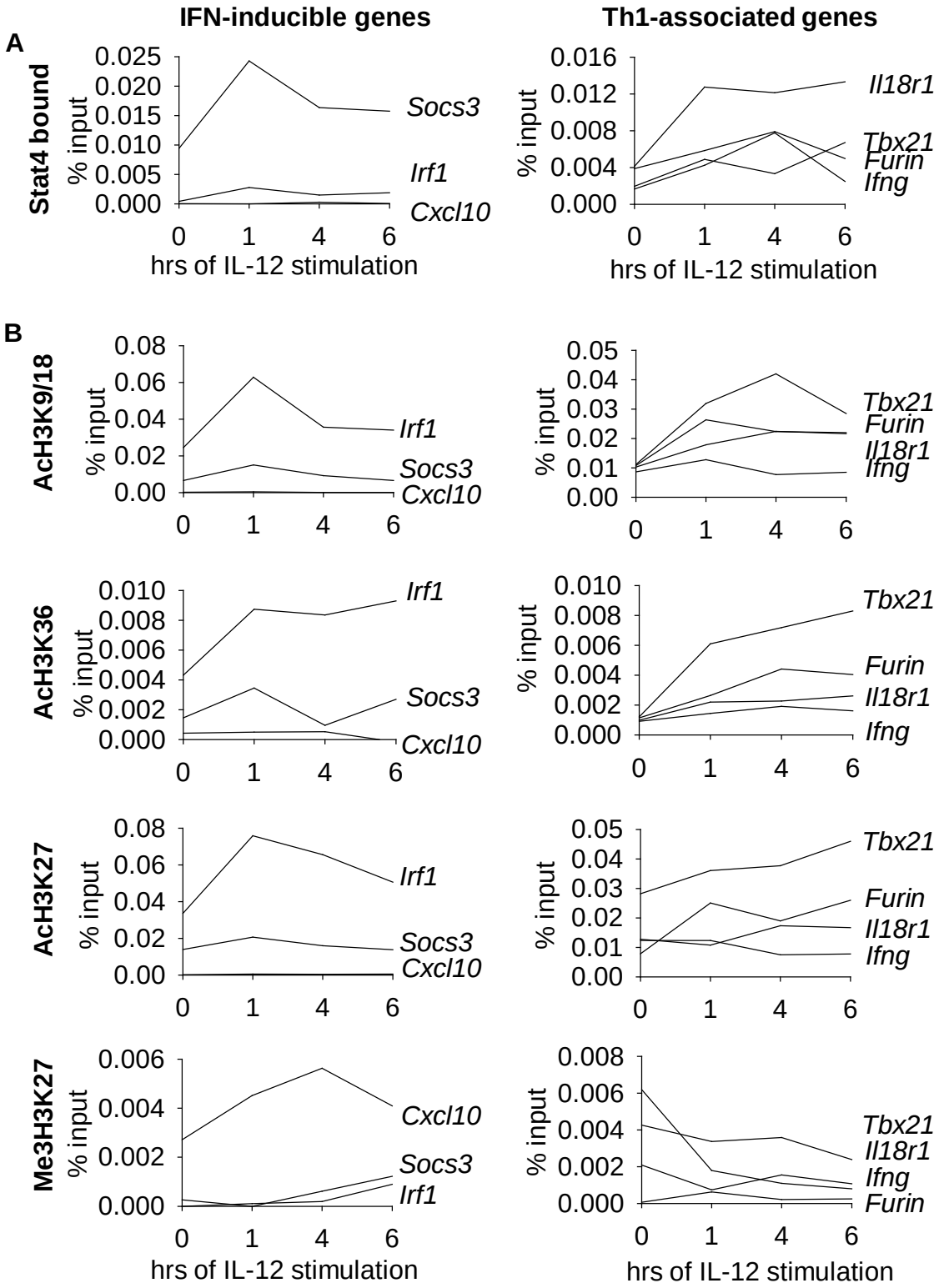

IFN-inducible genes
Th1-associated genes
A
Stat4 bound
% input
0.025
0.020
0.015
0.010
0.005
0.000
Socs3
Irf1
Cxcl10
0 1 4 6
hrs of IL-12 stimulation
0.016
0.012
0.008
0.004
0.000
Il18r1
Tbx21
Furin
Ifng
0 1 4 6
hrs of IL-12 stimulation
B
AcH3K9/18
0.08
0.06
0.04
0.02
0.00
Irf1
Socs3
Cxcl10
0 1 4 6
0.05
0.04
0.03
0.02
0.01
0.00
Tbx21
Furin
Il18r1
Ifng
0 1 4 6
AcH3K36
0.010
0.008
0.006
0.004
0.002
0.000
Irf1
Socs3
Cxcl10
0 1 4 6
Tbx21
Furin
Il18r1
Ifng
0 1 4 6
AcH3K27
0.08
0.06
0.04
0.02
0.00
Irf1
Socs3
Cxcl10
0 1 4 6
0.05
0.04
0.03
0.02
0.01
0.00
Tbx21
Furin
Il18r1
Ifng
0 1 4 6
Me3H3K27
0.006
0.004
0.002
0.000
Cxcl10
Socs3
Irf1
0 1 4 6
hrs of IL-12 stimulation
0.008
0.006
0.004
0.002
0.000
Tbx21
Il18r1
Ifng
Furin
0 1 4 6
hrs of IL-12 stimulation

Figure 18. STAT4 binding and histone modification pattern in IL-12-stimulated WT Th1 cells. (A-B) $CD4^{+}CD62L^{+}$ T cells were isolated from WT mice and cultured under Th1-polarizing conditions. After 5 days, cells were harvested, stimulated with IL-12 for the indicated times and used for analysis of STAT4 binding (A) and histone modification (B) analyses by ChIP assay. Data are representative of three independent experiments with similar results.

Although STAT4 binding showed the same transient induction at the IFN-inducible gene loci, there was more heterogeneity in STAT4-binding at the Th1-associated gene loci (Figure 18A). The patterns of H3K9/18 acetylation were similar to that in activated T cells, with transient induction in both sets of genes (Figure 18B). Acetylation at H3K36 was transient for the IFN-inducible genes, and demonstrated the same trend towards sustained increases, though with more heterogeneity than observed in activated T cells (Figure 18B). At H3K27 residues, IFN-inducible genes showed a transient increase in acetylation and generally increasing methylation (Figure 18B). In contrast, the Th1 associated genes demonstrated trends towards increased acetylation and decreased methylation at H3K27 residues (Figure 18B). Thus, STAT4 binding and changes in histone modifications are distinct among IL-12-inducible genes that have transient or sustained expression.

STAT4-dependent association of chromatin modifying enzymes at Th1 gene loci

To further define how STAT4 is required for IL-12-induced gene expression we examined the expression of Th1 genes in wild type and *Stat4*$^{-/-}$ cultures following IL-12 stimulation. In the absence of STAT4, Th1 cells have lower basal expression of Th1 genes, and IL-12-induced expression is completely absent

(Figure 19A). H3K4 methylation is induced by IL-12, and the induction is attenuated in *Stat4*$^{-/-}$ Th1 cells (Figure 19B). In contrast, H3K27 methylation is increased in STAT4-deficient Th1 cultures, compared to wild type cultures, and the amounts of methylation increased upon IL-12 stimulation (Figure 19B). This was concomitant with decreased association of the H3K27 demethylase Jmjd3, and increased association of Dnmt3a, the DNA methyltransferase known to associate with methylated H3K27 (Figure 19B) (Lindroth et al., 2004; Yu et al., 2007). Although our previous results suggested that Dnmt3a demonstrated increased association with Th1 loci in the absence of STAT4, these results suggest that IL-12-induced STAT4 reciprocally modulates Jmjd3 and Dnmt3a association at Th1 gene loci.

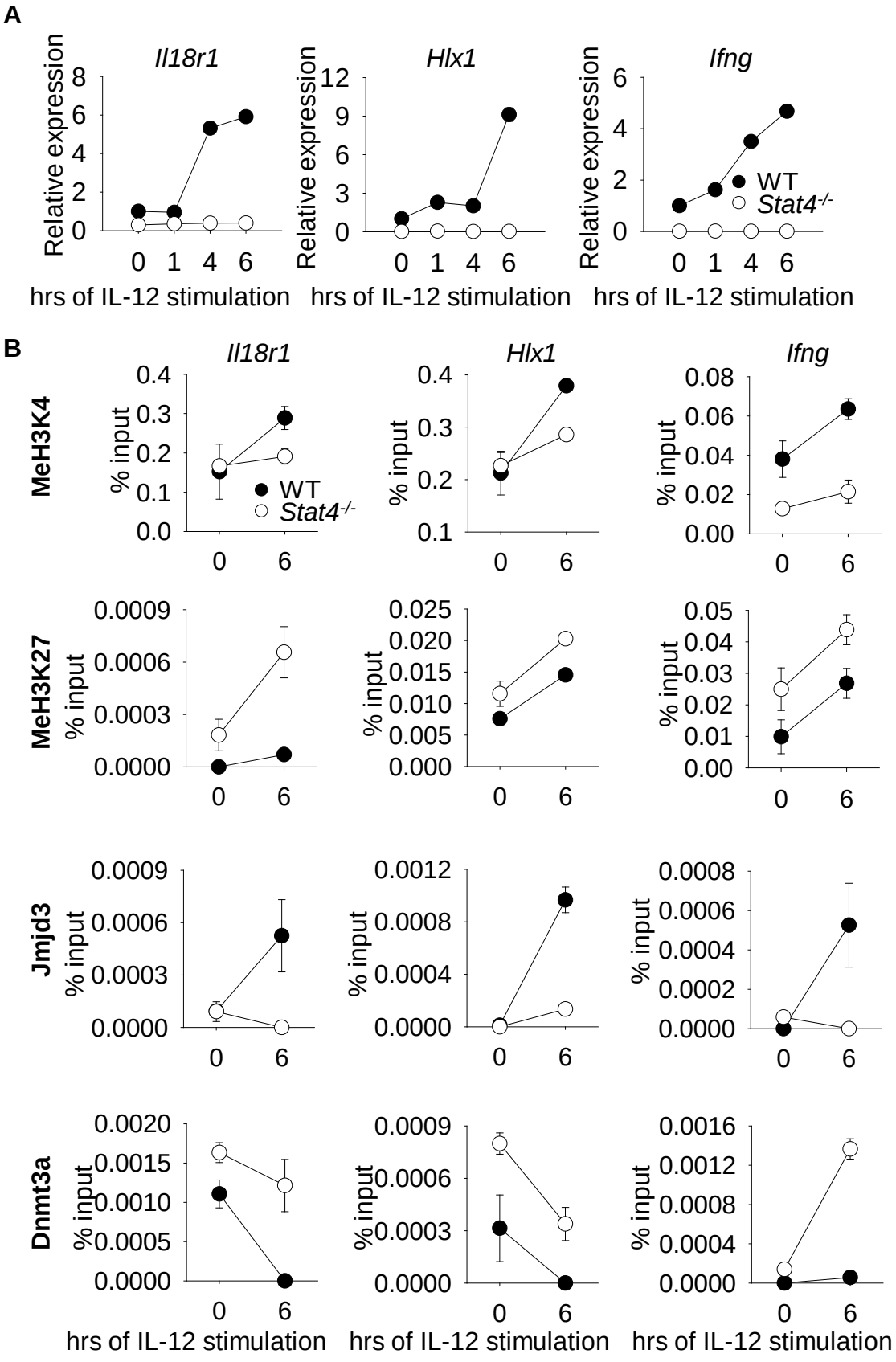

A
Il18r1
Hlx1
Ifng
Relative expression
WT
Stat4-/-
0 1 4 6
hrs of IL-12 stimulation
B
MeH3K4
MeH3K27
Jmjd3
Dnmt3a
% input
0 6

Figure 19. **Gene expression, histone modification, and chromatin modifying enzyme patterns in IL-12-stimulated WT and *Stat4*$^{-/-}$ Th1 cells.** Naïve $CD4^{+}CD62L^{+}$ T cells were isolated from WT or *Stat4*$^{-/-}$ WT mice and cultured under Th1-polarizing conditions. On day 5, cells were harvested, stimulated with IL-12 for the indicated time points, and gene expression was examined by qRT-PCR (A) or used for STAT4 binding, chromatin modifying enzymes and histone modification analyses by ChIP assay using primers specific for the promoters of the indicated genes (B). Data are average of replicate samples ± S.D. and representative of three independent experiments with similar results.

Jmjd3 facilitates IL-12-induced gene expression

To determine if Jmjd3 contributes to IL-12-induced gene expression in Th1 cells, we transfected wild type Th1 cells with control or*Jmjd3*-specific siRNA. We used intracellular staining to assess Jmjd3 expression and observed decreased mean fluorescence intensity without any effects on cell viability (Figure 20A-C). Reducing *Jmjd3* expression resulted in diminished IFNγ production after anti-CD3 or IL-12 stimulation (Figure 20D-E). Moreover, *Jmjd3* siRNA reduced IL-12-induced Th1 gene expression (Figure 20E). Transfection of Th1 cells with *Jmjd3*-specific siRNA resulted in decreased Jmjd3 associated with Th1 cytokine loci, and a corresponding decrease in H3K4 tri-methylation and increase in H3K27 tri-methylation (Figure 21). Decreasing *Jmjd3* expression also increased Dnmt3a association with Th1 gene loci (Figure 21). STAT4 binding at Th1 gene loci was not altered by*Jmjd3*-specific siRNA (Figure 21). These results support a pathway in which IL-12 induces STAT4-dependent H3K4 tri-methylation and Jmjd3 association with Th1 gene loci, which then decreases H3K27 tri-methylation, and limits Dnmt3a association with target loci.

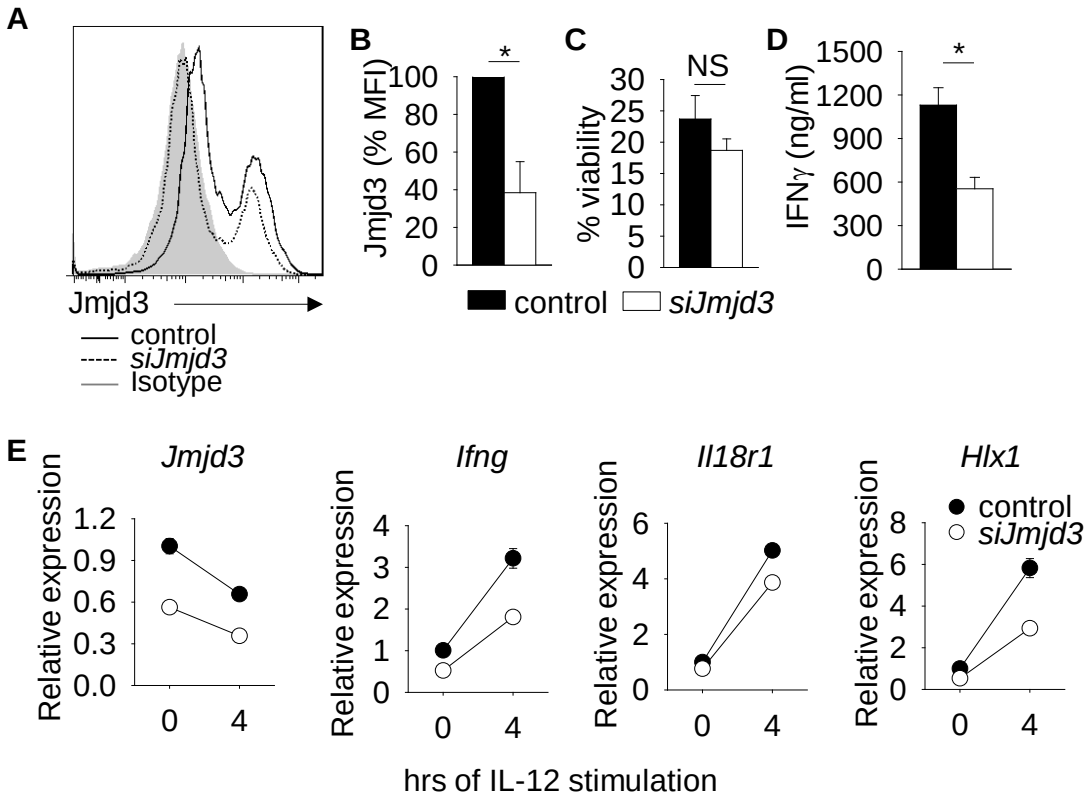


Figure 20. IL-12-induced gene expression requires Jmjd3. (A-E) Naïve $CD4^{+}CD62L^{+}$ T cells were isolated from WT mice and cultured under Th1-polarizing conditions. On day 5, cells were harvested, transfected with control or *Jmjd3*-specific siRNA. Cells were rested overnight and stimulated with anti-CD3 for 6 h, for Jmjd3 (with average of mean fluorescence intensity) and viability analyses by intracellular staining (A-C) or 24 h to measure cytokine production by ELISA (D). Transfected cells were stimulated with IL-12 for gene expression analysis by qRT-PCR (E). Data are average of three mice ± S.D. (A-D) or are average of replicated samples ± S.D. and representative of three independent experiments with similar results (E). *p<0.05. NS, not significant

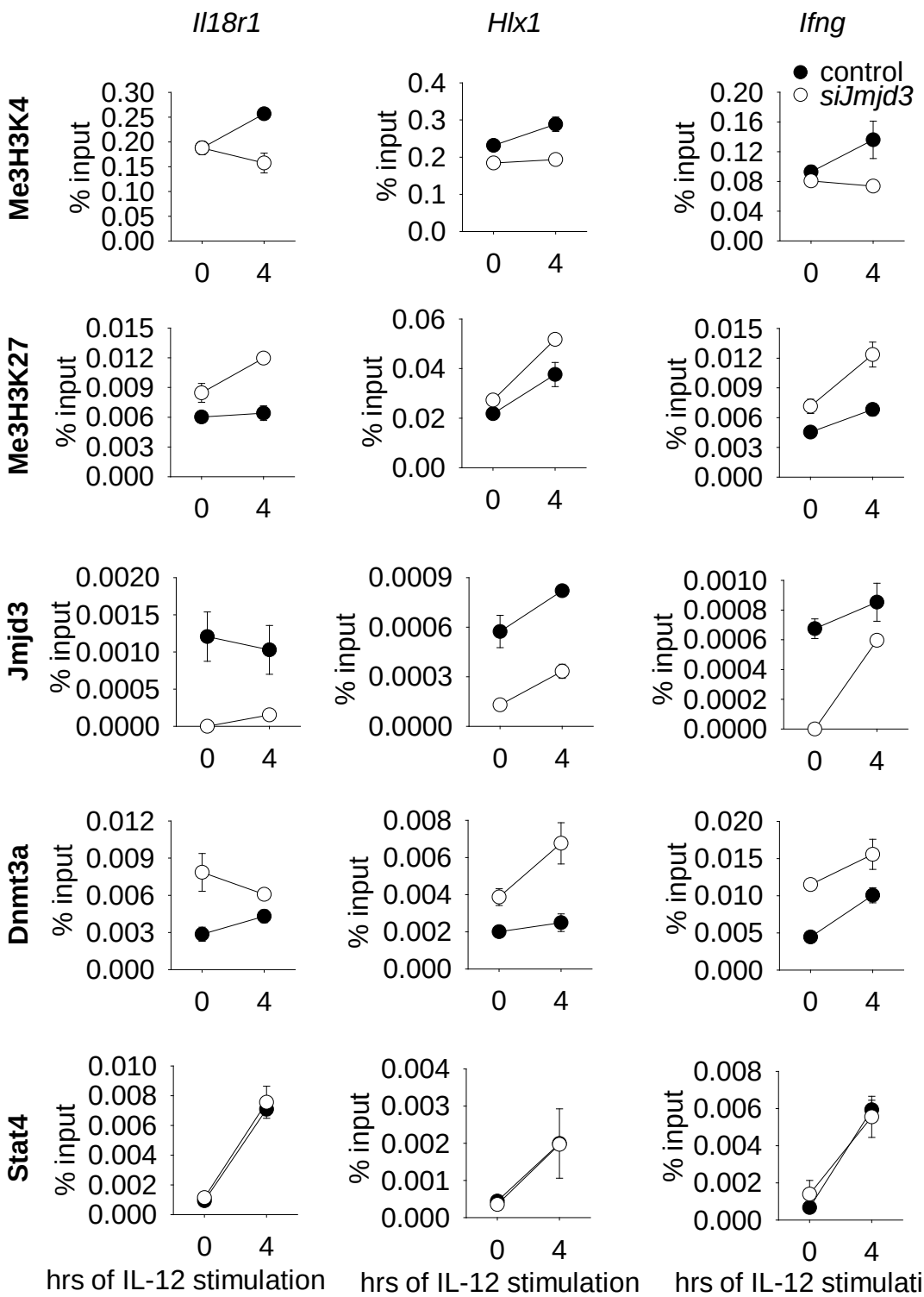


Figure 21. IL-12-induced chromatin modification requires Jmjd3. Naïve $CD4^{+}CD62L^{+}$ T cells were isolated from WT mice and cultured under Th1-polarizing conditions. On day 5, cells were harvested, transfected with control or *Jmjd3*-specific siRNA and stimulated with IL-12 before assessing chromatin modifying enzyme, histone modification and STAT4 binding by ChIP assay using

primers specific for the promoters of the indicated genes. Data are average of replicated samples ± S.D. and representative of three independent experiments with similar results

Dnmt3a negatively regulates Th1 gene expression

Based on the increased association of Dnmt3a with Th1 gene loci in the absence of STAT4, we tested whether elimination of a negative regulator, Dnmt3a, would rescue Th1 differentiation in *Stat4*$^{-/-}$ T cells. We mated *Stat4*$^{-/-}$ mice with *Dnmt3a*$^{fl/fl}$ CD4-Cre mice to generate compound mutant mice. Naïve CD4^{+} T cells were isolated from wild type, *Dnmt3a*$^{fl/fl}$ CD4-Cre positive and *Stat4*$^{-/-}$*Dnmt3a*$^{fl/fl}$ mice that were Cre-negative (STAT4-deficient, but expressing Dnmt3a) or Cre-positive (STAT4- and Dnmt3a-deficient) and differentiated under Th1, Th17, and regulatory T cell (iTreg) polarizing conditions. *Stat4*-deficient Th1 cells had diminished IFNγ production compared to wild type cultures when stimulated with anti-CD3 or PMA and ionomycin, although there was no significant effect on TNFα production (Figure 22A-B). We have previously seen that Dnmt3a-deficiency resulted in modest increases in IFNγ production (Yu et al., 2012) (Figure 22C). However, Th1 cells deficient in both STAT4 and Dnmt3a demonstrated greater production of IFNγ than *Stat4*$^{-/-}$ cells, assessed early using intracellular staining (Figure 22A). Recovery of IFNγ production was comparable to wild type cells in response to PMA and ionomycin, but was only partially restored when cells were stimulated with anti-CD3 (Figure 22A-C). This is consistent with the ability of PMA and ionomycin stimulation to overcome some of the effects of STAT4-deficiency (Afkarian et al., 2002; Good et al., 2009).

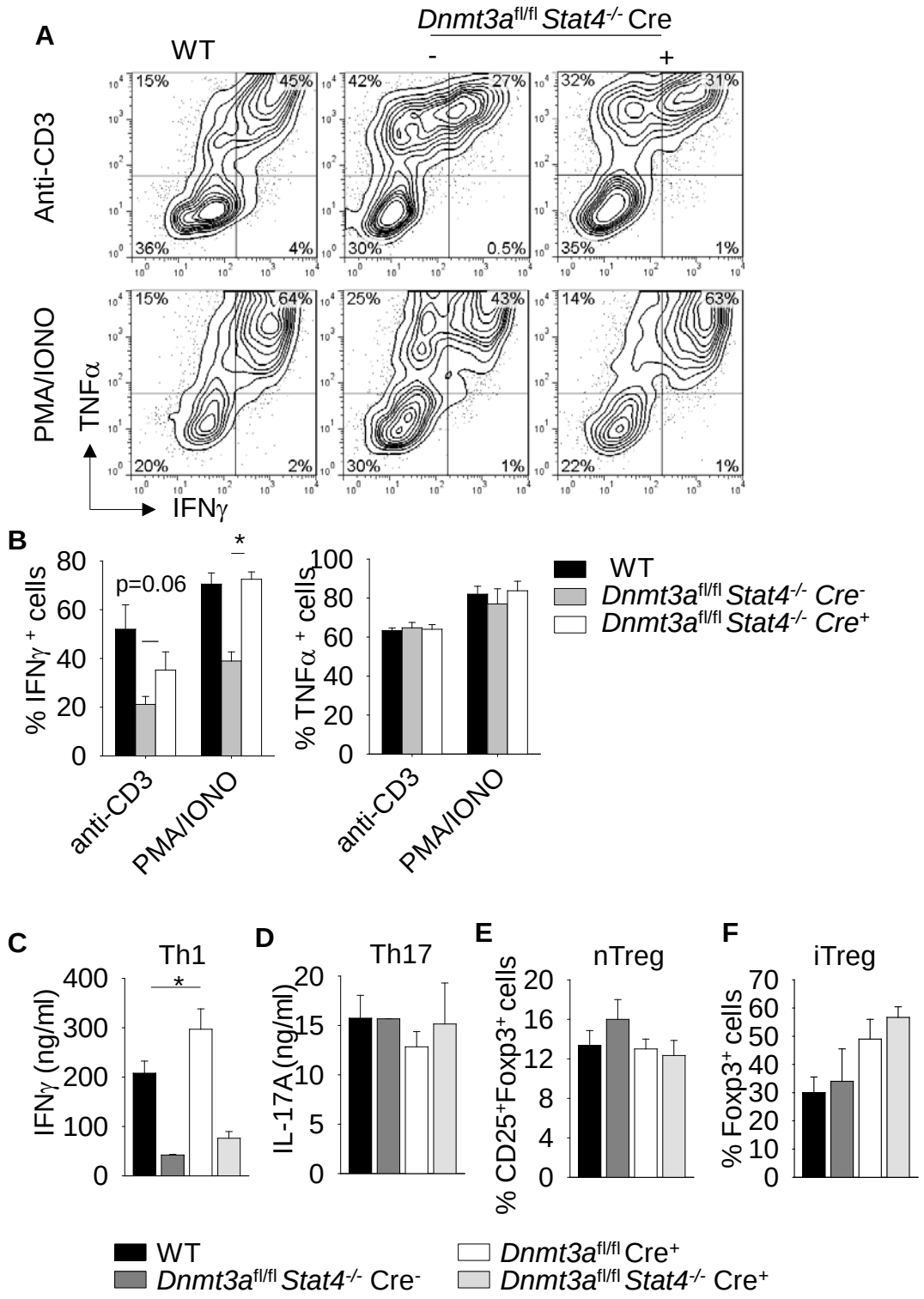


Figure 22. Dnmt3a is a negative regulator of Th1 genes. (A-D) Naïve $CD4^+CD62L^+$ T cells were isolated from WT, *Dnmt3a*$^{fl/fl}$ CD4-Cre positive or *Dnmt3a*$^{fl/fl}$ *Stat4*$^{-/-}$ CD4-Cre negative (-) or positive (+) mice and cultured under Th1, Th17 or iTreg polarizing conditions. (A-B) On day 5, Th1 cells were harvested, activated with anti-CD3 or PMA and ionomycin for 6 h before

assessing cytokine production by ICS, B, Averages of percent positive cells and mean fluorescence intensity for data in (A). (C-D) Day 5 Th1 and Th17 cells were harvested and activated with anti-CD3 for 24 h before assessing cytokine production by ELISA. (E-F) Splenocytes isolated from mice with indicated genotypes or day 5 *in vitro* generated regulatory T (Treg) cells were assessed for natural (nTreg, $CD4^+CD25^+Foxp3^+$) (E) or inducible ($Foxp3^+$) (F) Treg cells by intracellular staining. Data is gated on $CD4^+$ cells. Data are average ± S.D. of three independent experiments (A-F). *$p<0.05$

Th17 cell differentiation and *ex vivo* regulatory T cells (nTreg) were normal in cells deficient in both STAT4 and Dnmt3a compared to wild type cells in terms of IL-17A production (Th17) and the percentage of $CD4^+CD25^+Foxp3^+$ cells (nTreg) (Figure 22D-E). *In vitro* derived Treg cells from single Dnmt3a-deficient or double STAT4-and Dnmt3a-deficient naïve T cells demonstrated increased differentiation to Foxp3-expressing cells, compared to wild type cells (Figure 22F).

We then examined additional Th1 genes to determine if Dnmt3a had a similar negative effect on expression. *Stat4*$^{-/-}$ Th1 cells had diminished Th1 gene expression while Dnmt3a-deficiency resulted in modest increases in IFNγ production but minimal effects on the expression of other Th1 genes (Figure 23) (Yu et al., 2012). We observed partial recovery of *Etv5*, *Furin*, *Twist1*, *Il18r1,* and *Jmjd3* expression in Th1 cells deficient in both STAT4 and Dnmt3a, compared to wild type and *Stat4*$^{-/-}$ Th1 cultures (Figure 23). However, there was no recovery of *Hlx1*, *Runx3*, or *Tbx21* expression (Figure 23).

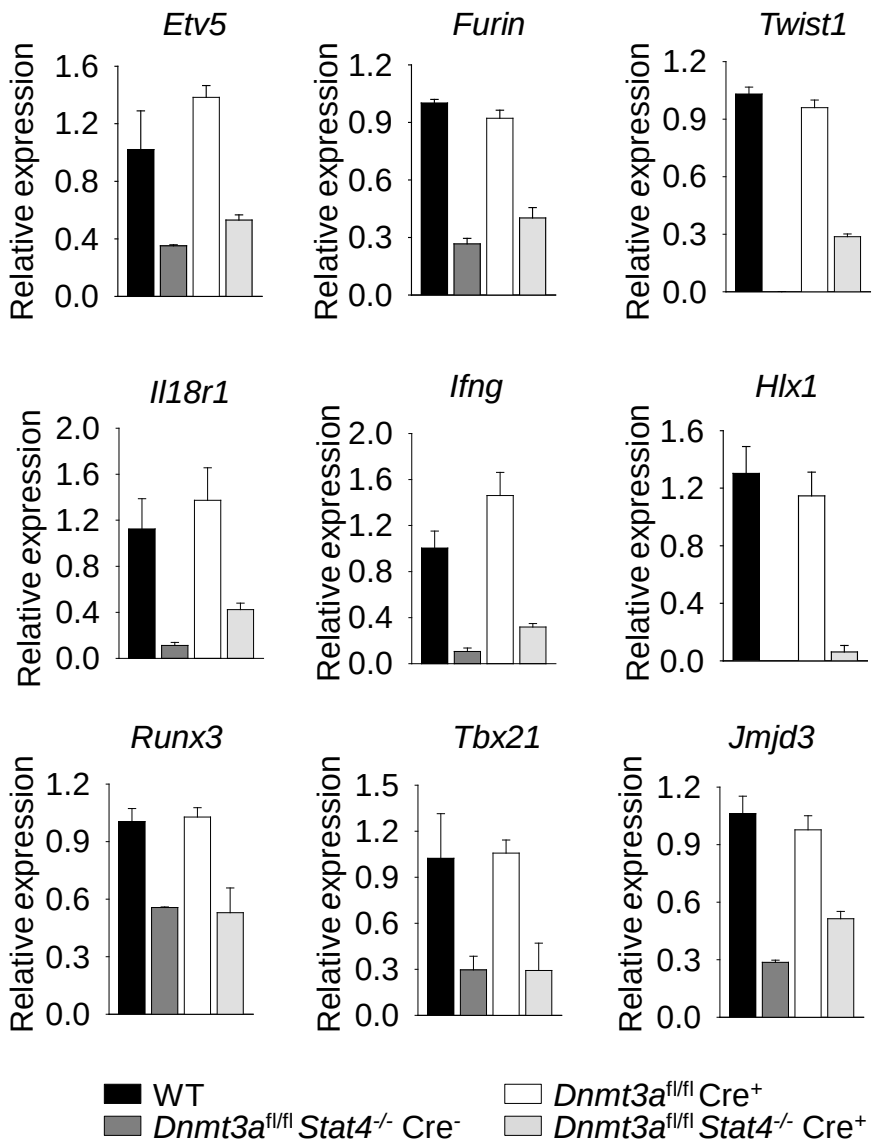


Figure 23. Dnmt3a is a negative regulator of Th1 genes. Naïve $CD4^+CD62L^+$ T cells were isolated from WT, *Dnmt3a*$^{fl/fl}$ CD4-Cre positive or *Dnmt3a*$^{fl/fl}$ *Stat4*$^{-/-}$ CD4-Cre negative (-) or positive (+) mice and cultured under Th1-polarizing conditions. Day 5 Th1 cells were used to examine gene expression by qRT-PCR before (*Etv5, Furin, Twist1, Il18r1, Hlx1, Runx3, Tbx21,* and *Jmjd3)* or after (*Ifng)* anti-CD3 reactivation. Data are average of replicated samples ± S.D. and representative of three independent experiments with similar results.

Since the recovery in STAT4-dependent IFNγ production and Th1 gene expression was only partial with simultaneous Dnmt3a deficiency, we wanted to determine if this effect was sufficient to enhance inflammation *in vivo*. To test

this, we used the myelin oligodendrocyte glycoprotein (MOG)-induced EAE model to compare the level of disease in wild type, *Dnmt3a*$^{fl/fl}$ Cre-positive *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-negative and *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive mice. Disease development in wild type and *Dnmt3a*$^{fl/fl}$ Cre-positive mice was indistinguishable, consistent with a minimal effect of Dnmt3a-deficiency on Th1 development *in vitro*. In agreement with previous reports (Chitnis et al., 2001; Mo et al., 2008b), mice deficient in STAT4 had minimal disease that developed much later than disease in wild type mice (Figure 24A-B). However, *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive mice demonstrated onset and paralysis that was intermediate to disease in wild type and *Stat4*$^{-/-}$mice (Figure 24B).The result correlated with increased CD4^{+}IFN$\gamma^{+}$and CD4^{+}IFNγ^{+}IL-17^{+} mononuclear cells isolated from brain in *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$Cre-positive mice compared to *Stat4*-deficient mice (Figure 24C). MOG-stimulated *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive splenocytes produced significantly more IFNγ and GM-CSF compared to *Stat4*-deficient cells (Figure 24D). Both *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$Cre-positive and *Stat4*-deficient mice had higher CD4^{+}IL-17^{+} mononuclear cells compared to wild type mice (Figure 24C). Similarly, MOG-stimulated *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive and *Stat4*-deficient splenocytes produced more IL-17A compared to wild type cells (Figure 24D).Thus, in the absence of Dnmt3a and STAT4, there is a partial recovery in inflammatory T cell function.

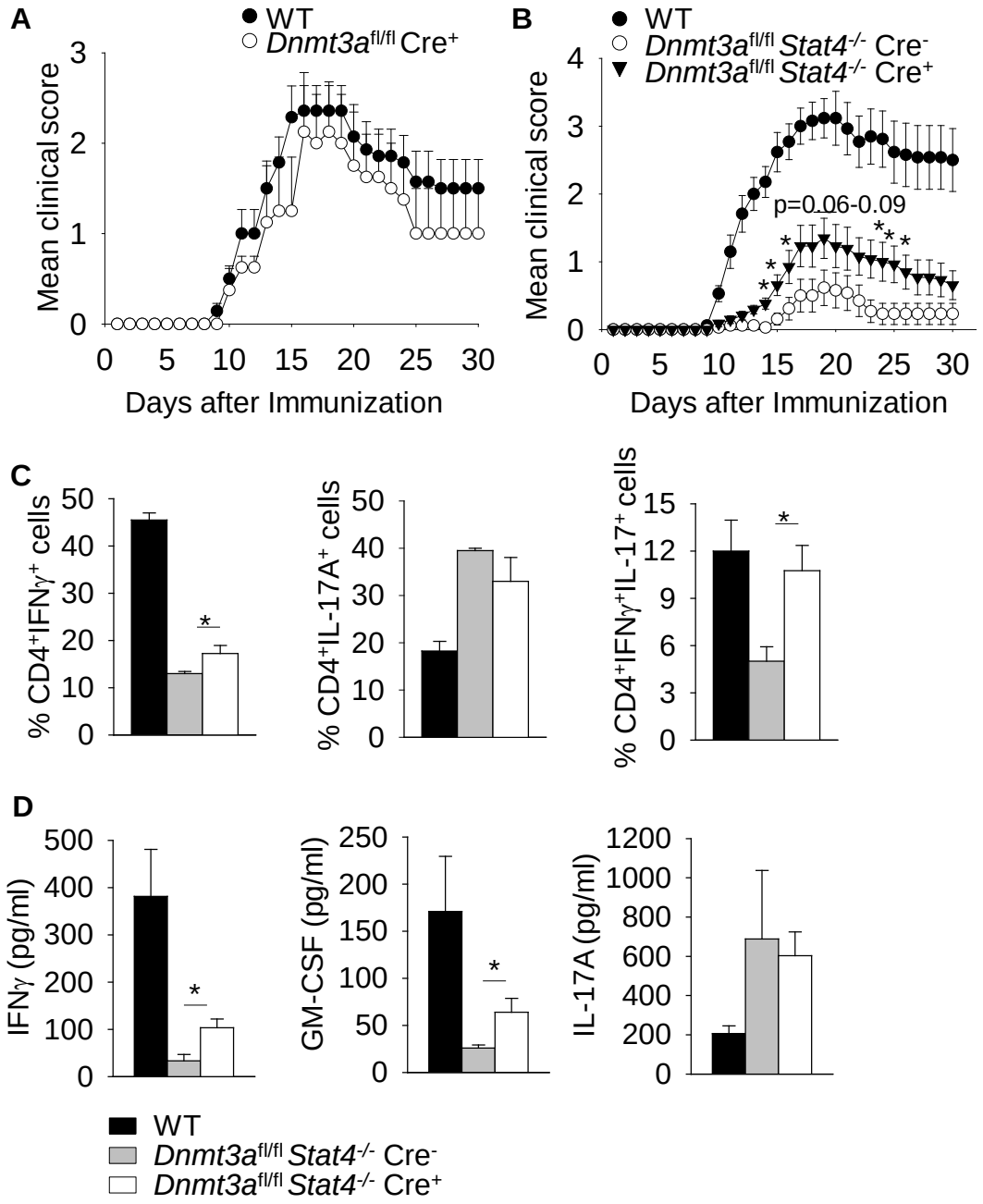


Figure 24. Mice with double deficiency in STAT4 and Dnmt3a had partial recovery in inflammatory T cell function. (A-B) Mean clinical score of MOG peptide (35-55)-induced EAE in WT, *Dnmt3a*$^{fl/fl}$ CD4-Cre positive (A) or in WT, *Dnmt3a*$^{fl/fl}$ *Stat4*$^{-/-}$ CD4-Cre negative or positive mice scored daily for 30 days (B). (C-D) Mice were sacrificed on day 14 and mononuclear cells were isolated from brain and stimulated with PMA and ionomycin for 6 h before staining for cytokine production (C) or isolated splenocytes were stimulated with MOG peptide for 48 h and cytokine production was measured using ELISA (D). Data are average ± S.E.M. of two independent (A-B, n=6-10 mice/group/experiment) or average ±

S.E.M. of 4 mice (C-D). *p<0.05 comparing *Dnmt3a*$^{fl/fl}$ *Stat4*$^{-/-}$ CD4-Cre negative or positive samples.

To further define how Dnmt3a was affecting Th1 gene expression, we examined acute IL-12 induced gene expression and histone modification. Although deficiency in Dnmt3a increased the basal level of gene expression in STAT4-deficient T cells following Th1 differentiation (Figure 22A-C, 23 and 25A), we did not observe a rescue of IL-12-induced gene expression in the absence of Dnmt3a (Figure 25A), suggesting that other STAT family members were not compensating for the function of STAT4. Histone modifications associated with activated or repressed genes were also altered at five regulatory elements across the *Ifng* locus, and at the *Hlx1* promoter. The amount of H3K27me3 at each site of the *Ifng* locus, and the *Hlx1* promoter was increased in the absence of STAT4 (Figure 25B-C). At three of the five sites in the *Ifng* locus and at the *Hlx1* promoter, deficiency of Dnmt3a and STAT4 decreased H3K27me3 to amounts close to those in wild type cells (Figure 25B-C). Conversely, H3K4me3 was decreased at the *Ifng* locus and the *Hlx1* promoter in the absence of STAT4. In Th1 cultures deficient in Dnmt3a and STAT4, three of the five sites in the *Ifng* locus, but not the *Hlx1* promoter, showed H3K4me3 amounts increased from *Stat4*$^{-/-}$ cells, although not to amounts seen in wild type cells. The results are consistent with previous reports of STAT4 binding to the *Ifng* locus (Figure 25A-C) (Wei et al., 2010).

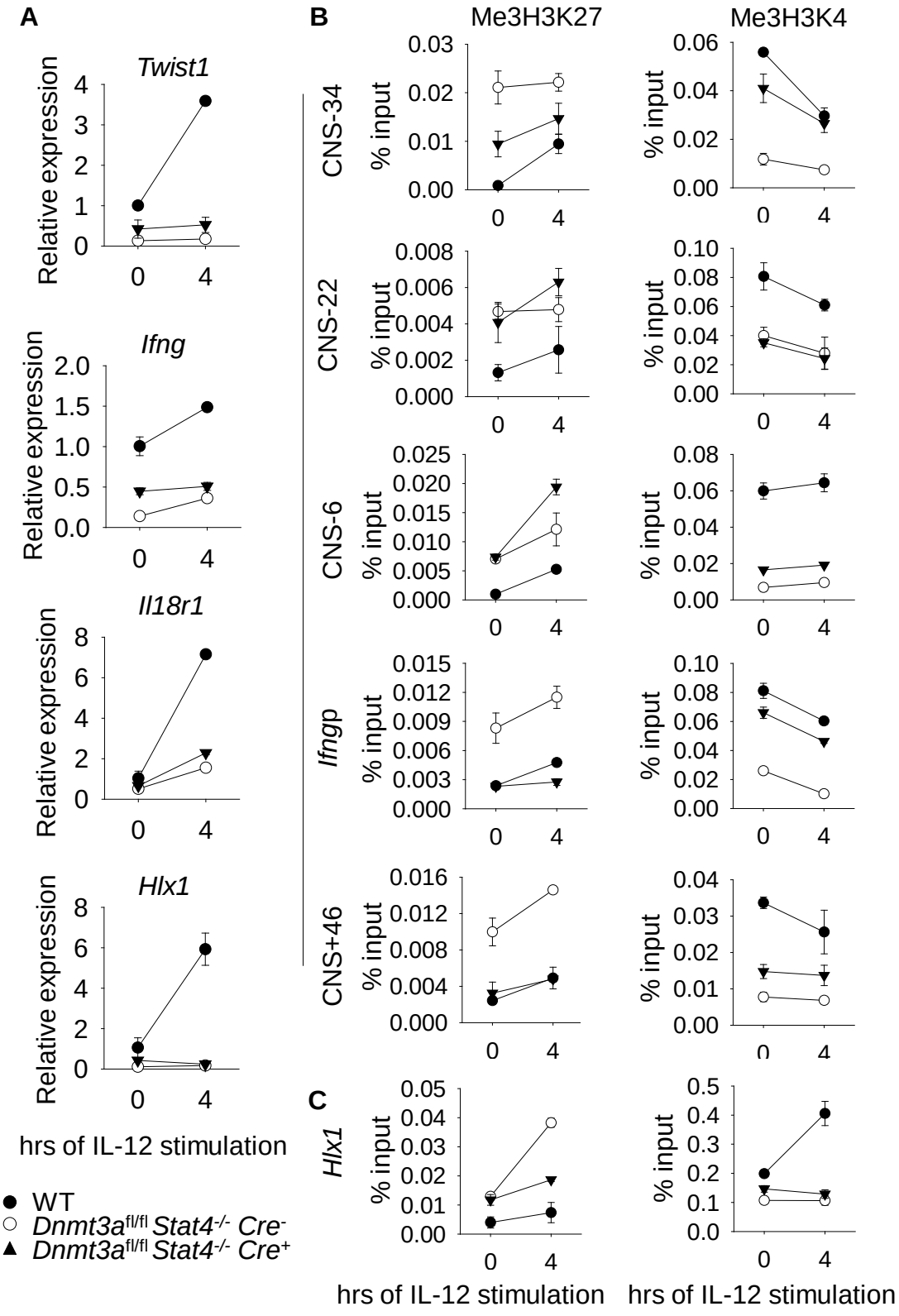

A
Twist1
Ifng
Il18r1
Hlx1
Relative expression
hrs of IL-12 stimulation
WT
Dnmt3afl/fl Stat4-/- Cre-
Dnmt3afl/fl Stat4-/- Cre+
B
Me3H3K27
Me3H3K4
CNS-34
CNS-22
CNS-6
Ifngp
CNS+46
% input
C
Hlx1
hrs of IL-12 stimulation
hrs of IL-12 stimulation

Figure 25. Gene expression and histone modification in the absence of *Dnmt3a.* (A-B) Naïve $CD4^+CD62L^+$ T cells were isolated from WT, *Dnmt3a*$^{fl/fl}$ *Stat4*$^{-/-}$ CD4-Cre negative or positive mice and cultured under Th1-polarizing conditions. On day 5, cells were harvested and stimulated with IL-12 for 4 hours before gene expression analysis by qRT-PCR (A) or histone modification analysis by ChIP assay at the *Ifng* regulatory elements (B) or the *Hlx1* promoter (C). Data are average of replicated samples ± S.D. and representative of three independent experiments with similar results.

Transcription factor regulation of Ifng in the absence of STAT4 and Dnmt3a

We then wanted to test whether Dnmt3a deficiency affected transcription factor binding at the *Ifng* locus using chromatin immunoprecipitation. At three sites that are known T-bet and Runx3 binding regions, we observed diminished or absent binding in *Stat4*$^{-/-}$ Th1 cells, compared to wild type cells (Figure 26). However, in Th1 cultures of cells lacking both STAT4 and Dnmt3a there was a partial recovery of binding by T-bet, and complete recovery of Runx3 binding at two of the three sites (Figure 26). Since the T-bet-Jmjd3 interaction is required for *Ifng* remodeling in differentiated Th1 cells (Miller et al., 2010), we then examined the binding of Jmjd3 at the *Ifng* locus. Paralleling T-bet binding, the binding of Jmjd3 at the *Ifng* locus was partially recovered in Th1 cultures of cells lacking both STAT4 and Dnmt3a, compared to *Stat4*$^{-/-}$ cells (Figure 26). This suggested that at least some of the recovery of *Ifng* expression in *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive Th1 cells was due to increased binding of *Ifng*-inducing transcription factors and histone modifying enzymes.

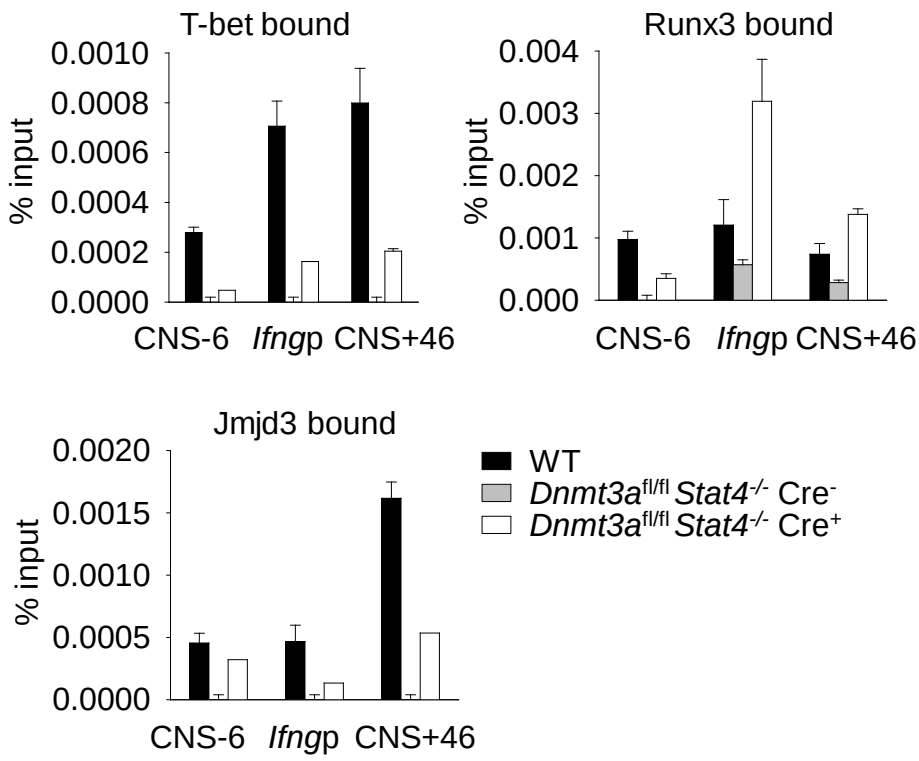


Figure 26. Transcription factor binding in the absence of *Dnmt3a.* Naïve $CD4^+CD62L^+$ T cells were isolated from WT, *Dnmt3a*$^{fl/fl}$ *Stat4*$^{-/-}$ CD4-Cre negative or positive mice and cultured under Th1-polarizing conditions. T-bet, Runx3, and Jmjd3 bound to the *Ifng* locus was analyzed by ChIP assay in Th1 cells. Data are average of replicated samples ± S.D. and representative of three independent experiments with similar results.

In addition to decreased binding of factors to the *Ifng* locus, there was also decreased expression of several factors required for *Ifng* expression including *Hlx1, Runx3,* and *Tbx21* in the absence of STAT4 (Figure 23). To determine if a combination of decreased Dnmt3a function and ectopic Th1 transcription factor expression would completely rescue IFNγ production, we used retroviral transduction to introduce transcription factor expression into *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive and Cre-negative Th1 cultures. Transduction of either *Hlx1* or *Tbx21* had modest effects on IFNγ production and no effects on TNFα production (Figure 27A). Transduction of Runx3 resulted in some recovery of IFNγ

production from *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-negative Th1 cells, but only modest effects on IFNγ production from *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive Th1 cells (Figure 27B). We reasoned that since *Hlx1* expression showed no recovery in *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$Cre-positive Th1 cells compared to *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-negative Th1 cells (Figure 23), and since T-bet binding was still diminished in *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$Cre-positive Th1 cells compared to *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-negative Th1 cells (Figure 26), double-deficient Th1 cells might be especially sensitive to ectopic expression of these two factors. Thus, we transduced *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive and -negative Th1 cells with retroviruses expressing T-bet and Hlx1. We observed that *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-negative Th1 cells demonstrated induction of IFNγ production, but that *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive Th1 cells demonstrated an even greater fold induction in IFNγ production (Figure 27C-D).

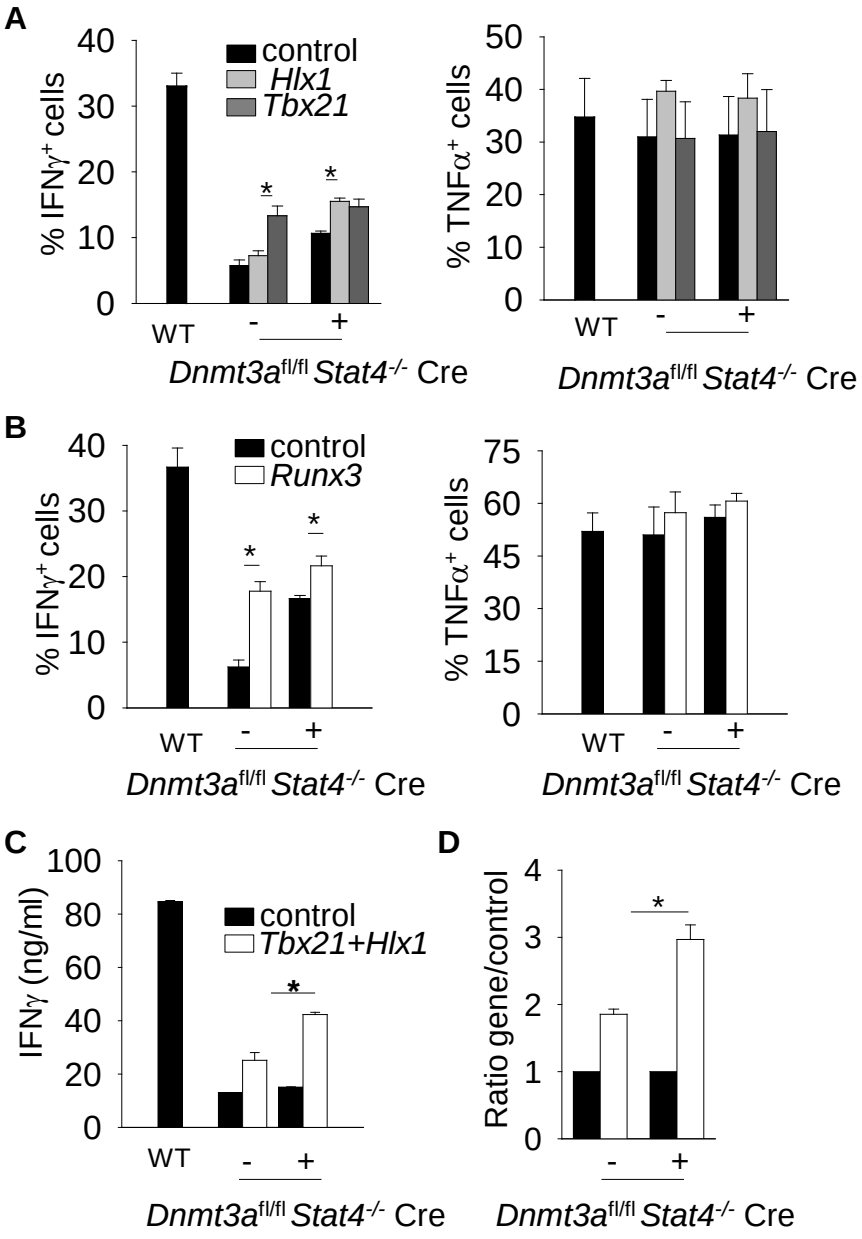


Figure 27. Ectopic Th1 transcription factor expression rescues IFNγ production. Naïve $CD4^+CD62L^+$ T cells were isolated from WT, *Dnmt3a*$^{fl/fl}$ *Stat4*$^{-/-}$ CD4-Cre negative (-) or positive (+) mice and cultured under Th1-polarizing conditions. On day 2, cells were transduced with retrovirus expressing MIEG-EGFP (MIEG), MIEG Hlx1-EGFP (Hlx1), or MIEG T-bet-EGFP (Tbx21) (A), or control vector Thy1.1 or Runx3-Thy1.1 (Runx3) (B), or both MIEG T-bet-EGFP (Tbx21) and MSCV Hlx1-Thy1.1 (Hlx1) or matching controls (C-D). After five days of differentiation, Th1 cells were activated with anti-CD3 for 6 h before measuring cytokine production by ICS. Average percentage of positive cells gated on transduced cells (A-B). (C-D) After five days of differentiation, doubly transduced Th1 cells were sorted, reactivated with anti-CD3 for 24 h, and cytokine production was measured by ELISA (C) with the fold induction in

cytokine production between *Dnmt3a*$^{fl/fl}$ *Stat4*$^{-/-}$ CD4-Cre negative and positive transduced Th1 cells compared to its control cells (D). Data are average ± S.D. of three independent experiments. *p<0.05

These results demonstrate that Dnmt3a represses gene expression in the absence of STAT4, and that in the absence of both factors, the *Ifng* locus is de-repressed and more sensitive to induction by additional *Ifng* trans-activators. Together, these studies reveal the negative regulatory role of Dnmt3a in Th1 gene expression.

Part II- Twist1 regulates *Ifng* expression in T helper 1 cells by interfering with Runx3 function

The effect of Stat4-target genes on IFNγ production in Th1 cells

In the previous section, we showed that STAT4 mediates its function in Th1 cells by facilitating increased histone acetylation and H3K4 methylation to induce gene expression, and by decreasing the association of DNA methyltransferases that repress gene expression. STAT4 is also required for the expression of several other transcription factors that contribute to Th1 gene expression including *Twist1, Hlx1, Tbx21, Erg3, Runx3,* and *Etv5* (Figure 23) (Thieu et al., 2008). Thus, we wanted to determine the role of these transcription factors in regulating cytokine production in Th1 cells. Ectopic gene expression including *Twist1, Hlx1, Tbx21, Egr3, Runx3,* and *Etv5* was performed on wild type Th1 cells, and IFNγ production was measured by intracellular staining. Overexpression of Twist1 in Th1 cells resulted in decreased IFNγ production compared to control cells (Figure 28). While overexpression of T-bet and Runx3 resulted in increased IFNγ production compared to control cells, ectopic Hlx1, Egr3, and Etv5 expression had modest effect on IFNγ (Figure 28). These results suggested that STAT4 regulates a number of transcription factors that have negative (Twist1) and positive (Hlx1, T-bet, Egr3, Runx3, and Etv5) effects on IFNγ in Th1 cells.

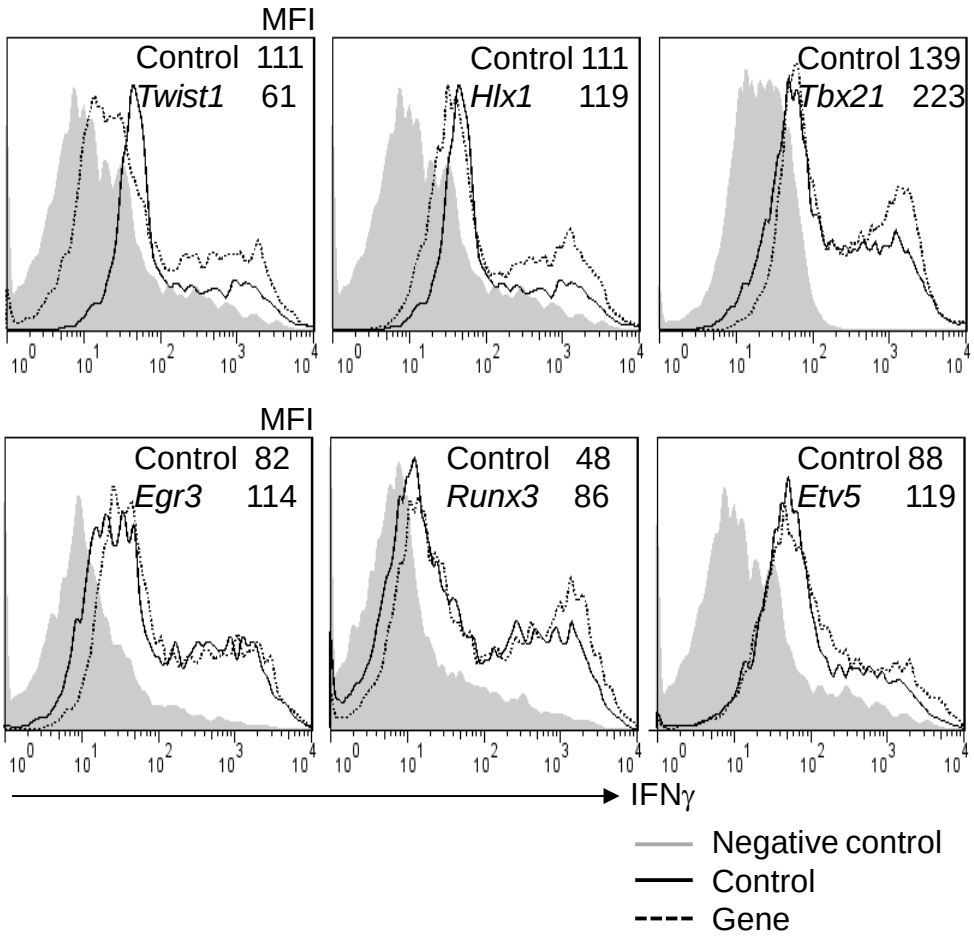


Figure 28. The effect of STAT4 target genes on Th1 cytokine production. Naïve $CD4^+CD62L^+$ cells were isolated from WT mice and differentiated under Th1 culture conditions. On day 2, cells were transduced with either control or *Twist1, Hlx1, Tbx21, Egr3, Runx3, or Etv5* expressing retroviruses. On day 5, cells were stimulated with PMA and ionomycin for 6 h before intracellular staining (ICS) for cytokine production with indicated mean fluorescence intensity. Data are gated on transduced cells. Data are representative of two to three independent experiments with similar results.

Twist1 is induced by Th1 cell activation

Despite Twist1 represses cytokine production in Th1 cells, how Twist1 regulates Th1 cytokine production remained unknown (Figure 28) (Niesner et al., 2008). To begin to define that mechanism, we determined the expression of Twist1 in T cell subsets. *Twist1* mRNA expression was highest in resting and activated Th1 cells,

compared to other T cell subsets (Figure 29A). Previous reports and our data identified *Twist1* as a STAT4 target gene (Niesner et al., 2008; Thieu et al., 2008) (Figure 23). Although *Twist1* mRNA expression was detected during differentiation, expression was dramatically reduced in *Stat4*$^{-/-}$ than wild type Th1 cells (Figure 29B). Stimulation of Th1 cells with anti-CD3 resulted in the induction of Twist1 mRNA and protein (Figure 29C-D). Thus, *Twist1* is expressed in the greatest amounts in Th1 cells compared to other T cell subsets and is induced by TCR stimulation.

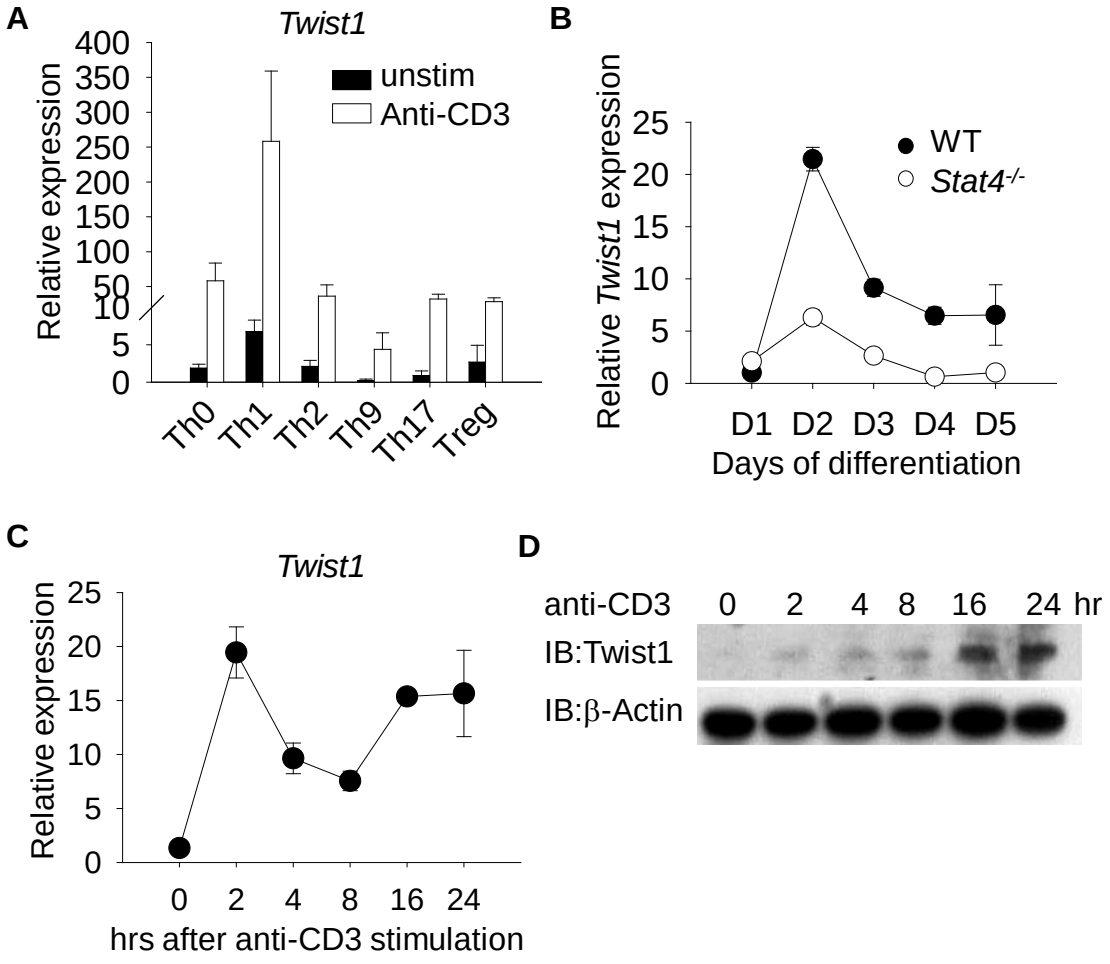


Figure 29. **Twist1 is expressed in activated Th1 cells.** (A) Naive WT $CD4^{+}CD62L^{+}$ T cells were differentiated under neutral conditions (Th0) or Th1, Th2, Th9, Th17 or Treg-polarizing conditions *in vitro. Twist1* mRNA expression in resting or anti-CD3 activated Th1 cells was determined by qRT-PCR. (B) Kinetics

of *Twist1* mRNA expression during Th1 differentiation in WT and *Stat4*$^{-/-}$ cells. (C-D) Kinetics of *Twist1* mRNA expression (C) and Twist1 protein (D) in Th1 cells after stimulation with anti-CD3 for 2, 4, 8, 16, and 24 h. Data are average ± S.D. of 3 mice; and representative of two independent experiments (A) or are average of replicate samples ± S.D and representative of two to three independent experiments with similar results (B-D).

Twist1 negatively regulates the Th1 transcription factor network

To define how Twist1 regulates Th1 cell function, Twist1 was ectopically expressed or targeted by shRNA in Th1 cells using retroviral transduction (Figure 30A, C). In agreement with a previous report (Niesner et al., 2008), ectopic Twist1 expression in Th1 cells reduced IFNγ and TNFα mRNA and protein levels, and decreasing *Twist1* expression resulted in increased IFNγ and TNFα production (Figure 30B, D). The differences in IFNγ and TNFα production in Th1 cells was not due to altered expression of other cytokines, including *Il4* and *Il17a* (Figure 30B). Coincident with decreased cytokine production, the expression of Th1-related transcription factors such as T-bet, Hlx1, and Runx3 were decreased upon ectopic Twist1 expression and increased upon *Twist1* shRNA transduction (Figure 30B, D).

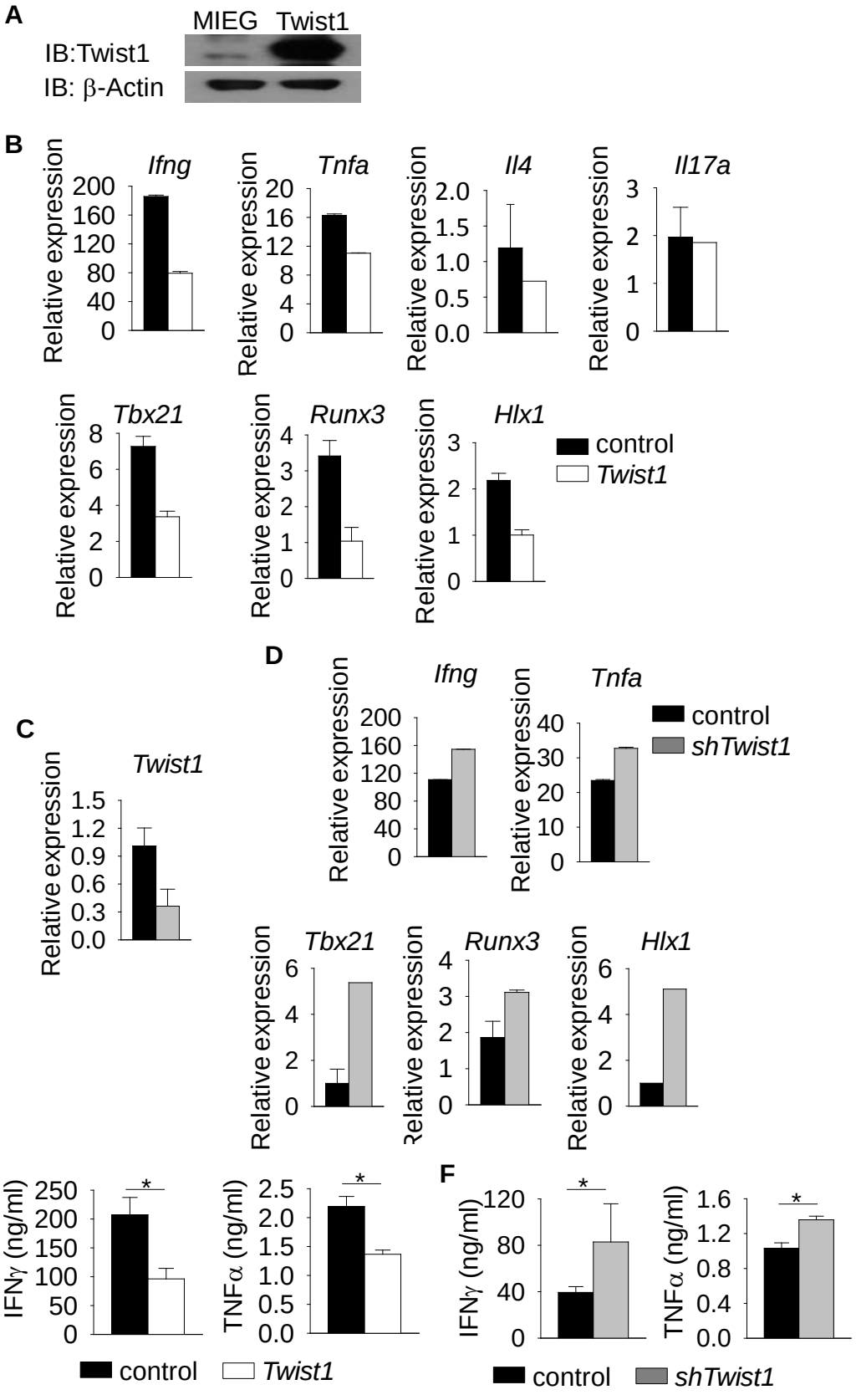

A
MIEG
Twist1
IB:Twist1
IB: β-Actin
B
Ifng
Tnfa
Il4
Il17a
Tbx21
Runx3
Hlx1
Relative expression
control
Twist1
C
Twist1
D
shTwist1
E
IFNγ (ng/ml)
TNFα (ng/ml)
F

Figure 30. **Twist1 negatively regulates Th1 gene expression and cytokine production**. (A-F) Naïve WT $CD4^+CD62L^+$ T cells were stimulated under Th1-polarizing conditions. On day 2, cells were transduced with control EGFP (MIEG) or retroviral vector expressing Twist1 and EGFP (Twist1) (A, B, E), or control or short hairpin Twist1 (shTwist1) (C, D, F). On day 5, GFP^+ cells were sorted for analysis. (A, C) Altered Twist1 protein expression in cells transduced with Twist1 (A) and altered *Twist1* expression in shRNA transduced Th1 cells (C). (B, D) Th1 gene expression in ectopic Twist1- (B) or short hairpin- (D) transduced cells were assessed by qRT-PCR before (*Tbx21, Runx3,* and *Hlx1)* or after *(Ifng, Tnfa, Il4,* and *Il17a)* 6 h re-stimulation with anti-CD3. (E-F) Sorted GFP^+ cells were stimulated with anti-CD3 for 24 h, supernatants were collected and analyzed for IFNγ and TNFα by ELISA. Data are mean ± S.D. of three independent experiments (E-F) or mean of replicate samples ± S.D. and representative of three independent experiments with similar results (A-D). *$p<0.05$

Since STAT4 is required for the expression of many Th1-specific genes (Figure 23) (Thieu et al., 2008), we examined whether Twist1 was a component in a feedback mechanism to control STAT4 activation. Phospho-STAT4, assessed by flow cytometry, was lower in Th1 cells transduced with Twist1-expressing retrovirus compared to vector control, following re-stimulation with increasing doses of IL-12 (Figure 31A). Decreased induction of pSTAT4 correlated with reduction in IL-18rα expression, a STAT4-target gene (Figure 31B) (Yu et al., 2008; Yu et al., 2007). Consistent with this result, reduced Twist1 expression resulted in increased IL-12-induced STAT4 phosphorylation (Figure 31C). We next examined the expression of genes that contribute to STAT4 activation. STAT4 mRNA and protein expression, and mRNA of suppressors of cytokine signaling (SOCS) and protein tyrosine phosphatase-Basophil like (PTP-BL) that negatively regulate STAT4 activation (Nakahira et al., 2007; Yamamoto et al., 2003) were not altered (Figure 31D, G). However, *Il12rb2* mRNA expression was decreased approximately 50% by ectopic Twist1 expression compared to vector

control (Figure 31E). In parallel, transduction of Th1 cells with retroviral Twist1 shRNA resulted in increased *Il12rb2* expression (Figure 31F).

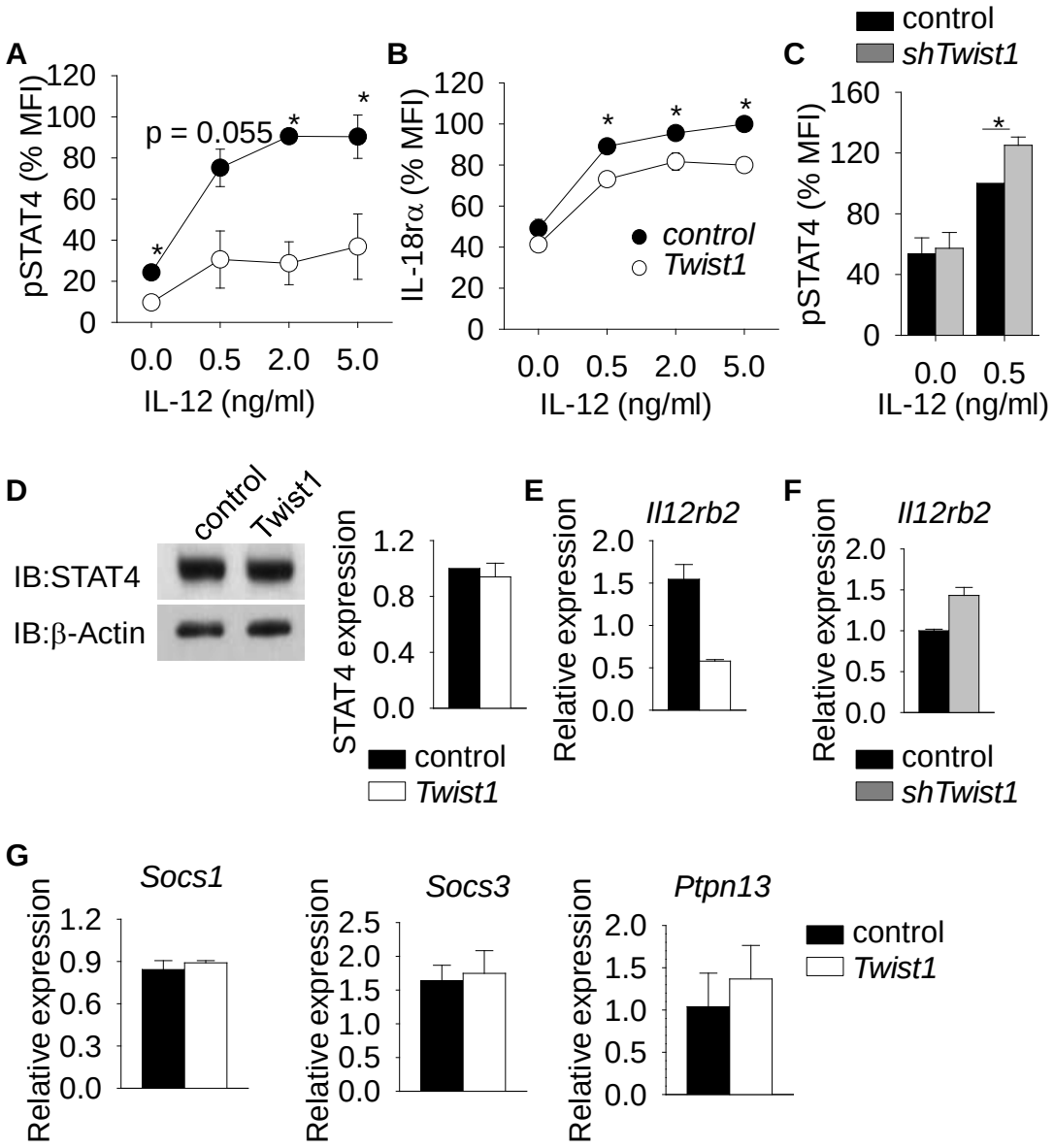


Figure 31. **Twist1 regulates *Il12rb2* expression and STAT4 activation**. (A-B) Naïve WT $CD4^+CD62L^+$ T cells were cultured and transduced as described in Figure 29. Transduced Th1 cells were stimulated with IL-12 for 4 h and GFP^+ cells were analyzed for phospho-STAT4 (pSTAT4) (A) and IL-18rα expression (B). (C) Phospho-STAT4 amounts were determined in control or shRNA transduced Th1 cells following sorting for GFP expression. (D) Total lysates from cells transduced with Twist1 were immunoblotted for STAT4 and β-Actin as a control and densitometry analysis of STAT4 protein is indicated. (E-G) Gene expression in Twist1 (E, G) or shTwist1 (F) transduced Th1 cells sorted for GFP

expression. Data are mean of three to four independent experiments ± S.D. (A-C) or the average of replicate samples ± S.D. and representative of two to three independent experiments with similar results (D-G). *p<0.05

Runx3 rescues the inhibitory effect of Twist1 independent of T-bet and STAT4

Since Twist1 regulates multiple genes in Th1 cells, we wanted to determine if a single factor could rescue the inhibitory effect of Twist1. Initially, we asked whether ectopic expression of IL-12Rβ2 could compensate for the repressive effect of Twist1 by co-transducing Th1 cells using transduction of both IL-12Rβ2 and Twist1. IL-12Rβ2 surface expression was decreased in cells with ectopic Twist1 expression but was higher in double transduced cells, compared to vector control (Figure 32A). Recovery of IL-12Rβ2 expression resulted in increased IL-12-induced phospho-STAT4 (Figure 32B). Despite recovery of phospho-STAT4 (Figure 32B), ectopic IL-12Rβ2 expression was unable to rescue IFNγ production, but recovered TNFα production (Figure 32C). Co-expression of T-bet and Twist1 resulted in higher T-bet expression compared to vector control but failed to recover IFNγ and TNFα production (Figure 32D-E). Thus, although recovery of IL-12 signaling was able to induce TNFα in the presence of Twist1, neither recovery of IL-12 signaling nor T-bet expression was able to compensate for the effects of Twist1 on IFNγ production.

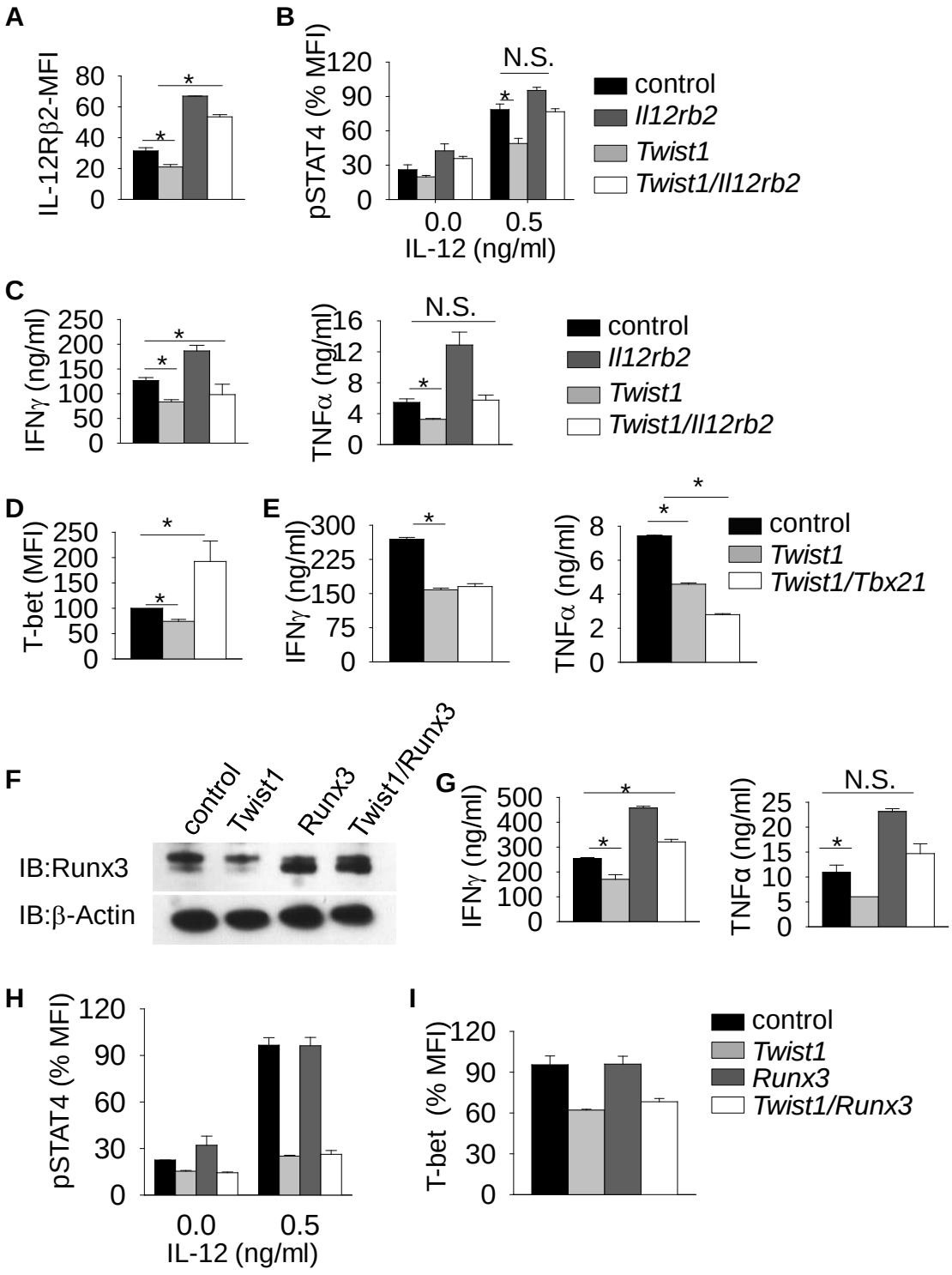


Figure 32. Ectopic Runx3 expression compensates for the repressive activity of Twist1 in Th1 cells. (A-I) Naïve WT $CD4^{+}CD62L^{+}$ T cells were stimulated under Th1-polarizing conditions. On day 2, cells were transduced with control retrovirus vectors or (A-C) retroviral vector expressing Twist1-GFP and IL-12Rβ2-hCD4 (D, E) Twist1-hCD4 and T-bet-GFP (F-I) Twist1-GFP and

Runx3-Thy1.1. Th1 cells were stained for IL-12Rβ2 by surface staining (A) or phospho-STAT4 with or without IL-12 stimulation by ICS (B, H), or T-bet by ICS (D, I). Analysis was performed by gating on doubly transduced cells. (F) Whole cell lysates were extracted from sorted double positive Th1 cells and were immunoblotted for Runx3 and β-Actin as a control. (C, E, G) Double positive cells were sorted and re-stimulated with anti-CD3 for 24 h. Supernatants were collected before IFNγ and TNFα production was measured by ELISA. Data are mean of three to four independent experiments ± S.D (A-E, G) or averages of replicate samples ± S.D. and representative of two to three independent experiments with similar results (F, H-I). *$p<0.05$. N.S., not significant

Since Runx3 regulates IFNγ independently of T-bet and STAT4 (Yagi et al., 2010), we hypothesized that regulation of Runx3 by Twist1 might be a critical target. Ectopic Twist1 expression resulted in decreased Runx3, and retroviral expression of Runx3 resulted in higher Runx3 level in double transduced cells compared to control cells (Figure 32F). IFNγ and TNFα production in Runx3/Twist1-transduced cells was comparable to that of control transduced cells (Figure 32G). The recovery of IFNγ and TNFα production by Runx3 in Th1 cells was independent of T-bet and STAT4 since there were no recovery of T-bet expression or phospho-STAT4-positive cells (Figure 32H-I). Thus, although Twist1 can negatively regulate many Th1 genes, the recovery of T-bet and IL-12Rβ2 expression (and as a consequence, STAT4 phosphorylation) did not compensate for the effects of Twist1. These results suggested that Runx3, or a Runx3 induced-gene, is at least one of the important Twist1 targets in the regulation of IFNγ.

Twist1 exists in a complex with Runx3 or T-bet

Twist1 inhibits osteoblast differentiation by interacting with the Runt domain of Runx2, a highly conserved domain shared with related proteins including Runx3 (Bialek et al., 2004). We hypothesized that Twist1 physically interacts with Runx3 and inhibits its regulatory function. Using immunoprecipitation of extracts from resting and early activated Th1 cells we observed that Twist1 co-purified with Runx3, and confirmed the association between Runx3 and T-bet in Th1 cells (Figure 33A) (Djuretic et al., 2007). We also found an association between T-bet and Twist1 following precipitation with T-bet antibody (Figure 33B).

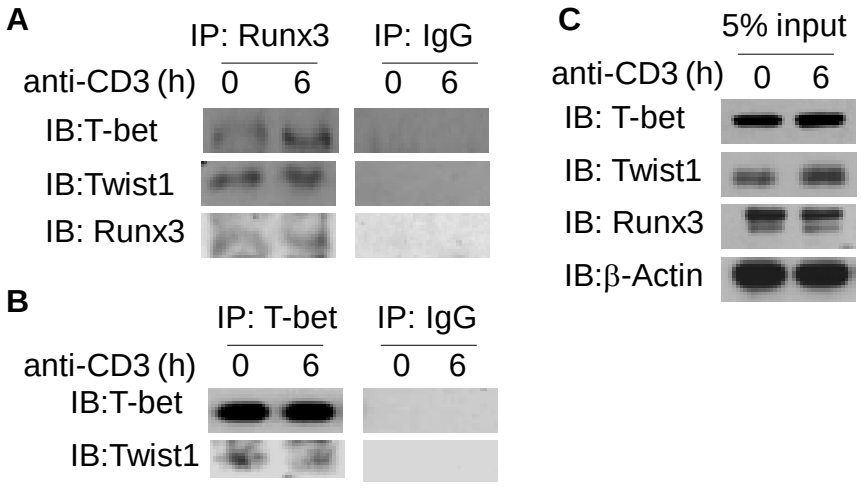


Figure 33. Twist1 physically interacts with Runx3 and T-bet. (A-C) Whole cell lysates were extracted from activated WT Th1 cells. Immunoblots were performed for the indicated proteins following immunoprecipitation with control or Runx3- (A) or T-bet- (B) antibodies as indicated. Input for immunoprecipitation is indicated on the right (C).

To examine the function of Runx3 and T-bet in the absence of Twist1, *Twist1*$^{fl/fl}$ mice (Chen et al., 2007) were mated with mice carrying a CD4-Cre transgene to generate mice with *Twist1*-deficient T cells (*Twist1*$^{fl/fl}$ CD4-Cre^{+}). We confirmed the absence of Twist1 expression in *Twist1*-deficient Th1 cells (Figure 34A-B).

Twist1-deficiency did not alter normal lymphocyte development in thymus, spleen, and lymph nodes (Figure 34C). *Twist1*-deficient Th1 cells produced significantly greater amounts of IFNγ on a per cell basis and in frequency of IFNγ-positive cells compared to wild type cells (Figure 35A-D). We also confirmed the increase in gene expression of *Tbx21, Runx3,* and *Il12rb2* in *Twist1*-deficient Th1 cells, compared to WT Th1 cells.

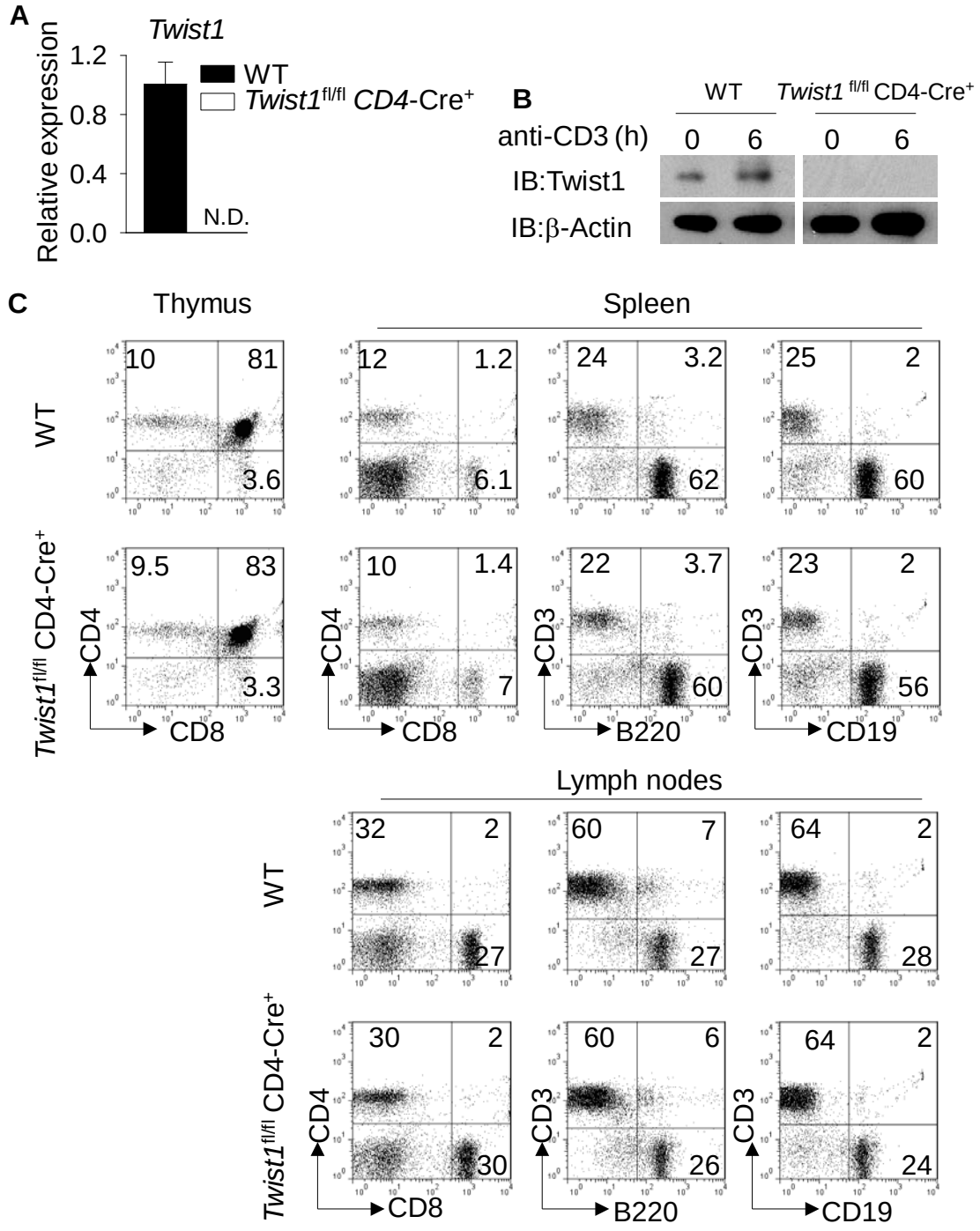


Figure 34. Generation of mice with *Twist1*-deficient T cells. Naïve $CD4^+CD62L^+$ T cells from WT and *Twist1*$^{fl/fl}$ CD4-Cre^+ were stimulated under Th1-polarizing conditions. (A) Resting Th1 cells were used to measure *Twist1* expression by qRT-PCR. (B) Twist1 protein expression in WT and *Twist1*-deficient Th1 cells after reactivated with anti-CD3 for 6 h. (C) Total cells were isolated from thymus, spleen, and lymph nodes of WT and *Twist1*-mutant mice and stained for cell surface markers. Results are the average ± S.D. of replicated

samples and are representative of three to four independent experiments with similar results. N.D., not detectable

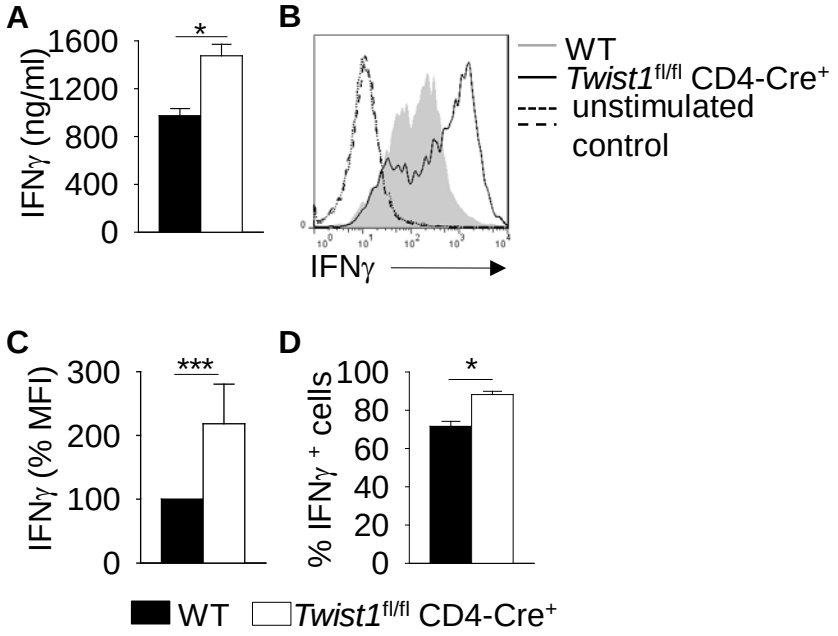


Figure 35. *Twist1*-deficient Th1 cells produce significantly more IFNγ than WT Th1 cells. (A-D) Naïve $CD4^+CD62L^+$ WT or *Twist1*$^{fl/fl}$ CD4-Cre^+ T cells were cultured under Th1-polarizing conditions. IFNγ production was measured by ELISA (A) after reactivating with anti-CD3 for 24 h or ICS (B-D) after reactivating with PMA and ionomycin for 6 h. (C-D) Graphs indicate the mean fluorescence intensity (C) or average percent IFNγ-positive cells (D) of Th1 cells from mice of the indicated genotype. Results are the average ± S.E.M. of three to four independent experiments with similar results. *p<0.05, ***p<0.001

We next tested the effect of Twist1 on Runx3 and T-bet DNA binding by DNA affinity precipitation assay using extracts from *Twist1*-mutant mice and littermate control (WT) activated Th1 cells. Extracts from WT and *Twist1*-deficient Th1 cells were incubated with biotinylated oligonucleotides containing a Twist1-binding sequence. Although T-bet and Runx3 were precipitated with DNA-bound Twist1 in WT cell extracts, T-bet and Runx3 were absent from precipitates of Twist1-deficient extracts, further supporting the interaction of these transcription factors and suggesting that interactions can occur when Twist1 is bound to DNA (Figure

36A-B). We observed that *Runx3* and *Tbx21* mRNA expression increased in *Twist1*-deficient Th1 cells compared to WT cells, although there was a modest increase at protein level, suggesting the importance of protein complex formation in the function of Twist1. Since there is an association between Twist1, Runx3, and T-bet, we tested if, in the absence of Twist1, there would be greater binding of Runx3 and T-bet to oligonucleotides containing Runx3 and T-bet specific binding sites. In the absence of Twist1, we detected greater binding of Runx3 and T-bet to the respective oligonucleotides compared to WT samples (Figure 36C-E, lane 2 vs. 1). When the binding sites of Runx3, T-bet or both were mutated, the binding of Runx3 and T-bet was diminished, regardless of Twist1 expression (Figure 36C). We then tested if Twist1 mediates its inhibitory effect in Th1 cells by interfering with the T-bet-Runx3 interaction. Using Runx3 immunoprecipitation, we detected a Runx3 and T-bet interaction in the absence of Twist1 (first lane), although this interaction was diminished in the presence of Twist1 (Figure 36F). These results suggested that the decrease in IFNγ production in Th1 cells might not only be due to the reduction in *Runx3* gene expression but also due to diminished association and DNA binding activity of Runx3 and T-bet.

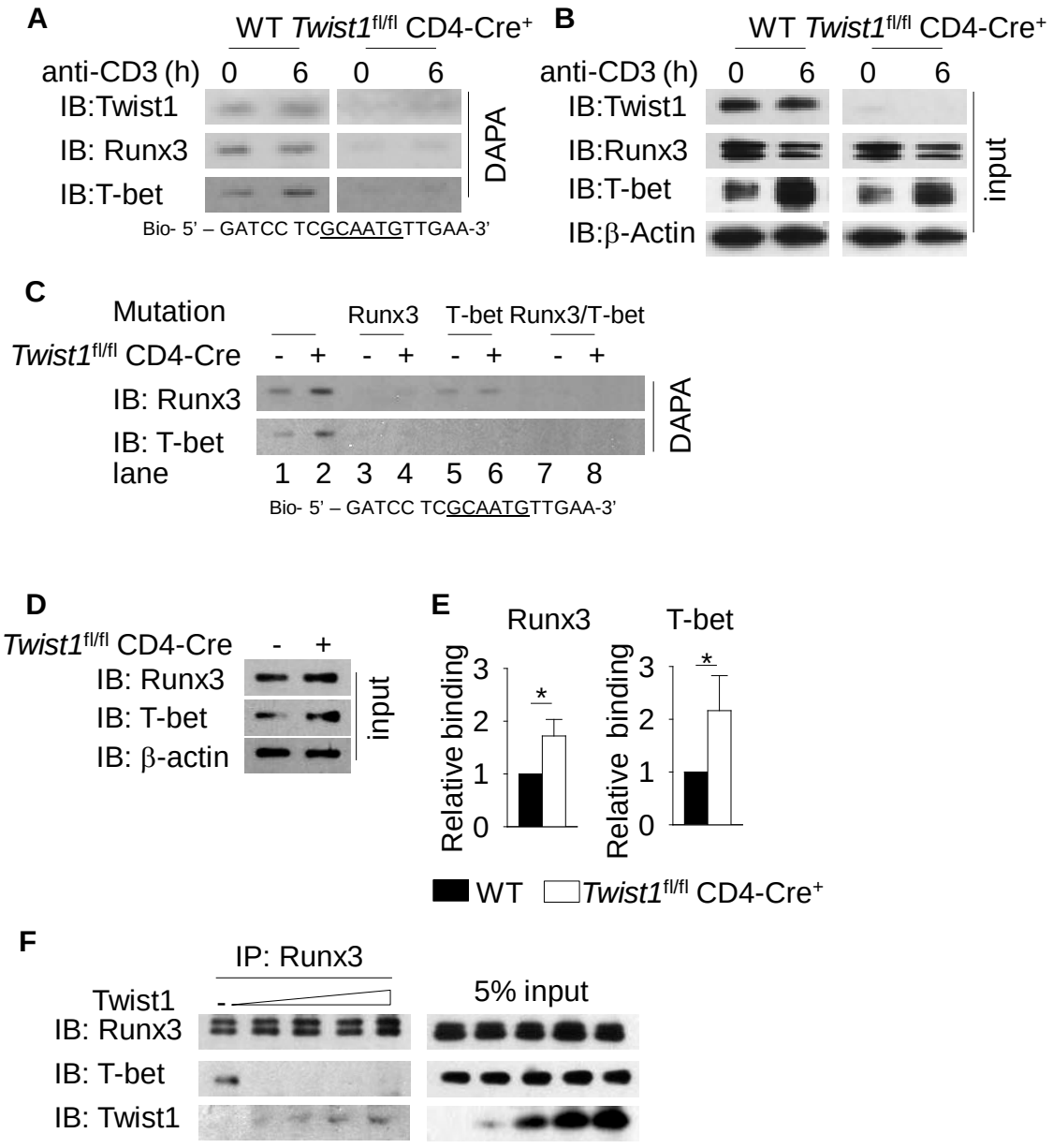


Figure 36. Twist1 physically interacts with Runx3 and T-bet. (A-F) Naïve $CD4^+CD62L^+$ WT or *Twist1*$^{fl/fl}$ $CD4\text{-}Cre^+$ T cells were cultured under Th1-polarizing conditions. (A-B) Nuclear extracts from activated (A-B) or resting (C) WT and *Twist1*$^{fl/fl}$ $CD4\text{-}Cre^+$ Th1 cells were incubated with biotinylated oligonucleotides containing Twist1-specific binding site (A) or Runx3-T-bet specific binding sites (wild type or mutant as indicated) (C). (B, D) Immunoblots of precipitated proteins, with densitometry measurements for (E). (F) Mixture of whole cell lysates collected from 293T cells transfected with constructs expressing Runx3, T-bet or Twist1 were incubated at 4^{o}C overnight. Immunoblots indicate proteins immunoprecipitated with Runx3. Results are average ± S.D. (E) of three independent experiments or representative of three or more independent

experiments with similar results (A-D, F). *p<0.05. DAPA, DNA Affinity Precipitation Assay

Twist1 interferes with the binding of Runx3 and T-bet to the Ifng locus

Twist1 interferes with T-bet and Runx3 DNA binding, however we observed only modest binding of Twist1 to the *Tbx21*, *Ifng,* and *Runx3* promoters (Figure 37A). Thus, we hypothesized that the interaction of Twist1 with Th1 transcription factors decreased their binding to target genes including *Ifng*. T-bet and Runx3 regulate *Ifng* gene expression by binding to several conserved non-coding sequences (CNS) (Yagi et al., 2010). To examine T-bet and Runx3 binding in the absence of Twist1, we performed ChIP assay using wild type and *Twist1*-deficient Th1 cells and examined the binding at previously documented regions including CNS-34, CNS-6, *Ifng* promoter, and CNS+46 of the *Ifng* gene (Figure 37B) (Schoenborn et al., 2007; Yagi et al., 2010), using ChIP for T-bet or CBF-β, the binding partner of Runx3 (Yagi et al., 2010). Our data showed that more T-bet was bound in *Twist1*-deficient Th1 cells than in wild type cells at the *Ifng* promoter and CNS+46 while there were no difference at CNS-34 and CNS-6 (Figure 37C). In contrast, more CBF-β was bound at CNS-34 and CNS-6 in *Twist1*-deficient Th1 cells compared to littermate controls (Figure 37C). This result suggested that Twist1 interferes with T-bet and CBF-β-Runx3 complex binding at specific regulatory regions of *Ifng* gene. Since T-bet and Runx3 bind to many CNS regions of the *Ifng* locus, and the association between T-bet and Runx3 is required for optimal *Ifng* expression (Djuretic et al., 2007; Yagi et al., 2010), it is likely that Runx3 binding to the *Ifng* locus enhances T-bet binding to

regulatory elements. This is consistent with the recovery of IFNγ production in the Runx3 transduction experiment (Figure 32G).

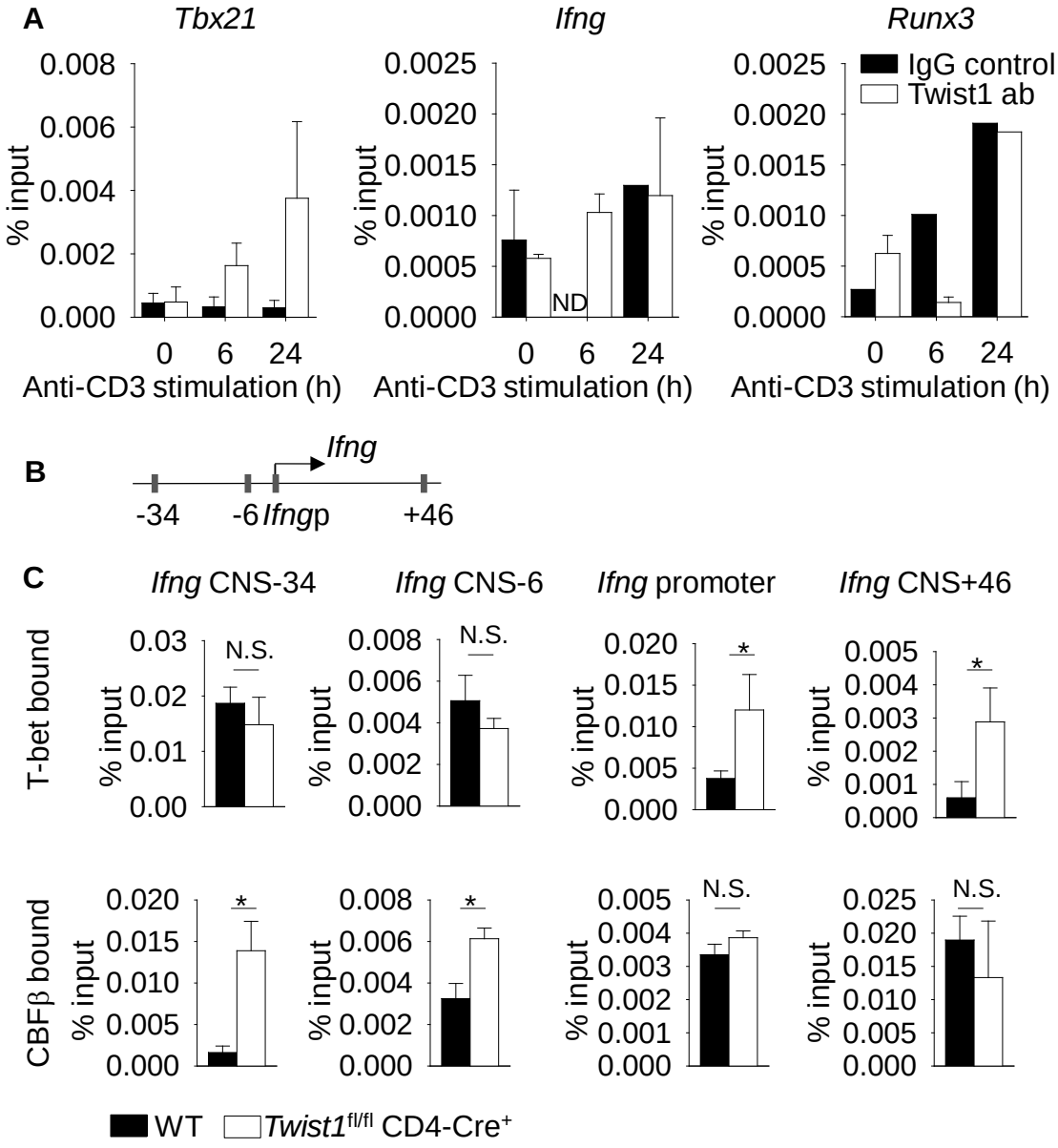


Figure 37. Twist1 interferes with transcription factor binding at the *Ifng* locus in Th1 cells. (A) Differentiated WT Th1 cells were stimulated with anti-CD3 for 6 and 24 h and used for ChIP analysis by qPCR using Twist1 antibody. (B) Representation of the *Ifng* locus indicating conserved non-coding sequences (CNS) used for analysis. (C) Naïve $CD4^+CD62L^+$ T cells from WT and *Twist1*$^{fl/fl}$ CD4-Cre^+ were stimulated under Th1-polarizing conditions. ChIP assays were performed on Th1 cells using T-bet and CBF-β antibodies. *p<0.05. N.D., not detectable

The effect of Twist1 on T-bet and CBF-β-Runx3 binding at distinct elements suggested that interfering with T-bet-Runx3 interactions might also alter chromatin looping at the *Ifng* locus (Hadjur et al., 2009; Sekimata et al., 2009). To determine whether Twist1 interferes with chromatin looping at the *Ifng* locus, a chromosome conformation capture (3C) assay was performed with wild type and *Twist1*-deficient Th1 cells that examined the interactions among *Ifng* CNS regions using an established assay (Figure 38) (Hagege et al., 2007; Sekimata et al., 2009) (Figure 39A-B). Using three different anchor points, our results showed increased crosslinking frequency between CNS-34, CNS+46, and the *Ifng* promoter, and increased crosslinking of CNS-6 with CNS-34 and CNS+46 (Figure 39C-E). The distance between the *Ifng* promoter and CNS-6 is too short to provide consistent results in this assay. Crosslinking of the CNS region at -71 was not altered. Together, these results suggest that Twist1 regulates *Ifng* expression by altering the binding of the Th1 transcription factors T-bet and Runx3, and altering the conformation of the *Ifng* locus.

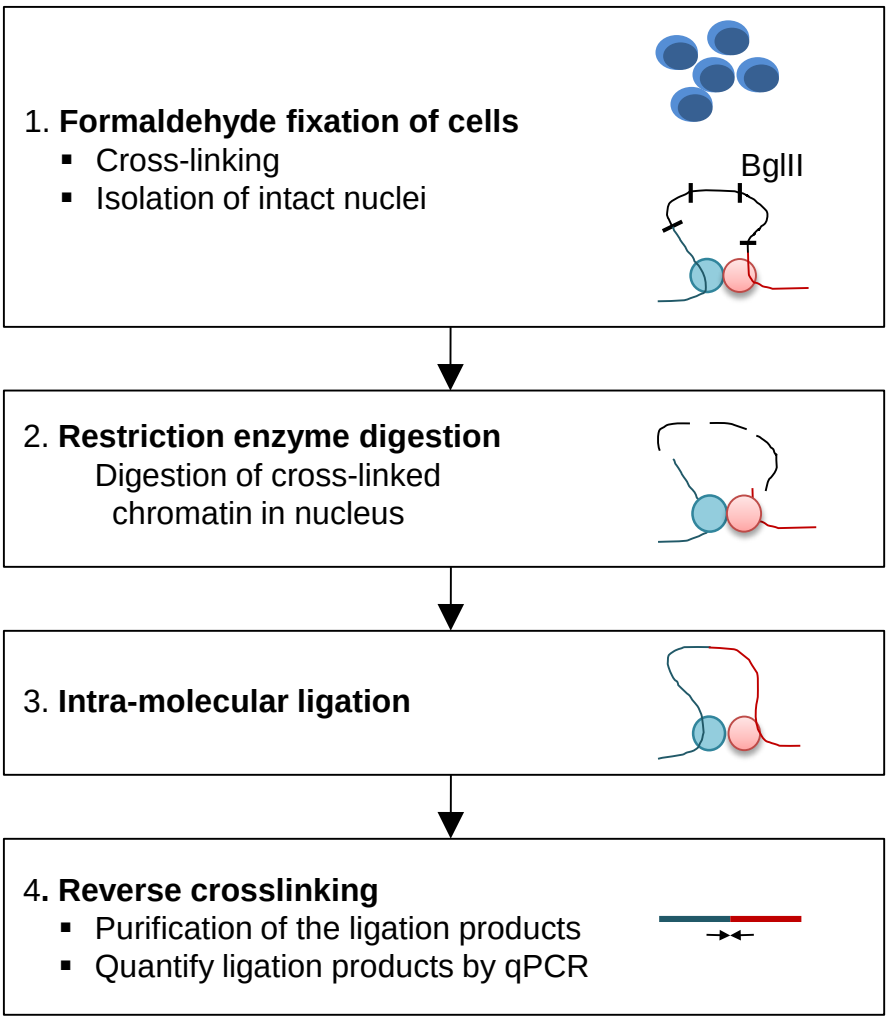


Figure 38. Chromosome conformation capture (3C) assay: methodology

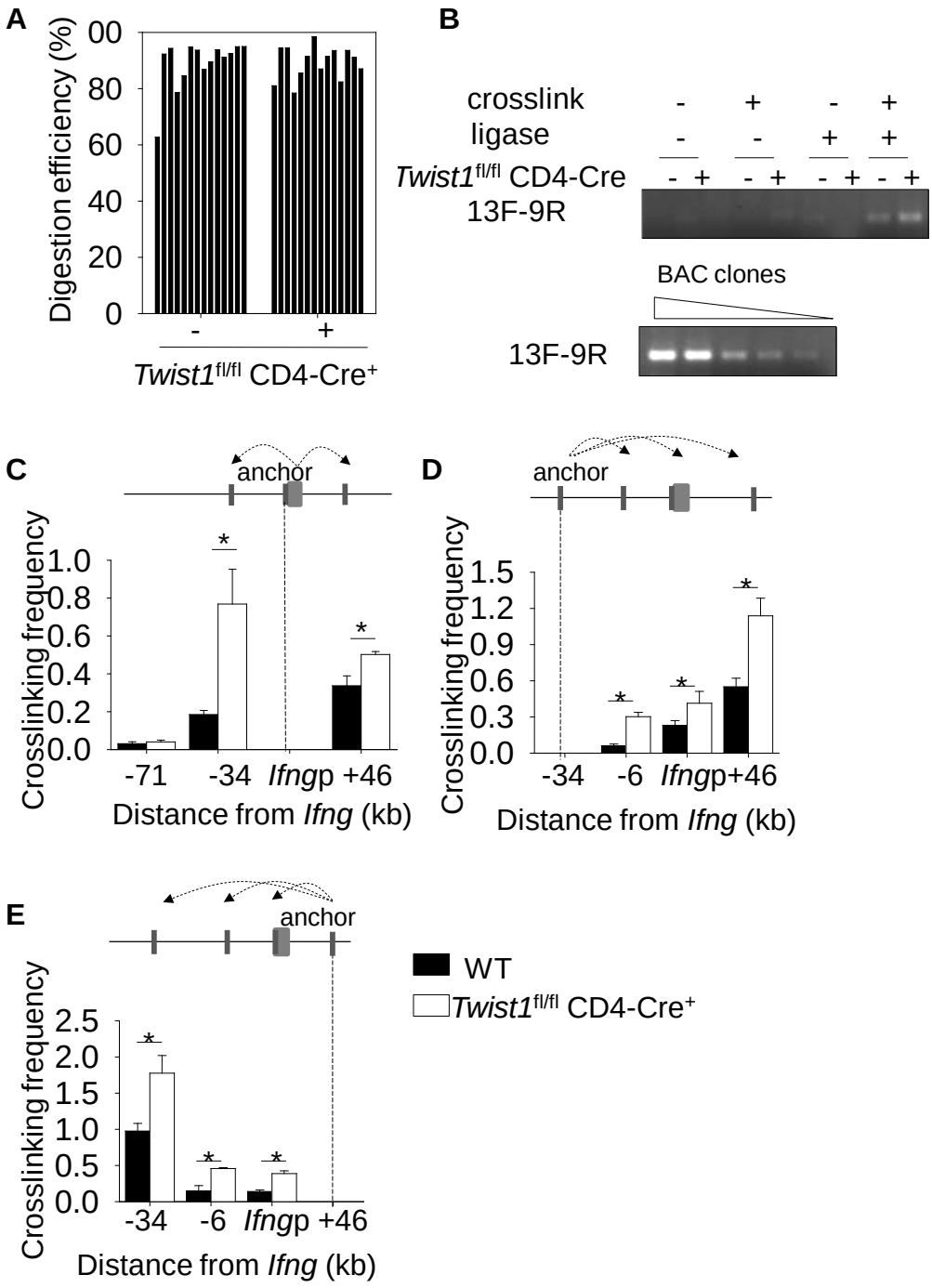


Figure 39. Twist1 interferes with chromatin conformation at the *Ifng* locus in Th1 cells. (A-E) Naïve $CD4^{+}CD62L^{+}$ T cells from WT and *Twist1*$^{fl/fl}$ CD4-Cre^{+} were stimulated under Th1-polarizing conditions. (A) Th1 cells were fixed and digested with *BglII* enzyme. Undigested and digested samples were subjected for qPCR using primer pairs spanning the restriction sites. %Digestion was calculated using the formula: $100 - 100/2^{((CtR - CtC)D - (CtR - CtC)\,UND)}$; D: digested, UND: undigested, R: restriction site, C: internal control. D, Th1 cells as described above were fixed (crosslinked) and/or ligated as indicated. (B) qPCR was

performed using primer pair 13F-9R. PCR products were run on 2% agarose gel. BAC clones were titrated for qPCR and used as control. (C-E), Chromosome conformation capture (3C) assay showing the relative cross-linking frequencies between the *Ifng* promoter (C), CNS-34 (D) or CNS+46 (E) as the fixed anchor fragments and other *BglII* fragments containing the indicated CNS regions. Results are the average ± S.D. of replicated samples and are representative of four independent experiments with similar results. *p<0.05

The Twist1-Runx3 interaction is required to regulate Ifng

A mutation in Twist1 at amino acid 192 from Serine to Proline (Twist1cc) results in diminished interaction between Twist1 and Runx2 (Bialek et al., 2004). Thus we wanted to examine whether Twist1 S192P (Twist1cc) could interact with Runx3. DAPA using extracts from cells transfected with Runx3, WT Twist1 (Twist1), and Twist1cc expressing vectors that were incubated with biotinylated oligonucleotides containing a Twist1-binding sequence demonstrated decreased interaction between Runx3 and Twist1cc (Figure 40A-B). The mutation in Twist1cc did not affect the association of Twist1 with T-bet confirmed by co-immunoprecipitation experiment (Figure 40C).

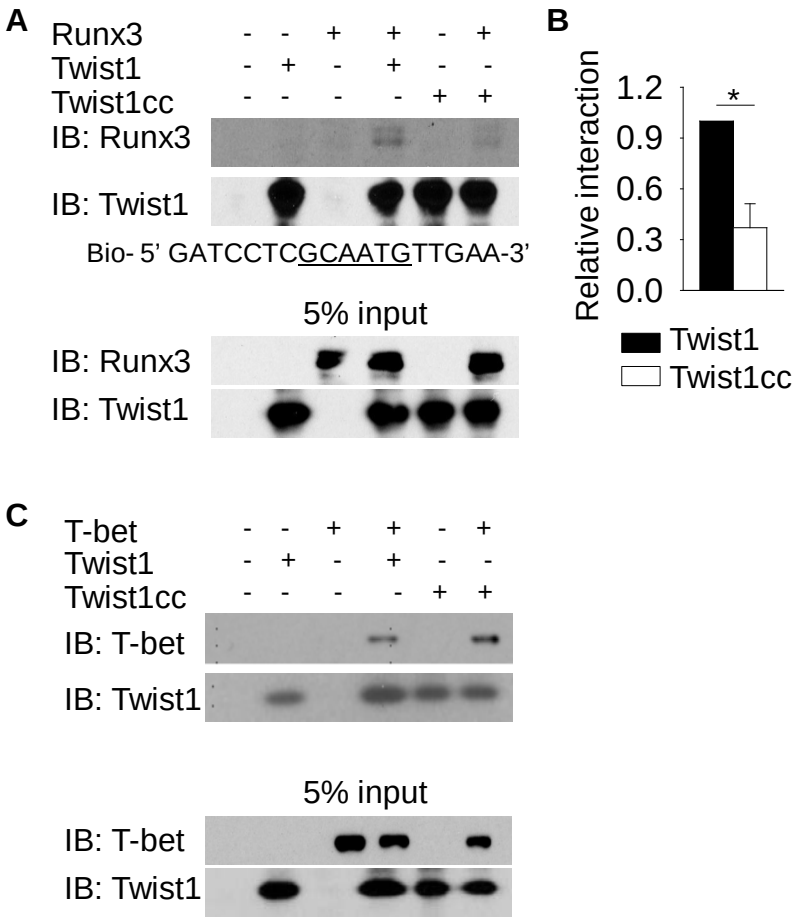


Figure 40. Twist1cc poorly interacts with Runx3. (A, B) Nuclear extracts from 293T cells co-transfected with either Flag-tagged Twist1 or Flag-tagged Twist1cc, and with or without Runx3, were incubated with biotinylated oligonucleotides containing Twist1-specifc sequence. Immunoblot demonstrates protein expression of Twist1 and Runx3 (A) with densitometry measurements (B). (C) 293T cells co-transfected with either Flag-tagged Twist1 or Flag-tagged Twist1cc and with or without T-bet. Immunoblots indicate proteins immunoprecipitated with T-bet. *$p<0.05$

The mutant Twist1 provided a tool to mechanistically distinguish the effects of Twist1 that rely on interactions with Runx3 at the protein level, versus effects independent of Runx3. To test the effects of mutant Twist1, a retrovirus expressing Twist1cc was introduced into Th1 cells, and cytokine production and gene expression were analyzed. Introduction of Twist1cc did not repress IFNγ

production in Th1 cells compared to vector control and wild type Twist1-transduced cells (Figure 41A-F). Importantly, Twist1cc was able to repress expression of several Th1 genes including *Il12rb2, Runx3,* and *Tbx21* as effectively as wild type Twist1 (Figure 41F). To further define the function of Twist1cc in Th1 cells, we utilized a mouse mutant strain termed Charlie Chaplin (*Twist1*$^{cc/wt}$) that encodes Twist1 S192P and results in hindlimb polydactyly (Bialek et al., 2004; Krawchuk et al., 2010). We mated *Twist1*$^{cc/wt}$ with *Twist1*$^{fl/fl}$ CD4-Cre^{+} mice generating *Twist1*$^{fl/wt}$ CD4-Cre^{+} and *Twist1*$^{fl/cc}$ CD4-Cre^{+} (*Twist1*$^{fl/cc}$) mice that have T cells expressing one wild type or one mutated allele of *Twist1*, respectively. IFNγ production in *Twist1*$^{fl/cc}$ Th1 cells was increased compared to control cells (Figure 41G-I). These results demonstrate although Twist1cc retains some repressive function in Th1 cells independent of Runx3, Twist1 control of IFNγ production is primarily through association with Runx3.

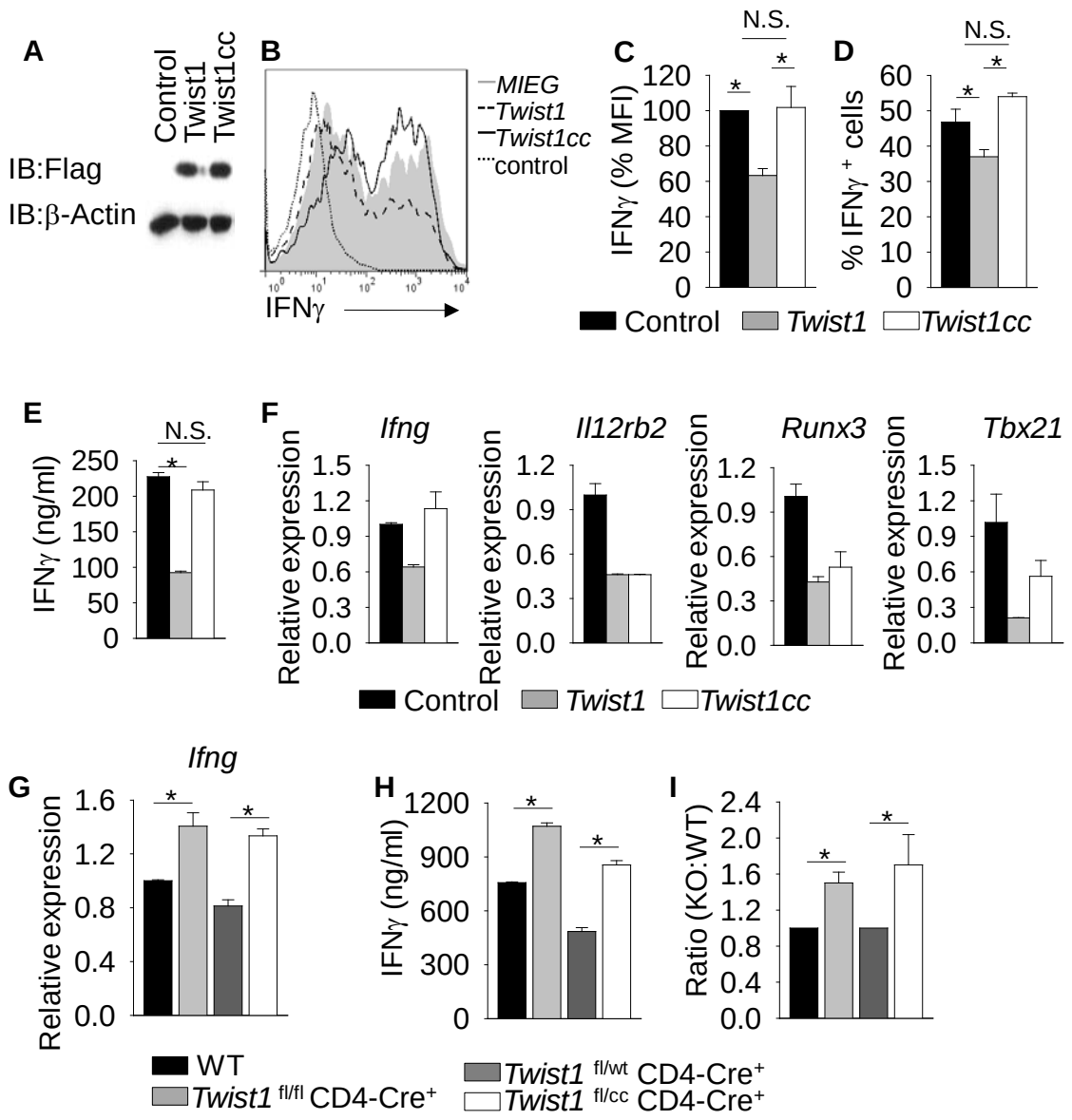


Figure 41. The Twist1-Runx3 interaction is required for regulation of *Ifng*. (A-I) Naïve $CD4^+CD62L^+$ T cells from WT were cultured under Th1-polarizing conditions and were infected with retrovirus expressing Twist1 or Twist1cc. (A) Twist1 protein was assessed in transduced Th1 cells using immunoblot, with β-actin as a control. (C-E) IFNγ production was assessed by intracellular staining and gating on GFP^+ populations following 6h of activation with anti-CD3 (B-D) or by ELISA following 24 h stimulation with anti-CD3 of sorted GFP^+ populations (E). (C-D) Graphs indicate the mean fluorescence intensity (E) or average percent IFNγ-positive cells (D) of each transduced population. (F) Th1 gene expression in ectopic Twist1 or Twist1cc expression was assessed by qRT-PCR before (*Il12rb2, Runx3,* and *Tbx21)* or after *(Ifng)* 6 h re-stimulation with anti-CD3. (G- I), Naïve $CD4^+CD62L^+$ T cells from mice of the indicated genotypes were cultured under Th1-polarizing conditions. Th1 cells were stimulated with anti-CD3 for 6 h or 24 h respectively for testing *Ifng* expression by qRT-PCR (G)

or IFNγ production by ELISA with the ratio of production from *Twist1*-deficient or *Twist1* mutant Th1 cells to the respective controls (H-I). Data are mean of three to four independent experiments ± S.D, *p<0.05 (A-E), or are mean of replicate samples ± S.D. and representative of two independent experiments with similar results (G-I). *p<0.05. N.S., not significant

Part III-Twist1 limits Th17 and Tfh cell development by repressing *Il6ra*

STAT3-activating cytokines induce Twist1 expression

Having demonstrated that Twist1 negatively regulated cytokine production in Th1 cells (Figure 30) (Pham et al., 2012), we next wanted to determine if Twist1 had effects in other T helper cell subsets. We compared cytokine production from *in vitro* polarized cultures of naïve $CD4^+$ T cells from wild type and mice carrying a conditional mutant allele of *Twist1* in T cells. As shown previously, Th1 cells display increased production of IFNγ (Figure 42A). Cytokine production by Th2 and Th9 cells, percentages of $Foxp3^+$ *in vitro* derived Treg cells, and percentages of natural Treg (nTreg; $CD4^+CD25^+Foxp3^+$) cells were similar between wild type and *Twist1*-deficient cultures (Figure 42A-B). In contrast, there was a marked increase in IL-17 production from Th17 cultures (Figure 42A).

We first examined the regulation of Twist1 in Th17 cells. Since STAT3 directly binds to the *Twist1* promoter in breast cancer cells (Cheng et al., 2008), we speculated that STAT3 might induce Twist1 expression in Th17 cultures. Stimulation of wild type Th17 cells with IL-6 or IL-23 to activate STAT3, or IL-12 to activate STAT4, led to increased *Twist1* mRNA and protein expression compared to unstimulated cells (Figure 43A-B). Since *Twist1* expression in Th17 cells is lower than Th1 cells (Figure 29A) (Pham et al., 2012), we hypothesized that an inhibitory signal represses *Twist1* expression in developing Th17 cells. Indeed, IL-6 or IL-12 induced *Twist1* expression in activated $CD4^+$ T cells and this was decreased when TGF-β was added to the culture (Figure 43C). To

further confirm that *Twist1* is a STAT3 target gene in Th17 cells, gene expression was compared in activated wild type and *Stat3*-deficient $CD4^+$ T cells. As expected, IL-6 stimulated *Stat3*-deficient $CD4^+$ T cells were unable to induce *Twist1* expression compared to wild type cells, although expression was equally induced in IL-12-stimluated wild type and *Stat3*-deficient $CD4^+$ T cells (Figure 43C).

Given that the *Twist1* promoter contains STAT3 binding sites (Figure 43D) (Cheng et al., 2008), we wanted to determine if STAT3 could directly bind to the regulatory regions of *Twist1*. Differentiated Th17 cells were stimulated with cytokines known to activate STAT3, and binding was examined by ChIP assay. In Th17 cells, STAT3-activating cytokines, but not IL-12, resulted in STAT3 binding to the *Twist1* promoter, with greatest amounts in the proximal promoter segment (Figure 43E). These results suggested that STAT3-activating cytokines and TGF-β play opposing roles in regulating *Twist1* expression in Th17 cultures.

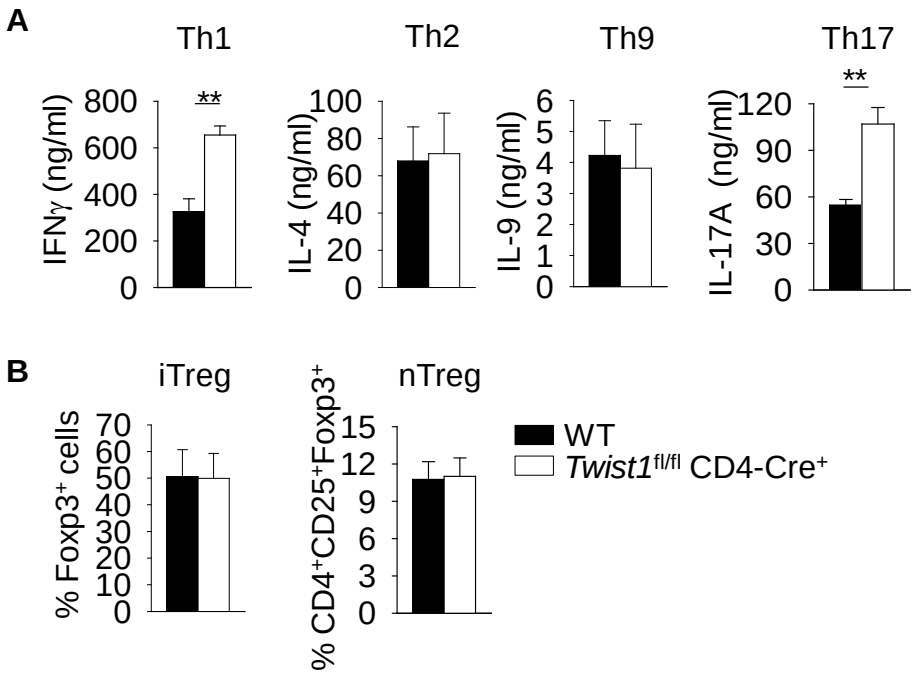


Figure 42. Characterization of T helper cell subsets in *Twist1*-mutant mice. (A) Naive WT and *Twist1*-deficient $CD4^+CD62L^+$ T cells were cultured under Th1, Th2, Th9, Th17, and regulatory T (iTreg) cells polarizing conditions. Th1, Th2, Th9, and Th17 cells were restimulated with anti-CD3 for 24 h to access cytokine production by ELISA. (B) Percentage of Foxp3 expression in inducible Treg following *in vitro* differentiation and percentage of natural Treg cells. Data are mean of four independent experiments ± S.E.M. **p<0.01

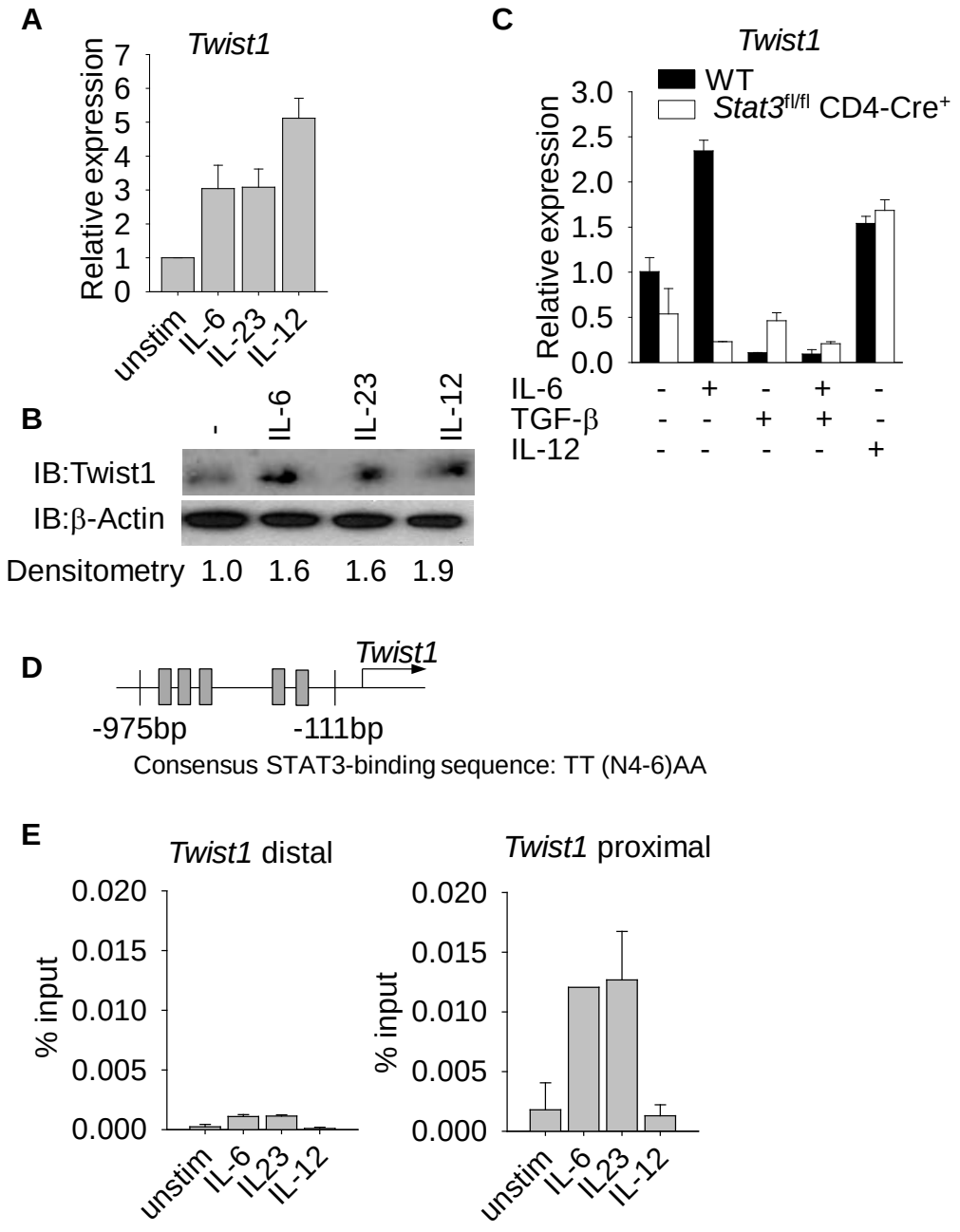


Figure 43. *Twist1* is regulated by STAT3 activating cytokines in Th17 cells. (A-B) Naive WT and *Twist1*-deficient $CD4^+CD62L^+$ T cells were cultured under Th17 polarizing conditions. On day 5, differentiated cells were rested or stimulated with IL-6, IL-23, or IL-12 for 2 h before gene expression analysis by qRT-PCR (A) and Twist1 expression by immunoblot with densitometry normalized against β-Actin (B). (C) Naïve wild type and *Stat3*-deficient $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence or absence of IL-6, TGF-β, or IL-12 and gene expression was analyzed by qRT-PCR after 3 d. (D) Schematic of the *Twist1* promoter containing STAT3 binding sites. (E) Cells

prepared as in (A) were used for ChIP analysis using STAT3 antibody. Data are mean of replicate samples ± S.D. and representative of three independent experiments with similar results.

Twist1 represses cytokine production in Th17 cells

To further demonstrate the ability of Twist1 to regulate the Th17 phenotype, we ectopically expressed Twist1 in Th17 cells and examined cytokine production. Ectopic *Twist1* expression in Th17 cells resulted in decreased IL-17A, IL-17F, and IFNγ production compared to control cells (Figure 44A-B). Consistent with observations in Figure 42A, *Twist1*-deficient Th17 cells produced more IL-17A, IL-17F, GM-CSF, and IFNγ than wild type cells, although IL-10 production was similar (Figure 44C-D).

Since TGF-β inhibits *Twist1* expression, and Th17 differentiation in the presence of IL-23 and absence of TGF-β results in highly encephalitogenic Th17 cells (Ghoreschi et al., 2010), we compared the differentiation of wild type and *Twist1*-deficient $CD4^+$ T cells in the presence or absence of TGF-β in Th17 cell culture conditions. Th17 cells derived in the absence of TGF-β had increased *Twist1* gene expression, compared to those derived under conventional Th17 conditions (Figure 44E). Moreover, *Twist1*-deficient Th17 cells derived in the absence of TGF-β had increased secretion of IL-17A, GM-CSF, and IFNγ (Figure 44F).

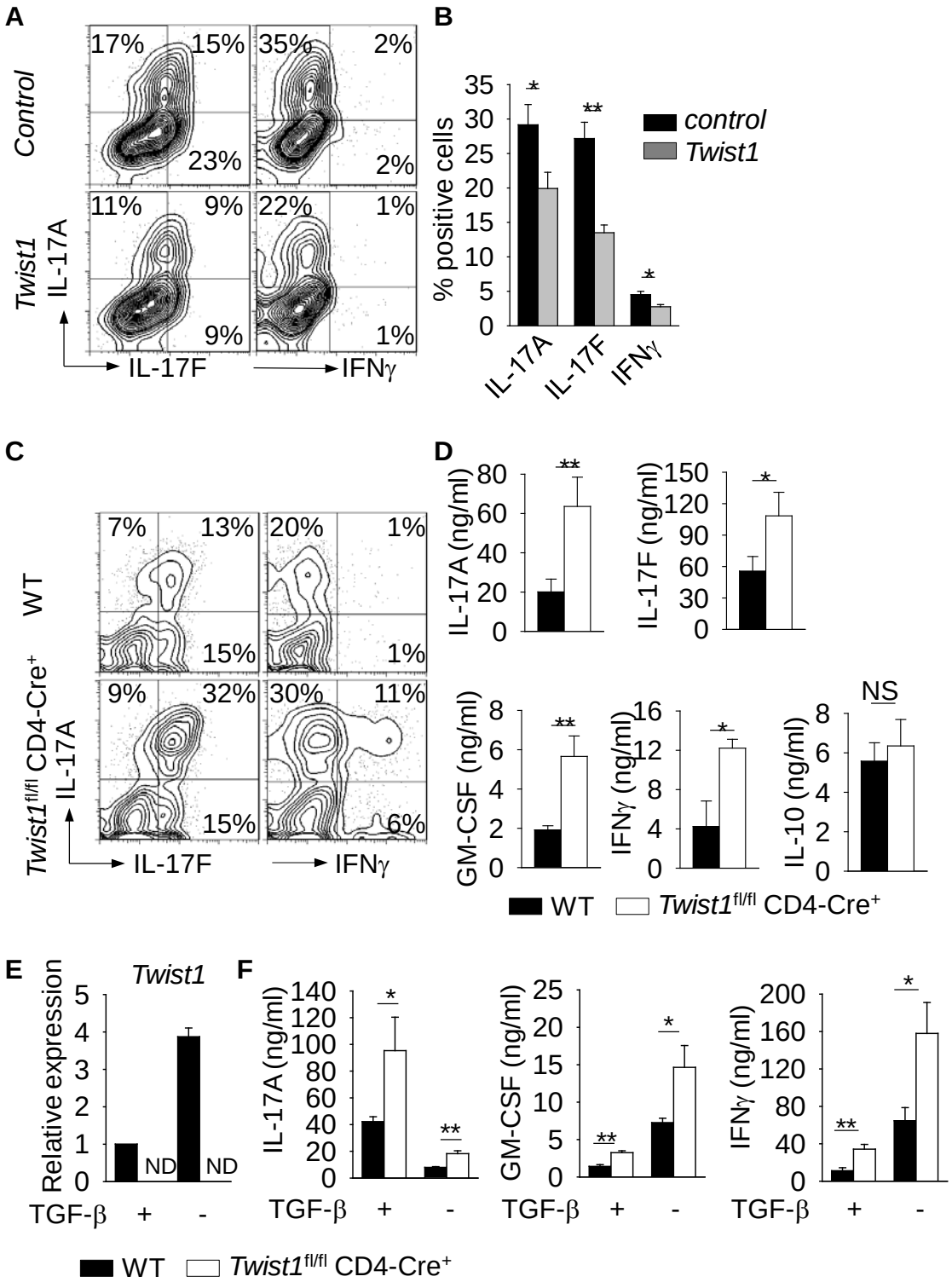


Figure 44. Twist1 suppresses cytokine production in Th17 cells. (A-B) Naïve $CD4^+CD62L^+$ cells were isolated from WT mice and differentiated under Th17 culture conditions. On day 2, cells were transduced with either control or Twist1-GFP (Twist1)-expressing retrovirus. On day 5, cells were stimulated with PMA and ionomycin for 6 h before intracellular staining (ICS) for cytokine production

(A) with the average of percent positive cells shown in (B). Data are gated on GFP^+ cells. (C-D) Differentiated WT and *Twist1*-deficient Th17 cells were stimulated with PMA and ionomycin for 6 h before ICS analysis (C) or anti-CD3 for 24 h for cytokine production measurement using ELISA (D). (E-F) Naïve wild type and *Twist1*-deficient $CD4^+$ T cells were cultured under Th17 polarizing conditions with or without TGF-β. On day 5, cells were left unstimulated for gene expression analysis by qRT-PCR (E) or reactivated with anti-CD3 for 24 h to assess cytokine production by ELISA (F). Data are mean of four to five independent experiments ± S.D (A-D, F), or are mean of replicate samples ± S.D. and representative of three independent experiments with similar results (E). *$p<0.05$, **$p<0.01$. N.S., not significant. N.D., not detectable

To demonstrate that Twist1 function is conserved in human Th17 cells, naïve $CD4^+$ T cells isolated from the peripheral blood of healthy individuals were differentiated into Th17 cells, transfected with siRNA encoding *TWIST1*, and assessed for gene expression. Knockdown of *TWIST1* in human Th17 cells resulted in increased *IL17A, IL17F,* and *IFNG* gene expression (Figure 45A). *TWIST1* knockdown in human Th17 cells also resulted in increased expression of the Th17-inducing genes *RORC, BATF,* and *MAF,* compared to control cells (Figure 45A). Messenger RNA for *Il17a, Rorc, Batf, and Maf* was similarly increased in *Twist1*-deficient Th17 cells compared to wild type cells (Figure 45B). Since each of these genes is a direct target of STAT3 (Bauquet et al., 2009; Brustle et al., 2007; Durant et al., 2010; Ivanov et al., 2006; Schraml et al., 2009), we tested whether binding of STAT3 to the promoters of these genes was altered. Consistent with gene expression data, we also observed increased STAT3 binding to gene promoters in *Twist1*-deficient Th17 cells compared to wild type cells (Figure 45C). Together, these data demonstrate that Twist1 impairs differentiation of mouse and human Th17 cells cultured in the presence or absence of TGF-β.

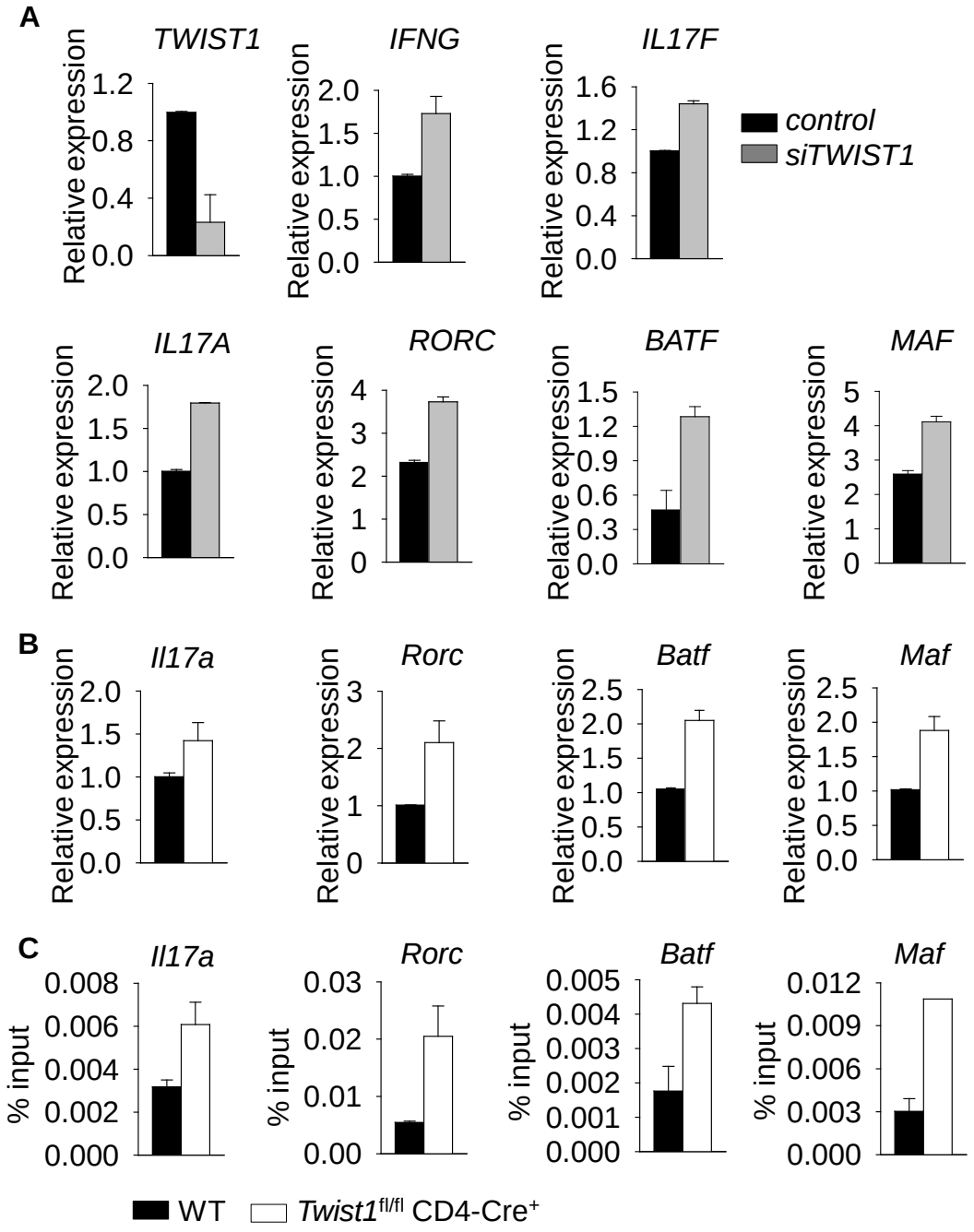


Figure 45. Gene expression in mouse and human Th17 cells in the absence of *Twist1.* (A) Naïve $CD4^+CD62L^+$ cells were isolated from PBMCs and differentiated under Th17 culture conditions. On day 5, cells were transfected with control or siRNA targeting *TWIST1,* rested overnight, and stimulated with anti-CD3 to assess gene expression by qRT-PCR. (B-C) Differentiated WT and *Twist1*-deficient Th17 cells were used for gene expression analysis by qRT-PCR before (*Rorc, Batf, and Maf*) or after (*Il17a*) 6 h anti-CD3 stimulation (B) and ChIP analysis using STAT3 antibody (C). Data are mean of replicate samples ± S.D. and representative of three independent experiments with similar results (A-C).

The Twist1-E47 interaction could potentially regulate cytokine production in Th17 cells

Twist1 has been shown to form a dimer complex with E47 protein, which is inhibited by the DNA-binding inhibitor Id3 (Castanon et al., 2001; Firulli et al., 2005; Hayashi et al., 2007). Interestingly, *Id3*-deficient mice have a defect in regulatory T cell generation and an enhancement in Th17 differentiation linked to the ability of E47 to induce *Rorc* expression (Maruyama et al., 2011). Maruyama et al. suggested that the ability of E47 to transactivate *Rorc* expression might require other factors downstream of IL-6 (Maruyama et al., 2011). In DAPA (Figure 36A) using nuclear *e*xtracts from WT and *Twist1*-deficient Th1 cells were incubated with biotinylated oligonucleotides containing a Twist1-binding sequence, we observed the binding of both Twist1 and E47 to oligonucleotides in WT samples (Figure 46A). However, E47 binding to oligonucleotides was significantly reduced in *Twist1*-deficient Th1 cells suggesting Twist1 might be required for E47 binding (Figure 46A). In addition, we detected increased E47 binding at the *Il17a* and *Rorc* promoters in *Twist1*-deficient Th17 cells compared to WT cells correlating with increased *Rorc* and *Il17a* expression (Figure 46B-C). There was no change in either *Tcfe2a* (encoding E47) or *Id3* expression (Figure 46C). The results suggested that the Twist1-E47 association could play a potential role in the regulation of Th17 cell differentiation.

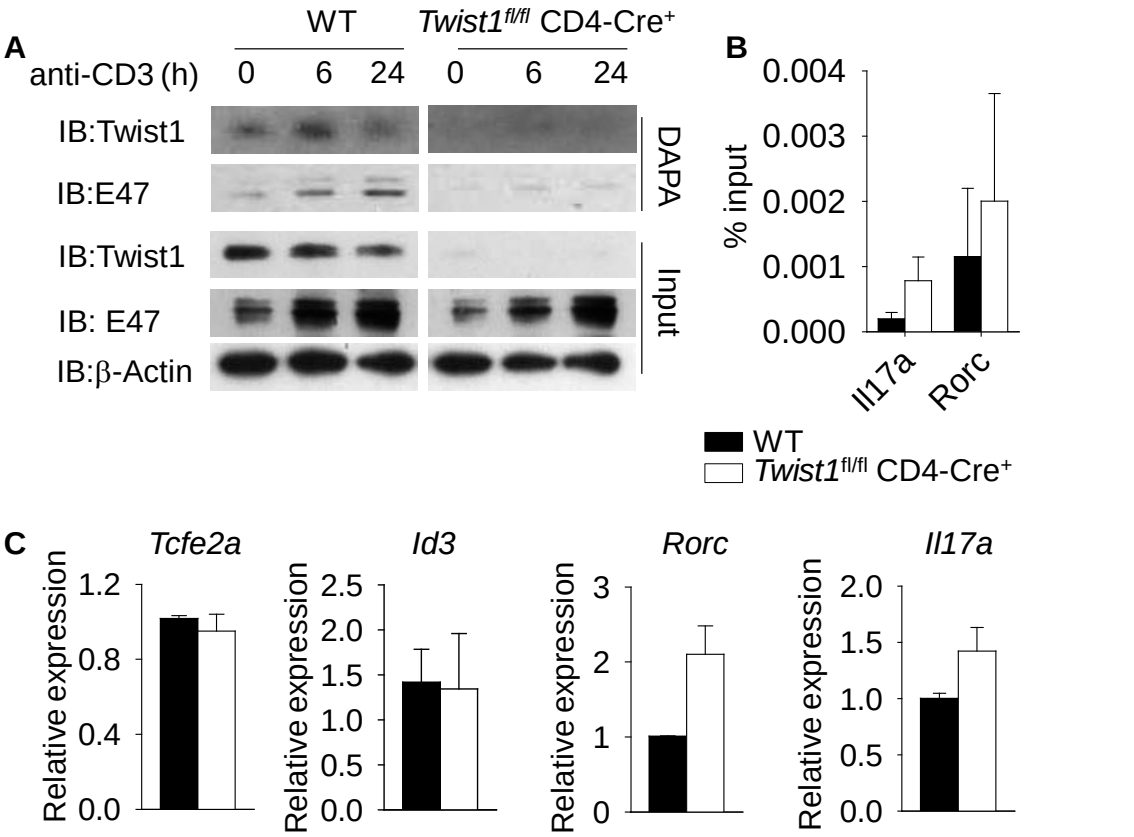


Figure 46. Twist1 interacts with E47 in Th1 cells. (A) Naïve $CD4^{+}CD62L^{+}$ WT or *Twist1*$^{fl/fl}$ CD4-Cre^{+} T cells were differentiated under Th1 culture conditions. Nuclear extracts from resting and activated WT and *Twist1*-deficient Th1 cells were incubated with biotinylated oligonucleotides containing Twist1-specific binding site and blotted for indicated proteins. (B-C) Naïve $CD4^{+}CD62L^{+}$ cells were isolated from WT and *Twist1*-mutant mice and differentiated under Th17 culture conditions. Day 5 differentiated Th17 cells were used for ChIP analysis at the *Il17a* and *Rorc* promoters using E47 antibody (B) or assessing gene expression by qRT-PCR before (*Tcfe2a, Id3* and *Rorc*) or after (*Il17a)* anti-CD3 stimulation (C). Data are mean of replicate samples ± S.D. and representative of three independent experiments with similar results.

Twist1 impairs IL-6-STAT3 signaling by repressing Il6ra expression

Twist1-deficiency resulted in increased binding of STAT3 to Th17 target genes, and the balance between STAT3 and STAT5 signaling is crucial in regulating Th17 cell differentiation (Yang et al., 2011). We hypothesized that Twist1 was altering cytokine signaling, and investigated the kinetics of phospho-STAT3 and

phospho-STAT5 during Th17 differentiation using wild type and *Twist1*-deficient naïve $CD4^+$T cells. The frequency of phospho-STAT3 was higher in *Twist1*-deficient Th17 cells on day 2 and day 3 compared to wild type cells, although phospho-STAT5 was comparable between the two cell types (Figure 47A). The increase in phospho-STAT3 but not phospho-STAT5 in *Twist1*-deficient Th17 cells correlates with higher IL-6Rα expression but similar IL-2Rα expression on days 2 and 3 compared to wild type cells (Figure 47B-C). *Il6st,* the gp130 chain of IL-6 receptor, and *Stat3* expression were similar in both wild type and *Twist1*-deficient Th17 cells, and *Il6ra* mRNA reflected the same pattern as protein expression (Figure 47C). Since IL-6Rα expression was increased at early time points, we examined cytokine production from Th17 cells during differentiation and observed the same increases of cytokine production from T cells that lack expression of Twist1 (Figure 47D).

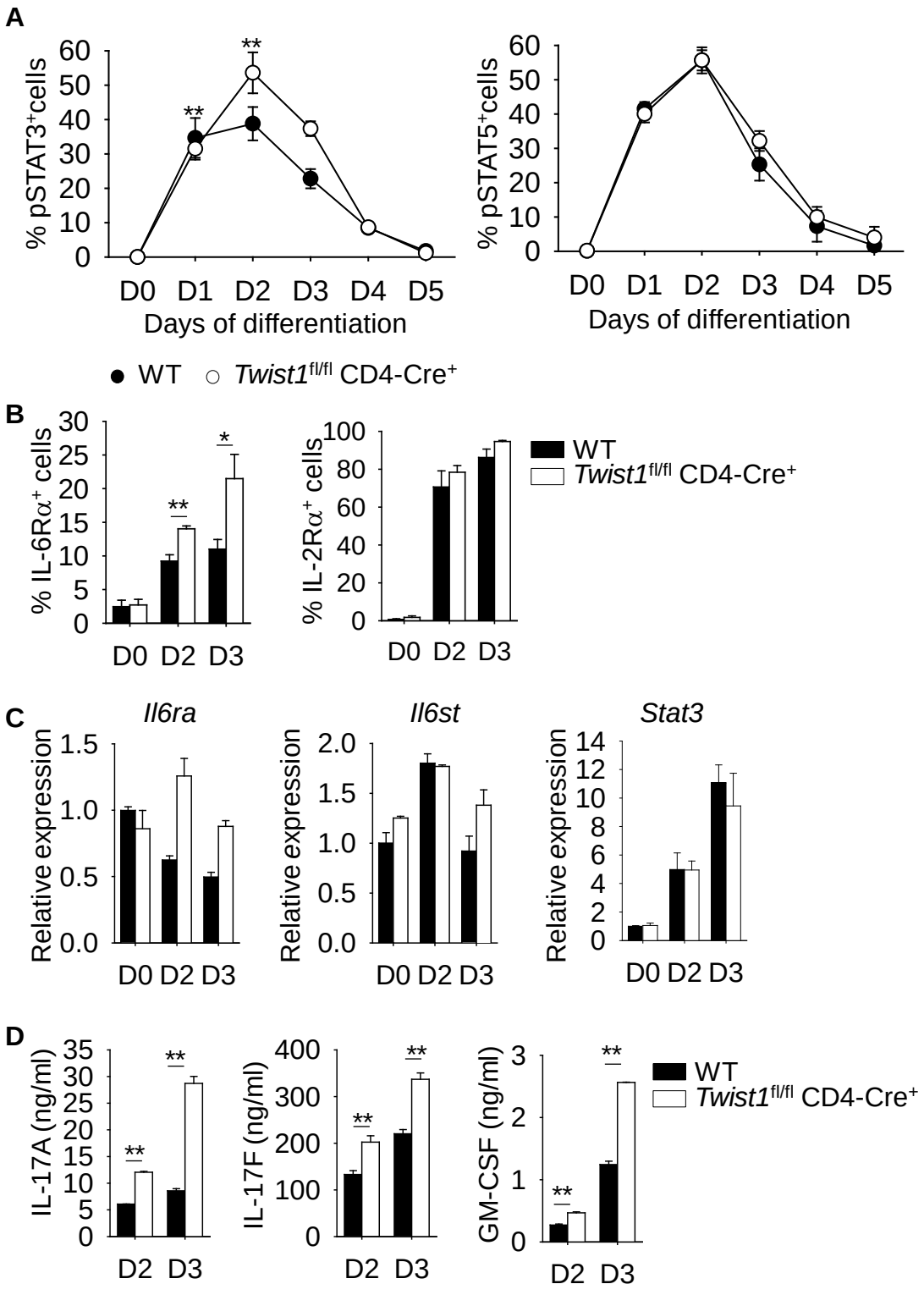


Figure 47. Twist1 impairs IL-6-STAT3 signaling in Th17 cells. (A-D) Naïve $CD4^+CD62L^+$ T cells were isolated from WT and *Twist1*-mutant mice and differentiated under Th17-polarizing conditions. The levels of phospho-STAT3 (pSTAT3) and phospho-STAT5 (pSTAT5) were measured by ICS each day (A).

T cells cultured under Th17 conditions for 2 or 3 days were used for surface marker analysis (B), gene expression analysis by qRT-PCR (C), or analysis of cytokine production after anti-CD3 stimulation (D). Data are mean of four independent experiments ± S.D. (A-B, D), or are mean of replicate samples ± S.D. and representative of three independent experiments with similar results (C). *p<0.05, **p<0.01

To test the requirement for STAT3 in this process, we treated wild type and *Twist1*-deficient Th17 cultures with an inhibitor of STAT3 activation during differentiation. Inclusion of the inhibitor decreased STAT3 phosphorylation at days 2, 3, and 5 of cultured wild type and *Twist1*-deficient T cells (Figure 48A). There was a corresponding dose-dependent decrease in IL-17 production at all time points (Figure 48B), with lower doses of the inhibitor resulting in similar IL-17 production in *Twist1*-deficient Th17 cells compared to untreated wild type cells (Figure 48B). Thus, STAT3 is required for the increased IL-17 production from *Twist1*-deficient Th17 cells.

Since IL-21 induced phospho-STAT3 and *Twist1* expression (Figure 49A), we wanted to determine whether Twist1 also has a negative effect on IL-21-STAT3 signaling. IL-21 was used instead of IL-6 to derive Th17 cultures with wild type and *Twist1*-deficient naïve $CD4^+$ T cells and similar analyses were performed. The level of phospho-STAT3 in *Twist1*-deficient Th17 cells was the same on day 2 and modestly increased on day 3 compared to WT cells, and the level of phospho-STAT5 was comparable between the two cell types (Figure 49B). *Il6ra* and transcription factor (*Rorc, Batf,* and *Runx1*) expression and IL-17A production were similar between wild type and *Twist1*-deficient Th17 cells

derived with IL-21 (Figure 49C-E). These results suggested that Twist1 specifically targets IL-6-STAT3 signaling in Th17 cells.

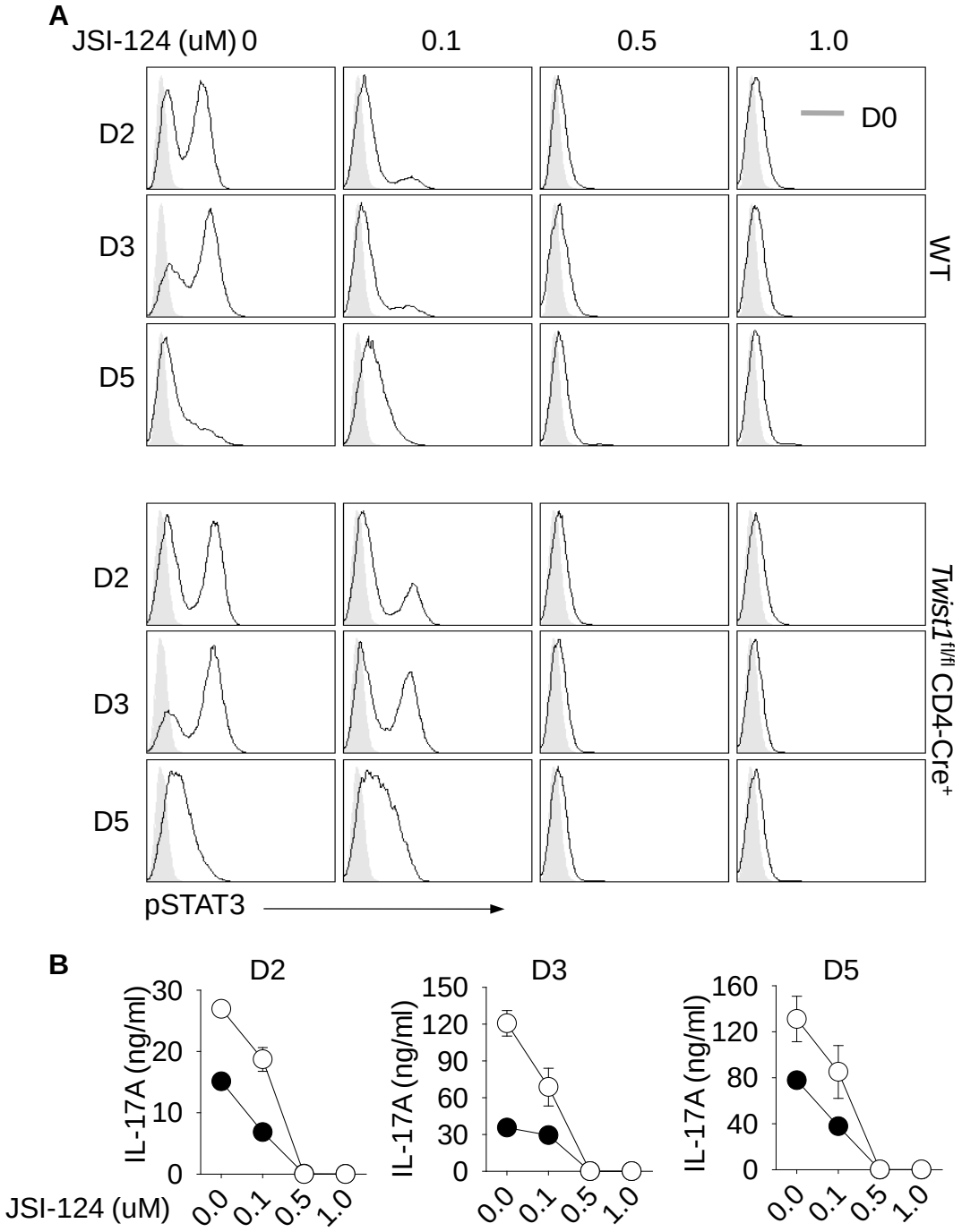


Figure 48. Inhibition of STAT3 activation reduced IL-17A production in *Twist1*-deficient Th17 cells. (A-B) Naïve $CD4^{+}CD62L^{+}$ T cells were isolated from WT and *Twist1*-mutant mice and differentiated under Th17-polarizing conditions with increased doses of STAT3 inhibitor (JSI-124). Cells were

harvested on days 2, 3, and 5 and used to measure the level of phospho-STAT3 (pSTAT3) by ICS (A) or restimulated with anti-CD3 to assess cytokine production by ELISA (B). Data are mean of replicate samples ± S.D. and representative of three independent experiments with similar results.

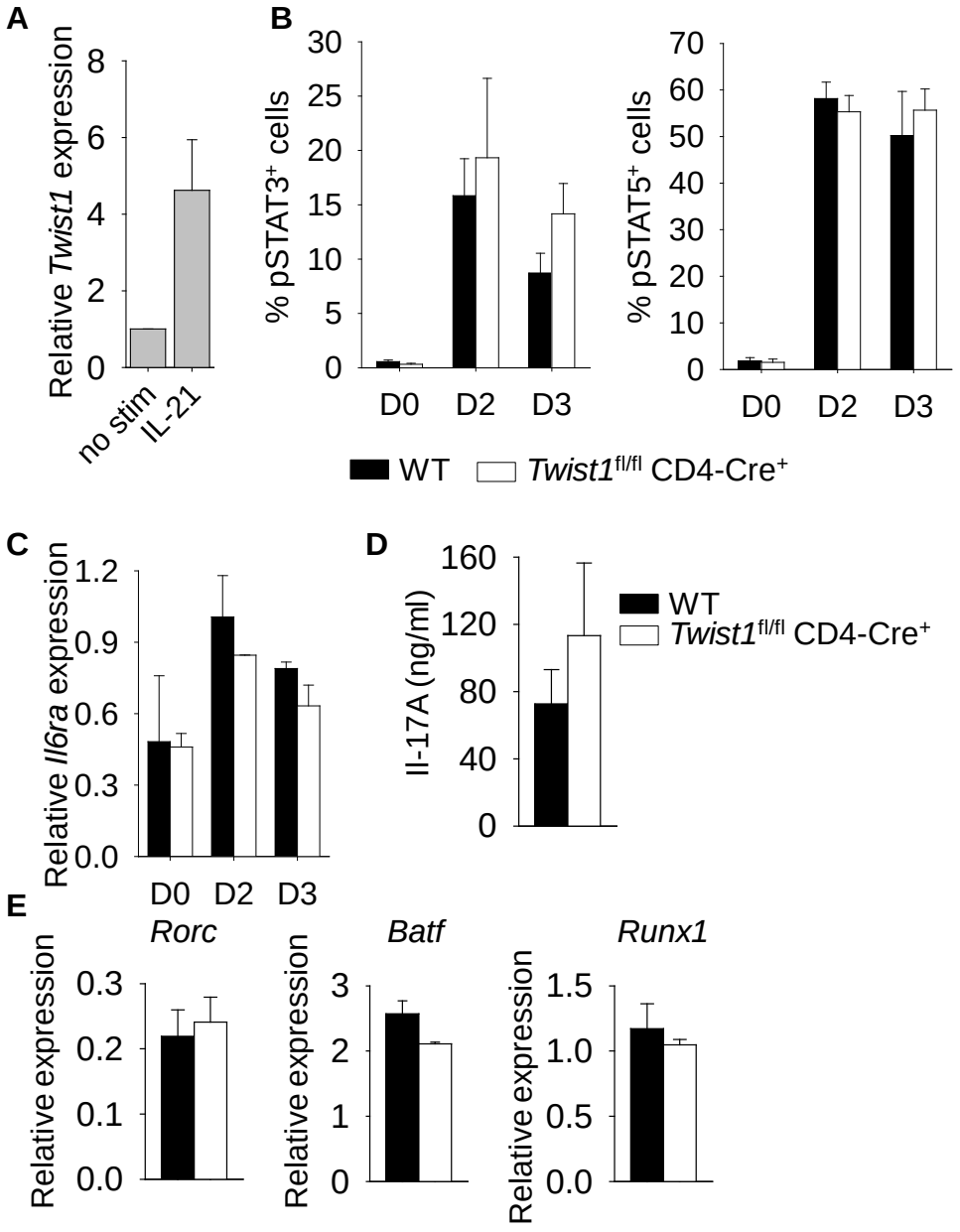


Figure 49. Twist1 does not affect IL-21-STAT3 signaling. (A) Naïve $CD4^+CD62L^+$ T cells were isolated from wild type and *Twist1*-mutant mice and differentiated under Th17 polarizing conditions. On day 5, cells were rested or stimulated with IL21 for 2 h before gene expression analysis by qRT-PCR. (B-E) Naïve $CD4^+CD62L^+$ T cells were isolated from wild type and *Twist1*-mutant mice and differentiated under Th17 polarizing conditions using IL-21 instead of IL-6. On days 2 and 3, cells were harvested, stained for pSTAT3 and pSTAT5 by ICS

(B), used for gene expression analysis by qRT-PCR (C). On day 5, cells were harvested, restimulated with anti-CD3 for 24 h and cytokine production was measured by ELISA (D) or rested for gene expression assessment by qRT-PCR (E). Data are mean of three independent experiments ± S.D (B-D), or are mean of replicate samples ± S.D. and representative of three independent experiments with similar results (A, E).

We next wanted to determine whether Twist1 represses *Il6ra* expression by directly binding to the E-box sites in the *Il6ra* promoter that is conserved in mouse and human genes (Figure 50A). When ChIP was performed using wild type and *Twist1*-deficient Th17 cells, the binding of Twist1 to the promoter of *Il6ra* was observed by days 2 and 3 in wild type cell cultures, with the peak of binding following the peak of Twist1 expression (Figure 50B-C). To further demonstrate the direct consequences of Twist1 binding to the *Il6ra* promoter, Jurkat T cells were transfected with an *IL6RA* luciferase reporter and a plasmid encoding Twist1. Notably, Twist1 repressed the transcriptional activity of the *IL6RA* promoter, but not an NFAT reporter, in a dose-dependent manner (Figure 50D).

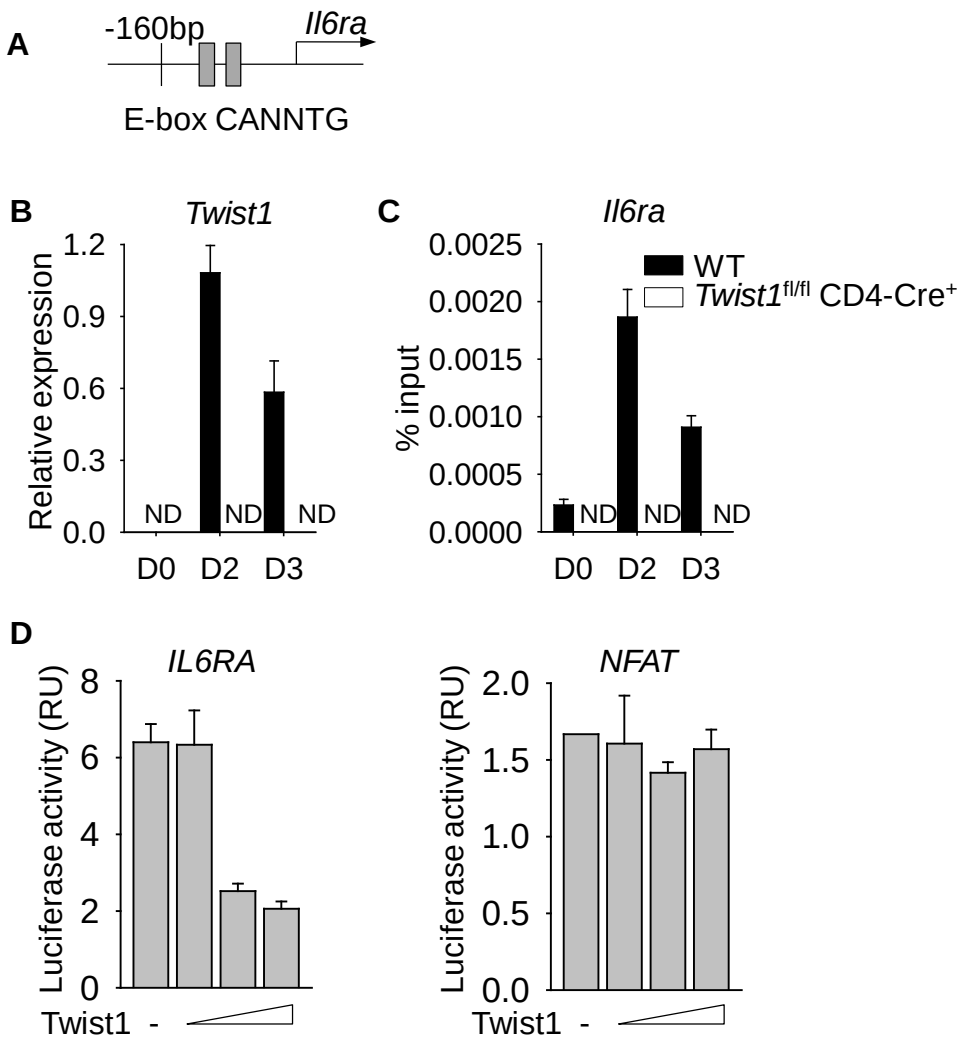


Figure 50. Twist1 represses *Il6ra* transcription. (A) Schematic of the *Il6ra* promoter containing Twist1 binding sites. (B-D) Naïve $CD4^+CD62L^+$ T cells were isolated from WT and *Twist1*-mutant mice and differentiated under Th17-polarizing conditions. T cells cultured under Th17 conditions for 2 or 3 days were used for gene expression analysis by qRT-PCR (B), or used for ChIP analysis using Twist1 antibody (C). (D) Luciferase activity in Jurkat T cells transfected with various concentrations of plasmid encoding Twist1 along with *IL6RA* or *NFAT* luciferase reporter, then activated for 6 h with PMA and ionomycin. Data are representative of three independent experiments with similar results. N.D., not detectable

Mice with Twist1-deficient T cells display an early onset of MOG-induced EAE

Although Th1 and Th17 cells have been demonstrated to be crucial in mediating the development of experimental autoimmune encephalomyelitis (EAE), the role of IFNγ and IL-17 in EAE disease has been controversial (Becher and Segal, 2011; Pierson et al., 2012). Recently, GM-CSF, produced by Th1 and Th17 cells, has been identified as a contributor to the development of EAE (Codarri et al., 2011; El-Behi et al., 2011). As Twist1 negatively regulates IL-17, GM-CSF, and IFNγ in Th17 cells (Figure 44) and IFNγ in Th1 cells (Figure 30) (Pham et al., 2012), we wanted to compare the development of myelin oligodendrocyte glycoprotein (MOG) peptide-induced EAE in wild type and *Twist1*$^{fl/fl}$ *CD4*-Cre^{+} mice. *Twist1*$^{fl/fl}$ CD4-Cre^{+} mice manifested earlier onset of MOG-induced EAE than wild type mice, although maximal severity and recovery were similar (Figure 51A). Increased disease resulted in a 26% increase in the area under the mean clinical disease score curve of *Twist1*$^{fl/fl}$ CD4-Cre^{+} mice, compared to control mice. The number of days with a mean clinical score greater than one was an average of 16.5 for control mice and 21 for *Twist1*$^{fl/fl}$ CD4-Cre^{+} mice, an increase of 27%. Earlier disease development correlated with an increase in CD4^{+}IL-17A^{+}, CD4^{+}IFNγ^{+}, and CD4^{+}IL-17A^{+}IFNγ^{+} mononuclear cells isolated from the brain of *Twist1*-mutant mice compared to wild type mice at day 12 (Figure 51B-C). In addition, MOG-stimulated *Twist1*-deficient splenocytes produced significantly more IL-17, GM-CSF, and IFNγ compared to wild type cells (Figure 51D). The earlier onset of MOG-induced EAE in *Twist1*-mutant mice is not likely due to a defect in regulatory T cells since *Twist1*-mutant mice have percentages of Treg

cells *in vivo*, and *in vitro* development of Treg cells, that are comparable to wild type mice (Figure 42B). Together, these data suggest that Twist1 limits the development of inflammatory T cell subsets and autoimmune disease.

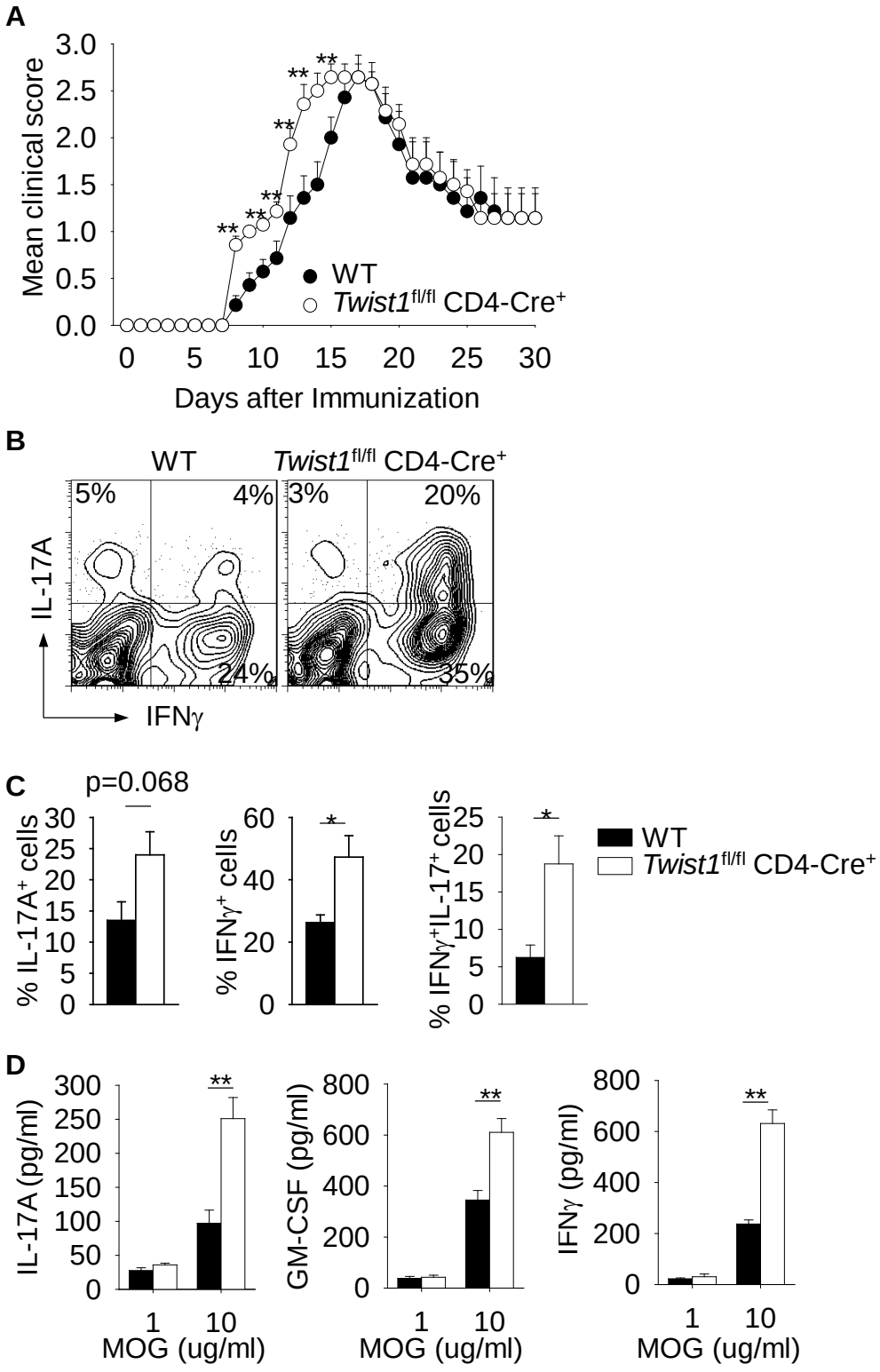


Figure 51. Early onset of EAE in the absence of *Twist1*. (A-D) WT and *Twist1*-mutant mice were immunized with MOGp(35-55) to induce EAE. Mean clinical score in MOG-induced EAE disease (A). On day 12, mononuclear cells

were isolated from brain and stimulated with PMA and ionomycin for 6 h to measure cytokine production by ICS (gated on $CD4^+$ T cells) (B,C) or splenocytes were stimulated with MOG peptide for 48 h and cytokine production was assessed by ELISA (D). Data are mean ± S.E.M. of 7 mice per group (A) or 4 mice per group (B-D) and representative of two independent experiments with similar results. *p<0.05, **p<0.01

Twist1 limits T follicular helper cell development

In the previous section, we demonstrated that Twist1 impacts IL-6 signaling by repressing *Il6ra* gene expression. In addition, it has been shown that IL-6-induced STAT3 signaling is required for Tfh development, we wanted to determine if Twist1-deficiency in T cells affected Tfh generation. Twist1 is expressed at greater amounts in Tfh cells ($CD4^+CD44^+CXCR5^+PD\text{-}1^+$) than in non-Tfh effector cells ($CD4^+CD44^+CXCR5^-PD\text{-}1^-$) or naïve T cells ($CD4^+CD44^-CD62L^+$) (Figure 52).

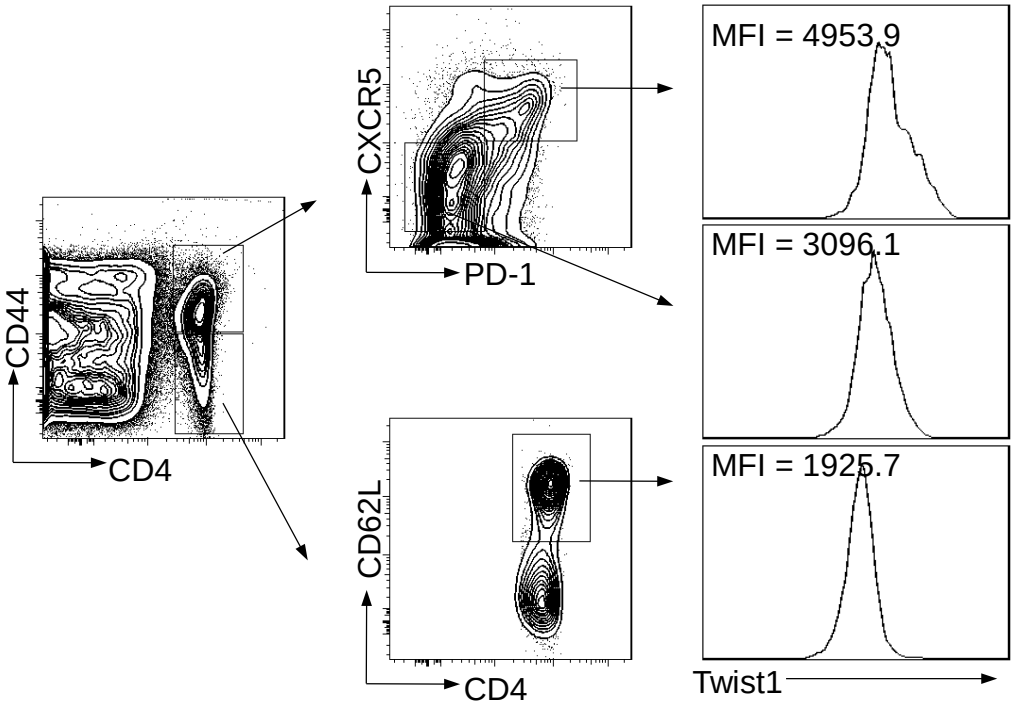


Figure 52. Twist1 expression in T follicular helper cells. Twist1 intracellular staining in Tfh cells ($CD4^+CD44^{hi}CXCR5^+PD\text{-}1^+$) compared to effector T cells ($CD4^+CD44^{hi}CXCR5^-PD\text{-}1^-$) and naïve T cells ($CD4^+CD44^{lo}CD62L^{hi}$) in day 9 SRBC-immunized WT mice.

We initially examined Tfh development in mice with EAE. Following immunization with MOG peptide, splenocytes from *Twist1*$^{fl/fl}$ CD4-Cre$^+$ mice had significantly more Tfh cells (defined as $CD4^+CXCR5^+PD\text{-}1^{hi}ICOS^+$) than wild type splenocytes (Figure 53A-B). To further explore the ability of Twist1 to regulate Tfh development, we immunized wild type and *Twist1*$^{fl/fl}$ CD4-Cre$^+$ mice with sheep red blood cells (SRBCs). As observed following MOG peptide immunization, SRBC immunization resulted in increased Tfh cell development in *Twist1*$^{fl/fl}$ CD4-Cre$^+$ mice, compared to wild type mice (Figure 53C-D). Percentages of Tfh cells in the absence of Twist1 were similarly increased defining cells with either ICOS

or Bcl-6 expression (Figure 53C-D). Moreover, in the absence of Twist1 there was an increase in the percentages of $CD4^{+}CXCR5^{+}PD\text{-}1^{hi}$ cells that were phospho-STAT3-positive and IL-6Rα-positive, and the amount (MFI) of IL-6Rα expression (Figure 53C-D). We then sorted Tfh and non-Tfh cells from SRBC-immunized wild type and *Twist1*$^{fl/fl}$ *CD4*-Cre^{+} mice to examine changes in gene expression following normalization for the increased Tfh cell number in the absence of Twist1. Consistent with flow cytometry (Figure 52), *Twist1* was expressed in greater amounts in the Tfh population than in non-Tfh cells, and no *Twist1* mRNA was detected in Cre^{+} cells (Figure 53E). We observed little difference in gene expression of *Batf, Bcl6,* and *Irf4* between wild type and *Twist1*$^{fl/fl}$ CD4-Cre^{+} cells in the non-Tfh population. In the Tfh population, the absence of Twist1 resulted in modest increases of *Batf* and *Bcl6*, and a more dramatic increase of *Irf4* (Figure 53E). Similar to observations in Th17 cells, the gene most increased in *Twist1*-deficient Tfh cells was *Il6ra* (Figure 53E).

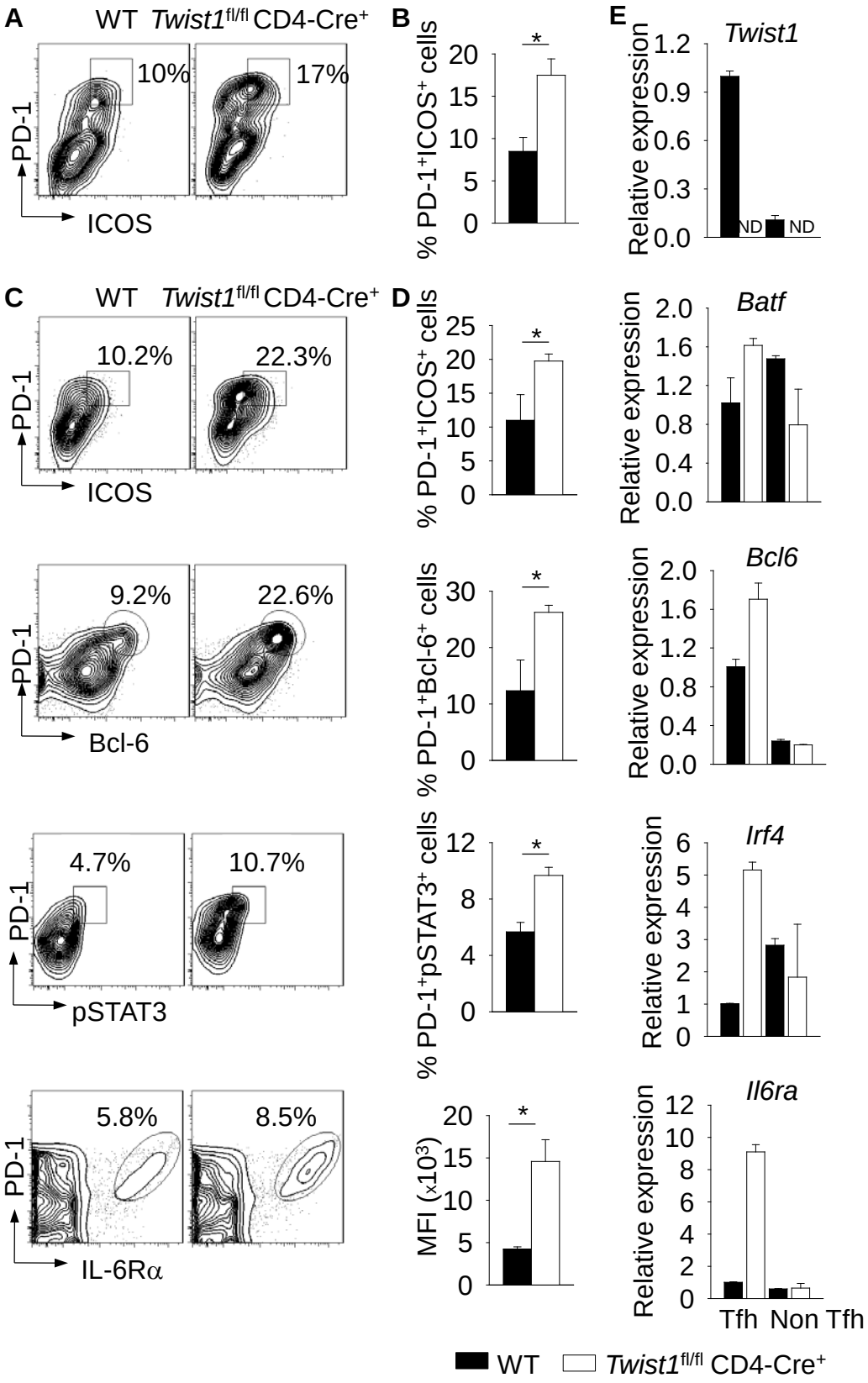

A
WT Twist1fl/fl CD4-Cre+
10%
17%
PD-1
ICOS
B
% PD-1+ICOS+ cells
C
10.2%
22.3%
D
9.2%
22.6%
Bcl-6
% PD-1+Bcl-6+ cells
4.7%
10.7%
pSTAT3
% PD-1+pSTAT3+ cells
5.8%
8.5%
IL-6Rα
MFI (x10³)
E
Twist1
Relative expression
ND
Batf
Bcl6
Irf4
Il6ra
Tfh
Non Tfh
WT
Twist1fl/fl CD4-Cre+

Figure 53. Mice with *Twist1*-deficient T cells have more T follicular helper cells. (A-B) WT and *Twist1*$^{fl/fl}$ CD4-Cre mice were immunized with MOGp(35-55) as in Figure 39. Twenty days following immunization, splenocytes were stained for T follicular helper (Tfh) cells (A) with the average percent PD-1^{+}ICOS^{+} cells in (B). (C-E) WT and *Twist1*$^{fl/fl}$CD4-Cre^{+} mice were immunized with SRBC. On day 9, splenocytes were analyzed by flow cytometry with percentages of PD-1^{+}ICOS^{+}, PD-1^{+}pSTAT3^{+}, and PD-1^{+}IL-6Rα^{+} cells indicated in (C), and the average percent positive cells shown in (D). Following immunization, cell populations were sorted for CD4^{+}CXCR5^{+}PD-1^{+}ICOS^{+} (Tfh) or CD4^{+}CXCR5^{-}PD-1^{-}ICOS^{-} (non-Tfh) and gene expression was analyzed (E). (A-D) Data are gated on CD4^{+}CXCR5^{+}. Data are mean ± S.E.M. of 4-5 mice per group and representative of two independent experiments with similar results (A-D), or are mean of replicate samples ± S.D. and representative of three independent experiments with similar results (E). *p<0.05. N.D., not detectable

We then tested whether T cells activated in the presence or absence of IL-6 (Tfh-like conditions) demonstrated Twist1-dependent regulation of Tfh genes. Addition of IL-6 to activated T cell cultures resulted in increased pSTAT3, increased STAT3 binding to the *Twist1* promoter, and increased Twist1 expression over 48 hours of culture (Figure 54A-C). Paralleling the induction of *Twist1* expression, Twist1 binding to the *Il6ra*, *Bcl6,* and *Icos* promoters was also induced by IL-6 (Figure 54D). Thus, as in Th17 cells, Twist1 is a component of a STAT3-inducible negative feedback loop in Tfh cells.

To determine the functional consequences of the increased Tfh cells that develop in mice with *Twist1*-deficient T cells we examined the development of germinal center B cells and antibody production following SRBC immunization. We observed a 3-fold increase in the percentages of germinal center B cells (defined as B220^{+}CD19^{+}Fas^{+} GL-7^{+}PNA^{+}) (Figure 55A-B). Analysis of SRBC-specific antibody production demonstrated increased serum IgG antibody titers in

Twist1$^{fl/fl}$ CD4-Cre^{+} mice, compared to wild type mice (Figure 55C). Isotype-specific analysis demonstrated greater IgG1 and IgG2a/c serum antibody titers in mice that lack Twist1 expression in T cells than in wild type cells. Thus, Twist1 limits Tfh development and humoral immunity.

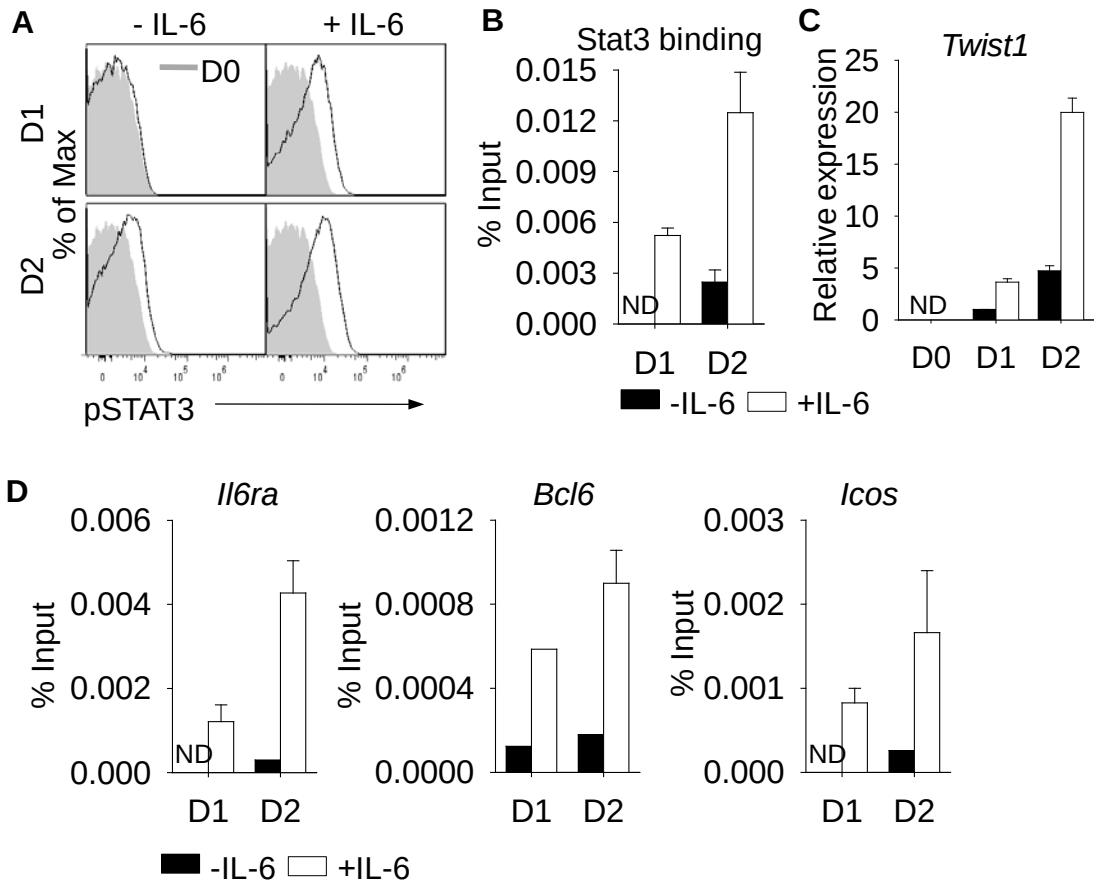


Figure 54. Twist1 binds to Tfh cell-associated genes. (A-D) Naïve WT CD4^{+}CD62L^{+} T cells were activated with or without IL-6 for 2 days. Cells were harvested daily to measure the level of phospho-STAT3 (pSTAT3) by ICS (A), analyze STAT3 binding to the *Twist1* promoter (B) or Twist1 binding to the indicated promoters (D) by ChIP assay, or to assess gene expression by qRT-PCR (C). Data are mean of replicate samples ± S.D. and representative of three independent experiments with similar results. N.D., not detectable

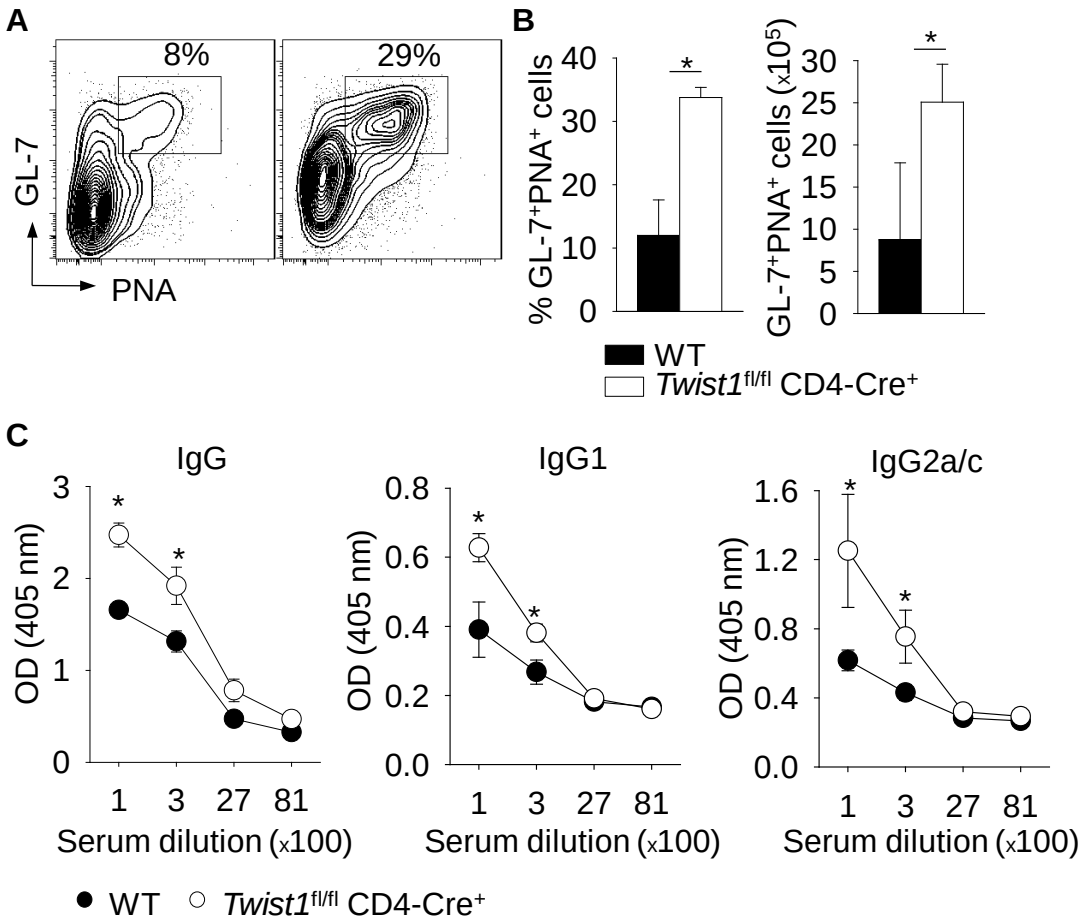


Figure 55. Twist1 represses germinal center B cells and antibody production in SRBC-immunized mice. (A-C) WT and *Twist1*$^{fl/fl}$ CD4-Cre^{+} mice were immunized with SRBC. On day 9, splenocytes were stained for germinal center B cells (A) with the average percent positive cells and total cell count shown in (B). Data are gated on B220^{+}CD19^{+}Fas^{+}. Serum from WT and *Twist1*-deficient mice was diluted and used to measure antibody titers by ELISA (C). Data are mean ± S.E.M. of 4-5 mice per group and representative of two independent experiments with similar results. *p<0.05

Part IV-Etv5 regulates T helper cell development

Etv5-deficient T cells display defects in T helper cell differentiation

Having demonstrated that Twist1 negatively regulates Th1 cell differentiation, we next wanted to study the role of Etv5, another STAT4-induced transcription factor, which has been shown to regulate IFNγ production in Th1 cells (Ouyang et al., 1999). However, the role of Etv5 in T helper cell development is still poorly understood. In order to study the role of Etv5 in T cells, we generated mice with *Etv5*-deficiency in T cells (*Etv5*$^{fl/fl}$ CD4-Cre^{+}) by mating *Etv5*$^{fl/fl}$ mice (Zhang et al., 2009) with mice carrying a CD4-Cre transgene. We confirmed the absence of *Etv5* expression in *Etv5*-deficient T cells using an antibody specific for C-terminal region (Figure 56A). Mice with *Etv5*-deficient T cells display normal lymphocyte development in thymus, spleen and lymph nodes compared to wild type littermate controls (Figure 56B). To define the role of Etv5 in T helper cell differentiation, we first examined Etv5 expression in T helper cell subsets. Etv5 expression was highest in resting and activated Th1 cells compared to other helper cell subsets at the message and protein levels (Figure 57A-B). Naïve CD4^{+} T cells from wild type and *Etv5*-mutant mice were used to generate Th1, Th2, and Th17, and cytokine production and gene expression were assessed. Interestingly, there were multiple defects in T helper cell differentiation. *Etv5*-deficient Th1 and Th2 cells produced more IFNγ and IL-4, respectively; while *Etv5*-deficient Th17 cells produced significantly less IL-17A compared to those of wild type cells (Figure 57C). Similar results were observed at the message level in *Etv5*-deficient T cells compared to wild type cells (Figure 57D). We next

examined the expression of transcription factors that are critical for the development of each subset. Surprisingly, *Gata3* and *Rorc* expression were similar between WT and *Etv5*-deficient Th2 and Th17, respectively (Figure 57E). *Etv5*-deficient Th1 cells expressed higher *Tbx21* compared to WT cells (Figure 57E).

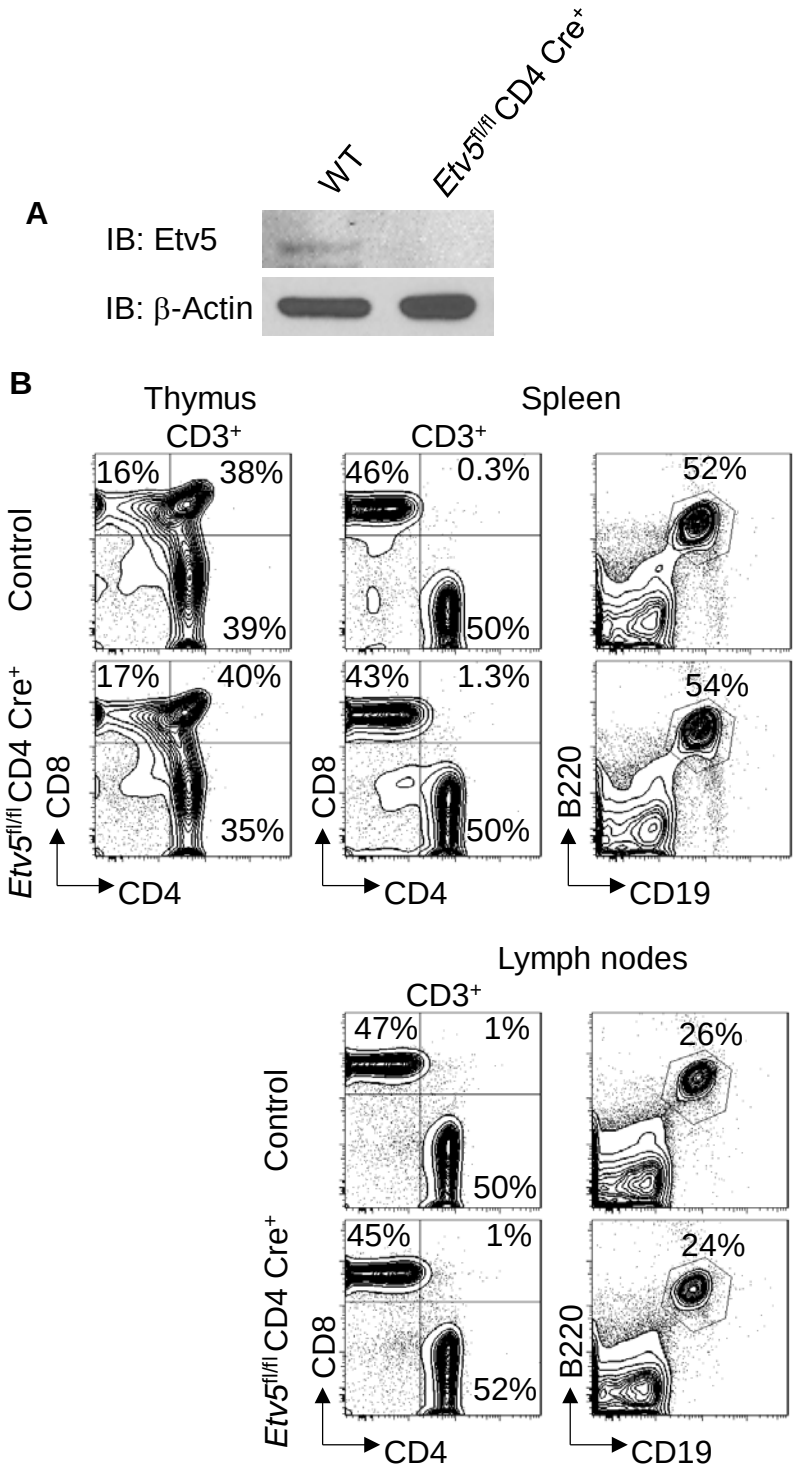


Figure 56. Characterization of mice with *Etv5*-deficient T cells. Naïve $CD4^+CD62L^+$ T cells from WT and $Etv5^{fl/fl}$ CD4-Cre^+ were activated with anti-CD3 and anti-CD28 for 5 days. Nuclear lystates were extracted and immunoblotted for Etv5 and β-Actin as a control. (B) Total cells were isolated from thymus, spleen and lymph nodes of WT and *Etv5*-mutant mice and stained for cell surface

markers. Data are representative of two independent experiments with similar results.

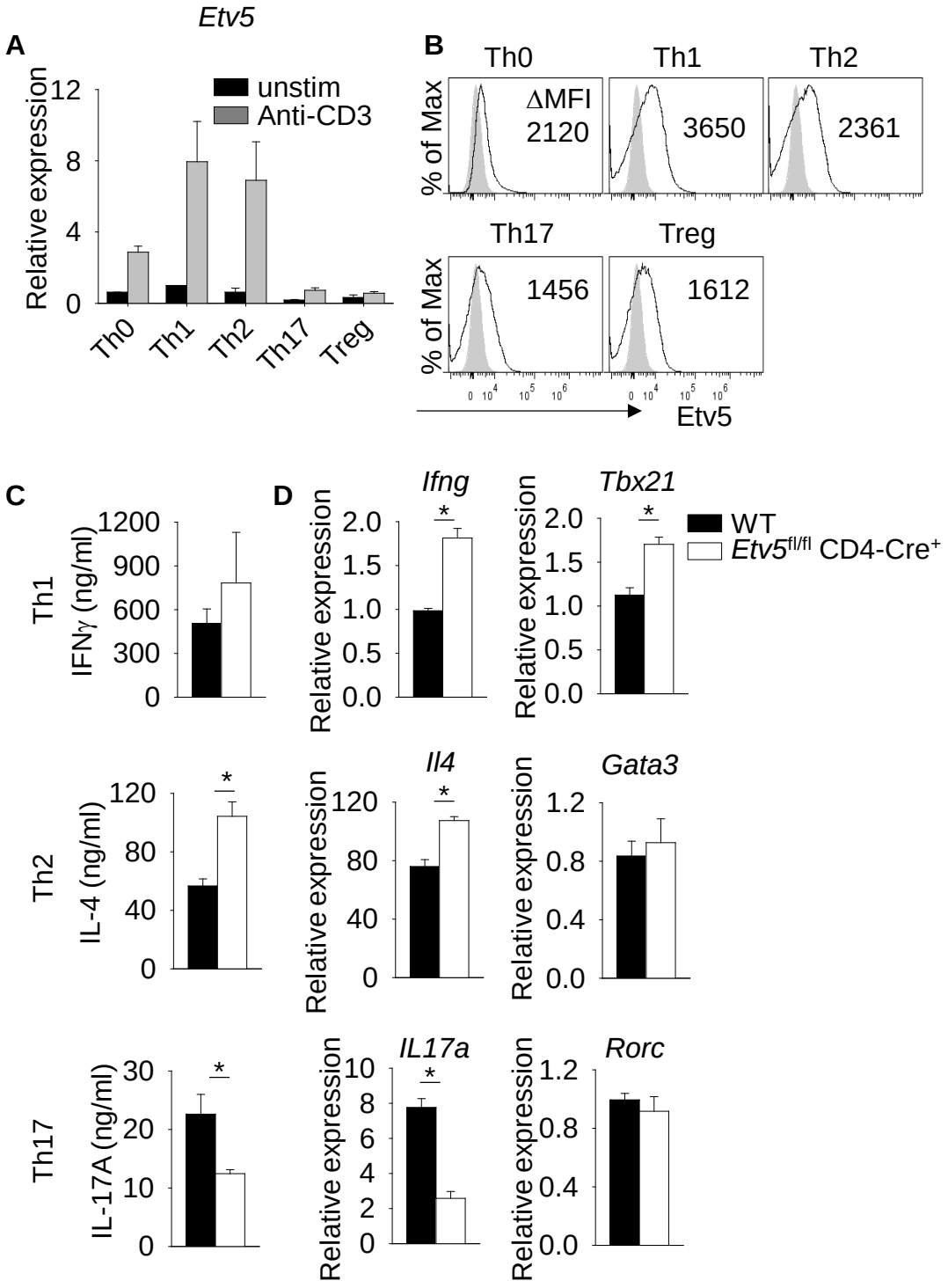


Figure 57. T helper cell differentiation in the absence of Etv5 in T cells. (A-D) Naive wild type and *Etv5*-deficient $CD4^+CD62L^+$ T cells were activated (Th0) or cultured under Th1, Th2, Th17, and regulatory T (Treg) cell polarizing conditions. Etv5 expression was measured in T helper subsets by qRT-PCR

before and after 6 h anti-CD3 stimulation (A) or by intracellular staining (ICS) (B). ΔMFI was calculated by subtracting the background from the signal of Etv5 antibody. Th0, Th1, Th2, and Th17 cells were used for assessing cytokine production by ELISA after 24 h anti-CD3 stimulation (C), and gene expression analysis before (*Tbx21, Gata3,* and *Rorc*) or after (*Ifng, Il4, and Il17a*) 6 h anti-CD3 stimulation by qRT-PCR (D). Data are gated on $CD4^+$ cells. Data are mean of four independent experiments ± S.E.M (A, C-D), or are representative of three independent experiments with similar results (B). *p<0.05

We next evaluated the percentages of *in vitro* generated (iTreg) and thymically derived natural regulatory T (nTreg) cells between WT and *Etv5*-mutant mice. The nTreg cell population (defined as $CD4^+CD25^+Foxp3^+$) in both lymph nodes and spleen was modestly but not significantly decreased in *Etv5*-mutant mice compared to WT mice (Figure 58A). Similarly, *Etv5*-deficient iTreg cells expressed less Foxp3 compared to WT cells at both message and protein levels (Figure 58B). Thus, mice that lack *Etv5* expression in T cells display multiple defects in T helper cell differentiation *in vitro*.

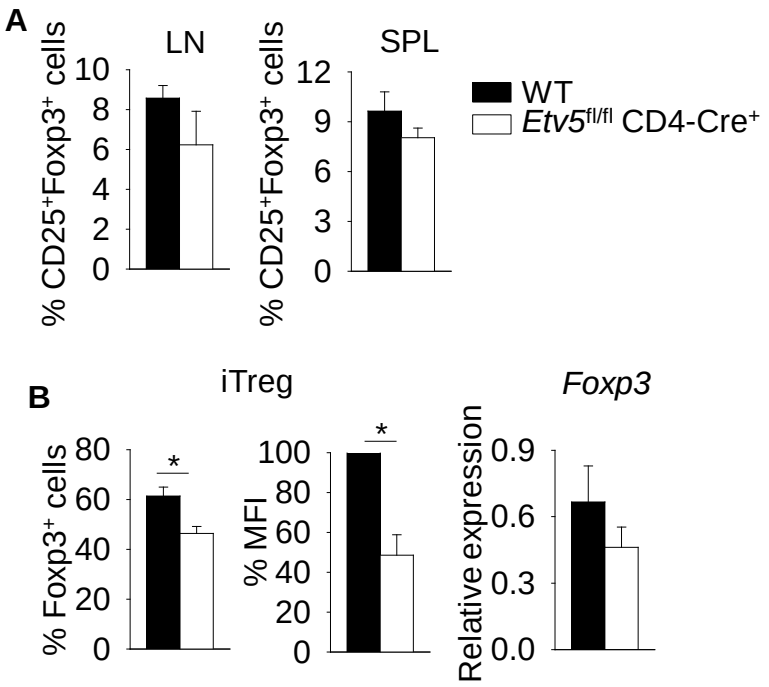


Figure 58. Regulatory T cell population in *Etv5*-mutant mice. (A-B) Cells isolated from lymph nodes (LN) and spleens (SPL) with indicated genotypes or day 5 *in vitro* generated regulatory T (Treg) cells were assessed for natural (nTreg, $CD4^+CD25^+Foxp3^+$) (A) or inducible ($Foxp3^+$) Treg cells by intracellular staining and qRT-PCR for *Foxp3* gene expression (B). Data are gated on $CD4^+$ cells. Data are mean of four independent experiments ± S.E.M. *p<0.05

Mice with Etv5-deficient T cells had reduced allergic inflammation

The data suggested that Etv5 plays a repressive role in Th1 and Th2 cell differentiation while promoting Th17 cell differentiation (Figure 57). To distinguish the importance of these effects *in vivo*, we performed house dust mite (HDM)-induced allergic inflammation in the lung, a model that is Th2- and Th17-dependent (Wilson et al., 2009b). We sensitized and challenged mice with intranasal HDM antigen for five weeks and assessed the inflammation. Interestingly, *Etv5*-mutant mice had significantly decreased numbers of total cells and specific cell populations in lung compared to wild type mice (Figure 59A). Analysis of the cellular composition of bronchioalveolar lavage (BAL) fluid

showed similar results (Figure 59B). Histological examination also demonstrated diminished inflammatory cell infiltrates in the lung, and decreased mucus production in the airways of *Etv5*-mutant mice compared to control mice (Figure 59C). Given that we observed the greatest fold reduction (> 5-fold) of the infiltrating neutrophils into the lung and BAL fluid in *Etv5*-mutant mice compared to wild type mice among all cell types (Figure 59A-B), we wanted to examine the expression of neutrophil attractant chemokines in total lung RNA. Consistent with the decrease in neutrophils, expression of *Cxcl2,* an IL-17-induced gene, was reduced in lung tissue from *Etv5*-mutant mice compared to control lung tissue (Figure 59D). In contrast there was no difference in *Cxcl1* expression (Figure 59D).

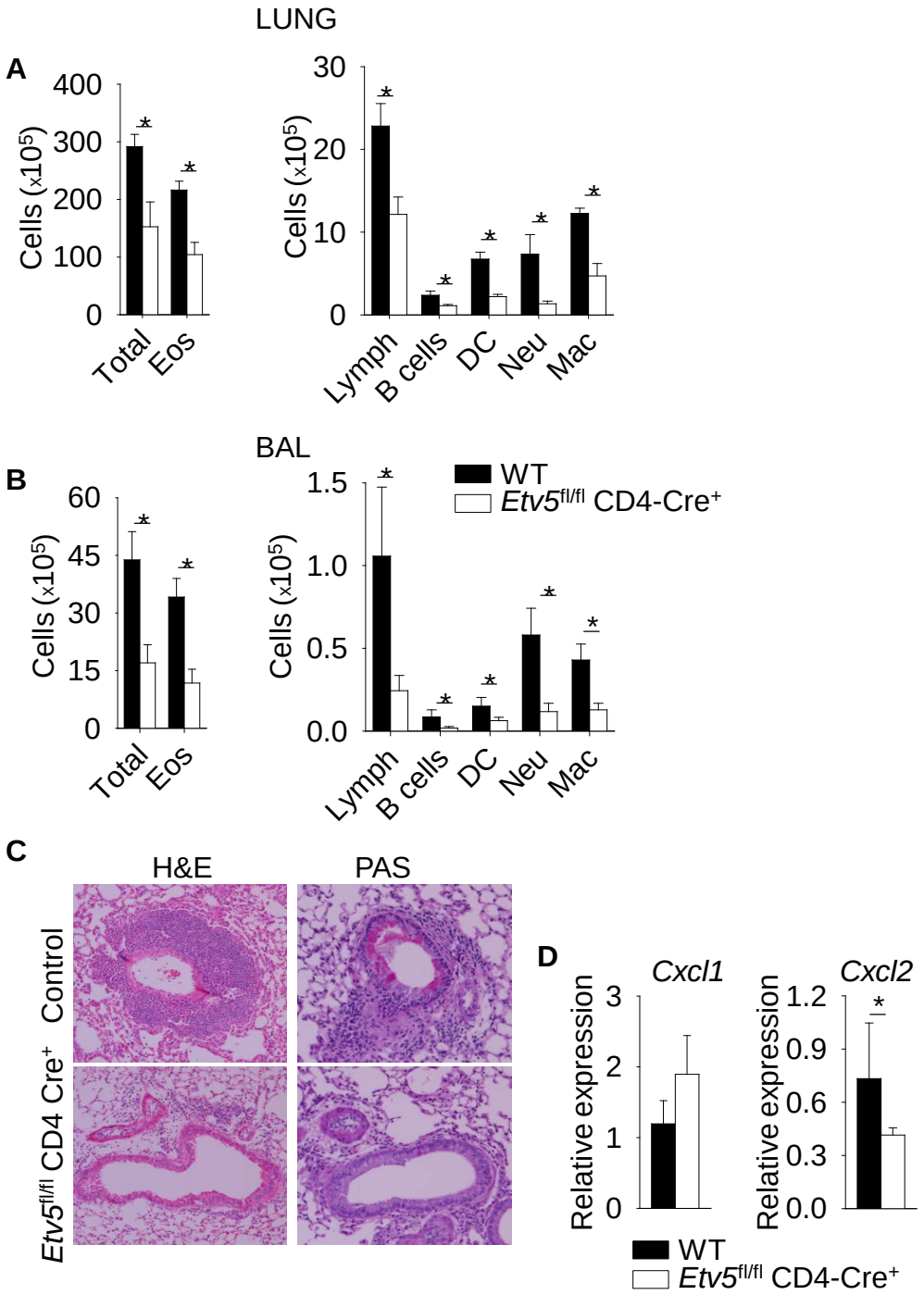


Figure 59. *Etv5*-mutant mice have reduced HDM-induced allergic inflammation. (A-D) Wild type and *Etv5*-mutant mice were immunized (i.n.) with HDM for five weeks to induce asthma. Inflammatory cells in the lung and BAL fluid of WT and *Etv5*-mutant mice: Eos, eosinophils; Lymph, lymphocytes; DC, dendritic cells; Neu, neutrophils; Mac, macrophages (A-B). Cell infiltration in the lungs and mucus in the airways of WT and *Etv5*-mutant mice evaluated by H&E and PAS staining (C). Total cells from lungs of WT and *Etv5*-mutant mice were used for gene expression analysis by qRT-PCR (D). Data are mean ± S.E.M. of 6

mice per group (A-D) and representative of two independent experiments with similar results. *p<0.05, **p<0.01

We next assessed the cytokine production in the lung and BAL cells of HDM-induced allergic inflammation in wild type and *Etv5*-mutant mice. Consistent with *in vitro* analyses (Figure 57), there were significant decreased frequency and numbers of IL-17A-producing $CD4^+$ T cells from the lung and BAL fluid of *Etv5*-mutant mice compared to wild type mice (Figure 60A-B). No change was observed in the frequency of IL-13-producing $CD4^+$ T cells from the lung and BAL fluid, although the cell number was decreased in *Etv5*-mutant mice compared to control mice (Figure 60A, C). The amount of IL-17A and IFNγ in the BAL fluid from *Etv5*-mutant mice was significantly decreased compared to BAL fluid of wild type mice, although similar amounts of IL-13 and IL-4 were observed (Figure 61). The frequency of Treg cells (identified by $CD4^+Foxp3^+$) and mean fluorescence intensity of Foxp3 in the lung and BAL cells were similar between wild type and *Etv5*-mutant mice (Figure 60D-F). We next assessed the generation of helper T cell responses in the periphery by stimulating splenic and mediastinal lymph nodes cells with HDM extract. IL-17A and IFNγ production were significantly reduced in *Etv5*-mutant mice compared to control mice, although there was no difference in the amount of IL-13 or IL-4 (Figure 61). Collectively, these results demonstrated that the critical role of Etv5 *in vivo* in response to an allergic allergen in experimental airway inflammation is the regulation of IL-17-secreting T cells.

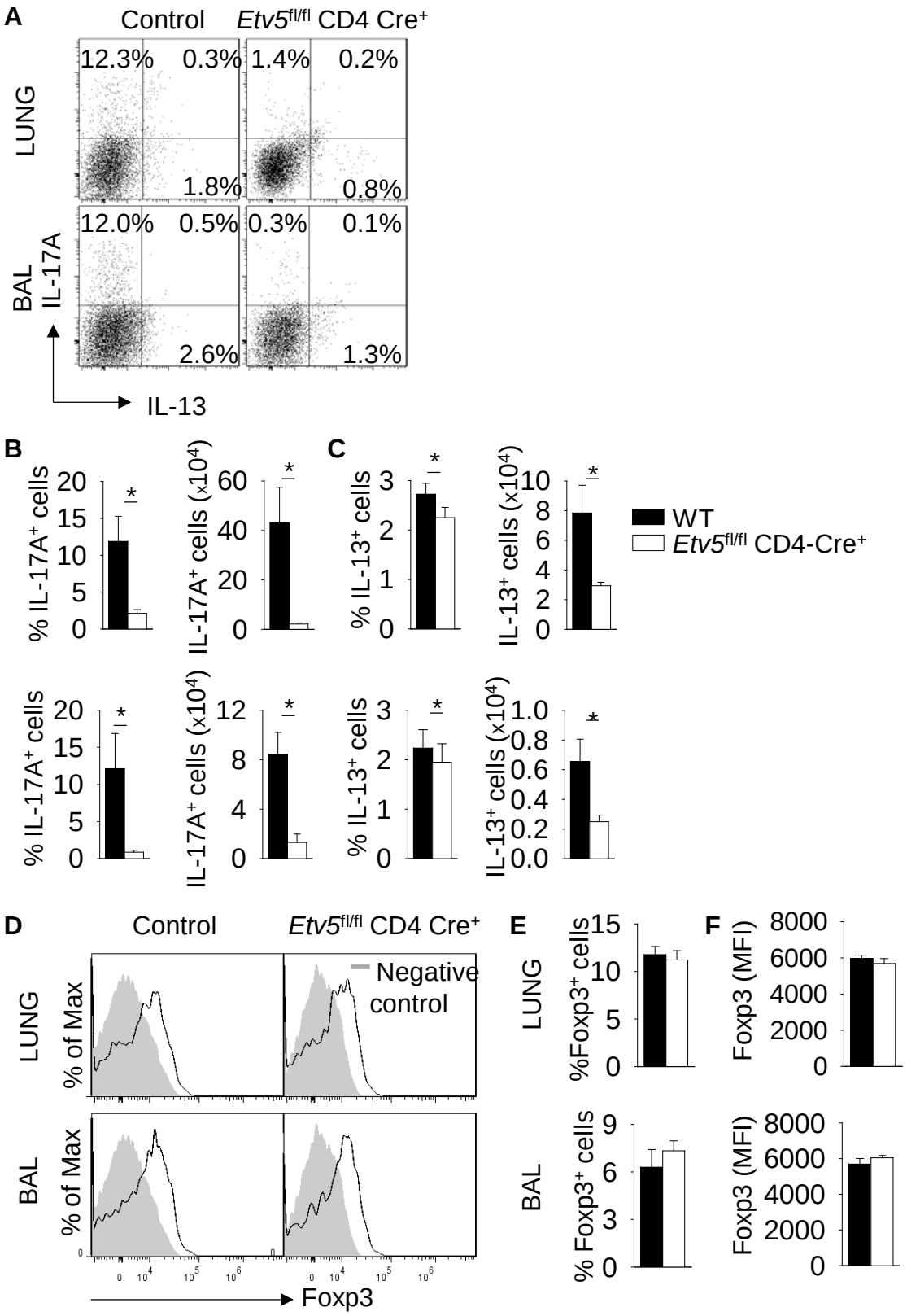

A
Control
Etv5fl/fl CD4 Cre+
LUNG
12.3%
0.3%
1.4%
0.2%
1.8%
0.8%
BAL
IL-17A
12.0%
0.5%
0.3%
0.1%
2.6%
1.3%
IL-13
B
% IL-17A+ cells
IL-17A+ cells (x10^4)
C
% IL-13+ cells
IL-13+ cells (x10^4)
WT
Etv5fl/fl CD4-Cre+
D
Control
Etv5fl/fl CD4 Cre+
LUNG
BAL
% of Max
Negative control
Foxp3
E
%Foxp3+ cells
% Foxp3+ cells
F
Foxp3 (MFI)

Figure 60. Etv5 regulates Th17 cells in HDM-induced allergic inflammation. (A-F) Wild type and *Etv5*-mutant mice were immunized (i.n.) with HDM for five weeks to induce asthma. Lung and BAL cells of HDM-induced asthma in WT and *Etv5*-mutant mice were stimulated with PMA and ionomycin for 6 h to assess cytokine production (A-C) and Foxp3 expression (D-F) by intracellular staining (ICS) with the average percent positive cells, cell number and mean fluorescence intensity (MFI) (B-C, E-F). Data are mean ± S.E.M. of 6 mice per group and representative of two independent experiments with similar results. *p<0.05

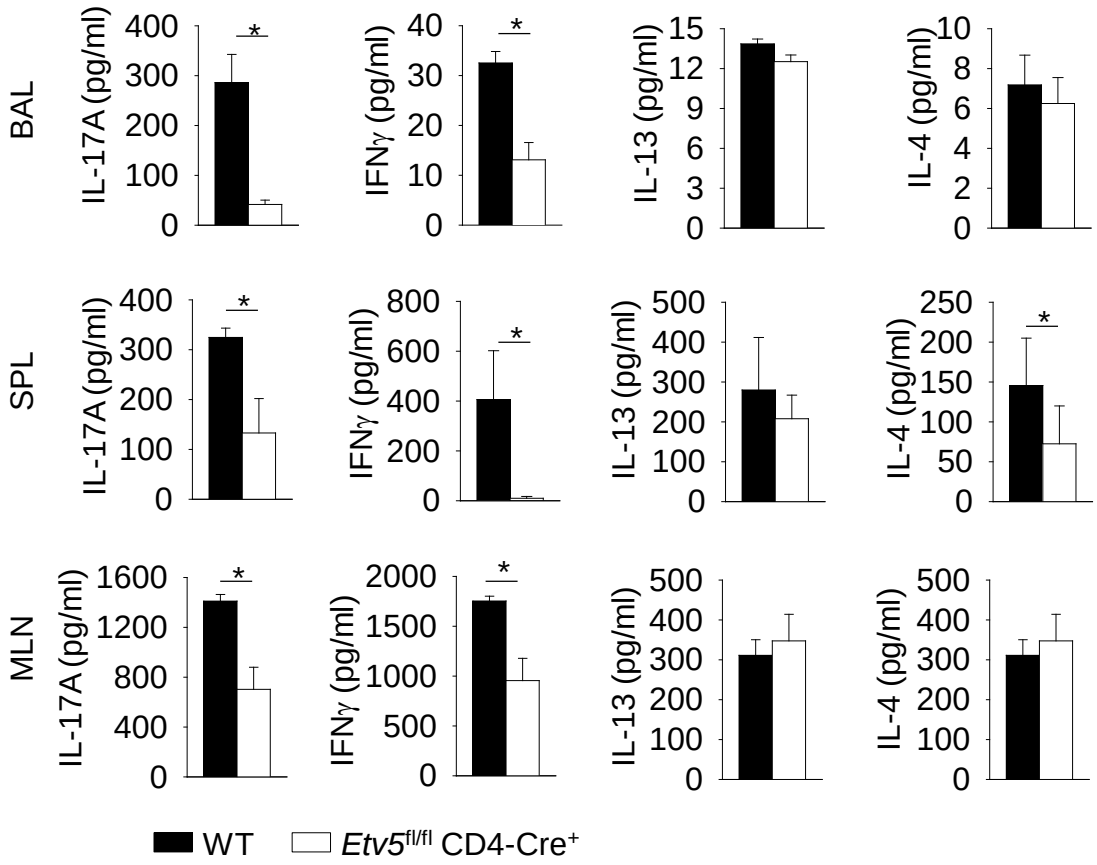


Figure 61. Etv5 regulates cytokine production in HDM-induced allergic inflammation. Cells from spleens (SPL) and mediastinal lymph nodes (MLN) from Figure 57 were stimulated with HDM for 5 days. Cell-free supernatant and BAL fluid were used to assess cytokine production by ELISA. Data are mean ± S.E.M. of 6 mice per group and representative of two independent experiments with similar results. *p<0.05

STAT3-activating cytokines induce Etv5 expression

Given that Etv5 positively regulates IL-17 *in vitro* and in the HDM-induced allergic inflammation (Figure 57C-D, 60), we wanted to more fully elucidate the ability of Etv5 to promote Th17 cell differentiation. We first assessed the kinetics of *Etv5* gene expression during Th17 cell differentiation. We observed increased *Etv5* expression 48 h after activation that gradually decreased over the subsequent three days of differentiation (Figure 62A). Since STAT4 regulates Etv5 expression in Th1 cells (Ouyang et al., 1999), we wanted to test whether STAT3 might induce Etv5 expression in Th17 cultures. Stimulation of wild type Th17 cells with IL-6 or IL-23 to activate STAT3, or IL-12 to activate STAT4, led to increased *Etv5* mRNA and protein expression compared to unstimulated cells (Figure 62B-C). Since *Etv5* expression in Th17 cells is lower than Th1 cells (Figure 57A), we hypothesized that an inhibitory signal represses *Etv5* expression in developing Th17 cells. As expected, IL-6 or IL-12 induced *Etv5* expression in activated $CD4^+$ T cells and this was decreased when TGF-β was added to the culture (Figure 62D). To further confirm that Etv5 is a STAT3 target gene, we treated activated wild type and *Stat3*-deficient T cells with either IL-6 or IL-12, and *Etv5* expression was assessed. As expected, IL-6 or IL-12 induced *Etv5* expression in activated $CD4^+$ T cells (Figure 62D). We next wanted to determine if STAT3 could directly bind to the STAT3 binding sites that are conserved in mouse and human genes (Figure 62E). Differentiated wild type Th17 cells were stimulated with cytokines known to activate STAT3, and binding was examined by Chromatin Immunoprecipitation (ChIP) assay. In Th17 cells,

STAT3-activating cytokines, but not IL-12 resulted in STAT3 binding to the *Etv5* promoter, in greater amounts at the distal than the proximal binding sites (Figure 62F). These results suggested that STAT3-activating cytokines and TGF-β play opposing roles in regulating *Etv5* expression in Th17 cultures.

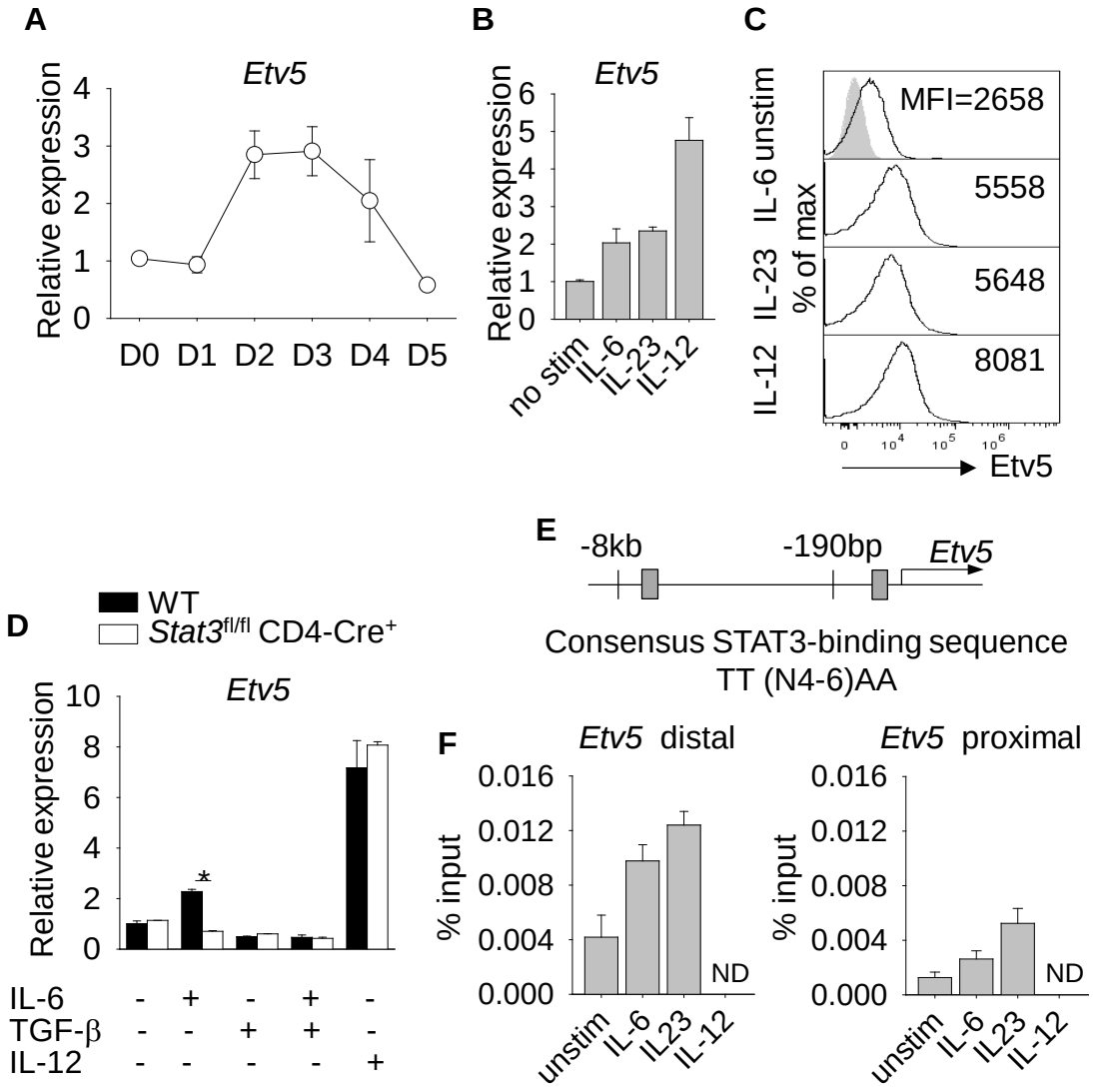


Figure 62. *Etv5* is regulated by STAT3 activating cytokines in Th17 cells. (A-C, F) Naive wild type $CD4^+CD62L^+$ T cells were cultured under Th17 polarizing conditions. Kinetics of *Etv5* gene expression during Th17 cell differentiation (A). Th17 cells were stimulated with IL-6, IL-23, and IL-12 for 2 h before gene expression analysis by qRT-PCR (B) or protein expression by ICS (C). (D) Naïve wild type and *Stat3*-deficient $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence or absence of IL-6, TGF-β, or IL-12 and gene expression was assessed by qRT-PCR after 3 d. (E) Schematic of the *Etv5* promoter containing STAT3 binding sites. (F) Cells prepared as in (B) were used for ChIP analysis using STAT3 antibody. Data are mean of replicate samples ± S.D. and representative of three independent experiments with similar results (A-F). *p<0.05. N.D., not detectable

Etv5 directly activates the Il17a-Il17f locus in Th17 cells

To further demonstrate the function of Etv5 in Th17 cell programming, we ectopically expressed Etv5 in Th17 cells and assessed cytokine production. Ectopic *Etv5* expression in Th17 cells resulted in increased IL-17A and IL-17F production compared to control cells (Figure 63A-B). To test whether Etv5 alone could induce IL-17 production in $CD4^+$ T cells, *Etv5* was ectopically expressed in non-polarized $CD4^+$ T cells (Th0). Ectopic *Etv5* expression in Th0 cells was able to induce IL-17A production compared to control cells (Figure 63C). Consistent with these observations, reduced *Etv5* expression in Th17 cells by transfecting T cells with siRNA targeting *Etv5* (Figure 63D) or using *Etv5*-deficient T cells (Figure 63E) resulted in increased IL-17A and IL-17F compared to control cells. GM-CSF production was not significantly different in control cells and cells that lack Etv5 expression (Figure 63D-E). We next examined the expression of transcription factors that are required for Th17 cell differentiation (Bauquet et al., 2009; Brustle et al., 2007; Durant et al., 2010; Ivanov et al., 2006; Schraml et al., 2009). Surprisingly, reduced *Etv5* expression did not alter gene expression of

Rorc, Batf, Maf, and *Irf4* (Figure 64A-B). We also noted a decrease in *Bcl6* expression in cells with reduced *Etv5* expression compared to control cells (Figure 64A-B).

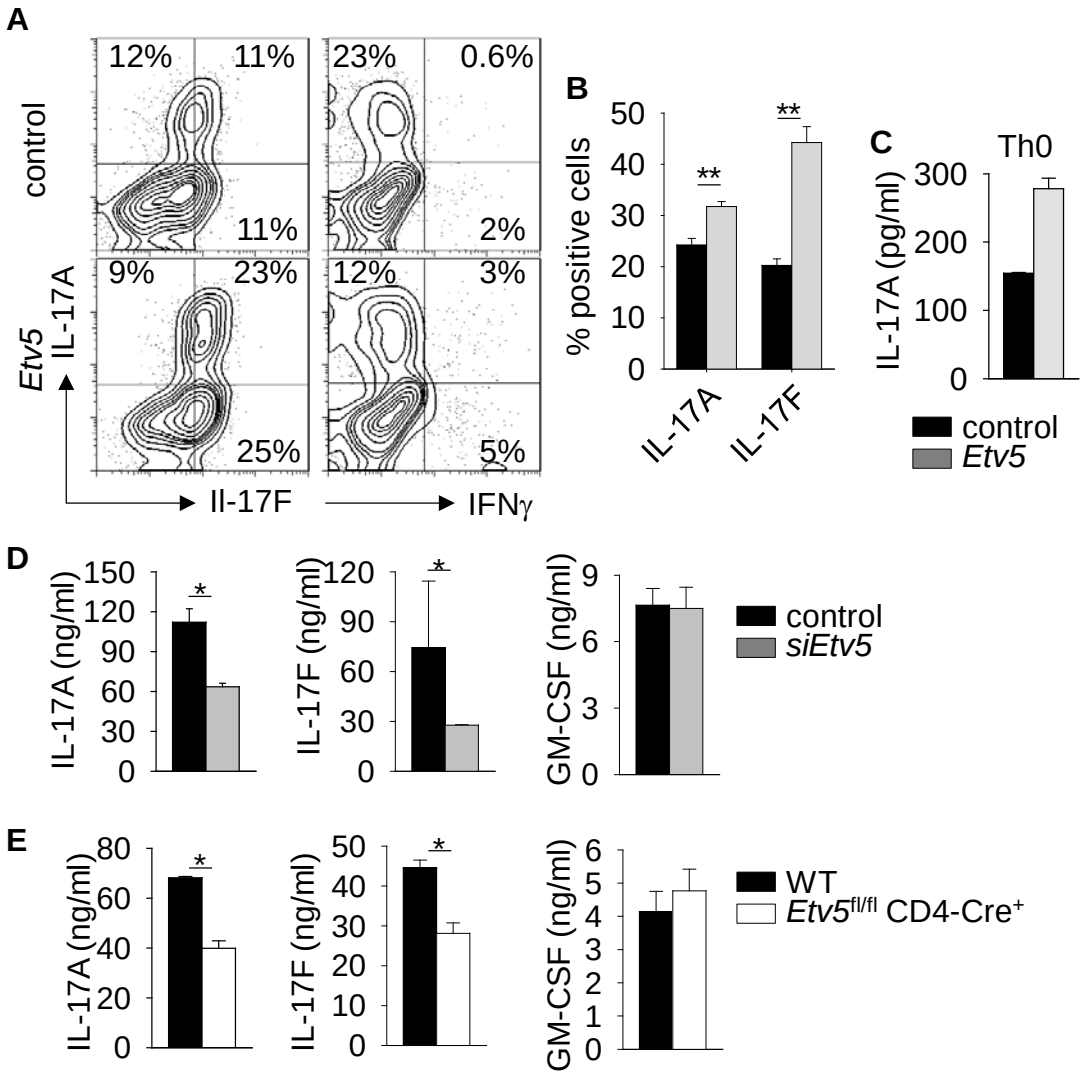


Figure 63. Etv5 promotes cytokine production in Th17 cells. (A-C) Naïve $CD4^+CD62L^+$ T cells were isolated from wild type mice and differentiated under neutral conditions (Th0) or Th17 culture conditions. On day 2, cells were transduced with either control or Etv5-YFP (Etv5)-expressing retrovirus. On day 5, Th17 cells were stimulated with PMA and ionomycin for 6 h before intracellular staining (ICS) for cytokine production (A) with the percent positive cells shown in (B). Data are gated on YFP^+ cells. Cytokine production from sorted YFP^+ Th0 cells was assessed by ELISA following 24 h stimulation with anti-CD3 (C). WT Th17 cells were transfected with control or siRNA-specific *Etv5,* rested overnight, and restimulated with anti-CD3 to assess cytokine production by ELISA (D). Wild

type and *Etv5*-deficient Th17 cells were restimulated with anti-CD3 to assess cytokine production by ELISA (E). Data are mean of four independent experiments ± S.E.M.(A-B, D-E) or are mean of replicate samples ± S.D. and representative of four independent experiments with similar results (C). *p<0.05, **p<0.01

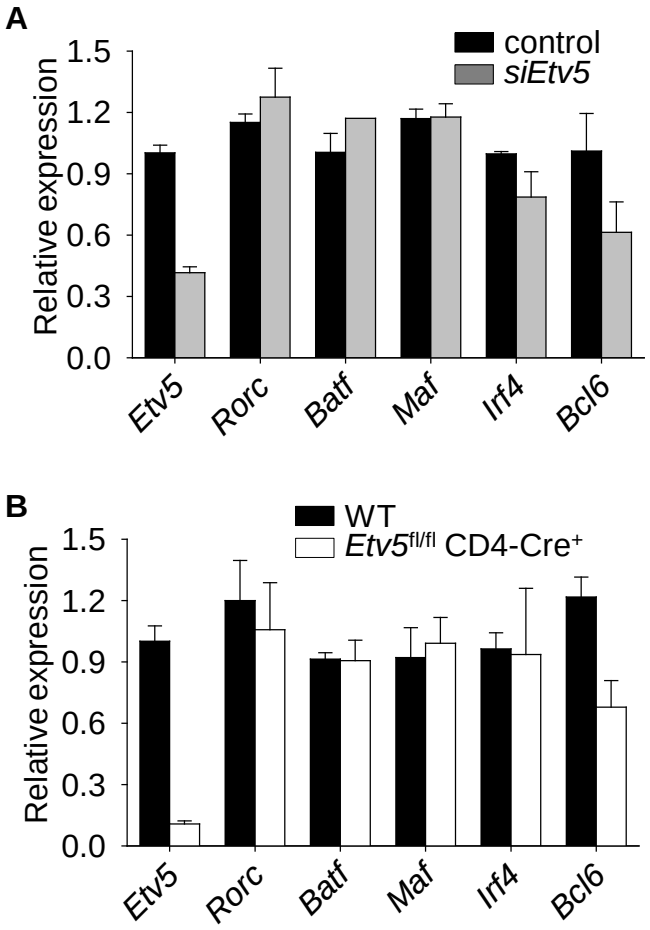


Figure 64. Etv5 does not regulate transcription factor expression in Th17 cells. (A) WT Th17 cells were transfected with control or siRNA-specific *Etv5* and rested overnight to assess gene expression by qRT-PCR. (B) Resting wild type and *Etv5*-deficient Th17 cells were used assess gene expression by qRT-PCR (B). Data are mean of replicate samples ± S.D. and representative of four independent experiments with similar results.

Since Etv5 did not impact any of the known regulators of IL-17A/F expression,

we tested whether Etv5 directly regulated expression of the *Il17a-Il17f* locus.

ChIP assay using differentiated Th17 cells revealed that Etv5 bound at several

sites across the *Il17a-Il17f* locus including the promoters and several conserved non-coding sequences (Figure 65A-B). We next transfected Jurkat T cells with *IL17A* and *IL17F* luciferase reporters and plasmid encoding Etv5 to demonstrate the direct consequences of Etv5 binding to the *Il17a-f* promoters. Notably, Etv5 promotes the transcriptional activity of the *IL17A* and *IL17F* promoters, but not an NFAT promoter, in a dose-dependent manner (Figure 65C). To define the modifications of histones correlated with active (H3K4 tri-methylation and H3K27 acetylation) and repressed (H3K27-trimethylation) gene expression at the *Il17a-Il17f* locus, we performed ChIP assays from control and *Etv5*-deficient Th17 cells. Interestingly, we observed decreased H3K4 methylation and H3K27 acetylation and increased H3K27 methylation across the *Il17a-Il17f* locus in the absence of Etv5 (Figure 66). In addition, the association of histone acetyltransferase p300 was decreased at the *Il17a-Il17f* locus in *Etv5*-deficient Th17 cells compared to wild type cells (Figure 66). These results suggested that Etv5 directly promotes IL-17 production, recruits histone modifying enzyme, and mediates changes at the *Il17a-Il17f* locus in Th17 cells.

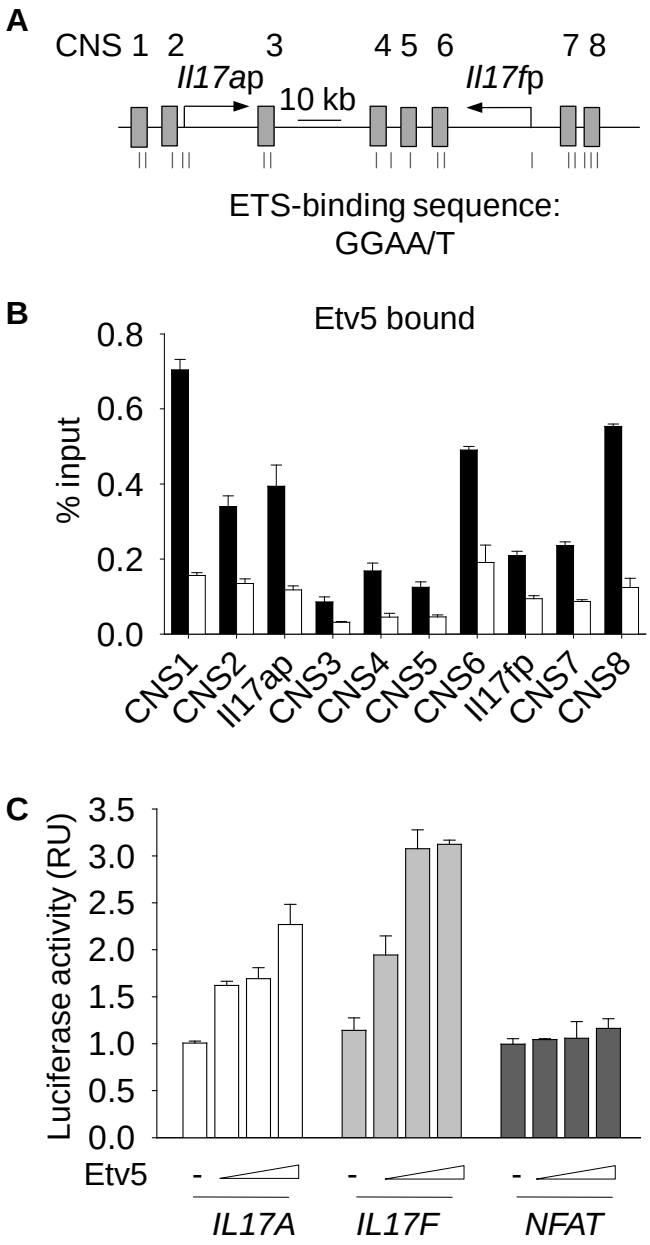


Figure 65. Etv5 binds the *Il17a/f* locus in Th17 cells. (A) Schematic of the *Il17a/f* locus containing ETS binding sites. (B) Naïve $CD4^{+}CD62L^{+}$ T cells were isolated from wild type mice and differentiated under Th17 culture conditions. ChIP analyses were performed using differentiated WT and *Etv5*-deficient Th17 cells to examine Etv5 binding to the *Il17a-Il17f* locus. (C) Luciferase activity in Jurkat T cells transfected with increased concentrations of plasmid encoding Etv5 along with *IL17A, IL17F,* or *NFAT* luciferase reporters, then activated for 6 h with PMA and ionomycin. Data were normalized to control samples. Data are mean of replicate samples ± S.D. and representative of three independent experiments with similar results.

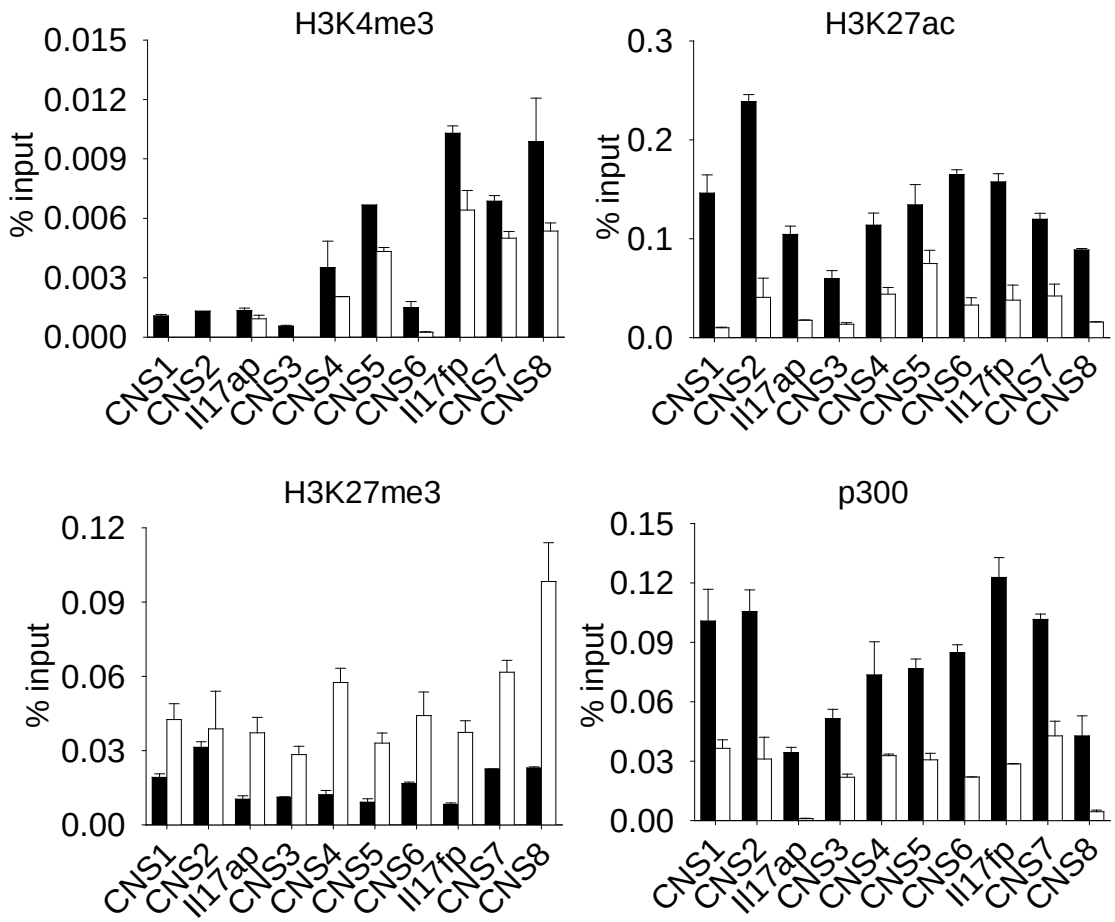


Figure 66. Etv5 mediates epigenetic changes at the *Il17a-Il17f* locus. ChIP analyses were performed using differentiated WT and *Etv5*-deficient Th17 cells to histone modification and the association of histone modifying enzyme at the *Il17a-Il17f* locus. Data are mean of replicate samples ± S.D. and representative of three independent experiments with similar results.

The opposing roles of Etv5 and Twist1 in controlling Th17 cell differentiation

We demonstrated that Twist1 and Etv5 reciprocally regulate IL-17 production in Th17 cells. Interestingly, there is a potential physical association between Etv5 and Twist1 (Zhang et al., 2010b). Therefore, we wanted to investigate whether Etv5 directly regulates IL-17 production or does so indirectly by interacting with Twist1 in Th17 cells. To test the idea, we transfected wild type and *Twist1*-deficient Th17 cells with siRNA targeting *Etv5* and examined gene expression

and cytokine production. siRNA knockdown of *Etv5* in wild type or *Twist1*-deficient Th17 cells decreased IL-17A and IL-17F production, with no effects on IFNγ and GM-CSF production compared to control siRNA cells (Figure 67A, B). The result suggested that Etv5 could modulate cytokine production in a Twist1-independent manner. Interestingly, decreased *Etv5* expression in wild type Th17 cells resulted in increased *Twist1* expression (Figure 67A). Etv5-dependent regulation of *Twist1* is likely direct as ChIP assay from Th17 cells demonstrated Etv5 bound to the *Twist1* promoter (Figure 67C).

Since Etv5 potentially forms a complex with Twist1, we next wanted to determine whether Etv5 could recover IL-17A production in Twist1 transduced Th17 cells. Ectopic *Twist1* or *Etv5* expression in wild type Th17 cells resulted in decreased or increased IL-17A production, respectively (Figure 67D). However, ectopic *Etv5* expression was not able to rescue IL-17A production in *Twist1* transduced Th17 cells (Figure 67D). Consistently, Etv5 could not prevent Twist1 from repressing the transcriptional activity of the *Il6ra* promoter (Figure 67E). Collectively, these results suggested that Etv5 positively regulates IL-17 production in Th17 cells, and that Etv5 and Twist1 reciprocally regulate gene expression of the opposing factor. However, Twist1 exerts a dominant role over Etv5 in controlling Th17 cytokine production.

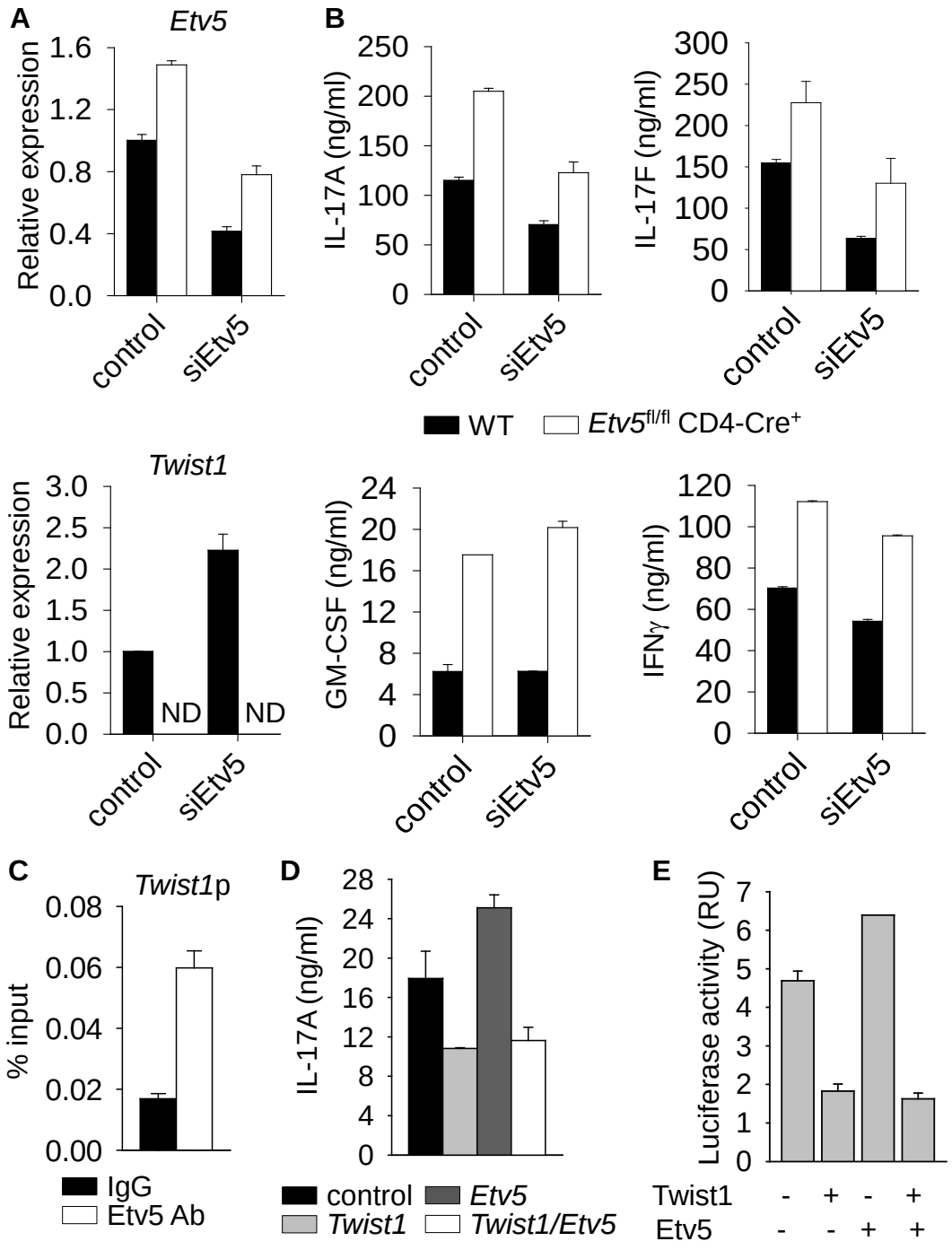


Figure 67. Etv5 controls IL-17 production in Twist1-dependent and -independent manners. (A-B) Naïve CD4$^+$ T cells were isolated from wild type and *Twist1*-mutant mice and differentiated under Th17 polarizing conditions. On day 2, cells were transfected with control or siRNA targeting *Etv5*, rested overnight, and analyzed for gene expression by qRT-PCR (A) or stimulated with anti-CD3 for cytokine production analysis by ELISA (B). (C) Day 5 differentiated wild type Th17 cells were used for ChIP analysis of the *Twist1* promoter using

Etv5 antibody. (D) Wild type Th17 cells were doubly transduced with control, Twist1-expressing, or Etv5-expressing retroviral vectors. Doubly transduced cells were sorted on day 5 and stimulated with anti-CD3 for 24 h before cytokine production analysis by ELISA. (E) Luciferase activity was measured in Jurkat T cells transfected with the combination of plasmid expressing Etv5, Twist1 and *IL6RA* luciferase reporter, following activation for 6 h with PMA and ionomycin. Data are mean of replicate samples ± S.D. and representative of three independent experiments with similar results. N.D., not detectable

Etv5 is required for T follicular helper cell development

Since *Bcl6* expression was decreased in Th17 cells that lack Etv5 expression (Figure 64), and because Bcl-6 is a master regulator of T follicular helper (Tfh) cell development, we wanted to determine if Etv5-deficiency in T cells affected Tfh generation. Etv5 is expressed at greater amounts in Tfh cells ($CD4^+CD44^+CXCR5^+PD\text{-}1^+$) than in non-Tfh effector cells ($CD4^+CD44^+CXCR5^-PD\text{-}1^-$) or naïve T cells ($CD4^+CD44^-CD62L^+$) (Figure 68). To examine the ability of Etv5 to regulate Tfh development, we immunized wild type and *Etv5*$^{fl/fl}$ CD4-Cre^+ mice with sheep red blood cells (SRBC). SRBC immunization resulted in dramatically reduced percentages of Tfh cells and cell number (defining cells with either ICOS or Bcl-6 expression) in *Etv5*$^{fl/fl}$ CD4-Cre^+ mice, compared to wild type mice (Figure 69).

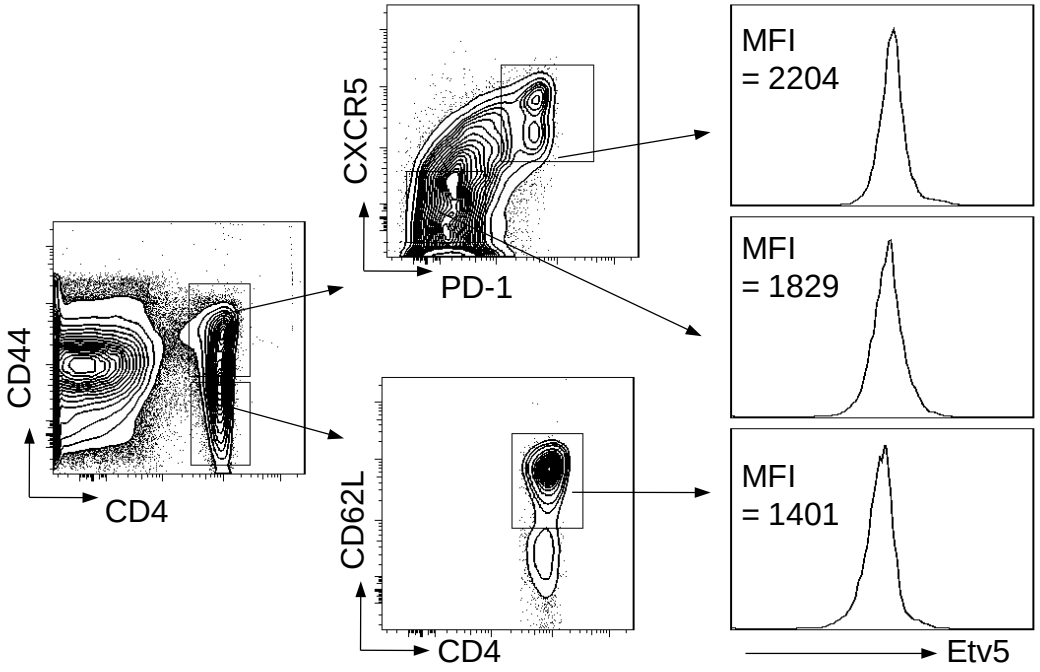


Figure 68. Etv5 expression in T follicular helper cells. Etv5 intracellular staining in Tfh cells ($CD4^{+}CD44^{hi}CXCR5^{+}PD\text{-}1^{+}$) compared to effector T cells ($CD4^{+}CD44^{hi}CXCR5^{-}PD\text{-}1^{-}$) and naïve T cells ($CD4^{+}CD44^{lo}CD62L^{hi}$) in day 9 SRBC-immunized WT mice.

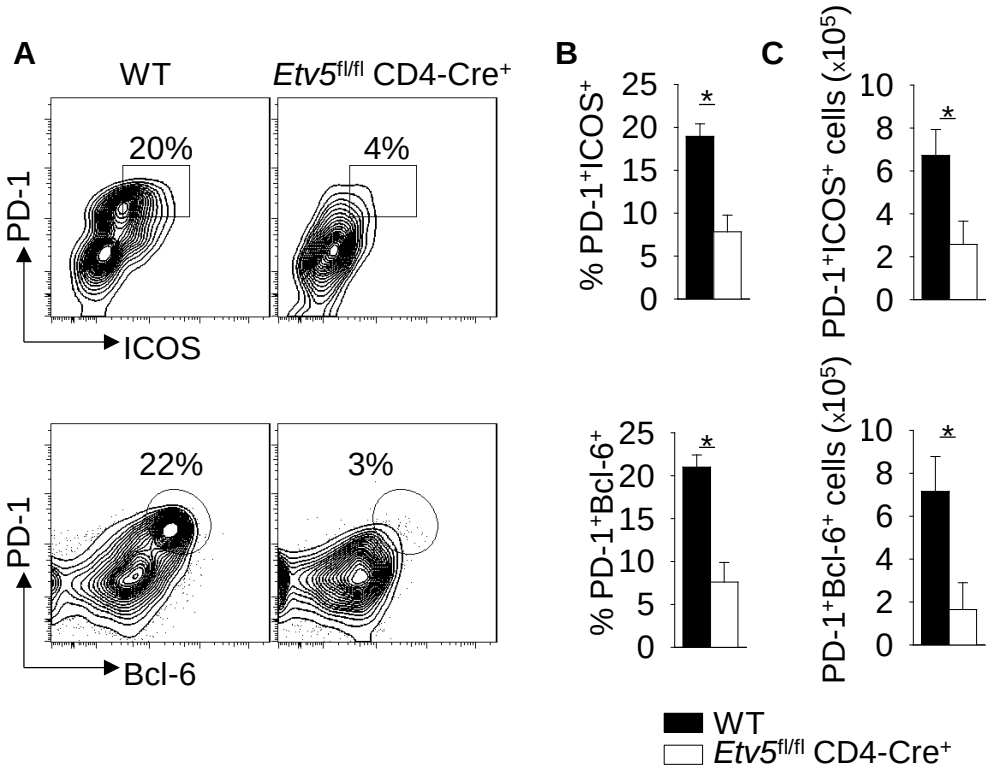


Figure 69. Mice with *Etv5*-deficient T cells have reduced T follicular helper cells. (A-C) WT and *Etv5*$^{fl/fl}$CD4-Cre^{+} mice were immunized with SRBC. On day 9, splenocytes were analyzed by flow cytometry with percentages of PD-1^{+}ICOS^{+} and PD-1^{+}Bcl-6^{+} cells indicated in (B), and the average percent positive cells shown in (C). Data are mean ± S.E.M. of 4-5 mice per group and representative of two independent experiments with similar results. *p<0.05

We then tested whether T cells activated in the presence of absence of IL-6 (Tfh-like conditions) demonstrated Etv5-dependent regulation of Tfh genes. Addition of IL-6 to activated T cell cultures resulted in increased pSTAT3, increased STAT3 binding to the *Etv5* promoter, and increased *Etv5* expression over 48 hours of culture (Figure 70A-C). Since we identified ETS binding sites in the *Bcl6* promoter that are conserved in mouse and human genes (Figure 70D), we examined the ability of Etv5 to bind to the *Bcl6* promoter. Paralleling the induction

of *Etv5* expression, Etv5 binding to the *Bcl6* promoter that contains conserved Etv5 binding sites was also induced by IL-6 (Figure 70E).

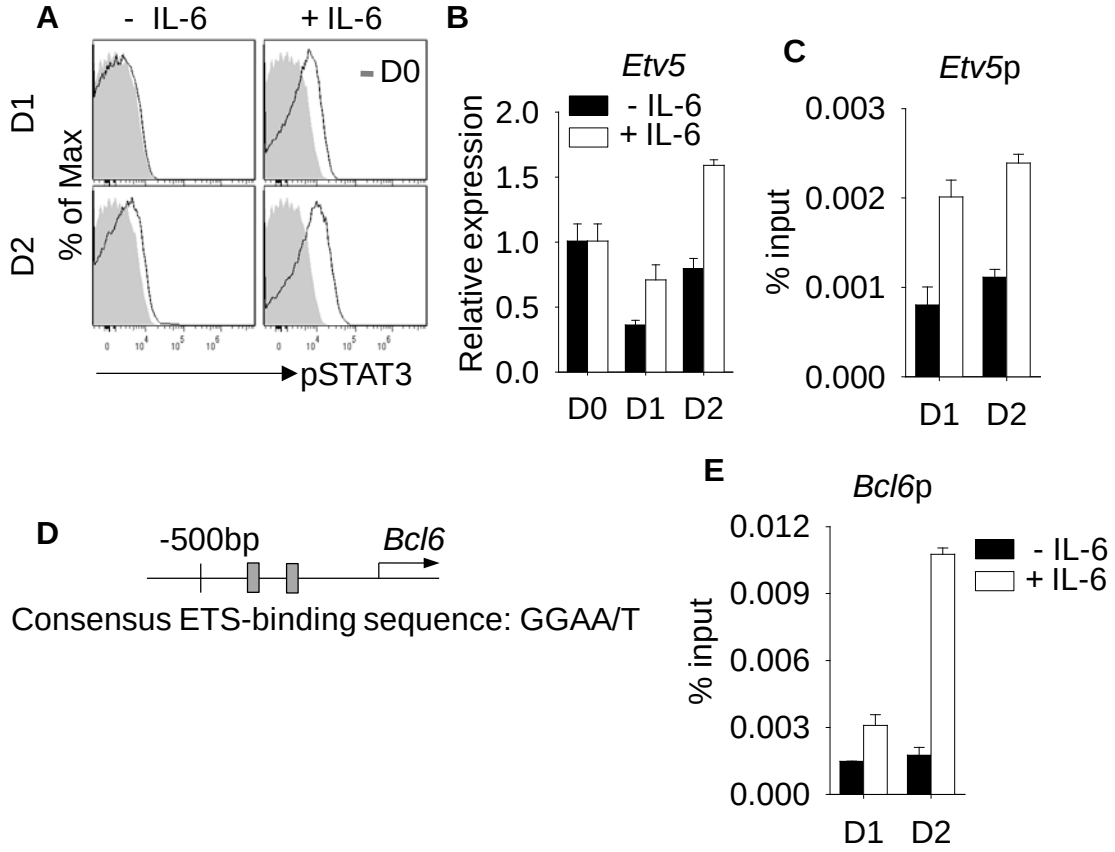


Figure 70. Etv5 binds the *Bcl6* promoter. (A-E) Naïve WT $CD4^+CD62L^+$ T cells were activated with or without IL-6 for 2 days. Cells were harvested daily to measure the level of phospho-STAT3 (pSTAT3) by ICS (A), analyze STAT3 binding to the *Etv5* promoter (C) or Etv5 binding to the *Bcl6* promoter (D-E) by ChIP assay, or to assess gene expression by qRT-PCR (B). Data are mean of replicate samples ± S.D. and representative of three independent experiments with similar results.

Finally, to determine the functional consequences of the decreased Tfh cells in mice with *Etv5*-deficient T cells we examined the development of germinal center B cells and antibody production following SRBC immunization. We observed a significant decrease in the percentages and total cell count of germinal center B cells (defined as $B220^+CD19^+Fas^+$ $GL\text{-}7^+PNA^+$ cells) (Figure 71A-B). Analysis of

SRBC-specific antibody production demonstrated decreased serum IgG antibody titers in *Etv5*$^{fl/fl}$ CD4-Cre^{+} mice, compared to wild type mice (Figure 71C). Isotype-specific analysis demonstrated reduced IgG1 and IgG2a/c serum antibody titers in mice that lack Etv5 expression in T cells than in wild type cells. Thus, Etv5 is required for Tfh development and humoral immunity.

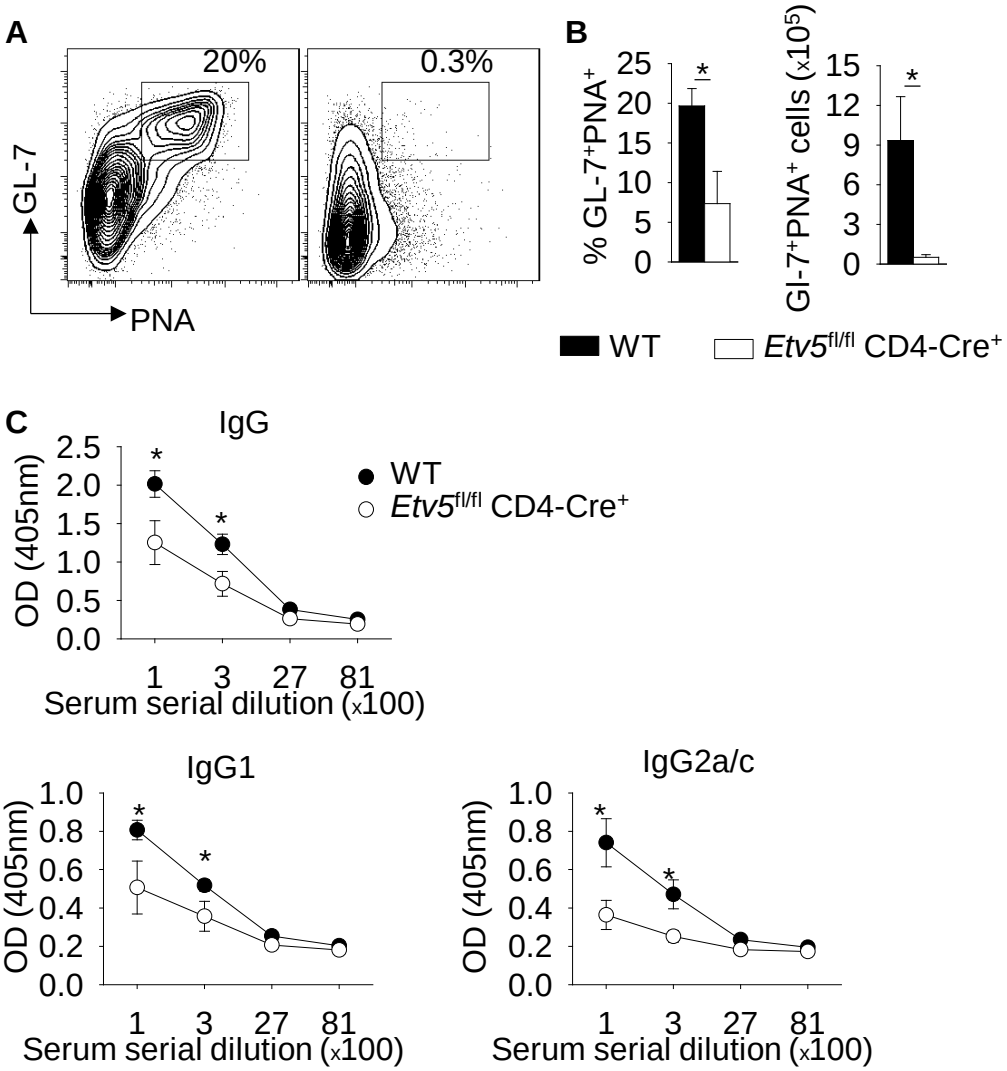


Figure 71. Etv5 promotes germinal center B cells and antibody production in SRBC-immunized mice. (A-C) WT and *Etv5*$^{fl/fl}$ CD4-Cre^{+} mice were immunized with SRBC. On day 9, splenocytes were stained for germinal center B cells (A) with the average percent positive cells and total cell count shown in (B). Data are gated on B220^{+}CD19^{+}Fas^{+}. Serum from WT and *Etv5*-mutant mice was diluted and used to measure antibody titers by ELISA (C). Data are mean ±

S.E.M. of 4-5 mice per group and representative of two independent experiments with similar results. *p<0.05

Etv5 promotes Th9 cell development

We observed Etv5 has a positive role in Th17 and Tfh cell development. We next examined the role of Etv5 in Th9 cell differentiation by first ectopically expressing Etv5 in Th2 and Th9 cells and assessing cytokine production. Ectopic *Etv5* expression in Th2 and Th9 cells resulted in increased IL-9 and decreased IL-4 production compared to control cells (Figure 72A). Consistent with these observations, reduced *Etv5* expression in Th2 and Th9 cells by or using *Etv5*-deficient T cells resulted in decreased IL-9 and increased IL-4 at the protein and message levels compared to control cells (Figure 72B-D). We next assessed the gene expression profiles that have been shown to be expressed highest in Th9 cells compared to other T helper cells in *Etv5*-deficient Th9 cells. There were no differences in *Irf4, Batf, Maf, Gata3, Ahr, Cxcl3, Ahrr, Ccr4, Ccr8, Fasl,* and *Itgae* expression between WT and *Etv5*-deficient Th9 cells (Figure 72E). Surprisingly, *Sfpi1* expression that encodes for PU.1 was significant higher in *Etv5*-deficient Th9 cells compared to WT cells (Figure 72E). We also detected decreased *Erg, Crem, Tnfsf13b*, *Il17rb,* and *Il1rn* expression in *Etv5*-deficient Th9 cells compared to wild type cells (Figure 72E). In the HDM-induced allergic inflammation described in Figure 61, the level of IL-9 in the BAL fluid was significantly reduced in *Etv5*-mutant mice compared to wild type mice (Figure 73). Similarly, stimulating splenic and mediastinal lymph nodes cells with HDM resulted in decreased IL-9 production in *Etv5*-mutant mice compared to wild type mice

(Figure 73). These data demonstrated that Etv5 is required for Th9 cell development and IL-9 production in an allergen-induced airway inflammation.

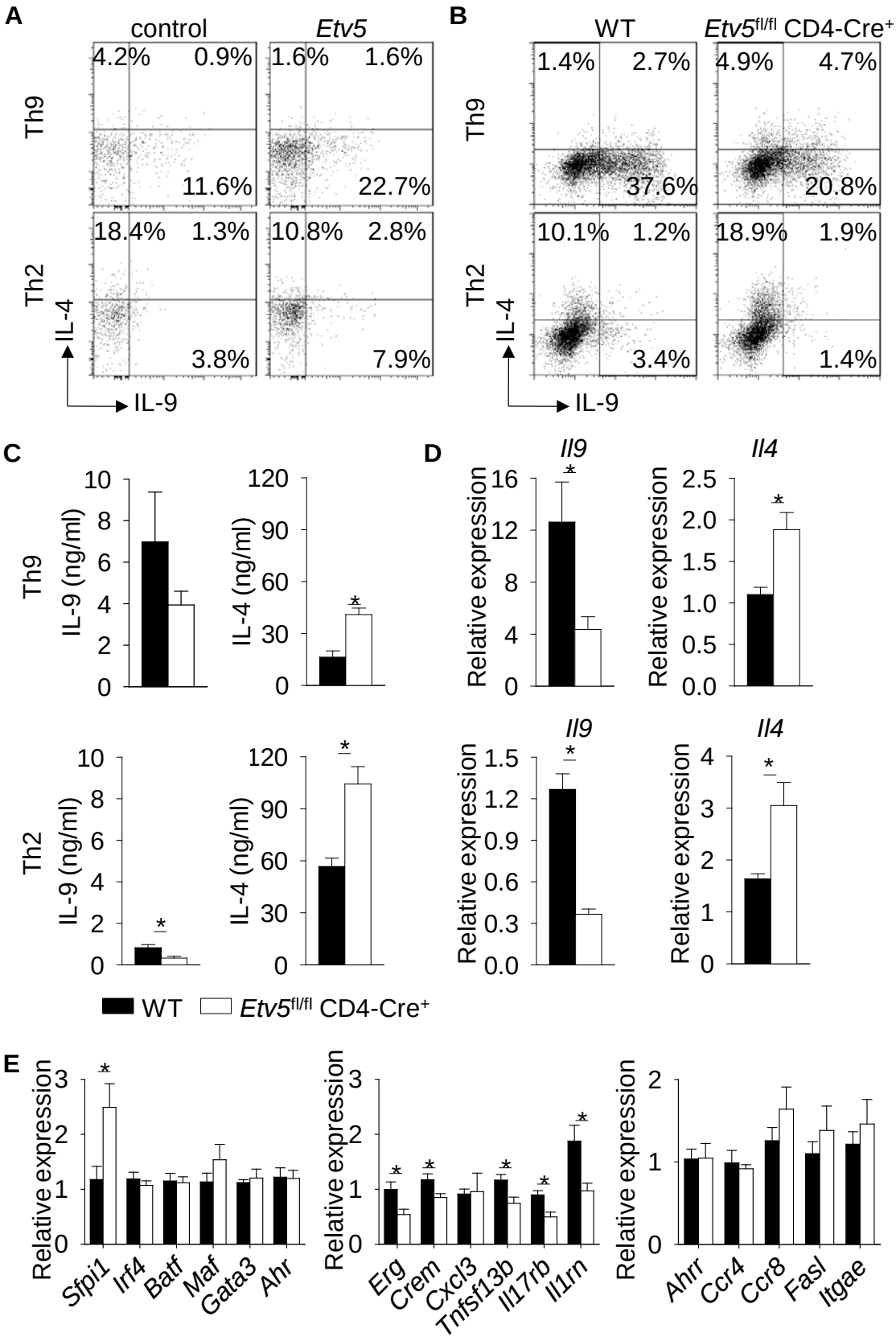

A
control
Etv5
Th9
Th2
4.2%
0.9%
11.6%
18.4%
1.3%
3.8%
1.6%
1.6%
22.7%
10.8%
2.8%
7.9%
IL-4
IL-9
B
WT
Etv5fl/fl CD4-Cre+
1.4%
2.7%
37.6%
10.1%
1.2%
3.4%
4.9%
4.7%
20.8%
18.9%
1.9%
1.4%
C
IL-9 (ng/ml)
IL-4 (ng/ml)
D
Il9
Il4
Relative expression
WT
Etv5fl/fl CD4-Cre+
E
Sfpi1
Irf4
Batf
Maf
Gata3
Ahr
Erg
Crem
Cxcl3
Tnfsf13b
Il17rb
Il1rn
Ahrr
Ccr4
Ccr8
Fasl
Itgae

Figure 72. Etv5 promotes cytokine production in Th9 cells. (A) Naïve $CD4^+CD62L^+$ T cells were isolated from wild type mice and differentiated under Th2 or Th9 culture conditions. On day 2, cells were transduced with either control or Etv5-YFP (Etv5)-expressing retrovirus. On day 5, cells were stimulated with PMA and ionomycin for 6 h before intracellular staining (ICS). Data are gated on YFP^+ cells. (B-E) Wild type and *Etv5*-deficient Th17 cells were restimulated with with PMA and ionomycin for 6 h before intracellular staining (ICS) (B) or anti-CD3 to assess cytokine production by ELISA (C) and gene expression by qRT-PCR (D-E). Data are mean of four independent experiments ± S.E.M. (C-E) or are mean of replicate samples ± S.D. and representative of four independent experiments with similar results (A-B). *p<0.05

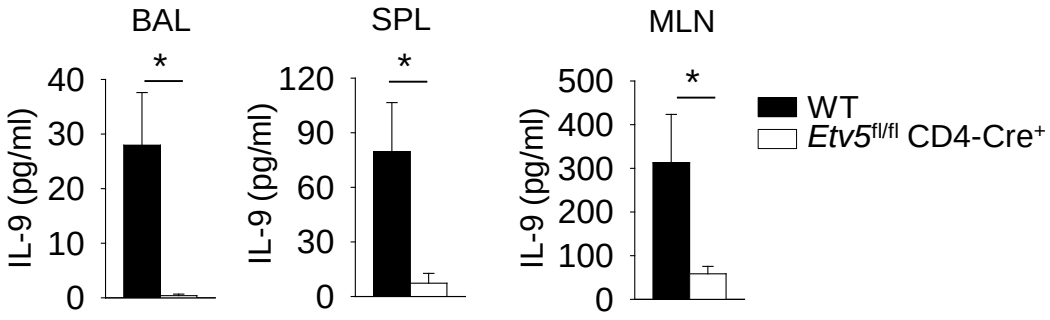


Figure 73. Etv5 regulates IL-9 production in HDM-induced allergic inflammation. Cells from spleen (SPL) and mediastinal lymph nodes (MLN) from Figure 57 were stimulated with HDM for 5 days. Cell-free supernatant and BAL fluid were used to assess cytokine production by ELISA. Data are mean ± S.E.M. of 6 mice per group and representative of two independent experiments with similar results. *p<0.05

DISCUSSION

$CD4^+$ T cell differentiation into cell lineage-programmed subsets requires the integration of multiple elements such as JAK/STAT signaling pathways, transcription factors, and epigenetic modification. Understanding how these signals are integrated during the T helper cell development will lead to greater insight into the regulatory networks that modulate differentiation.

The Signal Transducer and Activator of Transcription factor STAT4 is required for Th1 differentiation and inflammatory disease. STAT4 controls Th1 cell differentiation in many aspects such as promoting IFNγ expression, inducing transcription factor expression, recruiting and interacting with other factors at STAT4 target loci such as histone modifying enzymes and transcription factors to regulate gene expression. An additional mechanism for STAT4-dependent gene programming is to establish a de-repressed genetic state susceptible to transactivation by additional fate-determining transcription factors.

STAT4 not only induces the expression of transcription factors (T-bet, Runx3, and Hlx1) that promote Th1 cell differentiation but also modulates the process by inducing a negative feedback loop to control inflammatory response. STAT4 induces the expression of the transcriptional repressor Twist1 that impairs IL-12-STAT4 signaling and interferes with the transcriptional transactivation function of Runx3 and T-bet. Twist1 is not only involved in a STAT4-induced feedback loop in Th1 cells but also is a component of a STAT3-induced feedback loop in Th17

and Tfh cells that controls IL-6 signals by directly repressing *Il6ra*. Thus, Twist1 uniquely represses the function of Th1, Th17, and Tfh cells, and in mediating that repression without activation of additional subsets. In addition to Twist1, we identify Etv5 as a STAT3- and STAT4- target gene that has an important function in controlling T helper cell differentiation. In contrast to the repressive function of Twist1, Etv5 promotes Th9, Th17, and Tfh cell development with a modest effect in Th1 cell differentiation. Etv5 also plays important roles in the development of antigen-induced allergic airway inflammation, the formation of germinal center B cells, and antigen-specific antibody production.

Collectively, STAT proteins have a central role in controlling T helper cell development from promoting transcription factor expression, initiating epigenetic modification, and modulating regulatory mechanism. STAT proteins not only induce positive regulators that play critical roles in T helper cell differentiation, they also have self-regulatory mechanisms that shutdown the signaling pathway. As the result, CD4 T cells are tightly controlled over its differentiation and effector function. This is an essential aspect of understanding immune dysregulation and inventing therapeutic approach to targeting JAK/STAT signaling pathway in autoimmunity and allergic disease. Our studies specifically define IL-12-STAT4- and IL-6-STAT3- induced feedback (Twist1) and feed forward (Etv5) regulatory loops that impact multiple components of the Th1, Th9, Th17, and Tfh transcriptional networks and play a critical role in controlling both cell-mediated and humoral immunity. Thus, therapies aimed at altering negative (Twist1) or

positive (Etv5) factor, or delivering ectopically expressed Twist1 or Etv5, hold promise for modulating pathogen immunity, autoimmune disease, allergic inflammation and as an adjunct for limiting immune responses to viral-mediated gene therapy.

Part I- Opposing roles of STAT4 and Dnmt3a in Th1 gene expression

IL-12-induced STAT4 reciprocally modulates Jmjd3 and Dnmt3a association at Th1 gene loci

Although STAT4 is a critical factor in the development of Th1 cells and inflammatory immunity, a detailed understanding of how STAT4 programs gene expression has not been well documented. We define a pathway for the STAT4-dependent induction of Th1 gene expression. STAT4 binds to target loci and recruits histone acetyltransferases that mediate total histone acetylation (O'Sullivan et al., 2004; Yu et al., 2007) and acetylation of specific histone residues. STAT4 is required for the IL-12-inducible H3K4 methylation, and association of Jmjd3 with target loci (Figure 19). Diminished Jmjd3 expression results in decreased Th1 gene induction increased H3K27 methylation, and increased Dnmt3a association with target loci (Figure 21). This parallels data from Th2 cells that display decreased STAT4 expression accompanied by even greater Dnmt3a association and DNA methylation (Yu et al., 2008; Yu et al., 2007). STAT4 is required for the expression of several other transcription factors that contribute to Th1 gene expression including Hlx1. Despite decreased expression of several of these factors in *Stat4*$^{-/-}$ cells, double deficiency of STAT4

and Dnmt3a results in a partial increase in Th1 gene expression compared to *Stat4*$^{-/-}$ Th1 cultures. These results demonstrate that Dnmt3a plays an obligate role in repressing Th1 gene expression that is attenuated by the activity of STAT4. Overall, STAT4 functions by facilitating increased histone acetylation and H3K4 methylation to induce gene expression, and by decreasing the association of DNA methyltransferases that repress gene expression.

Compensatory role of Dnmt3a-deficiency in Stat4$^{-/-}$ *mice results in recovery of Th1 function*

Disease models can be complex and rely upon the balance of pro- and anti-inflammatory effector cells. EAE requires the function of Th1 and Th17 cells, and is inhibited by Treg cells (Pierson et al., 2012). Partial recovery of a Th1 phenotype in *Dnmt3a*$^{fl/fl}$ *Stat4*$^{-/-}$ Cre-positive cultures was recapitulated in an EAE disease model where *Dnmt3a*$^{fl/fl}$ *Stat4*$^{-/-}$ Cre-positive mice had increased clinical disease and IFNγ production compared to *Stat4*$^{-/-}$ mice. The contribution of Th17 cells to EAE in *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive mice is not limiting since there were no changes in IL-17-producing mononuclear cells, and *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive T cells had similar *in vitro* Th17 differentiation to *Stat4*$^{-/-}$ cultures. Although DNA methyltransferases have been reported to play an important role in the generation of regulatory T cells (Lal et al., 2009), and *in vitro* derived *Dnmt3a*$^{fl/fl}$ Cre-positive iTreg cultures had increased percentages of Foxp3^{+} cells compared to wild type cultures, *Dnmt3a*$^{fl/fl}$ Cre-positive mice displayed normal EAE development (Figure 24). IL-12 has also been shown to limit the

development of iTreg cells (O'Malley et al., 2009), although in the EAE inflammatory environment, there are many other cytokines that can modulate Foxp3 and compensate for the lack of STAT4. Moreover, if Tregs were increased in *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive mice, we would not expect to observe increased disease. Thus, it is likely that the partial recovery of Th1 target genes contributes to the observed EAE phenotype in *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive mice.

STAT4 function requires additional transcription factors

STAT4 is a multi-functional transcription factor with the ability to acutely activate gene expression in a short time span (within hours) and to program genes for lineage expression (lasting days). These intertwined functions likely involve both the chromatin remodeling mechanisms we have described here with the direct induction of genes via RNA polymerase II-dependent transcription. Although STAT4 can activate reporter genes in transient assays, suggesting a direct effect on rate of transcription, how it mediates these functions is not yet known. Importantly, despite the ability of IL-12 to activate several STAT proteins (Bacon et al., 1995; Jacobson et al., 1995), none can substitute for STAT4 in the acute induction of gene expression by IL-12 (Figure 19) or in IL-12-induced Th1 gene programming (Good et al., 2009). This suggests that STAT4 specifically interacts with other transcription factors at target loci to mediate gene induction. The specificity of these interactions might also distinguish the effects of STAT4 on the IFN-inducible genes, where there is only transient gene induction, from genes that are programmed for expression in committed Th1 cells. The identity of some

of these interacting factors is still unclear. STAT4 can interact with Jun family members (Nakahira et al., 2002; O'Sullivan et al., 2004) and co-operation with AP-1 complexes is possible. Previous work defining STAT4-interacting proteins identified a LIM domain-containing protein that regulated STAT4 stability and a tyrosine phosphatase that regulated STAT4 phosphorylation (Nakahira et al., 2007; Tanaka et al., 2005). Other interacting transcription factors have not been identified.

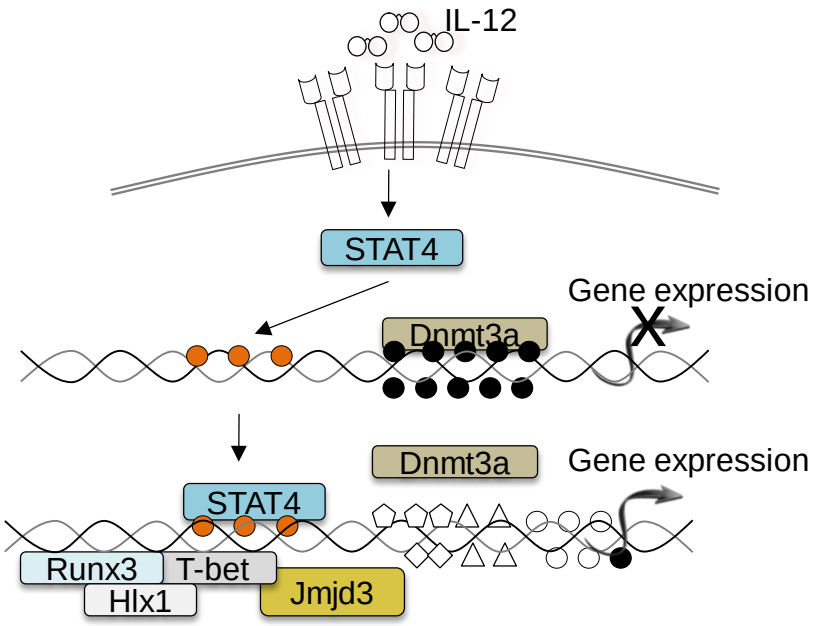
Although there is no evidence yet for physical interactions with other Th1-inducing transcription factors, it is clear that STAT4 functions in a network with these factors in Th1 cells. T-bet is the most obvious, where STAT4 induces expression of *Tbx21* and cooperates with T-bet in the induction of a subset of Th1 genes (Schulz et al., 2009; Thieu et al., 2008; Yang et al., 2007). Among the genes that both STAT4 and T-bet are required for, Runx3 and Hlx1 also contribute to expression of Th1 genes (Djuretic et al., 2007; Mullen et al., 2002). In this section, we tested the concept that in the absence of STAT4 acting as a positive regulator, eliminating a negative regulatory factor would lead to recovery of gene expression and differentiation. We observed a partial recovery of phenotype *in vitro* and function *in vivo* (Figure 22-24), suggesting that STAT4 has functions in addition to elimination of Dnmt3a association. Among those functions, STAT4 regulates chromatin remodeling in the absence of T-bet (Zhang and Boothby, 2006), and the induction of a negative regulatory loop that requires the transcription factor Twist1 (Niesner et al., 2008; Pham et al., 2012). Ectopic

expression of Runx3 or a combination of T-bet and Hlx1 were able to induce Th1 gene expression in *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive Th1 cultures to a greater extent than in *Stat4*$^{-/-}$ Th1 cultures, although still not to wild type expression (Figure 27). It is possible that reconstitution of all three factors would lead to complete recovery. However, it is also likely that STAT4 plays an indispensible role for initiating transcription at many Th1 target loci (Good et al., 2009), and not simply as an initial activator of the Th1 transcriptional network.

The cooperation of STAT4 and T-bet may be required for some of the effects observed in this section, since T-bet is also required for Jmjd3 recruitment to target loci (Miller et al., 2008; Miller et al., 2010). Indeed, we observed association of T-bet and Jmjd3 at the *Ifng* locus was increased in *Dnmt3a*$^{fl/fl}$*Stat4*$^{-/-}$ Cre-positive Th1 cells, compared to *Stat4*$^{-/-}$ Th1 cultures (Figure 26). However, Dnmt3a association with Th1 loci was increased more in *Stat4*$^{-/-}$ Th1 cells than in *Tbx21*$^{-/-}$ Th1 cells, suggesting that STAT4 has T-bet/Jmjd3-independent mechanisms to limit Dnmt3a association (Thieu et al., 2008). Since Dnmt3a interacts with unmethylated H3K4 and tri-methyl-H3K36, an additional possibility is STAT4-dependent recruitment of H3K4 methylases and H3K36 acetylases or demethylases (Chen et al., 2012; Li et al., 2007; Otani et al., 2009; Yu et al., 2007; Zhang et al., 2010a).

Collectively, STAT4 plays an indispensible role in controlling Th1 cell development from inducing Th1 target genes including transcription factors to

recruiting chromatin modifying enzyme complexes. Moreover, STAT4 likely interacts with these factors to limit DNA methylation and initiate gene transcription. A summary of STAT4 function in Th1 cells is shown in Figure 74.



Active histone marks

- ○ H3K4 Methylation
- △ H3K9/18 Acetylation
- ◇ H3K36 Acetylation
- ⬠ H3K27 Acetylation

Repressive histone mark

- ● H3K27 Methylation
- ● **STAT4 binding site**

Figure 74. STAT4 regulates gene expression by recruiting histone modifying enzymes and transcription factors to Th1 gene loci

Part II- Twist1 function in T helper cells

Twist1 negatively regulates Th1 cell differentiation through several mechanisms

Twist1 is expressed in Th1 effector memory cells and limits Th1-mediated inflammation in mouse models of delayed-type hypersensitivity and antigen-induced arthritis (Niesner et al., 2008). We have identified several mechanisms by which Twist1 modulates inflammatory cytokine production in Th1 cells. First, it negatively regulates the expression of Th1 genes. In macrophages, Twist1 has been shown to regulate TNFα and IL-1β by binding to E-boxes in gene promoters (Bialek et al., 2004). However, we did not observe Twist1 binding to the *Ifng*, *Tnfa*, or *Il12rb2* promoters, and only minimal binding to the *Tbx21* promoter. It is still possible that Twist1 binds directly to Th1 genes in regions other than the promoter that we tested. The second mechanism of Twist1-dependent gene regulation is through complex formation with Runx3. This mechanism appears to be the primary mechanism for regulating *Ifng*. Yet, the Twist1 S192P mutant that reduced interactions with Runx3 was still able to transcriptionally regulate other Th1 genes, suggesting that Twist1 has gene regulatory activity independent of Runx3. The direct regulation of genes other than *Ifng* might further impact the Th1 phenotype and further analysis will be required to further refine this regulatory network.

STAT3-induced a negative feedback loop that regulates Th17 and Tfh cell development

In addition to repressing cytokine production in Th1 cells, Twist1 also negatively regulates Th17 and Tfh cell development. Cytokines, including IL-6, induce the STAT3-dependent expression of Twist1, which then binds to the *Il6ra* promoter, repressing transcription and thus limiting IL-6 responsiveness and STAT3 activation. The ability of Twist1 to repress IL-6 signaling limits the development of Th17 cells and Tfh cells *in vivo* thereby controlling cell-mediated and humoral components of the immune response. This observation is consistent with recent findings that Twist1 can also regulate the cell fate decisions of multi potential cardiac neural crest between neurons and smooth muscle via its direct transcriptional repression of *Phox2b* (Vincentz et al., 2013).

Twist1 likely regulates T helper cell development through several mechanisms. We demonstrated that Twist1 inhibits cytokine production in Th17 and Tfh cells by repressing *Il6ra,* hence impairing IL-6-STAT3 signaling. STAT3-dependent mechanisms seem to be predominant, since impaired STAT3 signaling diminished the effects of Twist1-deficiency. Yet, through transcriptional repression, it is possible that Twist1 directly inhibits the expression of additional target genes in Th17 and Tfh cells.

Although Twist1 may regulate T helper subset development through several mechanisms, one paradigm that emerges is Twist1 being an essential

component of a cytokine-induced feedback loop. In Th1 cells, STAT4 induces Twist1, which subsequently decreases *Il12rb2* expression and STAT4 activation (Figure 31) (Pham et al., 2012). Similarly, in Th17 and Tfh cells, STAT3 induces Twist1, which represses *Il6ra* resulting in decreased STAT3 activation. In Th17 cells, and likely in Tfh cells as well, this alters the balance of activation between STAT3 and STAT5 that have opposing roles in both of these subsets (Johnston et al., 2012; Nurieva et al., 2008; Nurieva et al., 2012; Yang et al., 2011).Thus, Twist1 functions as a balancing factor that regulates signal integration.

Potential function of Twist1 dimer in T helper cell differentiation

Twist1 functions as either a homodimer or heterodimer with other bHLH factors, where the dimerization partners dictate the function (Castanon et al., 2001). Altering the balance between Twist1 and Hand2 association has a significant impact on limb and craniofacial defects in humans with Saethre-Chotzen syndrome (Firulli et al., 2005). Twist1 has been shown to form a dimer complex with E47 protein, which is inhibited by the DNA-binding inhibitor Id3 (Castanon et al., 2001; Firulli et al., 2005; Hayashi et al., 2007). Moreover, Th1 cells differentiated from *E2a*-deficient mice showed a decrease in IFNγ production compared to WT cells (Pan et al., 2004). Thus, it is possible that E12/E47 sequesters Twist1 in heterodimer complexes to inhibit its repressive function in Th1 cells. In the absence of E2A gene products, there is more “free” Twist1 to form homodimers and thus inhibit cytokine production. In addition, *Id3*-deficient mice have a defect in regulatory T cell generation and an enhancement in Th17

differentiation linked to the ability of E47 to induce *Rorc* expression (Maruyama et al., 2011). Maruyama et al. suggested that the ability of E47 to transactivate *Rorc* expression might require other factors downstream of IL-6 (Maruyama et al., 2011). Consistent with this, we observed an increase in E47 binding at the *Rorc* promoter in *Twist1*-deficient Th17 cells compared to WT cells, although there was no change in either *Tcfe2a* (encoding E47) or *Id3* expression. E2A and Id3 also have opposing roles in the generation of Tfh-like cells, and E2A contributes to germinal center B cell development, suggesting a similar role in this subset (Kwon et al., 2008; Miyazaki et al., 2011). Further studies need to be performed to dissect the relationship between Twist1, E47, and the lineage determining factors for the development of each subset.

Moreover, Twist1 can also function through non-canonical bHLH protein-protein interactions. We have previously shown that Twist1 inhibits IFNγ production by forming a complex with Runx3 through its Runt DNA binding domain and preventing it from binding DNA (Figure 33, 36-37) (Pham et al., 2012). Because Runx1 transactivates *Rorc* expression, it is possible that Twist1 interacts with Runx1, thus repressing *Rorc* expression. Whether Runx1 or Runx3 contribute to Tfh development has not been defined.

Twist1 is a novel repressor of Th1, Th17, and Tfh cell development

Many transcription factors contribute to the development of T helper cell lineages (Kanno et al., 2012; Zhou et al., 2009; Zhu et al., 2010). However, the majority of factors promote one lineage, at the expense of another. For example, GATA3 promotes Th2 differentiation as it inhibits the development of Th1 and Th17 cells (Usui et al., 2003; Yagi et al., 2010). In this respect, Twist1 is novel in that it represses Th1, Th17 and Tfh development, without any corresponding increase in cytokine secretion characteristic of other T helper subsets. In targeting these subsets, Twist1 regulates particular components of the inflammatory T cell-mediated immune response. The additional ability of Twist1 to limit B cell responses suggests that signaling pathways regulating Twist1 expression represent potential therapeutic targets for broad modulation of the immune response. A summary of Twist1 function in T helper subsets is shown in Figure 75.

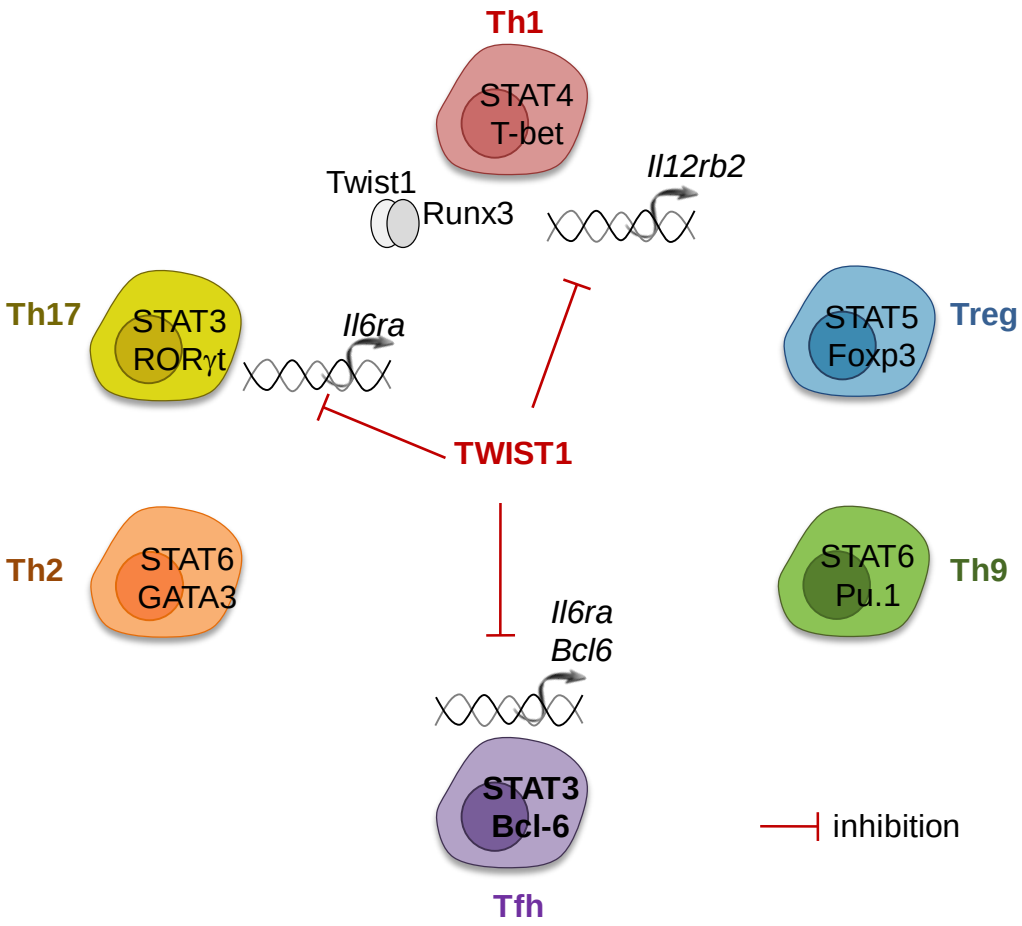


Figure 75. The function of Twist1 in T helper cell development

Part III-Etv5 regulates T helper cell development

Etv5 expression was detectable in all T helper cells with highest expression in Th1 and Th2 cells. Thus, it is possible that Etv5 might function in other T helper cell subsets. Our initial analysis suggested that Etv5 acts as a positive regulator in Th9, Th17, and Tfh cells and a negative regulator in Th2 cells. However, it remains unknown what determines Etv5 function in each T helper cell subset.

In Th1 cells, Etv5 has been shown to genetically cooperate with T-bet to promote IFNγ production (Ouyang et al., 1999). Surprisingly, we observed a modest increase in IFNγ production in *Etv5*-deficient Th1 cells compared to wild type cells (Figure 57). In addition, *Etv5*-deficient Th2, Th9, Th17, and Treg cells produced significantly more IFNγ compared to wild type cells, and that was correlated with increased T-bet expression (Figure 78). Our data suggested that Etv5 negatively regulates IFNγ production by repressing T-bet expression. Etv5 has been shown to physically interact with Twist1, a transcriptional repressor of Th1 cells (Pham et al., 2012; Zhang et al., 2010b) suggesting a possible mechanism in which Etv5-Twist1 complex represses T-bet expression in T helper cell subsets. We have shown that in addition to promoting Th17 cell differentiation, Etv5 directly binds to the *Bcl6* promoter and promotes Tfh cell differentiation. Because the ability of T-bet to represses Bcl-6 in Tfh cell differentiation (Nakayamada et al., 2011), Etv5 might indirectly regulate Tfh cell development by suppressing T-bet expression.

PU.1, an ETS transcription factor, has been shown to be the master regulator of Th9 cell subset (Chang et al., 2010). Interestingly, Etv5 also positively regulates IL-9 production in T cells (Figure 72). Since PU.1 and Etv5 recognize the same ETS consensus sequence, it will be interesting to determine whether there is a functional association between PU.1 and Etv5 in controlling IL-9 production in T cells. In addition, a subset of genes that are enriched and regulated by BATF in Th9 cells were decreased in *Etv5*-deficient Th9 cells compared to wild type cells (Figure 72E). Therefore, it is remained to be determined whether BATF and Etv5 control IL-9 production in T cells in a parallel pathway. Our data further add another transcription factor into a complex regulatory network in Th9 cells.

Ets-1, another member of ETS family transcription factors, has been shown to exert similar functional complexity as Etv5 in T helper cell differentiation. Ets-1 promotes Th1, Th2, and regulatory T cell development while inhibiting Th17 cell differentiation (Grenningloh et al., 2005; Moisan et al., 2007; Strempel et al., 2010). In addition, Ets-1 and Etv5 have been demonstrated to have opposing function in regulating sonic hedgehog (*Shh)* gene expression during limb development (Lettice et al., 2012). Thus, Etv5 might have functional collaboration or mutual antagonism with other members of Ets family transcription factors in regulating T helper cell development.

The requirement for IL-17 in asthma is not completely defined. Increased IL-17A and IL-17F are associated with severe asthma in patients (Al-Ramli et al., 2009).

IL-17 produced from activated $CD4^+$ T cells induces the expression of macrophage and neutrophil attracting chemokines that recruit inflammatory cells into the airways (Laan et al., 1999). However, in mouse models, IL-17 has differential effects during sensitization or challenge, and the requirement for IL-17 varies among model systems (Lajoie et al., 2010; Moreira et al., 2011; Nakae et al., 2002; Schnyder-Candrian et al., 2006; Yang et al., 2008a). Importantly, the house dust mite adjuvant-free model is dependent upon IL-17, in addition to Th2 cytokines. IL-17 production and neutrophil recruitment in the lung were increased following house dust mite sensitization and challenge (Phipps et al., 2009). Consistent with previous observations, we observed a significant decrease of the IL-17 production in the lung, BAL fluid, and in the peripheral lymphoid organs, as well as diminished neutrophil-attracting chemokines, and neutrophil recruitment to the lung in *Etv5*-mutant mice, compared to control mice. Although house dust mite sensitization and challenge elicits Th2 and Th9 responses (Figure 61, 73) (Phipps et al., 2009), the results demonstrated that IL-17 was the most abundant cytokine produced in the lung suggesting IL-17 production from T cells is critical for inflammation in this model. In the absence of Etv5 in T cells, there is diminished inflammation, and reduced IL-17 concentrations, without any corresponding effects on the amounts of Th2 cytokine production. In addition to decreased neutrophil recruitment to the lung, eosinophil number was also reduced. Since IL-17A and IL-17F are shown to have differential effects on eosinophil recruitment during antigen-induced airway inflammation (Yang et al., 2008a), further studies are needed to determine whether IL-17 is responsible for

the reduction in eosinophil number in the lung in our model. Our results suggest that Th17 production of IL-17 provides a unique contribution to the inflammatory milieu in the development of allergic pulmonary inflammation.

Our data show that Etv5-deficiency in T cells does not alter expression of Th17 transcription factors, or other Th17 cytokines, suggesting a restricted effect on expression of the *Il17-Il17f* locus. This is unique among transcription factors that promote Th17 development including RORγt, IRF4 and BATF that regulate multiple components of the Th17 genetic program (Brustle et al., 2007; Ciofani et al., 2012; Ivanov et al., 2006; Manel et al., 2008; Schraml et al., 2009). It is also important to note that Etv5 has a critical role in Th17 cytokine production despite expression of Etv5 not being specific for Th17 cells. This highlights that amount of expression of a transcription factor is not always the most important determinant of function. Our results further document that transcription factors that are not restricted to a particular subset still play obligate roles in defining cellular phenotype, in cooperation with lineage-specific factors.

These results demonstrate that Etv5 is a novel STAT3-induced transcription factor that directs expression of the *Il17-Il17f* locus in Th17 cells. This is consistent with a recent report that Etv5 also contributes to IL-17 production in γδ T cells, and suggests that Etv5 may have common functions among multiple IL-17-secreting T cell populations (Jojic et al., 2013). Our results further suggest that in addition to lineage regulators, such as *Rorc*, *Irf4* and *Batf* that regulate

multiple genes in a subset, there are additional transcription factors that are part of the T helper subset differentiation program that have a more restricted and specific function. Thus, Etv5 represents a unique contributing factor that positively regulates inflammatory responses in Th17 cells and promotes allergic airway inflammation

Collectively, we identified the ETS family transcription factor Etv5 downstream of STAT3 and STAT4 as a controller of T helper cell development. Etv5 promotes cytokine production that contributes to the antigen-induced airway inflammation. In addition, Etv5 plays a role in B cell responses and antibody production. A summary of Etv5 function in T helper subsets is shown in Figure 76.

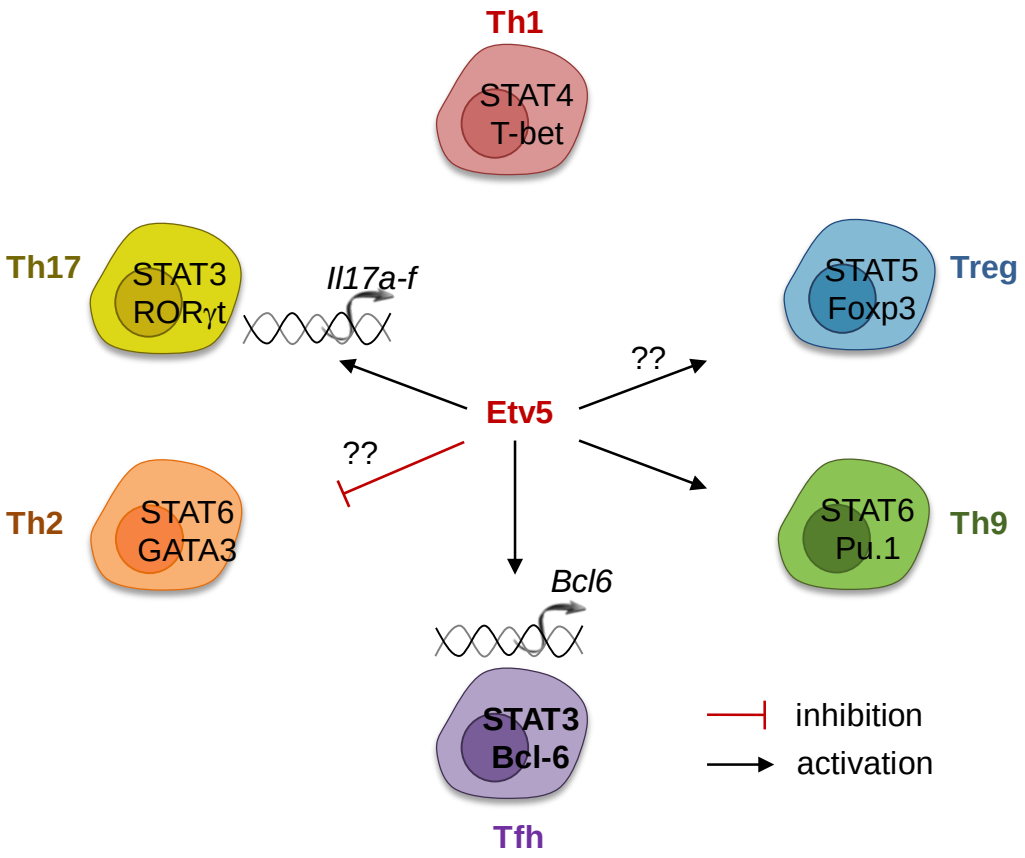


Figure 76. The function of Etv5 in T helper cell development

FUTURE DIRECTIONS

Part I- To define the role of Jmjd3 in gene regulation in Th1 cells

The apparent opposing function of STAT4 and Dnmt3a raises the question whether STAT4 is required to activate gene expression, or whether it functions solely by eliminating negative regulators or recruiting positive regulators of gene expression from target loci. In *Stat4*$^{-/-}$ deficient Th1 cells, we observed decreased Jmjd3 association at Th1 target genes (Figure 19). When Jmjd3 was knocked down in Th1 cells using siRNA targeting *Jmjd3*, we observed decreased IFNγ production compared to control cells (Figure 20). Our results suggest that Jmjd3 is recruited to Th1 gene loci by STAT4 to limit Dnmt3 association. Interestingly, we observed heterogeneous populations when wild type Th1 cells were co-stained with IFNγ and Jmjd3 (Figure 77) suggesting variable Jmjd3 expression in the Th1 cell population. Jmjd3 function is not limited to Th1 cells since knockdown of Jmjd3 in Th17 cells result in decreased IL-17 production (Ciofani et al., 2012). The result suggests that Jmjd3 might play an important role in other T helper subsets. These observations raise the question of how the expression of Jmjd3 integrates with other transcription factors in regulating gene expression and cytokine production in T helper cell subsets.

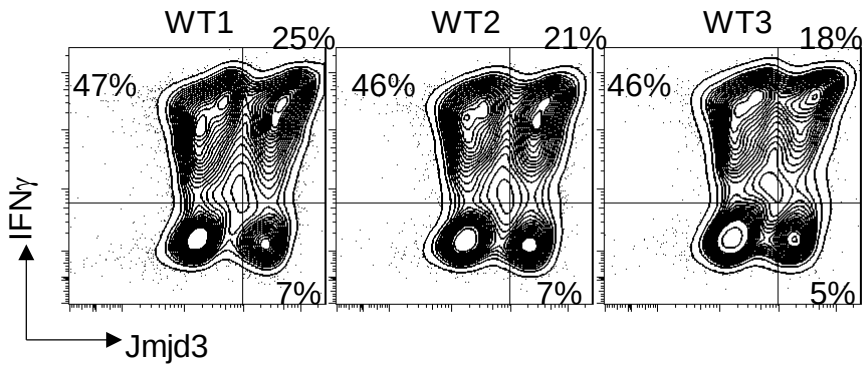


Figure 77. Jmjd3 expression in Th1 cells. Naïve $CD4^+CD62L^+$ T cells were isolated from wild type mice and differentiated under Th1 culture conditions. On day 5, cells were restimulated with PMA and ionomycin for 6 h before intracellular staining (ICS).

We will address the question with three approaches: first we will to determine the correlation between Jmjd3 and Th1 gene expression by generating Jmjd3 reporter mice and comparing gene expression and cytokine production profiles among different Jmjd3 populations in Th1 cells that include $Jmjd3^{neg}$, $Jmjd3^{lo}$, $Jmjd3^{mid}$, and $Jmjd3^{hi}$. Different Jmjd3 populations (neg, low, mid, and high expression) will be sorted to assess Th1 gene expression by qRT-PCR and cytokine production by ELISA. We will also examine the cell cycle and proliferation by CFSE to determine whether Jmjd3 has an effect on cell growth and survival. The second approach is to define the molecular mechanism of Jmjd3 in regulating Th1 cell differentiation. Subpopulation of Jmjd3 expressing Th1 cells will be sorted and the level of phospho-STAT1 and phospho-STAT4 will be assessed by ICS after restimulating the cells with IFNγ and IL-12, respectively. We will determine whether Jmjd3 interacts with STAT1 and STAT4 by performing co-IP and DAPA experiments using wild type Th1 cells. To examine how different amounts of Jmjd3 expression affects transcription factors

binding to Th1 gene loci, sorted cells as described above will be used to assess the ability of transcription factor (T-bet, Stat4, Hlx1, Runx3, and Twist1) bound to the regulatory elements of *Ifng* and other Th1 gene loci. Similarly, we will also examine histone modification and the association of histone modifying enzymes such as Dnmt3a at Th1 gene loci. The third approach is to define the role of Jmjd3 in other T helper subsets. We will examine Jmjd3 expression and the possibility of Jmjd3 associating with other STAT proteins (STAT3, STAT5, and STAT6) in Th2, Th9, Th17, Treg, and Tfh cells. We will generate mice with *Jmjd3*-deficient T cells by crossing *Jmjd3*$^{fl/fl}$ mice (Zhao et al., 2013) with CD4-Cre transgene mice to generate *Jmjd3*$^{fl/fl}$ CD4-Cre^{+}. Gene expression and cytokine production profiles for each T helper subset will be examined using wild type and *Jmjd3*-deficient T cells. Because Jmjd3 has a positive role in Th1 and Th17 cell differentiation, we will determine the effect of Jmjd3 *in vivo* using mouse models such as *Listeria monocytogenes*-induced Th1 development and HDM-induced airway inflammation (Th17).

These three approaches will elucidate the role of Jmjd3 in regulating gene expression in T helper cell differentiation.

Part II- Twist1 regulates T helper cell development

As shown in the previous section, STAT proteins are crucial factors in T helper cell differentiation that regulate a number of transcription factors involved in the process. Twist1 is a transcriptional repressor that is induced by STAT4 in Th1 cells and by STAT3 in Th17 and Tfh cells. Our results demonstrate that Twist1 negatively regulates subsets of genes shared by Th1 and Th17 cells (*Ifng* and *Tbx21)* or Th17 and Tfh cells (*Il6ra, Batf,* and *Maf*). Thus, we ask whether Twist1 regulates distinct gene expression in Th1, Th17, and Tfh cells that are responsible for its development. We will address the question with two aims: the first aim is to define gene expression profiles in T cells that lack Twist1 expression. We will perform a microarray assay using *in vitro* generated wild type and *Twist1*-deficient Th1, Th17, Tfh-like cells and Tfh cells from day 9 SRBC-immunized wild type and *Twist1*-mutant mice. The experiment will allow us to define genes that are distinctly regulated by Twist1 by Th1, Th7, and Tfh cells. Given that the ability of Twist1 to directly bind and repress gene expression, the second aim is to determine the direct target of Twist1 in regulating T helper cell differentiation. We will perform ChIP-seq assay to determine the ability of Twist1 binding to its target genes in Th1, Th17, and Tfh cells using wild type and *Twist1*-deficient T cells. We will perform ChIP and DAPA assays to validate ChIP-seq data and luciferase assay using Twist1 target genes to determine whether Twist1 could repress its transcriptional activity. The results will provide additional insight into the understanding of Twist1-mediated gene regulation in T helper cell differentiation.

Twist1 functions as a homodimer, or as a heterodimer with bHLH factors and non-bHLH factors. We observed Twist1 forms complexes with T-bet, Runx3, and E47 in Th1 cells (Figure 33 and 46). Thus, the Twist1 heterodimer plays curcial role in regulating T helper cell programming. We will determine the existence of other possible Twist1 interacting partners that are potentially involved in regulating T helper cell differentiation. Runx1 contains the Runt DNA binding domain that has crucial role in Th17 and Treg cell development (Kitoh et al., 2009; Lazarevic et al., 2011; Rudra et al., 2009). However, mice with *Twist1*-deficient T cells have defects in Th17 cell differentiation but not Treg cell differentiation. We want to determine whether the Twist1-Runx1 complex exists in Th17 cells using DAPA and co-IP assays. Similarly, we observed Twist1 interacts with E47 in Th1 cells, and that E47 binding to its target gene was decreased in the absence of Twist1 in Th17 cells (Figure 46). Thus, we will determine the association between Twist1 and E47 in Th17 cells using DAPA and co-IP assays. Given that a Twist1 and Etv5 interaction has been described (Zhang et al., 2010b), and that Twist1 and Etv5 have opposing function in T helper cell development, we want to determine whether there is an association between Twist1 and Etv5 in T helper cell subsets using DAPA and co-IP assays. Because *Twist1*-mutant mice have severe onset of MOG-induced EAE than wild type mice, we will mate *Twist1*$^{fl/fl}$ CD4-Cre mice with *Etv5*$^{fl/fl}$ CD4-Cre mice to generate compound mutant mice. We will perform MOG-induced EAE using wild type, *Twist1*-mutant, *Etv5*-mutant, and *Twist1*- and *Etv5*-doubly mutant mice. It will be interesting to see whether *Twist1*- and *Etv5*-doubly mutant mice develop

similar onset disease compared to wild type mice. These experiments will elucidate the interacting partners of Twist1 and their functions in regulating T helper cell development.

Part III-Etv5 regulates T helper cell development

To define the mechanism by which Etv5 promotes Th9 cell differentiation

We demonstrate that *Etv5*-deficient Th9 cells produce significantly less IL-9 compared to wild type cells (Figure 72B-D). *Etv5*-deficient Th9 cells also have less Th9-specific gene expression compared to wild type cells (Figure 72E). Moreover, in the HDM-induced airway inflammation model, we detect reduced IL-9 production by splenocytes and in the BAL fluid in *Etv5*-mutant mice compared to wild type mice (Figure 73).

Thus, our results suggest that Etv5 positively regulates IL-9 production in Th9 cells. We hypothesize that Etv5 is a novel transcription factor that promotes IL-9 production in T cells. We will address the hypothesis with two aims: the first aim is to define the mechanism in which Etv5 regulates IL-9 in Th9 cells. In this aim, we will determine whether Etv5 could directly bind and promote *Il9* expression using DAPA and luciferase assays. We will also determine the histone modification (H3K4me3, H3K27me3, H3K27ac, H4K5ac, H4K8ac, H4K16ac, H3K9/18ac, and H3K36ac), the association of histone modifying enzymes (p300, PCAF, and Gcn5), and transcription factor binding (PU.1, IRF4, and BATF) at *Il9* locus. Given that IL-9 is important for immune responses against worm infection (Faulkner et al., 1998; Richard et al., 2000), we will determine whether *Etv5*-mutant mice have worsened worm burden compared to wild type mice in the *Trichuris muris* model. Given that Etv5 and PU.1 belong to the ETS family of transcription factors, the second aim is to determine the cooperative effect of

Etv5 and PU.1 in regulating Th9 cell differentiation. We will perform the DAPA and luciferase assays to test the ability of Etv5 and PU.1 to cooperate and transcriptionally promote *Il9* expression. To test whether Etv5 and PU.1 genetically cooperate to regulate *Th9* cell development, we will ectopically express *Etv5* or *Sfpi1* in *Sfpi1*-deficient or *Etv5*-deficient Th9 cells, respectively; and examine cytokine production by ICS and ELISA and gene expression by qRT-PCR. To test whether both Etv5 and PU.1 are required for the optimal Th9 cell development, we will mate *Sfpi*$^{fl/fl}$ mice with *Etv5*$^{fl/fl}$ CD4-Cre mice to generate compound mutant mice. We will examine cytokine production, gene expression, histone modification, and the association of histone modifying enzymes at *Il9* locus using wild type, *Sfpi1*-deficient, *Etv5*-deficient, and *Sfpi1*- and *Etv5*-doubly deficient Th9 cells. We expect significantly decreased cytokine production and gene expression by *Sfpi1*- and *Etv5*-doubly deficient Th9 cells compared to *Sfpi1*-deficient and *Etv5*-deficient Th9 cells. We next want to test the requirement of Etv5 and PU.1 regulating IL-9 using *in vivo* disease models. We will test whether mice with *Sfpi1*- and *Etv5*-doubly deficient T cells have worsened worm burden compared to mice with *Sfpi1*-deficient or *Etv5*-deficient T cells in the *Trichuris muris* model. In contrast, because IL-9 is a key player in allergic airway inflammation, we can to test whether mice with *Sfpi1*- and *Etv5*-doubly deficient T cells have less inflammation compared to mice with *Sfpi1*-deficient or *Etv5*-deficient T cells in the OVA-induced airway inflammation model. The results will add additional insight into the transcription factor network in controlling Th9 cell development.

To define the mechanism in which Etv5 regulates Foxp3 function

We demonstrate that mice with *Etv5*-deficient T cells have less nTreg and iTreg compared to wild type cells (Figure 58B). Thus we hypothesize that Etv5 positively regulates Treg cell development. We will address the hypothesis with two aims: in the first aim, we will define the mechanism in which Etv5 promotes Treg cell differentiation. We will perform the suppression assay to test whether *Etv5*-deficient Treg cells adequately suppress effector T cells compared to wild type cells. We will perform ChIP assay testing the ability of Etv5 binding to the regulatory elements of the *Foxp3* locus using differentiated wild type and *Etv5*-deficient Treg cells. We will also perform luciferase assay to test whether Etv5 could repress the transcriptional activity at the *Foxp3* promoter. We will also determine the histone modification and the association of histone modifying enzymes at the *Foxp3* locus. In the second aim, we will define Treg function in the absence of Etv5 in T cells *in vivo* using disease model. Because *Etv5*-deficient T cells have increased IFNγ compared to wild type *in vitro* cultures (Figure 78A-B), we want test the ability of *Etv5*-deficient Treg cells to suppress inflammation *in vivo* in a T cell transfer model of colitis that induces strong Th1-mediated colitogenic inflammation. We will adoptively transfer *Il10*-deficient T cells together with wild type or *Etv5*-deficient Treg cells into Rag1-deficient mice and assess weight loss, changes in colon length, and histological analysis.

To define the role of Etv5 in Tfh cell development

We demonstrate that Etv5 directly binds to the *Bcl6* promoter, positively regulates its expression, and promotes Tfh cell development *in vivo* (Figure 69-70). Thus, we hypothesize that Etv5 is an upstream factor that positively regulates *Bcl6* expression and promotes Tfh cell differentiation. We will test our hypothesis with the approach that is to define the role of Etv5 in regulating Tfh cell differentiation. Given that *Etv5*-deficient and *Bcl6*-deficient mice have impaired Tfh cell development, we will generate Tfh-like cells *in vitro* and perform a microarray assay to compare gene expression profiles between *Etv5*-deficient and *Bcl6*-deficient T cells. This experiment will allow us to define subsets of gene that are distinctly regulated by Etv5 and Bcl6. Since we showed that Etv5 directly binds to the *Bcl6* promoter (Figure 70), we will further examine whether Etv5 is an upstream factor of Bcl-6 in regulating Tfh cell differentiation. We will ectopically express *Bcl6* in *Etv5*-deficient T cells *in vitro,* transfer to SRBC-immunized *Etv5*-deficient mice, and examine the formation of GC B cells. The results will add additional insight into the transcription factor network in Tfh cell development.

To define the mechanism in which Etv5 regulates Ifng expression in T helper cells

We show that *Etv5*-deficient Th2, Th9, Th17, and Treg cells express significantly higher *Ifng* and *Tbx21* expression compared to wild type cells, although deficiency has a with a modest effect in Th1 cells (Figure 78). We demonstrate

that knocking down T-bet using specific siRNA in *Etv5*-deficient Treg cells results in decreased IFNγ production compared to control cells and modest increased *Foxp3* expression compared to wild type cells (Figure 79). Thus, we hypothesize that Etv5 contributes to T helper cell identity by repressing *Tbx21* and *Ifng* expression.

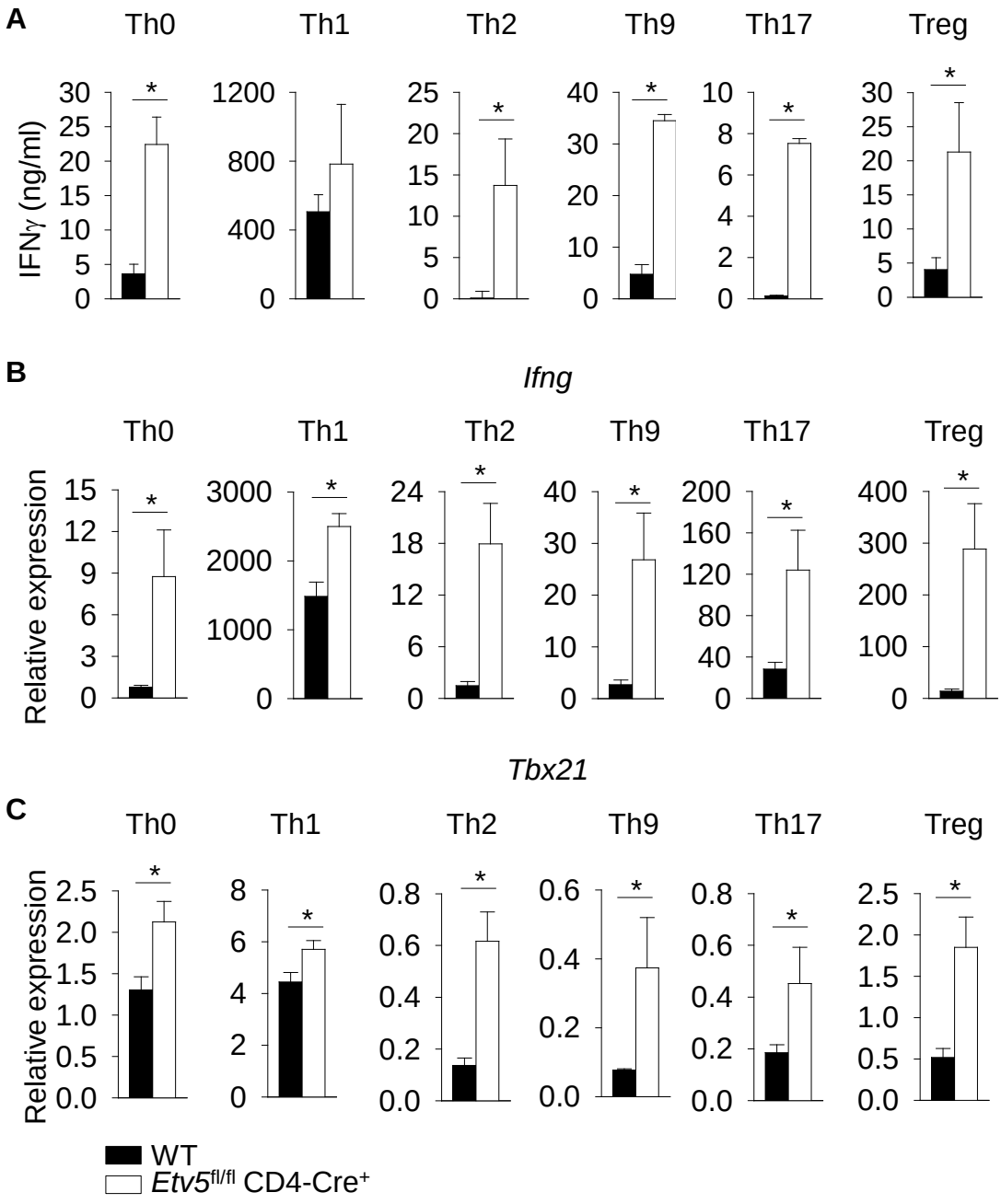


Figure 78*. Ifng* and *Tbx21* expression in *Etv5*-deficient T cells. (A-C) Naive wild type and *Etv5*-deficient $CD4^+CD62L^+$ T cells were activated (Th0) or cultured under Th1, Th2, Th9, Th17, and regulatory T (Treg) cell polarizing conditions. On day 5, cells were used for assessing IFNγ production by ELISA after 24 h anti-CD3 stimulation (A), and gene expression analysis before (*Tbx21*) (C) or after (*Ifng*) (B) 6 h anti-CD3 stimulation by qRT-PCR. Data are mean of four independent experiments ± S.E.M. *p<0.05

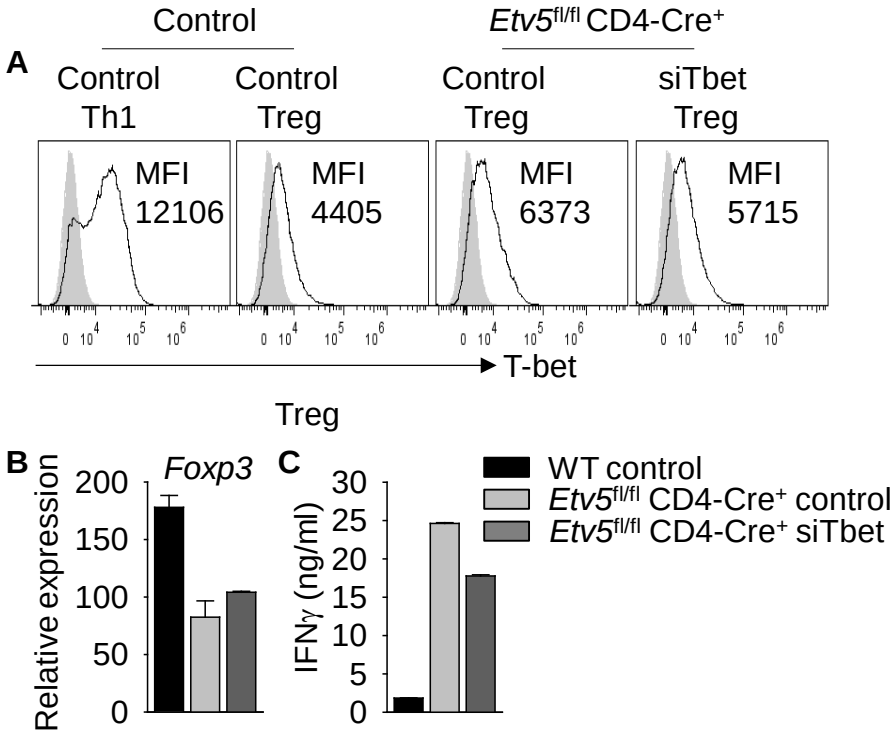


Figure 79. Etv5 represses *Ifng* and *Tbx21* expression in Treg cells. (A-C) Naïve $CD4^+CD62L^+$ T cells were isolated from wild type and *Etv5*-mutant mice and differentiated under Th1 or Treg cell culture conditions. On day 5, cells were transfected with control or siRNA-specific *Tbx21,* rested overnight, stained for T-bet expression by ICS (A), assessed for *Foxp3* expression by qRT-PCR (B), and restimulated with anti-CD3 to measure IFNγ production by ELISA (C). Data are mean of replicate samples ± S.D. and representative of four independent experiments with similar results.

We will test our hypothesis with two aims: in the first aim, we will determine the mechanism in which Etv5 regulates *Ifng* expression in non-Th1 cells. We will

perform ChIP assay to examine histone modification, the association of histone modifying enzymes, and T-bet binding at the *Ifng* locus in wild type and *Etv5*-deficient Th1, Th2, Th9, Th17, and Treg cells. To determine whether Etv5 negatively regulates IFNγ production in non-Th1 cells by repressing T-bet expression, we will perform similar analysis by assessing Etv5 binding at the *Tbx21* locus. We will mate *Etv5*$^{fl/fl}$ CD4-Cre positive with *Tbx21*$^{-/-}$ to generate *Etv5*$^{fl/fl}$ *Tbx21*$^{-/-}$ CD4-Cre positive with Cre-negative littermates as control mice. We will examine cytokine production, gene expression, histone modification, and the association of histone modifying enzymes at the *Tbx21* and *Ifng* loci using wild type, *Etv5*-deficient, *Tbx21*$^{-/-}$, and *Tbx21*- and *Etv5*-doubly deficient T cells. To determine the function of IFNγ-producing cells in *Etv5*-mutant mice, we will test the ability of *Etv5*-deficient IFNγ-producing T cells to mediate protective function against *Listeria monocytogenes* or *Toxoplasma gondii.* We will cross *Etv5*$^{fl/fl}$ CD4-Cre^{+} mice with IFNγ reporter mice (The Jackson Laboratory) to generate *Etv5*$^{fl/fl}$ CD4-Cre^{+}- IFNγ GFP mice. We will differentiate Th0, Th1, Th2, Th9, Th17, and Treg cells *in vitro* using *Etv5*$^{fl/fl}$ CD4-Cre^{+}- IFNγ GFP mice, sort IFNγ-producing T cells (GFP^{+}), and adoptively transfer to RAG2$^{-/-}$ recipients that have been infected with *Listeria monocytogenes* or *Toxoplasma gondii*. These experiments will allow us to determine whether IFNγ production by *Etv5*-deficient Th1, Th2, Th9, Th17, and Treg cells are capable of promoting protection against *Listeria monocytogenes* or *Toxoplasma gondii*.

In summary, these future studies not only dissect the role of Jmjd3, Twist1 and Etv5 in T helper cell subsets but also provide valuable information to the understanding of how transcription factor networks regulate T helper cell function and their roles in disease models. Different strategies can be employed to target transcription factors as a therapeutic approach such as small molecules targeting upstream activating pathways of transcription factors and DNA-binding activity. Therefore, Twist1 and Etv5 might be potential candidates for future therapy in autoimmune diseases and allergic inflammation.

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