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Functional Interplay Between Trans Factors and Cis-Elements that Govern Il17a&f Transcription

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FUNCTIONAL INTERPLAY BETWEEN *TRANS* FACTORS AND *CIS*-ELEMENTS THAT GOVERN *IL17A&F* TRANSCRIPTION

by

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ABSTRACT

Protective immunity against a variety of infections depends on the amplification and differentiation of naïve antigen-specific CD4 T cells. T helper 17 (Th17) cells are important for defending mucosal barriers from invading extracellular bacterial pathogens[3]. This effector CD4 lineage is characterized by production of the cytokines IL-17A, IL-17F, IL-21, and IL-22, which elicit influx of inflammatory cells to promote pathogen clearance[4-6]. The cytokines IL-6 and TGF-β are the principal factors that initiate Th17 lineage development[7,8], and the STAT3-inducing cytokines IL-21 and IL-23 assist and/or maintain the Th17 developmental program[9]. Recently, an additional cytokine has been demonstrated to encourage Th17 differentiation: IL-1β[10,11]. IL-1β impacts expression of Th17-associated genes very early in the course of Th17 lineage commitment[11], and in combination with IL-6 or IL-23, it can induce robust TCR-independent IL-17 production[12]. However, the mechanism(s) by which IL-1β exerts these effects are poorly understood.

We have examined the effects of IL-1 signaling upon Th17 chromatin conformation and transcription factor recruitment. We found that IL-1β induces activation of NF-κB proteins that bind critical genomic regulatory elements to affect *Il17* transcription. Our data suggest that IL-1β also modifies chromatin architecture, as its presence allowed for enhanced binding of the crucial Th17 transcription factor STAT3 to

regulatory sites in the extended *Il17a/f* locus. Additionally, IL-1β impacts proximal signaling events that result in amplification of STAT activation in response to cytokine signals in microenvironment. All of these mechanisms augment the ability of Th17 cells to respond effectively to pathogens and aid in host defense.

The long held belief that T helper cell differentiation results in irreversible lineage commitment with permanent heritable accessibility or silencing of cytokine genes has been undermined by recent studies demonstrating plasticity of helper T cell phenotypes[13,14]. We demonstrate that epigenetic instability at the *Ifng, Il17a/f,* and *Rorc* cytokine gene loci underlie the propensity of Th17 cells to convert to IFN-γ-producing cells under conditions of limited TGF-β[15]. This plasticity illustrates the dynamic interplay of transcription factors and epigenetic modifications that occur at cytokine gene loci in response to cytokine signals, which are of profound consequence for host defense.

DEDICATION

This work is dedicated to my son, Jackson Raul Whitley, who wishes to be a scientist and a doctor when he grows up. You brighten my every day and I love you more than words can express.

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List of Abbreviations

APC	Antigen-presenting cell
BATF	bZip ATF-like transcription factor
ChIP	Chromatin immunoprecipitation
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
FACS	Fluorescence-activated cell sort
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box protein p3
GATA3	GATA-binding protein 3
GFP	Green fluorescent protein
Hlx	H2.0-like homeobox transcription factor
IRF	Interferon-regulatory factor
Jak	Just another kinase
IEL	Intra-epithelial lymphocyte
IFN	Interferon
IL-	Interleukin-
LCR	Locus control region

LPL	Lamina propria lymphocyte
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll-a Protein
Rag	Recombination activating gene
RNA	Ribonucleic acid
ROR	Retioid orphan receptor
STAT	Signal transducer and activator of transcription
SRC	Sarcoma family kinase
SI	Small intestine
TCR	T cell receptor
Tg	Transgenic
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tissue necrosis factor
WT	Wild type

INTRODUCTION

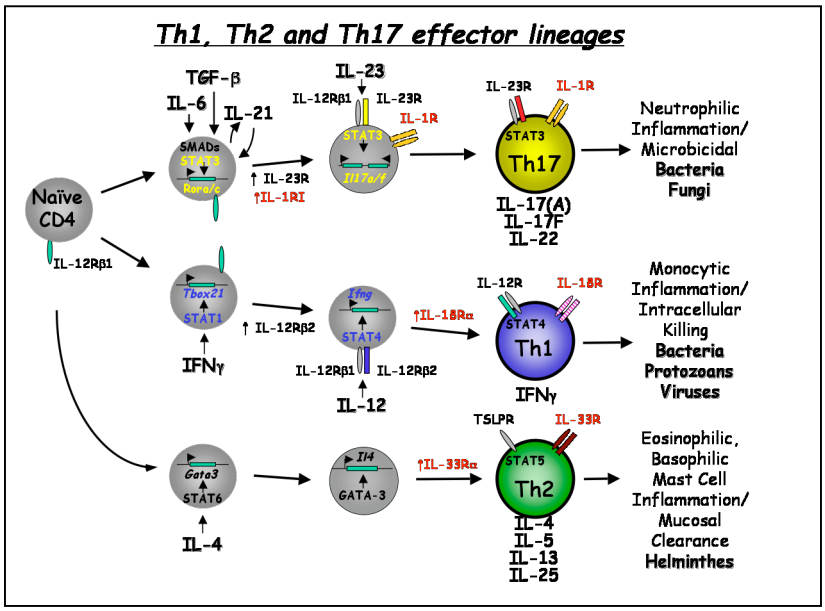
T helper Differentiation and Plasticity

Antigen-specific CD4 T cells are essential players in the adaptive immune response. T cell responses are initiated by specialized cells that are capable of processing antigens and presenting antigenic peptides bound to self major histocompatibility complex (MHC) proteins in the T-cell zone of secondary lymphoid organs where naïve T cells reside[1]. Only a T cell that expresses a T cell receptor (TCR) capable of recognizing a specific peptide–MHC (pMHCII) complex on an antigen presenting cell (APC) will undergo clonal expansion, effector differentiation, and potentially become a memory T cell. Following invasion of the body by a pathogen, APCs phagocytose microbial antigens and transport them to the T-cell zone of secondary lymphoid organs for presentation to T cells.

Priming of CD4 T-cell responses involves several different APC populations that sequentially present foreign pMHCII complexes to CD4 T cells[16]. These include lymph node resident $CD8^-$ dendritic cells (DCs), migratory DCs, monocyte-derived DCs, and B cells. As components of the innate immune system, these APCs also produce cytokines that influence T helper cell development. The cytokine milieu created by innate immune cells is central to induction of transcription factors that determine T helper cell fate[2,4].

T helper 1 cells emerge when Interleukin (IL)-12 impinges on the antigen-induced differentiation of a naïve CD4 cell[17,18]. Interferon gamma (IFN-γ) and/or IL-27 exposure leads to activation of the signal transducer and activator of transcription (STAT) 1 by developing T helper cells, and expression of the T-box transcription factor T-bet[19]. T-bet induces H2.0-like homeobox (Hlx) upregulation, and together these two transcription

factors synergize with those activated following TCR ligation to activate *Ifng* transcription and antagonize GATA-binding protein 3 (GATA3) expression[2]. These events culminate in IL-12 receptor expression, which allows Th1 precursors to respond to IL-12 secreted by antigen presenting cells and activate STAT4 and RUNX3[20]. Th1 cells function to protect the host from infections with intracellular pathogens such as viruses, and are critical for mounting cytotoxic T lymohocyte (CTL) responses that are key to effective elimination of such pathogens.



Antigen-induced differentiation in the context of IL-4 leads to T helper 2 cell development, which protects a host from infection with parasites. Naïve CD4 cells respond to IL-4 by activating STAT6 and transcribing *Gata3*. As a master regulator of Th2 development, GATA3 establishes a positive feedback loop that reinforces its own expression as well as that of the linked *Il4, Il5,* and *Il13* genes, collectively referred to as the Th2 cytokine locus. [21,22]

T-bet and GATA3 are termed master-regulatory proteins of the Th1 and Th2 lineages, respectively, on the basis of their ability to confer lineage-specifying attributes onto cells in the opposing lineage. Ectopic expression of T-bet in Th2 cells induces *Ifng* expression and down-regulation of Th2 cytokines[22]. Conversely, introduction of GATA3 into Th1 cells decreases IFN-γ production and induces IL-4, IL-5, and IL-13. Forced expression of GATA3 permits DNase hypersensitivity (HS) and histone acetylation around the *Il4* gene in the absence of STAT6[21]. These data suggest involvement of GATA3 and T-bet in directing epigenetic changes in cytokine loci during Th lineage commitment.

Epigenetics is the study of changes produced in gene expression by mechanisms other than changes in the underlying DNA sequence. Modification of the methylation status of DNA or acetylation of histones which comprise the tertiary structure of DNA termed chromatin, are examples of epigenetic changes. There are many epigenetic mechanisms that activate or silence gene expression. Chromatin-remodelling complexes, histone-modification enzymes, and polycomb silencing complexes are key for exerting epigenetic effects upon DNA. In combination with RNA polymerases and transcription factors, the other *trans* elements of chromatin, these factors help determine lineage fate by interacting with *cis* elements. *Cis*-elements are regulatory sequences within DNA that attract *trans* factors to promote or silence gene expression. Regulating the accessibility of *cis*-regulatory regions is one way in which TCR- and cytokine-induced signals affect cytokine gene transcription and T helper lineage commitment.[20]

Although gene promoters are necessary for initiation of transcription, it has been determined that *cis*-elements much more distal are what determine specificity and level of

gene expression[23]. Enhancers are *cis*-elements that augment transcription by promoting permissive epigenetic modifications, and silencers limit gene expression by inducing repressive modifications to chromatin. Other *cis*-regulatory elements include: insulators, locus control regions, and matrix attachment regions. Insulators create boundaries between adjacent genes, allowing them to be regulated independently of neighboring genes[24]. A gene locus control region (LCR) exhibits both insulator and enhancer function. LCRs have been functionally defined by their ability to permit copy number-dependent expression of transgenes. Matrix attachment regions are *cis*-elements that anchor chromatin loops to allow for interactions between DNA that form the basis of long-range gene regulation.[20]

Many *cis*-regulatory elements have been defined for the *Ifng* and Th2 cytokine loci[20-22,25,26]. DNase hypersensitivity mapping and chromatin immunoprecipitation (ChIP) have been the means by which most investigations of epigenetic regulation of DNA have been carried out. Increased sensitivity to cleavage by the enzyme DNaseI is a manifestation of epigenetic change indicative of nucleosome reorganization. DNase hypersensitive sites are typically located in regions of the genome that are highly accessible to sequence-specific DNA binding proteins.

Several DNase I hypersensitive regions have been identified within the coordinately regulated *Il4, Il5,* and *Il13* gene loci. Some DNase HS regions (HSS3 and HSIV) are present in the *Il4* locus of naïve CD4+ T cells, and many more emerge following Th2 differentiation, including sites located in the *Il4* and *Il13* promoters and introns[21,22]. An insulator element for the Th2 locus has been defined that is contained within the *Rad50* gene, which lies between *Il5* and *Il13* on murine chromosome 11[2].

Binding sites for the boundary element-associated factor CTCF as well as the Th2-specifying transcription factors STAT6, GATA3, and c-Maf have been identified within the Rad50 gene using chromatin immunoprecipitation, and multiple other hypersensitive sites that recruit these factors lie internal or proximal to the *Il4, Il5,* and *Il13* genes[21].

Conversely, most of the DNase HS regions identified for the *Ifng* gene are activation dependent[20,26]. Long-range hypersensitivity mapping of murine *Ifng* revealed that some DNase HS peaks are common to naïve, Th1, and Th2 cells (HS-70, -22, +19, +40, and +46). Accessibility of these regions in naïve CD4 cells suggests a possible role for them as foci for initiation of lineage-specific remodeling of *Ifng* during T helper differentiation. Th1 polarization induced multiple additional HS sites, including those at the *Ifng* gene promoter, and many that are coincident with conserved non-coding sequences (CNSs) at -54, -34, +17, +30, and +54[26]. A CNS is a non-coding segment of DNA conserved to at least 70% sequence identity between species, that is at least 100bp in length. In general most, but not all, DNase HS peaks correspond with CNSs. However, several HS regions reside in non-CNS sites in the extended *Ifng* locus[26]. Interestingly, different DNase HS patterns were observed for CNSs in the extended *Ifng* gene following TCR activation versus cytokine stimulation[26]. This may reflect a differential role for individual enhancers within the *Ifng* gene in response to distinct stimuli that induce IFN-γ.

There is acquired resistance of each cytokine locus to DNase I digestion and permissive histone acetylation that highlights the dichotomy central to T helper cell development: reciprocal regulation of effector cytokine genes[27]. Th1 differentiation is accompanied by favorable epigenetic changes across *Ifng*, whereas silencing of *Ifng*

transcription in Th2 cells is evident in the acquired resistance to DNase I digestion at *Ifng* CNSs +19, +40, and +46 with accompanying repressive histone modifcation (H3K27) at these and other *cis*-elements known to regulate *Ifng*.[20] As is the case in the Th2 cytokine locus, many transcription factors that induce Th1 development bind to *cis*-elements in the *Ifng* locus to regulate its expression. Previous work has characterized recruitment of the Th1-specifying factors STAT4 (CNSs +17/19, +46) and Runx3 (*Ifng* promoter) across the *Ifng* gene[20]. T-bet binding has been detected at *Ifng* CNSs-34, -22, +17/19, +30, and +46[19,28]. RelA, an NF-κB factor essential for acute *Ifng* transcription, binds to CNSs -34 and +40 upon TCR stimulation of Th1 cells, and to CNSs -34, -22, +46, and +54 following cytokine stimulation (IL-12 + IL-18)[26]. Two CTCF-binding elements define the outer boundaries of the *Ifng* locus, located at -70 and +66[28].

To summarize, studies to date have delineated multiple functional interactions between evolutionarily conserved *cis*-regulatory elements and important *trans* acting factors that dictate transcription of the reciprocally regulated *Ifng* gene and Th2 cytokine locus during T helper differentiation. This information has been the basis of the notion that the Th1 and Th2 effector subsets represent static, heritable phenotypes that are unequivocally passed onto daughter cells. The observation that Th1 or Th2 polarization of naïve CD4 T cells led to irreversible lineage commitment *in vitro* [29]that is associated with silencing of cytokines and transcription factors specific to the opposing Th lineage implied that T helper fate is irreversible. However, in recent years this dogma has been questioned, as characterization of a third lineage of effector T cells, termed T helper 17 (Th17) cells, which exhibit less stable production of its effector cytokines, has raised

questions about the degree to which T helper lineages represent stable, heritable phenotypes[13,14].

In addition to regulating transcriptional competence, it is becoming clear that epigenetic mechanisms enforce heritability and plasticity of chromatin conformation across cell division[15]. Although at the present time little is known why some T helper cells co-express cytokines or exhibit reversible silencing of lineage-inappropriate cytokines, the field is beginning to adopt a model of T helper cell development that takes into account the differences between phenomena observed *in vitro* and *in vivo* with respect to lineage commitment and stability[5]. New subsets of helper T cells continue to be recognized, including the IL-17-producing, IL-9-producing 'Th9' cells, and IL-22-producing 'Th22' cells that are more likely to represent a temporary epigenetic state than a distinct 'lineage' of effector CD4 cells[30]. Considerable controversy surrounds the definition of a lineage of T helper cells, as the nature of a lineage-committed T helper cell is demonstrably not as clear-cut as the original Th1/Th2 paradigm suggested.

By employing an IL-17F reporter mouse that permitted identification and isolation of Th17 cells that produced IL-17 and/or IFN-γ, our group discovered that the balance of cytokines in the local environment dictated whether progeny of Th17 cells retained high-level IL-17A/F production, or proceeded to silence IL-17 and produce copious IFN-γ[14]. Alternatively, cells could produce a heterogeneous mixture of both IL-17 and IFN-γ. Our results demonstrate that the Th17 lineage exhibits epigenetic instability underlying the capacity of 'committed' IL-17-producing Th17 effector cells to transition to predominantly IFN-γ-producing cells under conditions of TGF-β deprivation

or other changes to the cytokines present in the microenvironment. Regulatory T cells (Tregs) appear to possess a similar plasticity[13].

From these data, we surmise that the same epigenetic processes which allow programs of gene expression to be inherited by progeny cells, permit changes in gene expression in response to altered environmental signals. Such plasticity could be advantageous to the host in that they allow T cell responses to be stable and long-lasting, yet sufficiently adaptable to allow additional or alternative cytokine production when necessary.

Transcription Factor & Chromatin Dynamics at the *Il17a/f* Locus

T helper 17 (Th17) effector cells emerge when maturing progeny of naïve CD4 cells respond to the cytokines IL-6 and TGF-β in the local environment[7,30]. IL-6R signaling activates STAT3, an essential Th17-specifying factor, to enable upregulation of the IL-21 receptor and permit further STAT3 activation[3]. Together with TGF-β-induced signals, STAT3 induces the expression of retinoic-acid-receptor-related-orphan receptor-γt (RORγt), dubbed the master regulator of the Th17 developmental program. RORγt furthers Th17 differentiation by contributing to *Il21* upregulation. IL-21 acts as an autocrine factor, reinforcing its own actions on the Th17 pathway, and its actions are required for optimal expression of IL-23R[31]. IL-23, which was once believed to be an initiator of Th17 development, is now presumed to be important only at later stages of Th17 development, perhaps for the survival and expansion of committed Th17 effectors.

It has long been known that that specific absence of IL-23 protects animals from Th17- associated autoimmune disease[3]. Mice deficient in IL-23 succumb to *Citrobacter*

rodentium infection very rapidly, and develop disease in the experimental autoimmune encephalomyelitis (EAE) model[3]. Our own data suggests that IL-23 influences effector functions, for example acute cytokine production, that are critical to development of inflammation by Th17 cells.

STAT3 is of crucial importance to Th17 development, as its specific absence yielded a lack of IL-17 production and failure of cells to express *Il23r, Il22,* and other Th17-associated genes[32]. Importantly, CD4-specific deficiency of STAT3 in mice greatly decreased expression of RORγt and RORα, which are key Th17 lineage-specifying transcription factors. Interestingly, humans afflicted with hyper-IgE syndrome are now known to suffer from a mutation in the gene encoding STAT3 which prohibits its proper activation[33]. Not surprisingly, these patients exhibit profound deficiency of Th17 cells and experience general immune dysfunction. Three distinct Th17-inducing cytokines activate STAT3 upon binding to their Jak-associated receptor[8]. Activation of STAT3 by IL-6, IL-21, and IL-23 is clearly a major means by which these cytokines exert effects on IL-17 expression and Th17 lineage commitment.

STAT3 has been found to bind to regions in and around the *Il17a* and *–f* genes to directly regulate the coordinately expressed *Il17a* and *Il17f* genes[34]. Additionally, STAT3 binds the promoters of the *Il21, Il21r,* and *Il23r* genes[35] to exert global influence on Th17 gene expression. Multiple transcription factors that promote Th17 lineage specification are subject to direct regulation by STAT3: among them are Batf, IRF-4, and RORγt.

Batf is a member of the AP-1 transcription factor family that has been shown to be essential for Th17 differentiation. *Batf-/-* mice exhibit a complete loss of their Th17

compartment and are resistant to induction of EAE. Batf is known to heterodimerize with JunB to bind the promoters of *Il17a, Il17f, Il21,* and *Il22,* as well as two intergenic CNS elements in the *Il17a/f* locus. [36]

IRF-4, previously implicated in the regulation of *Gata3* expression and Th2 lineage commitment, is now known to regulate the *Il17a/f* and *Il21* genes as well. It was found that *Irf4* mice are resistant to EAE, and there is some data to support the premise that IRF-4 cooperates with STAT3 to induce RORγt expression. [30]

As RORγt is as a key regulator of Th17 development, its recruitment to CNS elements in the *Il17a/f* locus has been probed. RORγt was found to bind a CNS located 5kb upstream of the *Il17a* promoter, and has been proposed to cooperate with the transcription factor Runx1 at this site to promote *Il17* transcription. CNS A-5 is also subject to opposing regulation by the Treg-associated transcription factor Foxp3; these three factors were shown to physically interact at this location to exert influence on *Il17* expression.[37]

Despite fairly extensive knowledge about the transcription factor networks that direct Th17 lineage specification, there is very limited information about *cis*-elements they interact with to regulate *Il17a* and *–f* expression. Prior to the studies described herein, it was difficult to surmise how various transcription factors may interact to promote and sustain IL-17 expression. Our findings substantially extend the functional characterization of *cis*-elements regulating *Il17a* and *–f* transcription. Additionally, we describe two *trans* factors induced by IL-1 signaling that are co-recruited with STAT3 to several *cis*-elements that regulate *Il17a/f*. We find that in addition to inducing factors that directly regulate *Il17*, IL-1β facilitates enhances STAT3 activation and binding to

cis-elements, providing a basis for understanding the functional interplay between *cis* and *trans* factors at the extended *Il17a/f* locus.

IL-1β Enhances STAT3 Activation and Enables Cooperativity between STAT3 and NF-κB Factors in Regulating *Il17* Transcription

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ABSTRACT

IL-1 contributes to differentiation of Th17 cells from naïve CD4 precursors and supports TCR-independent production of IL-17. However, the means by which IL-1 regulates various aspects of Th17 development is poorly understood. Here we show that IL-1β cooperates with STAT3-inducing cytokines to incite potent inflammatory cytokine production by Th17 cells. We describe a novel mechanism by which signaling through IL-1 family receptors leads to enhanced STAT activation in Th17, Th1, and Th2 cells. We find that IL-1β assists STAT3 in regulating *Il17* transcription by facilitating its binding to key *cis*-elements that control IL-17 expression. Additionally, we find that the IL-1-induced NF-κB factors RelA and c-Rel are important contributors to Th17 differentiation. These transcription factors directly regulate the *Il17a/f* locus in cooperation with STAT3. Our findings reveal that IL-1 regulates proximal signaling events that greatly enhance STAT3 activation and promotes complex interactions between transcription factors and *cis*-regulatory elements that govern *Il17a* and *-f* gene transcription.

INTRODUCTION

Production of cytokines is a fundamental means by which CD4+ T helper cells aid establishment of adaptive immunity. A helper T cell acquires the ability to produce cytokine in response to two distinct stimuli: signaling through its T cell receptor (TCR) or contact with cytokine produced by innate immune cells[1,2]. The latter pathway is a mechanism enabling T helper cells to respond rapidly to pathogens present in local tissue environments. TCR-independent activation of T cells subverts the need for transport of antigens to lymphoid tissues for processing and presentation, and allows T cells to be activated directly by inflammatory stimuli in the microenvironment.

T helper 1 (T_H1) cells exhibit robust IFN-γ production in response to IL-12 and IL-18 treatment[3]. Similarly, Th2 cells produce IL-13 upon exposure to IL-2, IL-7, or TSLP in combination with IL-33[4]. An analogous innate activation pathway exists for Th17 cells, which induce IL-17 following treatment with IL-6, IL-21, or IL-23 together with IL-1β[4,5].

Several studies have evaluated the nature of the synergism between IL-12 and IL-18 in TCR-independent IFN-γ production[6-8]. IL-18 cannot itself induce *Ifng*; its role is in augmenting the effects of STAT4 on Th1 development[3]. IL-12 activates the transcription factors STAT4 and AP-1, and as an IL-1 family member, IL-18 has been demonstrated to activate the NF-κB, Erk, p38 MAP kinase (MAPK), and JNK MAPK pathways[9]. These signals converge on sequences in and around the gene encoding IFN-γ to induce its expression. Balabasubramani et al. (2010) described differential utilization of *cis*-elements in the regulation of *Ifng* contingent on the method of stimulus. Demonstration that IL-12-induced STAT4 is required to recruit the NF-κB factor RelA to key *cis*-

regulatory elements for enhancement of *Ifng* transcription reinforces the notion that one aspect of IL-12 and IL-18 synergy is at the level of DNA-binding.

IL-33 signals through the IL-1 receptor-related protein T1ST2 to stimulate expression of the cytokines IL-5 and IL-13 by Th2 cells[4,10]. This process depends upon STAT5 induction through IL-2/IL-7/TSLP signaling, as well as initiation of the p38 MAPK and NF-κB pathways by IL-33[4]. IL-33 upregulates expression of its own receptor to reinforce its effects, and this process requires STAT5 and GATAT3 induction by way of Jak/STAT signaling. Presumably these transcription factors also synergize in direct regulation of the Th2 cytokine genes.

Presently the mechanism by which engagement of the IL-1 receptor supports TCR-independent IL-17 production is undefined. While neither IL-6 nor IL-23 alone can elicit IL-17 production from Th17 polarized cells, in combination with IL-1β these cytokines are powerful inducers of IL-17 production[4,5,11]. The role of IL-1β in Th17 differentiation is not limited to its ability to permit TCR-independent IL-17 production, for it has been reported to impact very early events in the Th17 developmental pathway (Dong C). IL-1β facilitates Th17 development *in vitro* by inducing genes (*Irf4, Rorc, Il1r1,* and *Il23r)* that program Th17 differentiation[11]. Mice deficient in IL-1 receptor 1 (IL-1R1) have decreased Th17 responses *in vivo* and are resistant to EAE development[11,12]. Additionally, IL-1β can counteract the inhibitory influence of IL-2 on Th17 development[13].

In the current study we describe a role for IL-1β and IL-18 in promoting TCR-independent IL-17 production that depends upon STAT3 activation. We have uncovered a novel mechanism common to Th1, Th2, and Th17 cells whereby engagement of an IL-1

family receptor leads to enhanced proximal STAT activation. Our data suggest that the IL-1 induced Erk, p38, and JNK MAPK pathways are operative in reinforcement of STAT activation through effects on SOCS3 and Jak2. We report that multiple STAT-dependent enhancer elements govern *Il17a/f* expression, and demonstrate that IL-1β not only heightens IL-23-induced STAT3 binding to these elements; it enables recruitment of STAT3 to additional regulatory sites contained within the extended *Il17* locus. Finally, we demonstrate that IL-1β induces NF-κB factors to directly regulate *Il17a/f* transcriptional in collaboration with STAT3. To summarize, it is clear that in addition to its involvement in early Th17 differentiation events, IL1β serves dual functions in promoting acute transcription of *Il17a* and *-f*. Taken together, our results are suggestive of a generic mechanism by which signaling through IL-1 family receptors promotes cytokine production by T helper cells.

RESULTS

IL-1 and IL-18 synergize with IL-23 to induce IL-17 production.

IL-1β plays a critical role in IL-6 and TGFβ-initiated Th17 differentiation[5,11,14,15],as expression of IL-1R1 is necessary for Th17 development and induction of Th17-mediated autoimmunity. Consistent with a previous report[4], we observed that treatment of Th17 effector cells with IL-1 (α or β) can invoke IL-17A production independently of T cell receptor (TCR) stimulation. Interestingly, we discovered that the IL-1 family member IL-18 has a similar capacity to induce IL-17A. In combination with IL-23 these cytokines stimulate high-level IL-17A production akin to the robust induction of interferon-gamma (IFN-γ) by IL-12 and IL-18 stimulation of Th1 cells (**Fig 1b,c**). Upon

examination of spleens and mesenteric lymph nodes (MLN) of colitic C57BL/6 *Il10*$^{-/-}$ mice we observed that, similar to *in vitro* differentiated Th17 cells, colitogenic Th17 cells produced significant levels of IL-17A in response to stimulation with IL-23 plus IL-1 or IL-23 plus IL-18 **(Fig 1d,e)**.

Having noted that the IL-17 response to IL-1 plus IL-23 was greater than that of IL-18 plus IL-23 in Th17 cells, we sought to determine whether the dose response of Th17 cells to these cytokines was similar. Upon restimulation with 10ng/ml of IL-23 in combination with 10-fold titration of IL-1 or IL-18, maximum IL-17 production was evoked with 10^{-1}ng/ml of IL-1b, whereas up to 10ng/ml of IL-18 in the presence of IL-23 failed to induce a significant amount of IL-17A, regardless of whether IL-23 was added during initial Th17 differentiation **(Fig 1f)**. This result suggests that IL-1 is the dominant factor that synergizes with IL-23 to induce IL-17A by the TCR-independent pathway; at least 1000-fold higher levels of IL-18 are required for IL-17A production in Th17 cells. This also correlates with the lower level of IL-18R expression during Th17 polarization.

It has been demonstrated that IL-12 signaling through IL-12R activates STAT4 to induce IL-18 responsiveness in Th1 cells as a pathway to TCR-independent IFN-γ activation[3,7]. Previous reports have shown that STAT3 activation by IL-6 and IL-21 is critical for initial Th17 differentiation; there is severe impairment of Th17 development in *Stat3*$^{-/-}$ CD4+ T cells[16,17].

To define the involvement of STAT3 in TCR-independent pathway, we utilized small interfering RNA (siRNA) to suppress STAT3 in committed Th17 cells. Silencing of STAT3 in Th17 cells was verified by western blot 3 days following transfection. STAT3-silenced Th17 cells were then restimulated with different combinations of IL-1,

IL-18, and IL-23 in the absence of TCR. Production of IL-17A by Th17 cells subject to STAT3 silencing was significantly reduced **(Fig 1g)**, demonstrating that IL-23-driven STAT3 signaling is responsible for production of IL-17 via the TCR-independent pathway. This indicates that IL-23 in combination with IL-1 or IL-18 can impact Th17 function at a late stage of Th17 differentiation through activation of STAT3.

Profiling of Th17 chromatin conformation and CTCF/Rad21 occupancy

In an attempt to identify *cis*-elements that regulate *Il17a* and *Il17f* transcription we performed long-range DNase I hypersensitivity (HS) mapping on approximately 2.5 megabases of DNA flanking the murine *Il17a* and *Il17f* genes, as reported previously (Mukasa et al.2010). Prominent DNase I HS peaks localized to a ~200 kb region surrounding the *Il17a/f* genes. (**Fig 2a**) Naïve CD4+ cells as well as Th1 and Th2 cells were largely devoid of DNase I HS sites, but Th17 cells exhibited 13 discernable peaks that corresponded well with evolutionarily conserved non-coding sequences (CNSs). Many hypersensitivity peaks were present in resting polarized Th17 cells, indicating that the *Il17a/f* locus is poised for gene transcription following Th17 differentiation. Stimulation with phorbal ester (PMA) plus ionomycin (P+I) or anti-CD3 induced emergence of additional peaks at CNSs -97, -37, and +6 which suggests a role for these elements as stimulus-dependent *Il17a/f* enhancers. While TCR stimulation induced alterations in chromatin structure at the aforementioned CNSs, we found that cytokine stimulation (IL-23 + IL-1β) left the extended *Il17a/f* locus nearly identical to that of resting Th17 cells, as the locus is poised to transcribe *Il17a* and *Il17f* in the resting configuration.

An insulator is a DNA sequence that can act as a barrier to the influence of regulatory elements from neighboring genes. In an effort to identify *cis*-elements that possess insulator activity we assessed for binding to the CCCTC-binding factor (CTCF) and a subunit of its associated cohesin, Rad21. CTCF has been shown to be required for barrier and enhancer-blocking activities of many insulator elements[18], and cohesin is hypothesized to organize chromatin structure in a manner that facilitates insulator function[19]. We mapped CTCF and Rad21 sites by ChIP-chip on custom tiled microarrays (**Fig 2b**) and found that CTCF and Rad21 co-localized to only two sites in the extended *Il17a/f* locus: CNSs A-118 and F-13. Both of these regions correspond to DNaseI hypersensitive sites and represent evolutionarily conserved sequences. Their locations, flanking the *Il17a/f* locus outside of the most distal Th17-specific DNase hypersensitive regions, and situated in or adjacent to the neighboring genes *Pkhd1* and *Mcm3*, are consistent with a role for A-118 and F-13 as boundary elements. Although all CTCF-binding sites exhibited Rad21 binding, we detected additional prominent Rad21 binding sites near regions of DNase hypersensitivity (A-35, A+8) that did not correspond to areas of sequence conservation. It is possible that these regions represent foci of cohesin binding that attribute to cohesin's role in sister chromatid cohesin and serve a distinct function in gene regulation. As a regulator of higher order chromatin structure, cohesin is speculated to contribute to formation of chromatin loops[19], and therefore could be involved in a number of different interactions between *cis*-regulatory elements that facilitate gene regulation.

CTCF and Rad21 binding at CNSs A-118 and F-13 were marked in Th17 cells, but also evident in naïve CD4 and Th1 cells (**Fig 2c)**. Notably, CTCF and Rad21

occupancy were observed at several sites in the *Ifng* gene previously reported to bind CTCF[20]. (**supp fig 1**).

STAT3 is recruited to distal *cis*-regulatory elements to enhance TCR-independent *Il17a* and *Il17f* transcription

In an effort to ascribe a function to the *Il17a/f* CNS elements identified to be DNaseI hypersensitive, we utilized a novel promoter-reporter assay that involved transfection of primary murine Th17 cells. Various CNS elements were subcloned upstream of a fragment of *Il17a* or *Il17f* promoter, and readings of firefly luciferase from transfected cells were used as a surrogate for transcriptional activity. CNSs A-118, A-97, A-37, A+10, A+28, and F-7 exhibited enhancement of promoter activity upon restimulation with anti-CD3 (**Fig 3a**), indicating an ability of these *cis* elements to augment TCR-driven *Il17a* gene transcription. Similar data was obtained for all CNSs relative to a 295bp fragment of the *Il17f* promoter (data not shown). Enhancer activity was retained for CNSs A-97, -37, +10, and +28 under conditions of cytokine restimulation (**Fig 3b**), with an observable increase in luciferase activity following addition of IL-1β over IL-23 alone.

We examined the enhancer CNS sequences for predicted transcription factor binding sites, and discovered that a composite STAT/Bcl6 site was unique to the four *cis* elements that enahanced cytokine-driven *Il17* transcription (**supp fig 2**), with CNS A+10 also possessing overlapping NFAT and NFκB sites. Based on this information, as well as the knowledge that STAT3 is integral to *Il17a/f* transcription, we mutated the putative STAT sites contained within CNSs A-97, A-37, A+10, and A+28.. The mutations

diminished cytokine-driven enhancer activity for all of the CNSs tested (**Fig 3c**). This demonstrates that these *cis* regulatory elements have the capacity to bind STAT3 *in vitro* and suggests that they serve as foci for acute transcriptional regulation following cytokine stimulus.

Previous studies have demonstrated STAT3 binding to *cis* elements spanning 90kb of the extended *Il17a/f* locus.[21,22] To confirm these results and assess for recruitment of STAT3 to genomic elements more distal, we performed STAT3 chromatin immunoprecipitation analysis across a region encompassing ~180kb of sequence surrounding *Il17a* and *Il17f*. Consistent with a previous report [22], we detected STAT3 binding at CNSs A+10, A+28, and the *Il17f* promoter following stimulation with IL-23. (**Fig 3d**) We discovered that the presence of IL-1β had a profound effect on STAT3 occupancy across the locus, as treatment with IL-23 plus IL-1β not only caused substantial increases in the degree of STAT3 recruitment to the aforementioned regions; it allowed emergence of additional STAT3 binding sites at A-97 and the *Il17a* promoter (**Fig 3d**). These results indicate that the enhancer CNSs A-97, A+10, A+28 and the *Il17a* and *Il17f* promoters bind STAT3 *in vivo* to regulate *Il17a/f* transcription, and IL-1R signaling enhances binding of STAT3 to genomic regulatory elements participating in *Il17a/f* transcription.

IL-1β induces the NF-κB factors RelA and c-Rel to participate in regulation of *Il17a* and *Il17f* transcription in cooperation with STAT3.

IL-1 receptor (IL-1R) signaling is initiated when IL-1α or IL-1β binds to the IL-1R1 subunit, enabling recruitment of an accessory receptor subunit, IL-1RAP. Juxtaposition

of TIR domains on the cytoplasmic tails of the receptor subunits allows recruitment of myeloid differentiation primary response protein 88 (MYD88), and subsequently IL-1R-associated kinase 4 (IRAK4), TNFR-associated factor 6 (TRAF6), and additional downstream signaling molecules[23]. Activation of IL-1R signaling typically results in activation of the NF-κB and MAP kinase pathways[24].

To determine if IL-1β treatment of Th17 cells resulted in NF-κB activation, and if so, how the kinetics of NF-κB activation compared with those of STAT3 activation, expression of these proteins were assessed over a timeframe spanning 4 hours following cytokine stimulation. (**Fig 4a**) We found that IL-1β treatment indeed triggered NF-κB nuclear import, but the addition of IL-23 did not support or enhance NF-κB signaling. Nuclear translocation of the NF-κB proteins RelA, c-Rel, and p50 took place within ten minutes of cytokine exposure, and STAT3 tyrosine phosphorylation occurred concordantly. Peak expression of NF-κB proteins in the nucleus of Th17 cells paralleled STAT3 activation, and both NF-κB and STAT3 expression began to decline between two and four hours following cytokine stimulus, revealing that these pathways have very similar kinetics and duration of action in Th17 cells.

The NF-κB proteins RelA (p65), RelB, c-Rel, p50, and p52 exist in the cytoplasm as inactive dimers bound to inhibitors of κB (IκB) proteins. Upon ligand binding to IL-1R cytoplasmic TIR domains bind and activate the adaptor protein MyD88 to induce phosphorylation-dependent release of NF-κB proteins from the IκB complex and subsequent transport into the nucleus. Here the proteins bind to NF-κB response elements in DNA and recruit transcriptional co-activators to affect expression of select genes. The *Il2* gene is a known target of RelA and c-Rel regulation[25,26]. More recently c-

Rel has been reported to be obligatory for *Il21* gene expression in T cells[27]. IL-21 has an essential function in Th17 differentiation; it can induce IL-23R expression and promote autocrine IL-21 production to maintain the expansion of developing Th17 cells[11,16,28].

We made use of mice possessing a conditional deletion of RelA, as described in Balasubramani et al. 2010[8], as well as mice deficient in c-Rel (*Rel*-/-) to evaluate the ability of Th17 cells to produce IL-17 in the absence of these factors. Both RelA and c-Rel deficiency result in decreased IL-17 production (**Fig 4b).** We found that despite existing data that c-Rel is critical for *Il21* regulation, addition of exogenous IL-21 did not correct the deficit in IL-17 production observed in *Rela*$^{fl/fl}$.*CD4*.cre+ or *Rel-/-* mice. Consistent with previous reports we found IFN-γ expression to be reduced in *Rela*$^{fl/fl}$.*CD4*.cre+ and *Rel-/-* Th1 cells, however, cytokine production by Th2 and induced T-reg (iTreg) cells was not impaired by deficiency of RelA or c-Rel (**supp fig 3b**). Although Th17 proliferation was unaffected by the absence of RelA or c-Rel (Fig 6b), *Rel-/-* Th1 cells had severely impaired proliferative capacity (**supp fig 3a**). IL-17A protein production was decreased during primary culture and upon all modes of restimulation of Th17 effector cells. Real-time PCR analysis of 3 day-polarized Th17 cells revealed that mRNA expression of several Th17-associated genes is reduced in *Rela*$^{fl/fl}$.*CD4*.cre+ and *Rel-/-* mice, including *Il17f, Rorgt, Il21,* and *Il22.* (**Fig 4c)** This indicates that RelA and c-Rel impact early events in Th17 differentiation as well as acute transcription of *Il17* in polarized effectors.

To determine if RelA and c-Rel directly regulate the *Il17a* and *Il17f* genes, or merely affect another facet of the Th17 differentiation program, we performed ChIP analysis to assess binding of these factors across the extended *Il17a/f* locus. (**Fig 4d**) We

found that like STAT3, RelA and c-Rel bind to the CNS A+10 enhancer element following cytokine or TCR stimulation to affect *Il17a/f* transcription. RelA also bound weakly to CNS A+28, another potential focus of interaction with STAT3. RelA and c-Rel binding were detected at the proximal CNS elements A-5 and F-7 under TCR stimulation only. c-Rel weakly and/or variably bound to CNSs A-15, F-13 and the *Il17a/f* promoters, located a relatively short distance away. Taken together, these data point to a complimentary role for the NF-κB factors RelA and c-Rel in STAT3-mediated regulation of *Il17a/f* transcription.

In vivo regulation of *Il17a/f* by the NF-κB factor RelA

Infection with the murine enteric pathogen *Citobacter rodentium* induces attaching-effacing lesions of the colon that depend on the Th17 cytokines IL-17A, IL-17F, and IL-22 for development[29]. These cytokines are also important for clearance of the microbes, as several antimicrobial responses are induced by IL-17 and IL-22 in the gut-associated lymphoid tissues. IL-22 has been shown to be required for secretion of C-type lections of the RegIII family, and production of β-defensins is dependent upon IL-17A and IL-17F[30]. To assess for an impact of RelA on development of a Th17 response *in vivo*, we orally challenged *Rela*$^{fl/fl}$.*CD4*.cre+ and WT littermate mice with *C. rodentium* and evaluated the percentages of colonic CD4+ T cells expressing IL-17, IL-22, and IFN-γ both at peak infection (day 8) and in uninfected *Rela*$^{fl/fl}$.*CD4*.cre+ and control mice for comparison. (**supp fig 4**) We observed that even at steady state (uninfected mice) *Rela*$^{fl/fl}$.*CD4*.cre+ mice exhibited a reduced fraction of IL-17+ lamina propria lymphocytes (LPLs) compared to control mice. Infection with *C. rodentium* exaggerated the difference, with

approximately a 10-fold decrease in IL-17 production by *Rela*$^{fl/fl}$.*CD4*.cre+ mice compared to their WT counterparts. Conversely, IL-22 expression was only mildly affected by the absence of RelA. Of note, the mean fluorescence intensity (MFI) was reduced significantly commensurate with reductions in IL-17A. However, despite previous reports describing a critical role for RelA in production IFN-γ[8], we did not observe substantial decreases in IFN-γ compared to WT in uninfected or *C. rodentium*-infected *Rela*$^{fl/fl}$.*CD4*.cre+ animals. These findings highlight the importance of RelA in Th17 development *in vivo.*

IL-1 family members augment cytokine-driven STAT activation

The STAT proteins are latent transcription factors that become phosphorylated upon specific tyrosine and serine residues in response to cytokine receptor ligation. Following phosphorylation the activated STATs dimerize and translocate to the nucleus where they regulate transcription.[31,32] IL-6, IL-21, and IL-23-induced STAT3 activation are required for Th17 differentiation[33], although the relative contributions of each of these cytokines throughout the Th17 differentiation program are not clearly defined. We examined the phosphorylation status of STAT3 in polarized Th17 cells treated with IL-23, with or without addition of IL-1β or IL-18. (**Fig 5a,b**) As expected, rapid tyrosine phosphorylation occurred upon IL-23 treatment, with peak STAT3 activation detected between 30 minutes to 1 hour following IL-23 addition. Interestingly, we observed enhanced STAT3 tyrosine phosphorylation with addition of IL-1β or IL-18, occurring 30 minutes to 1 hour following cytokine addition. This rapid increase in tyrosine phosphorylation was accompanied by enhanced serine phosphorylation of STAT3, which

is reportedly required for full transcriptional activation of STAT3[34,35]. IL-1β had a similar effect on IL-6 and IL-21-induced STAT3 (**Fig 5c,d**), indicating that IL-1β can directly regulate STAT3 phosphorylation irrespective of the inducing cytokine.

T helper cells from Th1 and Th2 lineages also respond to signals from a STAT activator plus an IL-1 family member to produce an effector cytokine in a TCR-independent fashion[4]. Given the observed effects of IL-1β and IL-18 on STAT3 activation, we next assessed whether the IL-1 family members IL-18 and IL-33 had comparable effects upon IL-12-driven STAT4 activation in Th1 cells and IL-2-driven STAT5 activation in Th2 cells, respectively. Indeed, addition of IL-18 amplified IL-12-induced STAT4 phosphorylation (**Fig 5e**), although it should be noted that this effect was only observable at doses of IL-12 lower than 1ng/mL. Accordingly IL-33 enhanced IL-2-induced STAT5 activation (**Fig 5f**) leading us to conclude that signals received through an IL-1 family receptor together with a Jak/STAT-inducing signal synergize to evoke robust STAT activation. This may serve as a general mechanism by which effector CD4 cells can potently activate STAT-dependent transcription to produce cytokine independently of TCR stimulation.

IL-1β enhances STAT3 tyrosine and serine phosphorylation through regulation of Jak/STAT and MAP kinase signaling pathways

STAT activation occurs after cytokine binding stimulates receptor-associated tyrosine kinases of the Janus kinase family (Jaks). Jaks associate with proline-rich regions of type I and type II cytokine receptor signaling subunits[31,32,36]. When cytokine encounters the ligand-binding chain of its receptor the receptor complex undergoes a conformational

change that allows Jaks to transactivate each other and phosphorylate tyrosine residues contained within the receptor cytoplasmic tails. The phosphotyrosine residues create a docking site for STAT proteins, which are subsequently phosphorylated by the activated Jaks. Maximal STAT activation requires phosphorylation on specific tyrosine and serine residues. Although Jak2 is known to be responsible for phosphorylating STAT3 at Tyr705, other tyrosine kinases have been reported to associate with cytokine receptors and contribute to STAT activation[37]. Additionally, it is possible that many signaling pathways are responsible for carrying out Ser727 phosphorylation of STAT3 in Th17 cells[34].

To determine the degree to which STAT3 activation and IL-17 production rely on various cell signaling intermediaries, we pretreated Th17 cells with compounds that specifically inhibit nuclear factor-κB (NF-κB; PDTC), c-Jun N-terminal kinase (JNK; SP600125), Jak2 (AG490), phosphatidylinositol-3-kinase (PI3K; LY294002), mitogen activated protein (MAP) kinase kinase (MEK/Erk; PD98059), Src family tyrosine kinases (PP2), and p38 MAP kinase (SB203580), then stimulated with IL-23 plus IL-1β or anti-CD3 and quantified STAT3 phosphorylation and IL-17 production (**Fig 6a,b**). When we compared IL-17A production from Th17 cells subjected to cytokine stimulation versus those stimulated through TCR, we found that in both cases IL-17 production was impaired following treatment with PI3K and Src family kinase inhibitors. Important roles for PI3K and Src family kinases as proximal intermediaries of signals from the T cell receptor complex and CD28 co-stimulatory molecule can account for the drastic reduction in IL-17 observed following inhibition of these kinases in the context of anti-CD3 stimulation (**Fig 6b**). A less profound role for NF-κB in TCR-driven IL-17

production was suggested by the effect of the inhibitor PDTC. NF-κB activation is one of the downstream consequences of T cell receptor signaling, so this result is consistent with a more minor role for NF-κB proteins in regulating TCR-induced IL-17 production.

In the absence of Jaks, cytokines are unable to initiate downstream signaling events[37,38]. This can explain the drastic reduction in IL-17 seen following Jak2 inhibition (**Fig 6a**). However a second class of cytokine receptor-associated tyrosine kinases are activated upon IL-23 binding to IL-23R. Src family kinases (SFKs) are required to activate the full range of intracellular signaling events downstream of cytokine receptors[37], and contribute to maximal tyrosine phosphorylation of STAT proteins. In most experiments we did not observe an appreciable effect of SFK inhibition on STAT3 tyrosine phosphorylation (**Fig 6c,d**), although PP2 treatment did reduce IL-17 production following cytokine stimulation (**Fig 6a**). This suggests that Src family kinases affect *Il17* transcription by a mechanism other than affecting STAT activation. The same is true for PI3K, whose inhibition produced a considerable decrease in IL-17 production, but did not impact STAT3 phosphorylation events. It is possible that PI3K activation of Akt is important for IL-17 production because this kinase activates a number of transcription factors and is involved in a variety of cellular functions including cell survival, proliferation, and protein synthesis. Although it did not achieve statistical significance, we did note a modest decrease in cytokine-driven IL-17 production following NF-κB inhibition. p38 MAP kinase (MAPK) inhibition, like that of Jak2, affected both STAT3 activation and IL-17 production (**Fig 6a,c,d**). Jak2 is critical for STAT3 tyrosine phosphorylation, but our data suggests that IL-1β-induced signals can also affect STAT3 tyrosine phosphorylation. Inhibition of Erk, JNK, and p38 MAPK led to dimunition of

STAT3 tyrosine phosphotylation (**Fig 6c,d**). The MAP kinase cascade can be activated by antigen receptor or co-stimulatory signals, but IL-1α and IL-1β are known to be potent inducers of MAPK signaling[39]. Additionally, p38 MAP kinase appears to play a role in STAT3 phosphorylation at serine 727. This result is supported by previous reports which indicate that MAP kinases perform phosphorylation of STATs on serine residues (ref) to aid in their activation. The MAP kinase cascade can be activated by antigen receptor or co-stimulatory signals, but IL-1α and IL-1β are known to be potent inducers of MAPK signaling[39,40]. Initiation of MAPK signaling that culminates in enhanced STAT3 tyrosine and serine phosphorylation is an additional way in which IL-1β can augment STAT3 activation and IL-17 production to reinforce Th17 development.

To further investigate the mechanism by which IL-1β enhances STAT3 tyrosine phosphorylation, we next assessed whether Jak2 phosphorylation was impacted by IL-1 signaling. **(Fig 6e)** Although IL-23 + IL-1β-induced Jak2 phosphorylation was equivalent to that induced by IL-23 alone ten minutes following cytokine exposure, by thirty minutes we consistently observed a 2-3 fold increase in normalized Jak2[pYpY1007/1008] expression that reveals a positive effect of IL-1β on Jak2 phosphorylation and subsequent STAT3 activation. Enhancement of Jak2 phosphorylation had dissipated after one hour of cytokine stimulus, although Jak2[pYpY1007/1008] levels remained elevated at this time point compared to cells left unstimulated.

Excessive action of cytokines can be harmful to an organism. Several mechanisms exist to rapidly extinguish cytokine signaling and prevent cytokine overproduction. The Jak/STAT signaling pathway has three classes of negative

regulators that inhibit its amplification at various levels[41,42]. Protein tyrosine phospatases (PTPs), ex. SHP-1 and T cell PTP (TC-PTP), are constitutively expressed proteins that regulate phosphorylation. These phosphatases contain SH2 domains through which they associate with cytokine receptor subunits and deactivate receptor kinases including Jaks[38]. Protein inhibitors of activated signal transducers and activators of transcription, termed PIAS proteins, are also constitutively present in cells, but their inhibitory interaction with STATs requires cytokine stimulation. PIAS3 inhibits the function of STAT3 by interfering with its DNA binding ability[41]. The third family of negative regulators is termed suppressor of cytokine signaling, or SOCS proteins[43]. In contrast to PTPs and PIAS proteins, SOCS proteins are induced rapidly following cytokine signaling. They coordinate classic feedback inhibition of Jak/STAT signaling. SOCS proteins both interfere with STAT activation by competing for binding to activated cytokine receptors and inhibit JAK activity through their kinase inhibitory region (KIR)[42].

SOCS3 is a principal regulator of STAT3-activating cytokines. Its importance in constraining IL-23-mediated STAT3 phosphorylation has been demonstrated in Th17 cells[44], and we therefore wished to examine its expression in response to IL-23 in the presence or absence of IL-1β. Although it is possible that IL-1β exerts direct effects on Jak2 to amplify its activation of STAT3, we considered SOCS3 to be an attractive target for interference by IL-1β. We measured *Socs3* mRNA abundance in Th17 cells either left unstimulated or treated with IL-23, IL-1β, or both for up to two hours. We found *Socs3* mRNA expression to be maximal thirty minutes following IL-23 treatment, and did not detect *Socs3* induction by IL-1β treatment alone. **(Fig 6f)** Under the condition of IL-

23 plus IL-1β treatment we observed diminished *Socs3* mRNA expression compared with IL-23 alone at all time points. This result suggests that inhibition of *Socs3* transcription is the mechanism by which IL-1β enhances Jak2 tyrosine phosphorylation. Altered Jak2 phosphorylation has profound consequences for downstream STAT3 activation and ultimately transcription of the STAT3 target genes *Il17a* and *Il17f*.

DISCUSSION

In this study, we have performed a functional survey of conserved non-coding sequences (CNSs) spanning 200kb across the murine *Il17a* and *Il17f* genes. In so doing we have identified six new *Il17a/f* enhancer elements, with a clear dichotomy of utilization in response to TCR and cytokine stimuli. CNSs A-97, -37, +10, and +28 exhibited STAT-dependent enhancer activity that underscores their importance in cytokine-induced transcriptional activation. CNSs A-97, +10, +28, and the *Il17a* & *-f* promoters were found to directly interact with STAT3 to regulate *Il17a/f*. Although CNS A-37 did not bind STAT3 *in vivo* in response to IL-23/IL-1β stimulation of 6-day polarized Th17 effectors, its ability to potently enhance cytokine-driven *Il17a/f in vitro* and the presence of a well-conserved putative STAT binding site suggests that perhaps this element has interactions with STAT3 at another stage of Th17 development and/or in response to different stimuli.

Co-recruitment of the NF-κB factors RelA and c-Rel to STAT3-binding sites within the *Il17* locus implies that such sites are regulatory nodes that facilitate interactions between transcription factors. This supposition is bolstered by our data illustrating Rad21/cohesin binding in close proximity to CNSs A+10 and A-37. CTCF

and Rad21 have incontrovertible roles in facilitating chromatin interactions by looping DNA, and the presence of a CTCF or Rad21binding site near an enhancer element likely denotes a site of physical association between regulatory DNA sequences and *trans* factors bound to them. We have defined the limits of the *Il17* locus by revealing CTCF and Rad21 occupancy of two *cis*-elements flanking the genes adjacent to *Il17a* and *–f*. These insulator/boundary elements are likely brought into physical contact with one or more proximal CNS elements and/or the *Il17a* and *–f* promoters to carry out transcriptional regulation, because CNS A-118 exhibits the capacity to enhance *Il17* expression.

Cooperativity between IL-1β-induced NF-κB factors and STAT3 at *Il17 cis*-regulatory elements underlies one mechanism of synergy between IL-1β and STAT3-inducing cytokines in IL-17 production. The fact that IL-1β treatment augmented binding of IL-23-induced STAT3 to genomic regulatory elements and allowed for additional foci of STAT3-DNA interaction to emerge, leads us to postulate that IL-1R signaling affects chromatin structure. We infer that IL-23 + IL-1β stimulation positions the *Il17a/f* locus for greater accessibility to transcription factors; therefore, it would be interesting to examine the effect of IL-1β on epigenetic modifications in the *Il17* locus in future studies.

The fact that deficiency of IL-1-induced RelA and c-Rel affected expression of several Th17-associated genes and greatly diminished IL-17 production early in the course of Th17 development indicates that IL-1 signaling influences Th17 programming as well as acute transcription of *Il17* via NF-κB activation. NF-κB signaling has been reported to potentiate IL-6-induced IRF4 activation[33], and thereby influence Th17

specification through promoting RORγt expression. This indirectly supports activation of STAT3, which is crucial for *Il17* transcription and Th17 development. Mice and humans deficient in STAT3 have severely impaired Th17 differentiation[13,17,45,46]. This is because in addition to directly binding to regulatory sites within the *Il17* locus, STAT3 controls expression of multiple genes involved in Th17 lineage specification, among them *Rorc, Irf4, Batf, Il21, Il21r, Il23,* and *Il23r*[21].

Given the extreme importance of STAT3 to the Th17 developmental pathway, our observation that IL-1R signaling augments STAT3 activation purports that IL-1β can exert significant influence over both differentiation and transcriptional events. By revealing that IL-1R signaling enhanced STAT3 activation through suppression of SOCS3 and amplification of Jak signaling, we have identified a novel mechanism for proximal regulation of STAT3 by IL-1β and IL-18. Moreover, we show that this pathway is not unique to Th17 cells; it is operative in Th1 and Th2 lineages in response to activation of Jak/STAT signaling combined with engagement of an IL-1 family receptor.

The p38 MAPK and NF-κB pathways have been established to be of importance in TCR-independent production of IFN-γ and IL-13[4,6,7]. IL-18 signaling induces expression of GADD45β, which selectively augments cytokine-induced IFN-γ by regulating MAPK-extracellular signal-regulated kinase (Erk) kinase 4 (MEKK4). GADD45β activation of p38 MAPK is essential for IL-12 + IL-18 to induce IFN-γ, although p38 MAPK is not required for transcription of *Ifng* downstream of TCR signaling[6]. Yang et al[6]. demonstrated that GADD45β and p38 activation were not sufficient for induction of IFN-γ, concluding that additional cytokine-activated

transcription factors, such as STAT4 and/or NF-κB are required to induce transcription of *Ifng*. Likewise, IL-33/STAT5-induced production of IL-13 depended upon NF-κB and p38 MAPK signaling, but did not require nuclear factor of activated T cells (NFAT)[4].

We demonstrate a role for the IL-1-induced p38 MAP kinase pathway in serine and possibly tyrosine phosphorylation of STAT3 in Th17 cells. Serine phosphorylation of STAT3 is reported to be required for its full activation and optimal DNA-binding activity[35]. However, tyrosine phosphorylation is sufficient to allow STAT dimerization and translocation to the nucleus for transcriptional regulation[34]. Our data indicates that inhibitors to all three MAPK pathways (Erk, JNK, p38) downstream of IL-1R signaling influenced STAT3 tyrosine phosphorylation. Further, we show data suggesting that *Socs3* is a target of these pathways. SOCS3 expression is induced rapidly following STAT3 activation and inhibits STAT3 activation by classic negative feedback regulation. SOCS3 is known to be an important negative regulator of Th17 development, as CD4-specific SOCS3 deficiency allows for more IL-17 production and enhanced Th17 development *in vitro* and *in vivo*[44]. By inhibiting SOCS3, IL-1β can temporarily allow Jak/STAT signaling to proceed unchecked, enhancing STAT3 activation. Regulation of such proximal signaling events in cytokine-induced *Il17* transcription can allow IL-1 family cytokines to exert powerful influence over both acute effector cytokine transcription and T helper cell development.

In summary, our findings significantly extend knowledge about regulation of the *Il17a* and *Il17f* cytokine genes. We demonstrate that interplay between STAT3 and NF-κB factors directly regulate these genes downstream of IL-23 and IL-1β signaling, and IL-1β is able to enhance the influence of STAT3 on the *Il17a/f* locus through multiple

mechanisms. These events are of relevance to host defense, as they facilitate rapid cytokine-induced mobilization of T helper cells by the innate immune system in response to pathogens.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6, B6.OT-II TCR transgenic (OT-II), B6.129S1-*Il12b*$^{tm/Jm}$/J (IL-12p40-/-), B6.*Il10*-/-, and B6.FVB-Tg (EIIa-cre)C5379Lmgd/J mice were purchased from Jackson Laboratories and/or bred at the University of Alabama at Birmingham . The generation of *Il17f*$^{Thy1.1/Thy1.1}$ reporter mice were described previously[47], as were the creation of *Rela*$^{fl/fl}$.*Cd4*-cre+ mice[8]. B6.*Rel*-/- mice used in some studies were a kind gift from Ranjan Sen (National Institute on Aging, NIH). All animal breeding and experimentation were carried out in accordance with institutional regulations.

CD4+ T cell Isolation and Culture

Spleens and lymph nodes were isolated from the indicated strains of mice, and CD4+ T cells were purified by positive selection with magnetic beads (Invitrogen) before culture with OVA peptide (5ug/mL) or anti-CD3 (2.5ug/mL), and irradiated CD4-depleted splenocytes at a ratio of 1:7 (Th17) or 1:5 (Th1 and Th2) CD4 cells/APCs. T cells were cultured in Iscove's media containing 10% FBS, 100 IU/mL penicillin, 100mg/mL streptomycin, 1mM sodium pyruvate, 1x non-essential amino acids, 2.5 mM β-mercaptoethanol, 2mM L-glutamine. Th17 polarizations were carried out using

neutralizing antibodies to IFN-γ and IL-4 (10ug/mL), with addition of 20ng/mL IL-6 (R&D systems), 2-5ng/mL rhTGF-β1 (R&D). Th1 cultures were supplemented with 10ng/mL IL-12 and 10ug/mL anti-IL-4, and Th2 cultures were performed with 1000U/mL IL-4 and 10ug/mL anti-IFN-γ. In some experiments cells were stimulated with plate-bound anti-CD3 (clone 145-2C11) plus soluble anti-CD8 (clone 27.51, eBioscience) at the indicated concentrations, or underwent polarization with anti-CD3/CD28 coated beads (Invitrogen) according to manufacturer's instructions. In experiments where cells were treated with cell signaling inhibitors, the following reagents and concentrations were used: AG490 (50uM), LY294002 (20uM), SB203580 (5uM), SP600125 (1uM), PD98059 (20uM), PP2 (1uM), and PDTC (1uM). With the exception of PDTC (Sigma), all inhibitors were purchased from Calbiochem.

Plasmids, CD4 Cell Transfection, and Promoter-Reporter Assay

Il17a and *–f* promoter fragments were cloned into the pGL3 basic luciferase vector (Promega) using XhoI and HindIII restriction sites. Similarly, MluI and NheI restriction sites were used to cassette in various CNS elements with the primer sequences listed in the supplementary information. Clones for each construct were screened by restriction digest and sequenced to verify their authenticity. Th17 cells were generated as described and 5-6 following polarization between 1-3 million CD4 cells were transfected using mouse nucleofactor kit (Lonza) and an AMAXA electroporator. 20 micrograms of promoter-reporter construct DNA was administered to cells along with 1ug of pRL-TK to allow for normalization. Following transfection cells were either rested for 2-3 hours and restimulated with plate-bound anti-CD3 (1ug.mL), rested overnight and restimulated with

between 3-10ng/mL IL-23 +/- 10ug/mL IL-1β, or left unstimulated. The dual luciferase kit (Promega) was to perform the luciferase assay. Cells were lysed in passive lysis buffer and relative light units were assessed for both firefly and renilla using a Turner Systems TD 20/20 luminometer. Each transfection was performed in triplicate and data represent a minimum of three independent experiments.

Flow Cytometric Analysis

CD4 T cells were collected and where indicated, stimulated with PMA (50 ng/ml; Sigma) and ionomycin (750 ng/ml; Calbiochem) for 5 h in the presence of Golgi Plug (BD). Intracellular staining was performed as previously described [48]. LIVE/DEAD Fixable Green Dead Cell Stain (Invitrogen) was used to exclude dead cells in flow cytometric analyses extracellulary. Phycoerythrin (PE)-conjugated anti-CD90.1 (OX-7) and anti-IL-17A (TC11-18H10) were purchased from BD Biosciences; allophycocyanin (APC)-conjugated anti-IFNg (XMG1.2) and PE-Cy7-conjugated anti-CD4 (GK1.5) were purchased from eBioscience. Samples were acquired on an LSRII instrument (BD Biosciences) and data was analyzed using CellQuest Pro (BD Biosciences) or FlowJo software (Tree Star Inc.).

RNA isolation, cDNA systhesis, and Real-time PCR

mRNA was extracted from T cells using TRIZOL (Invitrogen) and treated with DNA-free (Ambion). cDNA synthesis was performed using Superscript III first-strand synthesis system (Invitogen). Real-time PCR was performed on a Bio-Rad iCycler with

Taqman primer pairs and probes specific for cDNAs of *Il23r*, *Il12rβ2, Ifng, Il17a*, *Il17f*, *Il22*, *Rorc, Il21,* and *β2-microglobulin (β2m)*. Primer sequences used were as described[47,49], with the following additional primers/probe: *Socs3* forward primer: AGTGCAGAGTAGTGACTAAACATTACAAGA; *Socs3* reverse primer: AGCAGGCGAGTGTAGAGTCAGAGT; *Socs3* probe: CGGCCTCCGAGGCGGCTCT. Reactions were run in triplicate and normalized to *β2m*expression.

DNase Hypersensitivity Mapping

DNase-ChIP samples were prepared as previously described[50]. In brief, nuclei were isolated form $5x10^7$ cells and subjected to digestion with the enzyme DNase I (Roche) over a range of concentrations (0-12 units). EDTA was added to halt DNA digestion, the nuclei were embedded in 1% InCert agarose (Lonza) at a 1:1 ratio of volumes preceding overnight inclubation at 37°C in LIDS buffer (1% lithium dodecyl sulfate, 10mM Tris-HCl pH7.5, 100mM EDTA). Agarose plugs were washed extensively in 50mM EDTA before digestion with T4 DNA polymerase (New England Biolabs). Blunted DNA fragments were then extracted, labled with biotinylated linkers, captured, and amplified by ligation-mediated PCR to prepare for hybridization to microarrays. Data was visualized using IGB browser[51].

Chromatin Immunoprecipitation

All ChIP experiments were performed using a ChIP assay kit (Millipore) as previously described[49]. Relative recruitment was determined using RT-PCR and ΔΔCt values were expressed as fold change over indicated reference cell type, or displayed as a percentage

of input DNA. Inputs were appropriately diluted to assist in normalization, and primers used were previously described[8,49], with the addition of the following: CNS A-118 forward: CCAAACCTAAACACAAGGAGAAATC, CNS A-118 reverse: TCTTGTGGCCAATATTTGCATT, CNS A-118 probe: CAGGCTGCGAAGACAACGCAGG; CNS F-13 forward: AGGGAAAATCCCCCAAGAGA, CNS F-13 reverse: TGTGGGCTTAGCTTCTGCATT, CNS F-13 probe: CCTGCTGCCACCTTGTGATGACTTGA; *Socs3* promoter forward: AGTGCAGAGTAGTGACTAAACATTACAAGA, *Socs3* promoter reverse: AGCAGGCGAGTGTAGAGTCAGAGT; *Socs3* promoter probe CGGCCGGGCAGTTCCAGGA. Antibodies used in ChIP include the following: STAT3 (Cell Signaling #9132), RelA (Millipore #06-418), c-Rel (Millipore #09-040), CTCF (Milliipore #07-729), and Rad21 (Abcam #ab992). Samples were prepared for ChIP-chip analysis as previously described[8].

ELISA and Luminex Assays

Th17 cells or ex vivo CD4 T cells of C57BL/6 or IL-10-/- were incubated with indicated cytokine such as IL-1b (10 ng/ml), IL-6 (20 ng/ml), IL-18 (50 ng/ml), IL-23 (10 ng/ml), TGF-b (5 ng/ml) and IL-12 (10 ng/ml), all purchased from R&D, in the presence or the absence of coated anti-CD3 (10 ug/ml) (clone 145-11). Supernatants were collected after 48 hrs and the production of IL-17A or IFNg was detected by color development (TM-Blue; Sigma) of HRP-avidin substrate (Vector) followed by incubation of antibodies directed against mouse IL-17A

(BD) or biotinylated anti-mouse IFNg (BD). The amounts of cytokine were determined from standard curves established with serial dilutions of recombinant murine IL-17A or IFNg (R&D). Luminex analysis was carried out using Milliplex mouse cytokine immunoassay kit (Millipore #MPXMCYTO70KPMX32) and plates were read using a Luminex 100^{TM} instrument.

Immunoblotting

Th cells were prepared as described above. After 4 (Th1) or 5 days (Th17 and Th2), viable CD4 T cells were purified on a Ficoll gradient and activated with IL-12 (0.1-0.5ng/ml) or IL-23 (4-5ng/ml) for indicated time. Cell lysates were prepared in lysis buffer (RIPA buffer; 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS) containing a protease & phosphatase inhibitor mixture (Pierce). Protein was quantified by Bradford Assay before equivalent amounts were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). Primary antibody directed against STAT3 (Cell Signaling #9132), phospho-tyrosine(705)-STAT3 (Cell signaling Technology #9145), phospho-serine(727)-STAT3 (Cell signaling Technology #9134), STAT4 (Santa Cruz, sc-486x), phospho-STAT4 (Invitrogen #71-7900), phospho-tyrosine(694)-STAT5 (Cell signaling Technology #9314), STAT5 (Cell signaling Technology #9358), RelA (Santa Cruz, sc-372x), c-Rel (Santa Cruz, sc-71x), NF-κB p50 (Santa Cruz, sc-1190x), or beta-actin (Abcam #6276). HRP-cojugated Donkey anti-rabbit or HRP-conjuaged anti-mouse antibody (Affinity Bioreagents) were used to detect target protein by ECL detection kit (GE Healthcare or Pierce SuperSignal Dura).

Small Interfering RNA (siRNA)

STAT3-specific siRNA (3 mg) (Qiagen) or Allstar negative control siRNA (3 mg) (Qiagen) was administrated to Th17 cells using mouse T cell nucleofector kit from AMAXA. Silencing STAT3 in Th17 cells was verified by western blot at 3 days following transfection. STAT3-silenced Th17 cells were restimulated with indicated cytokine(s) in the presence of or the absence of 10ug/mL plate-bound anti-CD3. After 48 hrs, supernatants were collected and the production of IL-17A was analyzed.

Statistical Analyses

Statistical significance was evaluated using the two-tailed unpaired t test. Unless indicated, all p values <0.05 were considered significant.

Supplemental Information

Supplemental data include four figures and associated legends and primer sequences.

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FIGURE LEGENDS

Figure 1: IL-23 acts synergistically with IL-1 or IL-18 to induce IL-17A production by *in vitro* and *in vivo* generated Th17 cells independently of TCR stimulation.

(A) FACS-sorted naïve CD4 T cells from OT-II TCR transgenic mice were cultured with irradiated *Il12b*$^{-/-}$ splenic feeder cells and 5 mg/ml OVAp for 7d under Th17-polarizing conditions (TGF-b, 5 ng/ml; IL-6, 20 ng/ml; anti- IFNg, 10 mg/ml; anti-IL-4, 10 mg/ml). Cells were harvested and restimulated with irradiated *Il12b*$^{-/-}$ splenic feeder cells in the presence of Th17-polarizing cytokines for an additional two rounds (7 d each) and stained intracelluarly for IL-17A and IFNg after PMA/ionomycin activation for 5h in the presence of monensin. **(B)** Viable CD4 T cells of (A) were purified on a Ficoll gradient and 2x10^5 CD4 T cells were restimulated with indicated cytokine(s) in the absence of TCR stimulation either for 48 hrs to perform ELISA for IL-17A (B) or for 12hrs (the addition of monensin during the final 5hrs) to stain intracellulary for IL-17A and IFNγ **(C)**. **(D)** CD4 T cells were isolated from spleens and mesenteric lymph nodes of either B6 **(left panels)** or colitic IL-10-deficient mice **(right panels)** and stimulated with PMA plus ionomycin stimulation for 5 h in the presence of monensisn before intracellular cytokine staining for IL-17A and IFNg. **(E)** Isolated CD4 T cells (2x10^5) were stimulated with indicated cytokine(s) for 48hrs to measure the IL-17A concentration by ELISA. Error bars represent means ±s.d. of triplicate determinations. Flow cytometry plots are gated on CD4+ cells and the numbers represent the percentages of cells in the each quadrant. Data are representative of at least three independent experiments. **(F)** FACS-sorted naïve CD4 T cells from OT-II TCR transgenic mice were Th17 polarized as in (A). Viable CD4 T cells were purified on a Ficoll gradient and 2x10^5 cells were restimulated with consistent

10 ng/ml IL-23 with titrated dose of IL-1 or IL-18 for 48h and ELISA was performed to measure IL-17A concentration. Error bars represent means ±s.d. of triplicate determinations. Data are representative of at least three independent experiments. **(G)** Viable CD4 T cells of (A) were purified on a Ficoll gradient and 3ug of STAT3-specific or negative control siRNA (Qiagen) was administrated to Th17 cells using AMAXA transfection. 3d following administration cells were treated with the indicated cytokines for 48h and ELISA was performed to measure IL-17A concentration. Error bars represent means ± s.d. of triplicate determinations. Data are representative of at least three independent experiments.

Figure 2: DNase-hypersensitivity profiles and CTCF/Rad21 binding at the murine *Il17a-Il17f* loci of naïve, Th1, Th2, and Th17 cells.

(A) Long range DNase hypersensitivity mapping was performed on naïve, Th1, Th2, and Th17 cells. CD4+ T cells isolated from C57/BL6 mice were cultured under Th1 polarizing conditions for 5 days or in Th2 differentiation media for 2 weeks. CD4+ cells isolated from *Il17f*$^{Thy1.1/Thy1.1}$ reporter mice were used for Th17 polarization. After 6 days of culture Thy1.1+ Th17 cells were isolated and rested overnight prior to restimulation. Harvested cells were either left unstimulated (rest), restimulated with PMA+ionomycin (P+I), subject to anti-CD3 treatment (αCD3), or stimulated with rIL-23 + rIL-1β (IL-23 + IL-1β) before DNaseI digestion, labeling, and hybridization to custom arrays for DNase-chip analysis. DNaseI hypersensitivity profiles are displayed using IGB browser (Affymetrix) and are aligned with a corresponding VISTA plot demonstrating percentage similarity between mouse and human sequences. CNSs are defined as non-coding

regions of at least 100bp in length exhibiting greater than 70% sequence homology between species. Positions of CNSs are relative to the transcription start of the mouse *Il17a* or *Il17f* gene. **(B)** ChIP-chip analysis of CTCF and Rad21/cohesin occupancy across the extended *Il17a-Il17f* loci. Thy1+ Th17 cells generated from *Il17f*$^{Thy1.1/Thy1.1}$ reporter mice underwent chromatin immunoprecipitation (ChIP) with an antibody directed against murine CTCF or Rad21. ChIP samples and input material were subject to whole genome amplification and hybridized to custom tiled arrays. Regions of CTCF and Rad21 binding are visualized using IGB browser (Affymetrix). **(C)** CTCF and Rad21ChIP analysis in naïve, Th1, and Thy1+ Th17 cells. Results are the mean +/- SEM of duplicate determinations and are representative of two independent experiments; results were quantified using real-time PCR and data are expressed as the percentage of input DNA recovered.

Figure 3: Distal *cis* elements regulate *Il17a* and *Il17f* transcription in a STAT3-dependent manner. A fragment of the *Il17a* or *Il17f* promoters was linked to a firefly luciferase reporter in the presence or absence of an additional sequence corresponding to a CNS element in the extended *Il17a-Il17f* locus. Plasmids were transfected into 5 day polarized primary murine Th17 cells along with a renilla luciferase vector to control for transfection efficiency. Transfected cells were rested 14-18 hours and either left unstimulated or restimulated with anti-CD3 for 4 hours **(A)**, or 10ug/mL cytokine **(B** and **C)** for 1 hour. Luciferase readings were taken and data are expressed as relative light units, normalized to renillla luciferase activity. Hatched boxes indicate predicted STAT binding sites, and solid boxes indicate predicted NFκB binding elements. **(C)** Luciferase

activity of Th17 cells transfected with reporter constructs with ('X' in box) or without (open box) mutated STAT binding sites. Data (mean +/- SEM of duplicate samples) are representative of at least three independent experiments. **(D)** CD4+ T cells were isolated from *Il17f*$^{Thy1.1/Thy1.1}$ reporter mice and either assayed directly or grown under Th17 conditions for 6 days. Following Thy1+ isolation and overnight rest, ChIP was performed with antibody directed against STAT3 or IgG in cells that were either left unstimulated, stimulated with IL-23 (3ng/mL) or IL-23 plus IL-1β (10ug/mL) for 1 hour, or PMA + ionomycin for 4 hours. Real-time PCR was performed on immunoprecipitated DNA using primer sets designed to detect the indicated CNS elements and promoter regions. The *Socs3* and *16*S ribosomal promoters were used as positive and negative controls, respectively. qPCR values were normalized to input DNA and values representing relative STAT3 binding are expressed as n-fold increase versus naïve CD4. Results are the mean +/- SEM of two to five experiments. #$p<0.05$ and *$p<0.01$ versus STAT3 recruitment to the *16S* ribosomal promoter (16Srp).

Figure 4: IL-1β induces NF-κB factors that collaborate with STAT3 to regulate *Il17a/f* transcription.

(A) B6 CD4+ T cells were Th17 polarized for 5 days before live cell isolation was performed using a Ficoll gradient and cells were rested overnight. On day 6 Th17 cells were restimulated for the indicated time frames with IL-23 (5ng/mL) and/or IL-1β (20ng/mL) and nuclear extracts were prepared. Nuclear translocation of P-Tyr-STAT3, P-Ser-STAT3, and the NF-κB factors RelA, c-Rel, and p50 were evaluated by immunoblotting. Blots were stripped and reprobed with β-actin as a loading control.

(B) FACS-sorted naïve CD4+ T cells from *Rela*$^{fl/fl}$.*Cd4.cre*+, *Rel*-/-, or WT B6 littermate mice were labeled with CFSE and cultured under Th17 polarizing conditions for 3 days. Cells were stimulated with PMA plus ionomycin stimulation for 5 h in the presence of monensin before intracellular cytokine staining for IL-17A. Proliferation was assessed by CFSE. Flow cytometry plots are gated on live CD4+ cells and the numbers represent the percentages of cells in the each quadrant. Data is representative of at least three independent experiments. **(C)** Naïve CD4+ T cells from the indicated mice were cultured under Th17 conditions for 3 days as in (A) (**left panel**) and assayed directly or 5 days (**right panel**), and assayed following restimulation (48 hours) as indicated. The amount of IL-17 in culture supernantants was quantified by ELISA. **(bottom panel)** FACS-sorted naïve CD4+ from *Rela*$^{fl/fl}$.*Cd4.cre*+, *Rel*-/-, or WT B6 mice were cultured under Th17 conditions for 72 hours and then processed for mRNA quantification by real-time PCR for the indicated genes. Data was normalized to beta-2 microglobulin and is expressed as relative difference (n-fold). (**D**) CD4+ T cells were isolated from C57Bl/6 mice and either assayed directly or grown under Th17 conditions for 6 days. Following Ficoll separation and overnight rest, ChIP was performed with antibody directed against NF-κB p65 (RelA) **(A)** or IgG in cells that were either left unstimulated, stimulated with 10ug/mL of cytokine (IL-1β or IL-23 plus IL-1β) or PMA + ionomycin for 2 hours. The ChIP assay was performed on RelA-deficient Th17 cells generated from *Rela*$^{fl/fl}$.*CD4*.cre+ mice for comparison. Real-time PCR was performed on immunoprecipitated DNA using primer sets designed to detect the indicated CNS elements and promoter regions. The *Il2* and *16S* ribosomal promoters were used as positive and negative controls, respectively. qPCR values were normalized to input DNA

and values representing relative RelA binding are expressed as n-fold increase versus unstimulated Th17. Results are the mean +/- SEM of three to six indepenent experiments. *p<0.05 and #p<0.01 versus RelA recruitment to the 16Srp **(E)** c-Rel ChIP analysis on Th17 cells either left unstimulated or restimulated with PMA + ionomycin, IL-1β, or IL-23 + IL-1β for 2 hours. Data were normalized to input DNA and are expressed relative to resting Th17 cells. The *Il2* and 16S ribosomal promoters were used as positive and negative controls. Results represent mean +/- SEM of at least three independent experiments. *p<0.05 and #p<0.01 versus c-Rel recruitment to the 16Srp.

Figure 5: Signals from IL-1 family cytokines converge with Jak/STAT signals to enhance STAT activation in Th17, Th1, and Th2 cells.

(**A-D**) B6 CD4+ T cells were cultured with anti-CD3/CD28 (αCD3/CD28) and irradiated CD4(-) antigen presenting cells (APCs) in the presence of IL-6 (20ng/mL), TGF-β (2ng/mL), anti-IFNγ (10ug/mL), and anti-IL-4 (10ug/mL) for five days to induce Th17 development. Following Ficoll separation to isolate viable cells, cells were rested overnight in neutral media. The following day after serum starvation for 2 hours cells were restimulation with 4ng/mL of IL-23 (A,B), IL-6 (C), or IL-21 (D) in the presence or absence of 20ng/mL IL-1β (A,C,D) or 40ng/mL IL-18 (B) for the indicated times. Cell lysates were harvested and immunoblotted with antibody directed against phospho-tyrosine(705)-STAT3 (P-Tyr-STAT3). Membranes were stripped and reprobed with anti-serine(727)-STAT3 (P-Ser-STAT3) (A) and anti-STAT3 (A-D) as a loading control. (**E**) Th1 cells were generated by culturing B6 CD4+ T cells for 4 days with αCD3/CD28 and irradiated CD4(-) APCSs in the presence of IL-12 (10ng/mL) and anti-IL-4

(10ug/mL). Viable cells were obtained as in (A-D) and rested for 4-6 hours before serum starvation (2h) and restimulation with IL-12 (0.1ng/mL) in the absence or presence of IL-18 (40ng/mL) for the indicated times. Phospho-tyrosine(693)-STAT4 (P-Tyr-STAT4) expression was analyzed by immunoblotting, and membrane was stripped and reprobed with anti-STAT4 as a loading control. (**F**) B6 CD4+ were cultured under Th2-polarizing conditions (αCD3/CD28 plus IL-4 (500U/mL) and anti-IFNγ (10ug/mL)) with irradiated feeder cells for 2 rounds of 7 days. Viable cells were rested overnight in neutral media before restimulation with 50U/mL IL-2 with or without addition of IL-33 (20ng/mL). Expression of phospho-tyrosine(694)-STAT5 (P-Tyr-STAT5) was assessed by immunoblot before membrane was stripped and reprobed with anti-STAT5 as a loading control. All data shown are representative of at least three independent experiments.

Figure 6: IL-1β modulates STAT3 phosphorylation through inhibition of SOCS3 and induction of MAP kinase signaling.

(**A**) B6 CD4+ T cells were cultured with irradiated splenic feeder cells and 2.5 ug/ml αCD3/CD28 for 5d under Th17-polarizing conditions as in figure 2. Cells were purified on a Ficoll gradient and rested overnight before $2x10^6$ CD4 T cells were serum starved for 1 hour then pre-treated with various cell signaling inhibitors for 1 hour. Following restimulation with IL-23 (5ng/mL) and/or IL-1β (20ng/mL) (**A**) or plate-bound anti-CD3 (1ug/mL) (**B**) for 14-18 hours, culture supernatants were harvested for analysis by ELISA for IL-17A protein production. Data represent mean +/- SEM from at least 2 independent experiments. Statistical significance is relative to IL-17 production by unstimulated cells (#$p<0.05$ and *$p<0.01$) (**C**) Th17 cells were generated and pre-treated with cell signaling

inhibitors as described in (A) before restimulation for 30 minutes with IL-23 (5ng/mL) +/- IL-1β (20ng/mL). Cell lysates were harvested and subjected to immunoblot analysis for P-Tyr-STAT3 or P-Ser-STAT3 the then membranes were stripped and reprobed with total STAT3 as a loading control. Phospho-STAT3 integrated band density values (IDV) were normalized to total STAT3 and expressed as fold change over unstimulated cells. Results are representative of three independent experiments. (**Left panel**) represents P-Try-STAT3; (**right panel**) P-Ser-STAT3. (**D**) Representative western blots are show for P-Tyr STAT3 (**left panel**) and P-Ser-STAT3 (**right panel**). (**E**) $2x10^7$ Th17 cells polarized for 6 days and processed as in (A) were serum starved, restimulated with IL-23, IL-1β, or IL-23 + IL-1β for the indicated times, then lysed and subjected to ELISA analysis that quantified both the active phosphorylated form of Jak2 (Jak2[pYpY1007/1008]) and total Jak2. Jak2[pYpY1007/1008] values were normalized to total Jak2 expression and data is expressed as fold change over unstimulated Th17 cells where unstimulated cells were assigned a value of 1. The graph represents duplicate values averaged from three independent experiments, where error bars indicate mean + SEM. (**F**) B6 CD4+ T cells were isolated and cultured under Th17 conditions. On day 5 cells viable cells were obtained with a Ficoll gradient and rested overnight in neutral media. On day 6 cells were restimulated with 3ng/mL IL-23 and/or IL-1β for the indicated times. Transcript levels of *Socs3* were normalized against β2-microglobulin and relative expression compared to unstimulated cells was calculated using the ΔΔCt method. Data represent mean +/- SEM from three independent experiments.

SUPPLEMENTARY INFORMATION

Supplementary Figure 1: Putative transcription factor binding site (TFBS) analysis of enhancer CNSs A-97, -37, +10, and +28 and *Il17a/f* promoters.

Syntenic regions of mammalian sequences for *Il17a/f* promoters and CNSs were aligned using ClustalW to exhibit clusters of highly conserved STAT/Bcl6, NFkB, NFAT, RORA, and VDR binding motifs. CNSs A-97, -37, +10, +28, and the *Il17a* promoter have a unique tandem STAT site coincident with an overlapping Bcl motif. # denotes a binding site located on the anti-sense DNA strand, and * denotes sense strand location. TFBS analyses was performed using Genomatix software (http://www.genomatix.de) and rVISTA.

Supplementary Figure 2: DNase-chip and CTCF and Rad21 ChIP-chip analysis across the *Ifng* locus.

B6 CD4+ T cells were differentiated under Th1 or Th17 conditions for 5 or 6 days, respectively. For DNase-chip analysis, day 5/6 cells were either left unstimulated or stimulated with anti-CD3/CD28 or PMA + ionomycin for five hours before processing and array hybridization. For CTCF and Rad21 ChIP-chip analysis, cells were left resting and prepared as described in figure 4. Recruitment of CTCF and Rad21 to *Ifng* was visualized using IGB broswer (Affymetrix) aligned with a VISTA plot comparing human and muring *Ifng* and surrounding region.

<u>Supplementary Figure 3</u>: Absence of RelA and/or c-Rel affects Th1 proliferation and cytokine production but does not compromise expression of Th2 and iTreg cytokines.

CD4+ T cells isolated from WT, *Rela*$^{fl/fl}$*.Cd4.cre+*, or *Rel*-/- mice were differentiated under Th1, Th2, or iTreg conditions (+/- addition of CFSE). (**A**) On day 3 of Th1 polarization proliferation was assessed by CFSE analysis. Plots are gated on CD4+ T cells. (**B**) On day 5 (Th1) or 7 (Th2, iTreg) of primary culture, cells were restimulated with1ug/mL plate-bound anti-CD3 and culture supernantants were harvested for ELISA. A luminex assay was used (Millipore) to quantify cytokine production by helper T cell subsets.

<u>Supplementary Figure 4</u>: RelA expression is required for maximal IL-17 production *in vivo*.

(A) Lamina propria lymhocytes (LPL) were isolated from uninfected (**top panels)** *Rela* $^{+/+}$*.Cd4.cre-* or *Rela*$^{fl/fl}$*.Cd4.cre+* animals or animals who underwent infection with the pathogen *Citrobacter Rodentium* (**bottom panels**) and were subjected to PMA + ionomycin stimulation for 5 h in the presence of monensin before intracellular cytokine and surface staining followed by flow cytometry. FACS plots are gated on live CD3+CD4+ lymphocytes. **(B)** IL-22, IL-17, IFN-γ production, and corresponding MFI (IL-17) as determined by FACS analysis is displayed graphically. Each dots represents cytokine production as a percentage of live CD3+CD4+ LPL for an individual mouse.

Primer Sequences for Cloning of CNSs & Promoter Fragments: Promoter-Reporter Assay:

All CNS fwd primers contained MluI sites, and all reverse primers included an NheI site

All promoter fragments were cloned using HindIII and XhoI restriction sites

Il17a promoter: 286bp fragment:

Fwd GCTACTCGAGGCAAAGCATCTCTGTTCAGC

Rev CGTAAAGCTTGCGTCCTGATCAGCTGGTGC

Il17f promoter: 295bp fragment

Fwd GCATCTCGAGAAAGGTAATGGGAGTGGAAG

Rev GCATAAGCTTGGTTTCTCCAATGGCTGCTTC

CNS A-118

Fwd CTTGCCATCTTTCCTTCTTG

Rev CTGTCTTGCCTTCAGTGC

CNS F-13

Fwd GAGACACAGGAAAGGAGAGG

Rev GGAGCAGAGATTACTCAATGACAG

CNS A-97

Fwd GTTTCTTGTGCCTTCTCTTG

Rev CAAGGTTGGGCATTGAGC

CNS F-7

Fwd GCAAGACTGGAAAGGAGAAACATC

Rev GCACAGCCTCTTCGTTTG

CNS A-60

Fwd GCCTAACTGTCAGAAAGTCACC

Rev GCTGAGTTCTTCTCCCCTTAC

CNS A-37

Fwd ATGGAGCATTTCAGCAGGC

Rev ATGCTTCCTGCCTTGATG

CNS A-5

Fwd ATCCTTCATCATAGCAGCC

Rev TGAATACTTGCGTGGCAG

CNS A+10

Fwd ACTTGCTGCTCTCACGGAAG

Rev CCTGAACAGAACACCAATGG

CNS A+28

Fwd GCTATCTCTCCAGCCCTAAG

Rev CAGGCTAATCTTGGGAATG

CNS A+23

Fwd CGTAACGCGTCAGAACAAGTCACCTGCTG

Rev CGTAGCTAGCCCTGTGATTTCCTCATTGG

CNS A+36

Fwd CGTAACGCGTTCCTACTGTGATGACCAGGC

Rev CGTAGCTAGCAGTCCATCCTCAATGTGGC

Primers used for mutation of predicted STAT binding sites:

CNS A+10 STAT mut

Fwd GTGCAGTGACTAAAAGGAGAGTCCTCGAGGATAAAGTAACCTACC

Rev GGTAGGTTACTTTATCCTCGAGGACTCTCCTTTTAGTCACTGCAC

CNS A+28 STAT mut

Fwd CCTGGCTGAGGAGAACGGAGAATCCCTTTGTGATCTTTCAGTCC

Rev GGACTGAAAGATCACAAAGGGATTCTCCGTTCTCCTCAGCCAGG

CNS A-97 STAT mut

Fwd CATCATACACTAATTGTGAGTGAACTTTGTAGCCTTTTGTAGATC

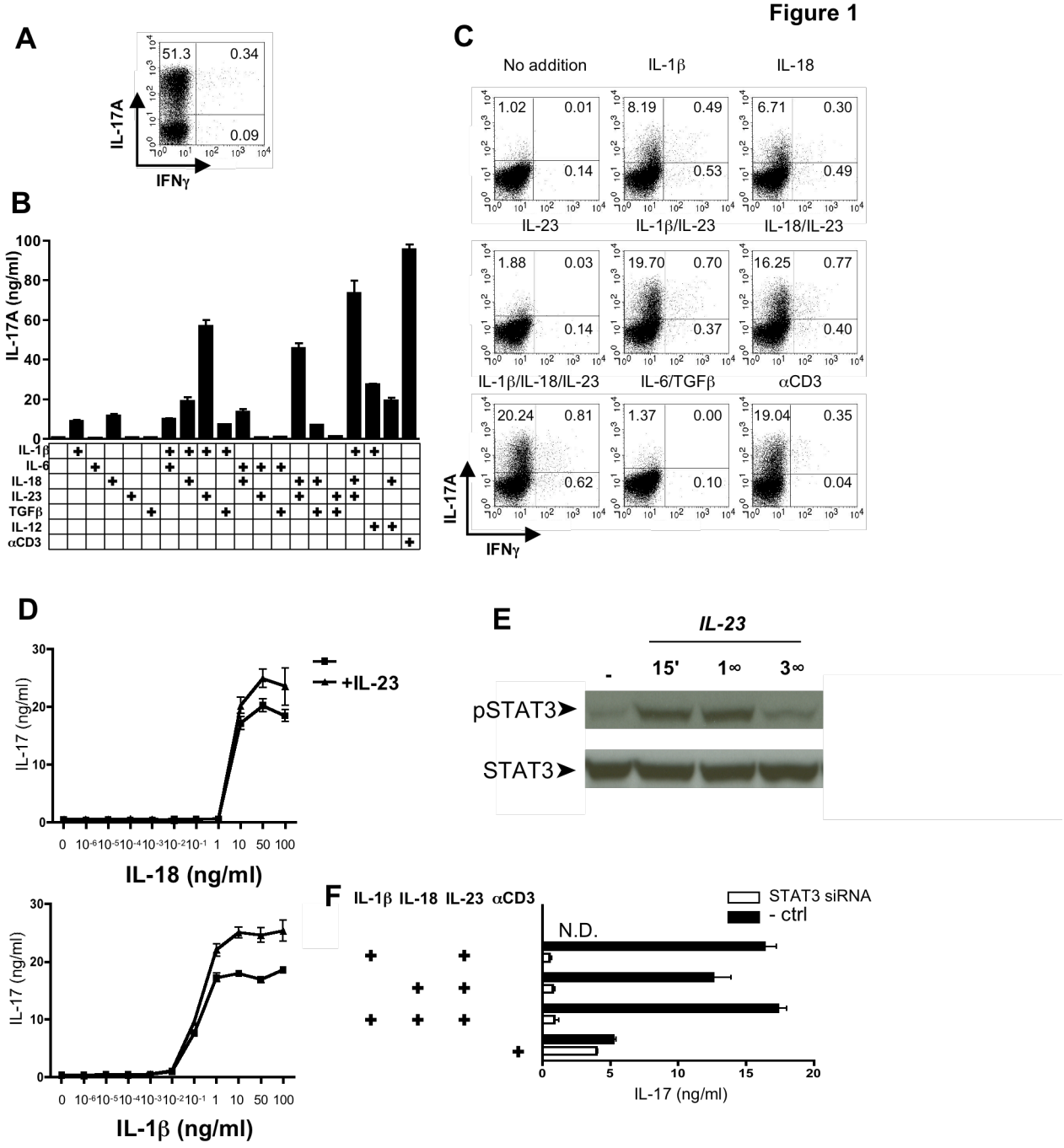
Rev GATCTACAAAAGGCTACAAAGTTCACTCACAATTAGTGTATGATG

CNS A-37 STAT mut

Fwd CAGAGGCCCTAGCCGCAAGCTGTCTGGACTCAGCTGGTCAAG

Rev CCTGACCAGCTGAGTCCAGACAGCTTGCGGCTAGGGCCTCTG

Figure 1


A
51.3
0.34
0.09
IL-17A
IFNγ
B
IL-17A (ng/ml)
IL-1β
IL-6
IL-18
IL-23
TGFβ
IL-12
αCD3
C
No addition
IL-1β
IL-18
1.02
0.01
0.14
8.19
0.49
0.53
6.71
0.30
0.49
IL-23
IL-1β/IL-23
IL-18/IL-23
1.88
0.03
0.14
19.70
0.70
0.37
16.25
0.77
0.40
IL-1β/IL-18/IL-23
IL-6/TGFβ
αCD3
20.24
0.81
0.62
1.37
0.00
0.10
19.04
0.35
0.04
IL-17A
IFNγ
D
IL-17 (ng/ml)
+IL-23
IL-18 (ng/ml)
IL-17 (ng/ml)
IL-1β (ng/ml)
E
IL-23
-
15'
1∞
3∞
pSTAT3
STAT3
F
IL-1β IL-18 IL-23 αCD3
STAT3 siRNA
- ctrl
N.D.
IL-17 (ng/ml)

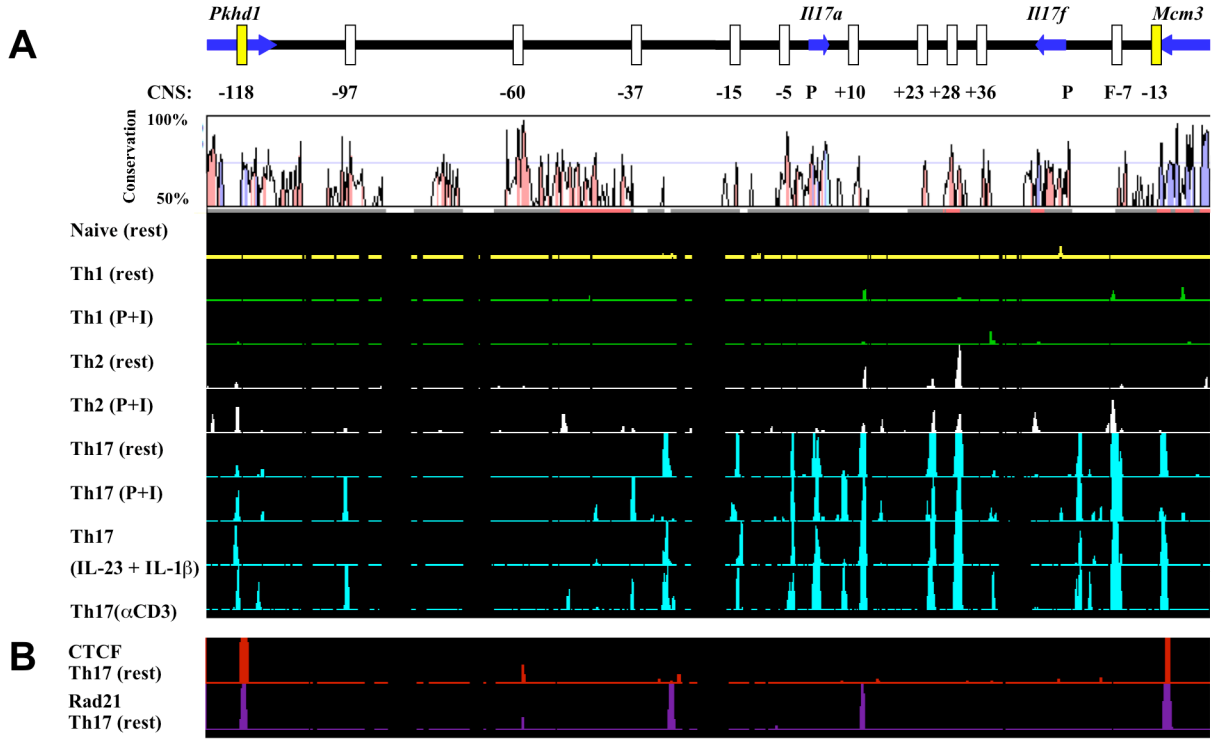

A
Pkhd1
Il17a
Il17f
Mcm3
CNS:
-118
-97
-60
-37
-15
-5
P
+10
+23
+28
+36
P
F-7
-13
Conservation
100%
50%
Naive (rest)
Th1 (rest)
Th1 (P+I)
Th2 (rest)
Th2 (P+I)
Th17 (rest)
Th17 (P+I)
Th17
(IL-23 + IL-1β)
Th17(αCD3)
B
CTCF
Th17 (rest)
Rad21
Th17 (rest)

Figure 2

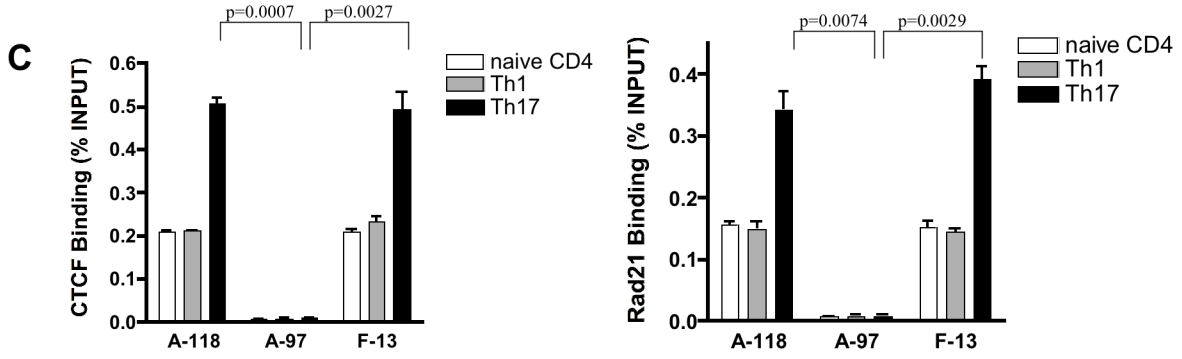
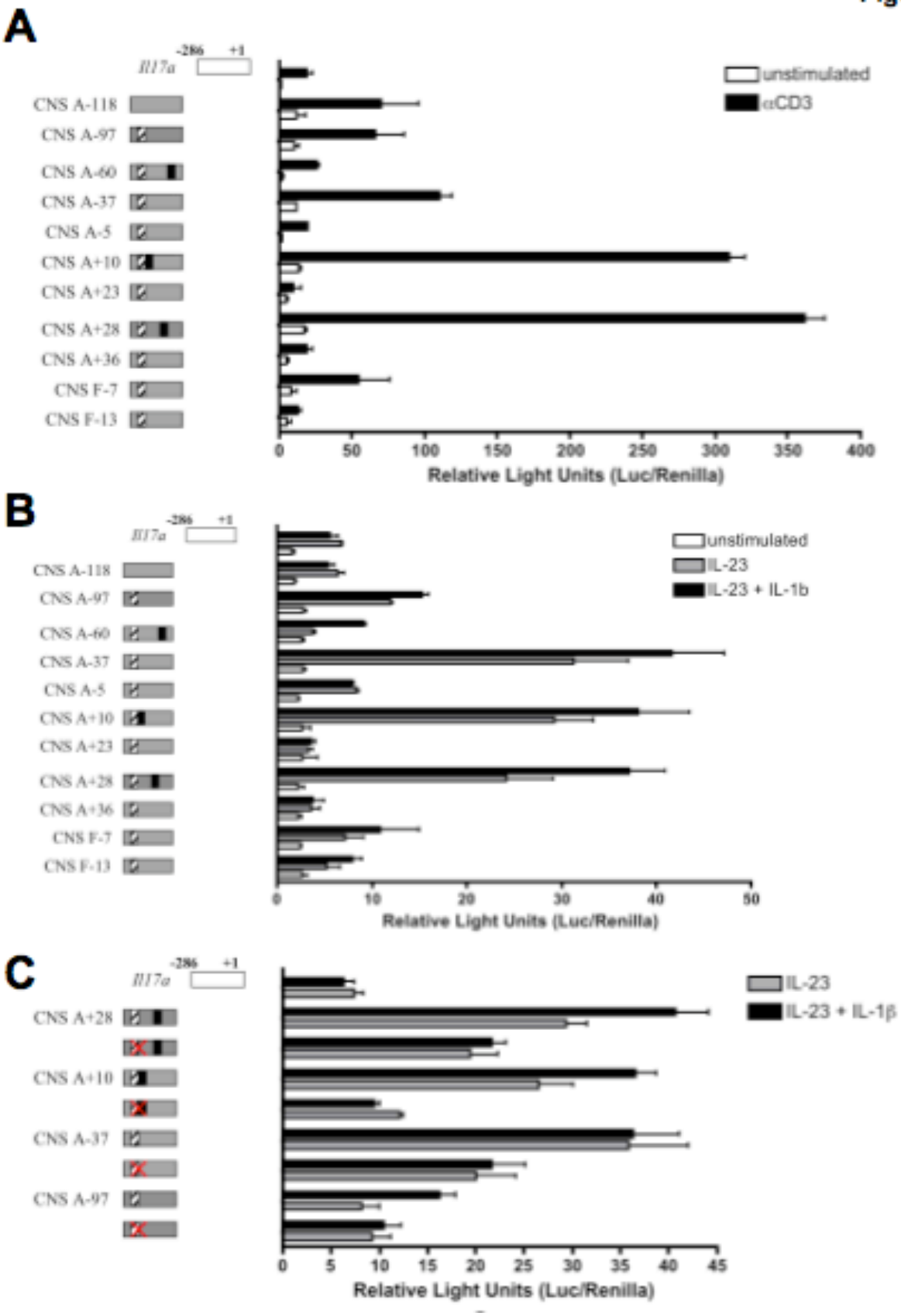

C
p=0.0007
p=0.0027
CTCF Binding (% INPUT)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A-118
A-97
F-13
naive CD4
Th1
Th17
p=0.0074
p=0.0029
Rad21 Binding (% INPUT)
0.4
0.3
0.2
0.1
0.0
A-118
A-97
F-13
naive CD4
Th1
Th17

Figure 3


A
-286
+1
Il17a
CNS A-118
CNS A-97
CNS A-60
CNS A-37
CNS A-5
CNS A+10
CNS A+23
CNS A+28
CNS A+36
CNS F-7
CNS F-13
unstimulated
αCD3
0
50
100
150
200
250
300
350
400
Relative Light Units (Luc/Renilla)
B
-286
+1
Il17a
CNS A-118
CNS A-97
CNS A-60
CNS A-37
CNS A-5
CNS A+10
CNS A+23
CNS A+28
CNS A+36
CNS F-7
CNS F-13
unstimulated
IL-23
IL-23 + IL-1b
0
10
20
30
40
50
Relative Light Units (Luc/Renilla)
C
-286
+1
Il17a
CNS A+28
CNS A+10
CNS A-37
CNS A-97
IL-23
IL-23 + IL-1β
0
5
10
15
20
25
30
35
40
45
Relative Light Units (Luc/Renilla)

D

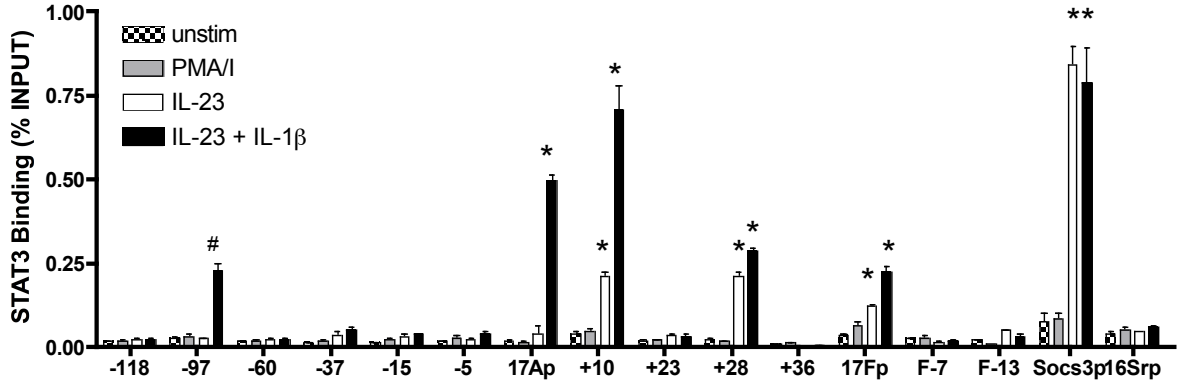

STAT3 Binding (% INPUT)
1.00
0.75
0.50
0.25
0.00
unstim
PMA/I
IL-23
IL-23 + IL-1β
#
*
*
*
*
*
*
*
**
-118
-97
-60
-37
-15
-5
17Ap
+10
+23
+28
+36
17Fp
F-7
F-13
Socs3p
16Srp

Figure 4

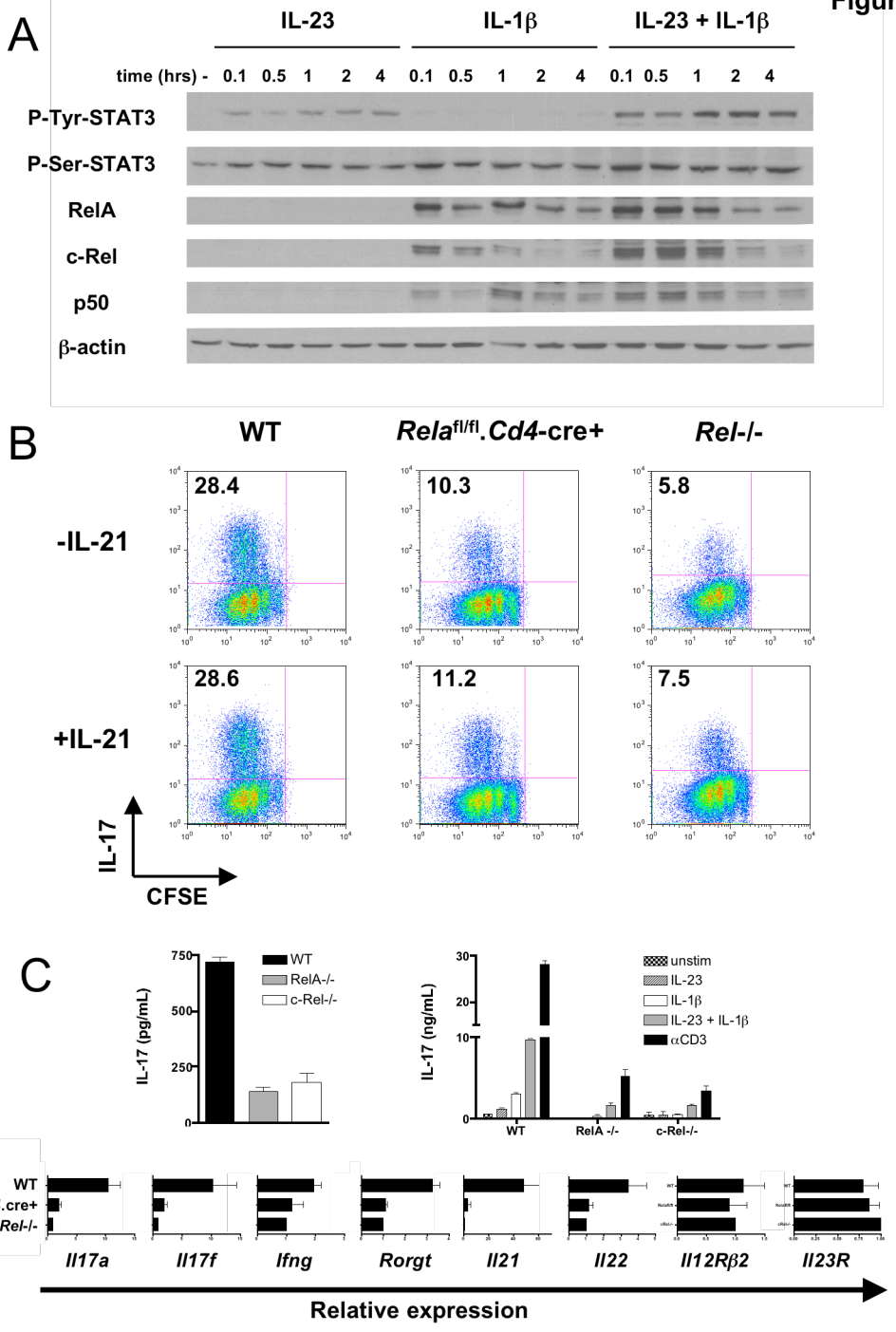
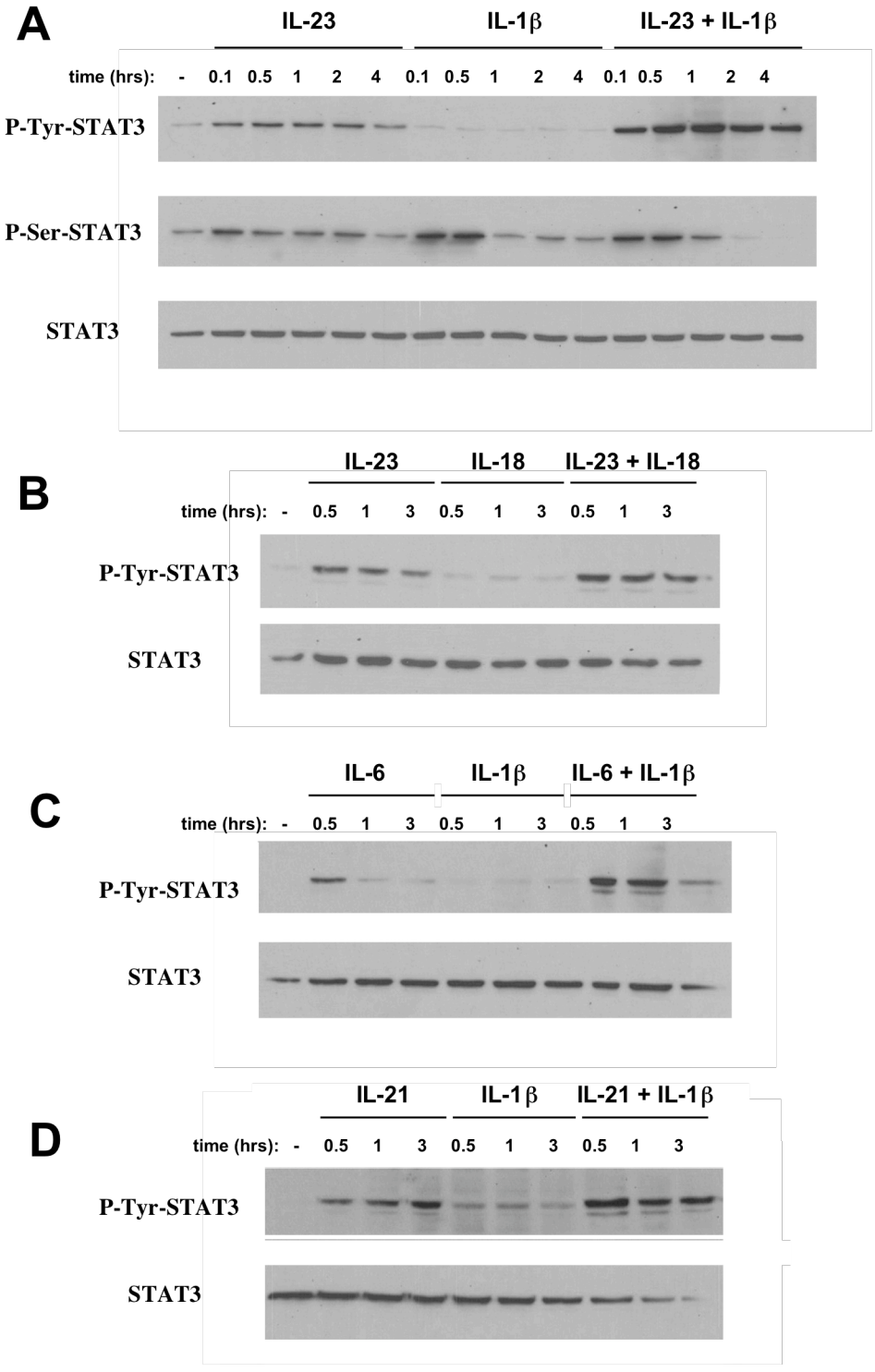
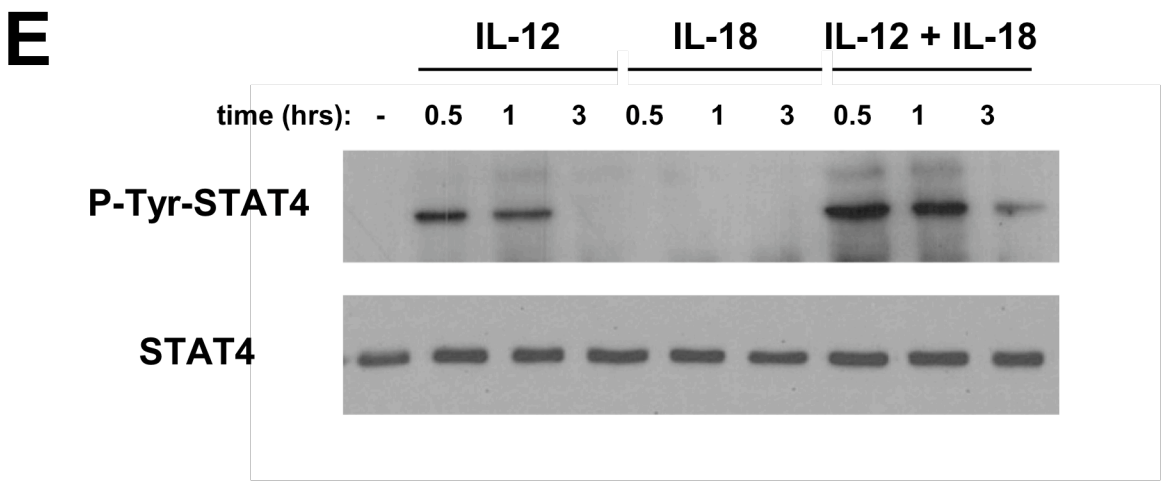

A
IL-23
IL-1β
IL-23 + IL-1β
time (hrs) - 0.1 0.5 1 2 4 0.1 0.5 1 2 4 0.1 0.5 1 2 4
P-Tyr-STAT3
P-Ser-STAT3
RelA
c-Rel
p50
β-actin
B
WT
Rela fl/fl .Cd4-cre+
Rel-/-
-IL-21
+IL-21
28.4
10.3
5.8
28.6
11.2
7.5
IL-17
CFSE
C
IL-17 (pg/mL)
WT
RelA-/-
c-Rel-/-
IL-17 (ng/mL)
unstim
IL-23
IL-1β
IL-23 + IL-1β
αCD3
WT
RelA -/-
c-Rel-/-
WT
Rela fl/fl .CD4.cre+
Rel-/-
Il17a
Il17f
Ifng
Rorgt
Il21
Il22
Il12Rβ2
Il23R
Relative expression

Figure 5


A
IL-23
IL-1β
IL-23 + IL-1β
time (hrs): - 0.1 0.5 1 2 4 0.1 0.5 1 2 4 0.1 0.5 1 2 4
P-Tyr-STAT3
P-Ser-STAT3
STAT3
B
IL-23
IL-18
IL-23 + IL-18
time (hrs): - 0.5 1 3 0.5 1 3 0.5 1 3
P-Tyr-STAT3
STAT3
C
IL-6
IL-1β
IL-6 + IL-1β
time (hrs): - 0.5 1 3 0.5 1 3 0.5 1 3
P-Tyr-STAT3
STAT3
D
IL-21
IL-1β
IL-21 + IL-1β
time (hrs): - 0.5 1 3 0.5 1 3 0.5 1 3
P-Tyr-STAT3
STAT3


E
IL-12
IL-18
IL-12 + IL-18
time (hrs): - 0.5 1 3 0.5 1 3 0.5 1 3
P-Tyr-STAT4
STAT4

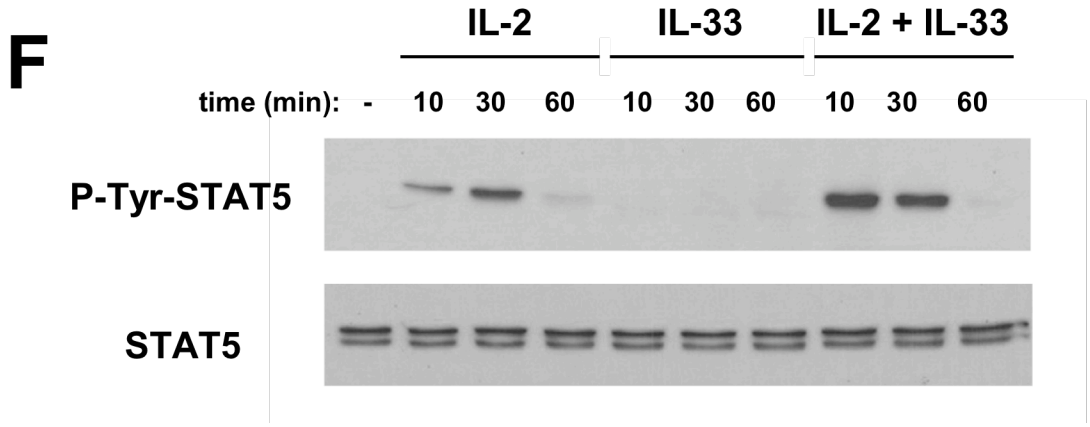
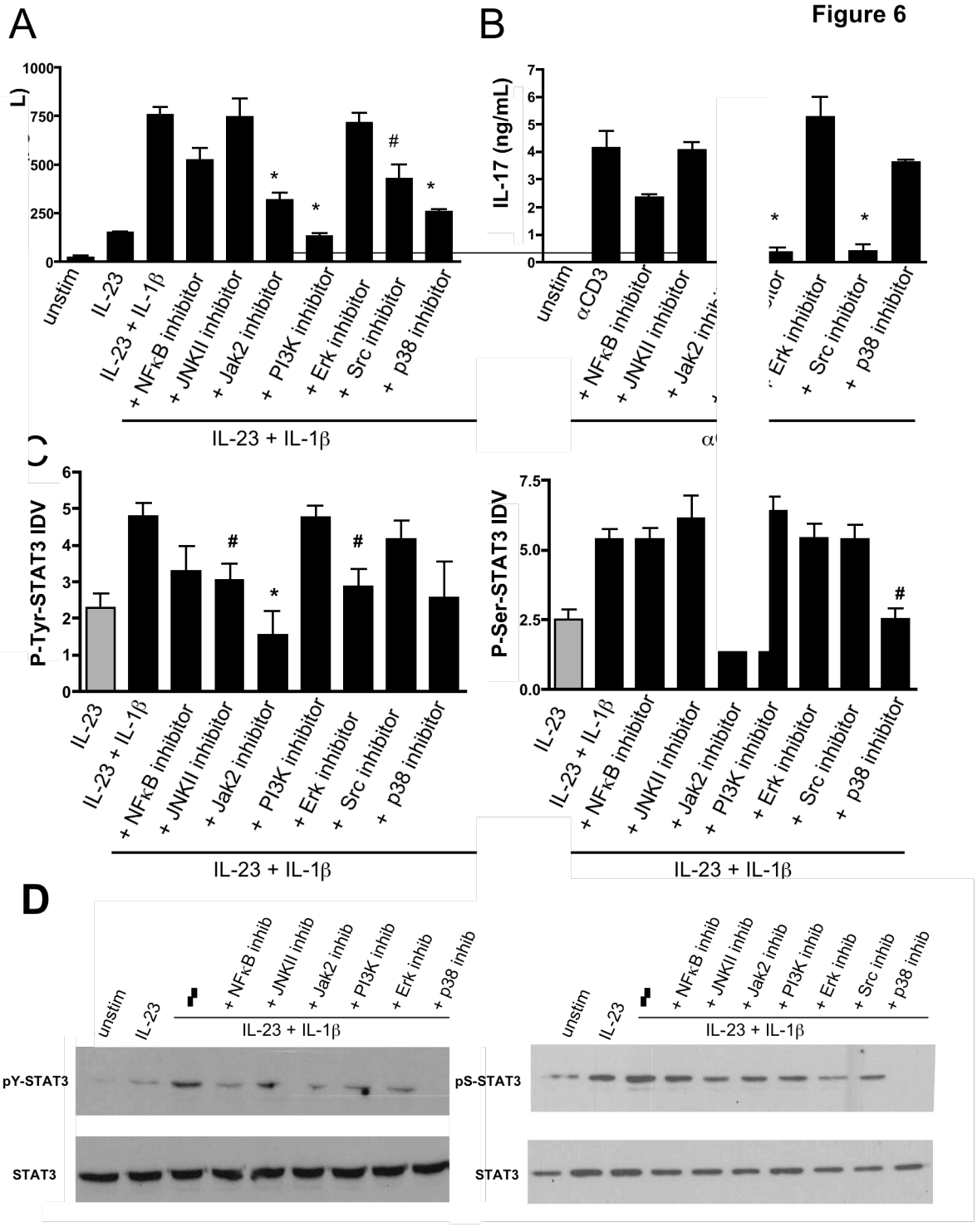
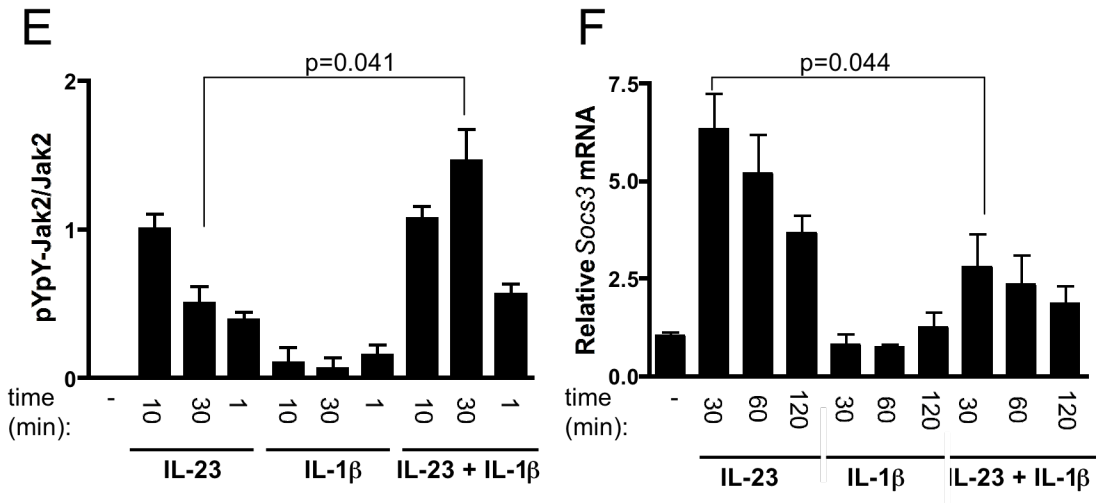
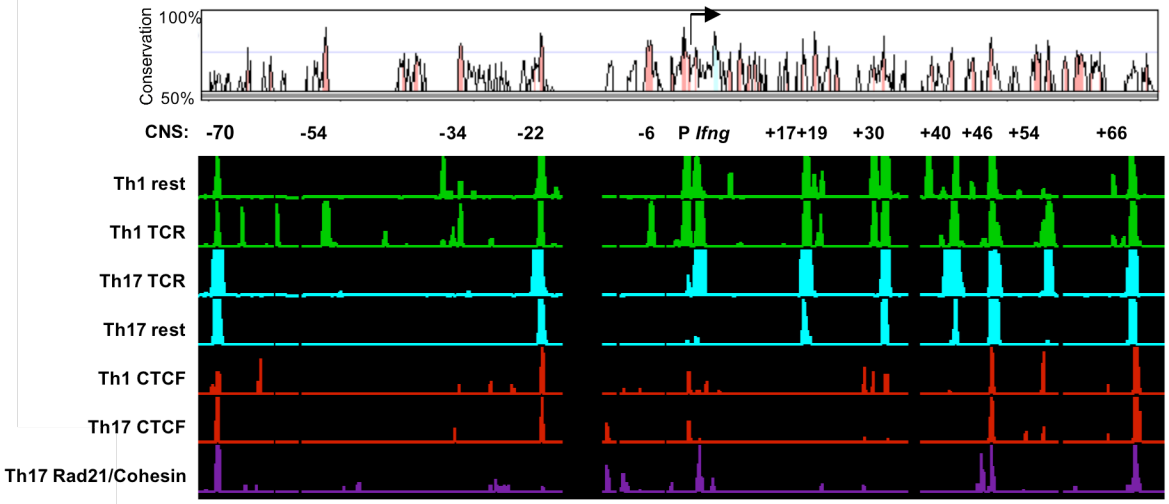

F
IL-2
IL-33
IL-2 + IL-33
time (min): - 10 30 60 10 30 60 10 30 60
P-Tyr-STAT5
STAT5

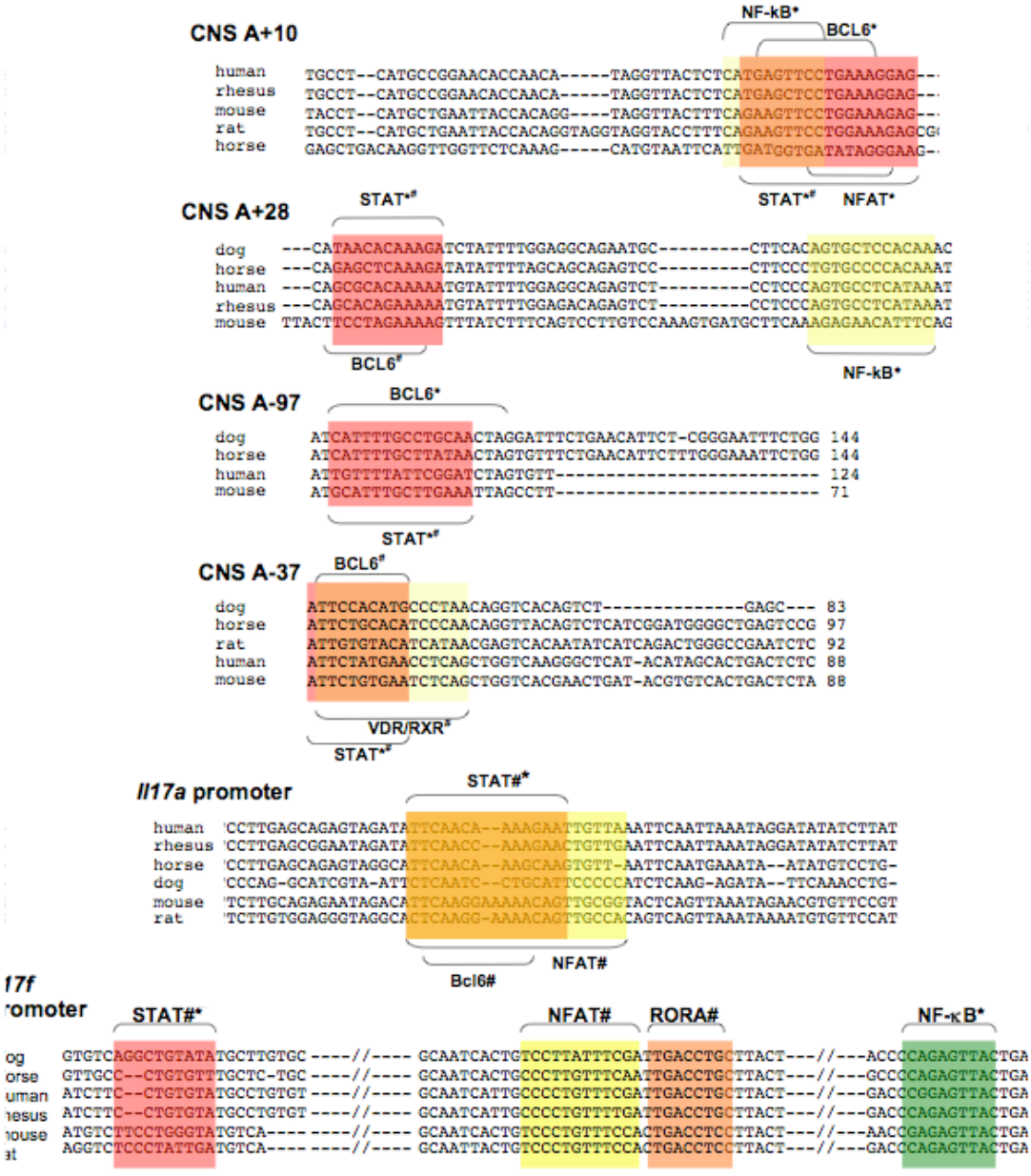
Figure 6


A
B
IL-17 (ng/mL)
unstim
IL-23
IL-23 + IL-1β
+ NFκB inhibitor
+ JNKII inhibitor
+ Jak2 inhibitor
+ PI3K inhibitor
+ Erk inhibitor
+ Src inhibitor
+ p38 inhibitor
αCD3
P-Tyr-STAT3 IDV
P-Ser-STAT3 IDV
D
pY-STAT3
STAT3
pS-STAT3
+ NFκB inhib
+ JNKII inhib
+ Jak2 inhib
+ PI3K inhib
+ Erk inhib
+ Src inhib
+ p38 inhib

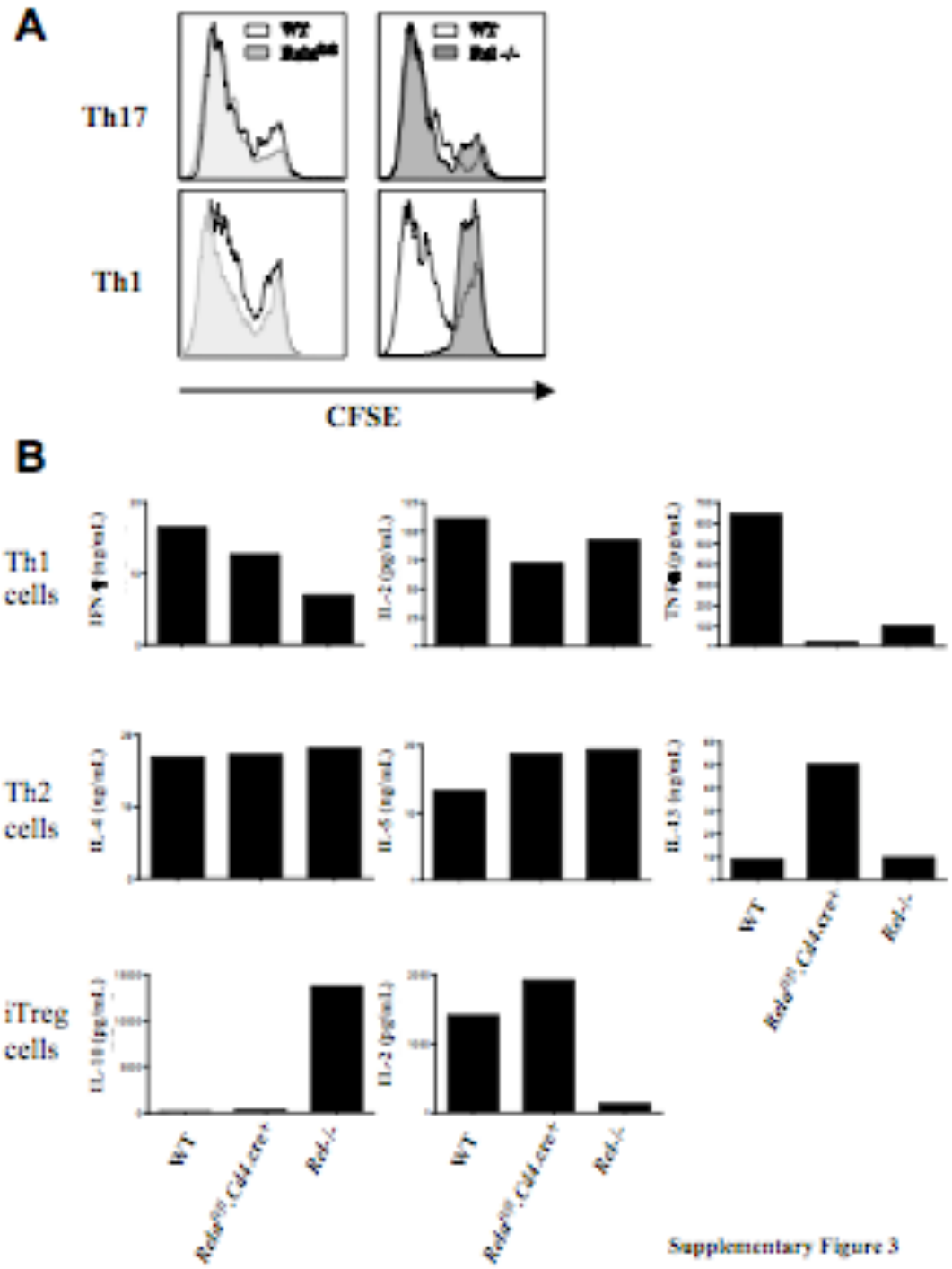

E
p=0.041
pYpY-Jak2/Jak2
2
1
0
time (min):
-
10
30
1
10
30
1
10
30
1
IL-23
IL-1β
IL-23 + IL-1β
F
p=0.044
Relative Socs3 mRNA
7.5
5.0
2.5
0.0
time (min):
-
30
60
120
30
60
120
30
60
120
IL-23
IL-1β
IL-23 + IL-1β



Supplemental Figure 1



Supplementary Figure 2



Supplementary Figure 3

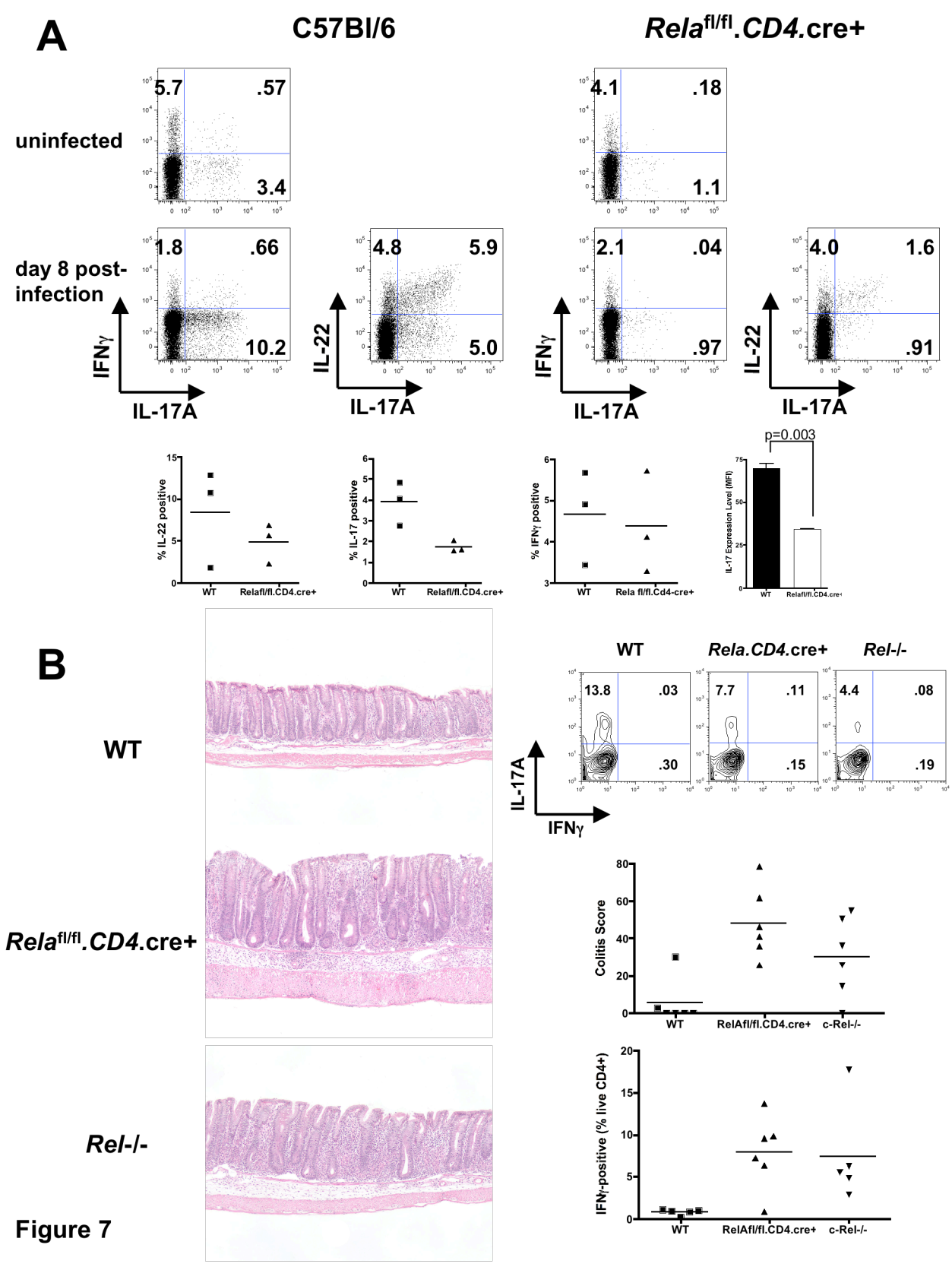

A
C57Bl/6
Rela fl/fl .CD4.cre+
uninfected
day 8 post-infection
5.7
.57
3.4
4.1
.18
1.1
1.8
.66
10.2
4.8
5.9
5.0
2.1
.04
.97
4.0
1.6
.91
IFNγ
IL-17A
IL-22
% IL-22 positive
% IL-17 positive
% IFNγ positive
IL-17 Expression Level (MFI)
p=0.003
WT
Relafl/fl.CD4.cre+
Rela fl/fl.Cd4-cre+
B
WT
Rela.CD4.cre+
Rel-/-
13.8
.03
.30
7.7
.11
.15
4.4
.08
.19
Rela fl/fl .CD4.cre+
Rel-/-
Colitis Score
RelAfl/fl.CD4.cre+
c-Rel-/-
IFNγ-positive (% live CD4+)

Figure 7

Epigenetic Instability of Cytokine and Transcription Factor Gene Loci Underlie Plasticity of the T helper 17 Lineage

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*These authors contributed equally to this work.
S.K.W. contributed data towards figures 1&4

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Format adapted for dissertation

ABSTRACT

Epigenetic modifications of key cytokine and transcription factor gene loci accompany T helper (Th) lineage specification, and are proposed to be responsible for heritable patterning of gene expression by mature T cells. The recognition of phenotypic plasticity of Th17 cells raises questions about stability of the chromatin structure of key genes in Th17 cells. We performed comparative analyses of epigenetic modifications in Th17 cells before and after induction of divergent late developmental fates promoted by restimulation of Th17 precursors by interleukin (IL)-12, which promotes a Th1-like transition with dominant IFN-g gene expression and extinguished IL-17A and IL-17F gene expression, or TGF-b, which promotes retention of a Th17 phenotype. Th17-committed precursors, which were found to have multiple lineage-specific DNase I hypersensitivity sites and permissive histone modifications across the extended *Il17a/f* locus, also showed extensive permissive epigenetic modifications within the *Ifng* locus as well, but lacked critical permissive modifications. IL-12 stimulation of Th17 cells rapidly induced STAT4- and T-bet-dependent permissive and repressive epigenetic changes in the *Ifng* and *Il17a/f* loci, respectively, and also induced repressive chromatin modifications of the *Rorc* locus that resulted in sustained down-regulation of ROR γ t transcripts, loss of which appeared to underlie the reversal of permissive epigenetic modification of the *Il17a/f* locus. These findings reveal substantial instability of the

chromatin structure in key transcription factor and cytokine genes of Th17 cells and support a model of Th17 lineage plasticity in which cell-extrinsic factors modulate Th17 cell fates through differential effects on the epigenetic status of Th17 lineage transcription factors.

INTRODUCTION

The development of distinct effector $CD4^+$ T cell subsets from multipotential, antigen-naïve precursors enables the adaptive immune system to best match the host response to distinct pathogen threats. Effector T cell subsets are distinguished on the basis of the unique cytokines they produce in response to antigen rechallenge. Th1 cells secrete interferon (IFN)-γ and are involved in host defense against intracellular bacteria and viruses, whereas Th2 cells produce interleukins 4, 5, and 13 (IL-4, IL-5, and IL-13) and play a role in protection against helminthic parasites (Mosman and Coffman, 1989; Murphy and Reiner, 2002). Th17 cells are characterized by production of IL-17A, IL-17F and IL-22 and appear to be important in host protection against extracellular bacteria

and fungi (Korn et al., 2009; McGeachy and Cua, 2008; Stockinger and Veldhoen, 2007; Weaver et al., 2007).

A prominent feature of effector $CD4^+$ T cell development is the network of positive and negative feedback mechanisms of cytokine and transcription factors, which reinforce differentiation of one subset and hamper development of others. Th1 development is promoted by IFN-γ and IL-12, which activate signal transducer and activator of transcription 1 and 4 (STAT1 and STAT4), respectively. In concert with signaling through the TCR, activated STAT1 induces the Th1 lineage transcription factor, T-bet, which interferes with Th2 differentiation while potentiating *Ifng* expression and inducing *Il12rb2* expression, thereby conferring responsiveness to IL-12 that further enhances *Ifng* expression via STAT4 (Djuretic et al., 2007; Kaplan et al., 1996; Murphy and Reiner, 2002; Szabo et al., 2000; Thierfelder et al., 1996). Th2 cell differentiation is driven by IL-4-mediated STAT6 activation, which synergizes with TCR signals to up-regulate the transcription factor GATA3 (Zheng and Flavell, 1997). GATA3 induces its own expression while it inhibits Th1 development by repressing IFN-γ and STAT4 expression, and potentiates transcription of *Il4, Il5* and *Il13* by various mechanisms, including epigenetic remodeling of the Th2 cytokine gene cluster (Ansel et al., 2006; Ho et al., 2009). Th17 development is initiated by transforming growth factor (TGF)-β and IL-6, and is inhibited by the Th1 cytokine IFN-γ and the Th2 cytokine IL-4 (Harrington

et al., 2005; Park et al., 2005). IL-21, produced by developing Th17 cells in response to IL-6-induced STAT3 signaling, serves as an autocrine factor for Th17 development by further activating STAT3 and inducing the lineage-specific transcription factors, ROR γ t and RORa, which induce *Il23r* expression and confer responsiveness to IL-23 (Ivanov et al., 2006; Korn et al., 2007; Nurieva et al., 2007; Yang et al., 2008; Zhou et al., 2007).

While lineage-specific transcription factor networks are important in specifying $CD4^+$ T cell differentiation, heritable and stable programs of gene expression are reinforced through epigenetic processes that include post-translational modifications of nucleosomal histones (eg, methylation, acetylation, phosphorylation, ubiquitylation), DNA methylation, and changes in higher-order chromatin structure (Ansel et al., 2006; Wilson et al., 2009). These epigenetic modifications regulate the accessibility of DNA elements to lineage-specific and basal transcription factors, and can be divided into permissive or repressive modifications that favor transcriptional activation or silencing of genes, respectively. Although the potential diversity of histone and DNA modifications are great, *cis*-regulatory elements of active or recently transcribed genes are typically characterized by the acetylation of histones H3 and H4 at various amino acid residues (H3Ac and H4Ac), and by methylation of H3 lysine residue 4 (H3K4) with one, two, or three methyl groups (H3K4me1, H3K4me2, and H3K4me3). Conversely, transcriptionally silent genes are characterized by methylation H3 lysine residue 27

(H3K7) with two or three methyl groups (H3K27me2 and H3K27me3), especially in facultative, but not constitutive, heterochromatin of tissue-specific gene loci (Wang et al., 2008; Wilson et al., 2009).

Th1, Th2, and Th17 cells show distinct epigenetic modifications at lineage-specific cytokine gene loci that are associated with the rapid and efficient production of effector cytokines in recall responses. At the *Ifng* locus, naïve $CD4^+$ T cells acquire permissive H3K4me2, H3Ac, and H4Ac modifications at the promoter and distal regulatory elements when they differentiate into Th1 cells, whereas Th2 and Th17 cells lack these permissive modifications, having instead, increased repressive H3K27me3 modifications (Akimzhanov et al., 2007; Chang and Aune, 2005; Hatton et al., 2006; Schoenborn et al., 2007; Wei et al., 2009). At the *Il17a/f* locus, where the *Il17a* and *Il17f* genes are clustered on opposite DNA strands, Th17 cells show permissive H3K4 trimethylation (H3K4me3), but no repressive H3K27me3 modifications at the promoters of both genes, whereas Th1 and Th2 cells show the opposite pattern (Wei et al., 2009). Th17 cells are also reported to have increased permissive H3 acetylation at the *Il17a and Il17f* promoters and several conserved non-coding sequences (CNSs) in the *Il17a/f* locus in comparison to Th1 and Th2 cells (Akimzhanov et al., 2007).

The epigenetic modifications at the *Il17a/f* and *Ifng* loci of Th17 cells described to date are consistent with their potential to produce high amounts of IL-17A and IL-17F,

but limited IFN-γ upon restimulation (Wei et al., 2009). Nevertheless, recent reports from our group and others indicate that there is substantial late developmental plasticity of Th17 cells (Lee et al., 2009; Lexberg et al., 2008). Thus, restimulation of in vitro-polarized Th17 cells by IL-12 induced rapid transition to a Th1-like phenotype marked by greatly enhanced production of IFN-γ and extinction of IL-17A and IL-17F (Lee et al., 2009; Lexberg et al., 2008). Similarly, a conversion of Th17-polarized cells to a Th1-like phenotype was observed in vivo in a transfer model of colitis (Lee et al., 2009), an antigen-specific ocular inflammation model (Shi et al., 2008), and transfer models of type I diabetes (Bending et al., 2009; Martin-Orozco et al., 2009). Although the mechanistic basis for the developmental plasticity of Th17 cells is incompletely understood, these findings suggest that the epigenetic modifications observed at the *Il17a/f* and *Ifng* loci might be particularly unstable.

Here we have performed comparative long-range DNase I hypersensitivity (HS) and histone modification analyses of the *Il17a/f* and *Ifng* loci in naïve, Th1 and Th17 cells, and in Th17 cells restimulated with TGF-b to maintain their phenotype or restimulated with IL-12 to deviate them to a Th1-like phenotype (Lee et al., 2009). Our findings reveal heretofore underappreciated remodeling of the *Ifng* locus in Th17 cells, and substantial reversibility of the chromatin structure of the *Il17a/f* locus in Th17 cells that appears linked to loss of RORgt expression downstream of IL-12-induced, STAT4- and

T-bet-mediated silencing of the *Rorc* gene. These findings provide a basis for the phenotypic plasticity of the Th17 lineage as well as the resistance of conventional and Th17-derived Th1-like cells to induction of *Il17a* and *Il17f* expression.

RESULTS

Identification of *cis*-regulatory elements in the *Ifng* and *Il17a/f* gene loci

Naïve CD4 T cells cells differentiated under Th17-polarizing conditions express low levels of the IL-12 receptor component, IL-12Rb2, and transition to Th1-like cells following restimulation in the presence of IL-12 and absence of TGF-b (Lee et al., 2009; Lexberg et al., 2008). IL-12 stimulation of polarized Th17 cells rapidly up-regulates *Ifng* expression, with a concomitant down-regulation of *Il17a* and *Il17f* expression. Although this transition is STAT4– and T-bet–dependent (Lee et al., 2009), details of the mechanism by which the rapid, IL-12-induced transition in cytokine expression phenotype might occur are undefined. To address this, we first identified potential regulatory elements at the *Ifng* and *Il17a/f* loci by long-range mapping of DNase I hypersensitivity mapping of naïve, Th1 and Th17 cells as a basis for delineating key *cis*-regulatory sites that might be targets of epigenetic modifications in the context of this phenotype shift (Boyle et al., 2008) (**Figure 1**).

For the *Ifng* locus, we analyzed an ~140 kb region flanking the *Ifng* gene and bordered by CTCF consensus sequences thought to represent insulator elements (Hadjur et al., 2009; Wallace and Felsenfeld, 2007; Wilson et al., 2009). DNase I hypersensitivity (HS) sites in activated Th1 cells co-localized well with conserved noncoding sequences (CNSs; ref. (Dermitzakis et al., 2005)) and the promoter (**Figure 1A**). Many HS sites

were shared with resting Th1 cells, while those at CNS-54, CNS-6, and CNS+54 were cell activation–dependent. Notably, naïve and Th17 cells, which don't express substantial amounts IFN-γ, also demonstrated some DNase I hypersensitivity sites shared with Th1 cells at the *Ifng* locus. In particular, activated Th17 cells showed a HS site pattern remarkably similar to activated Th1 cells, with the notable exceptions of CNSs -6 and +46, which were not hypersensitive in Th17 cells. The *Ifng* promoter of resting Th17 cells was DNase I–resistant, in accord with recently reported repressive histone modifications at this site (Wei et al., 2009). However, the promoter demonstrated substantial hypersensitivity following activation of Th17 cells. This suggests that the chromatin structure at the *Ifng* locus of Th17 cells is in a highly "poised" state for *Ifng* transcription, despite the limited production of IFN-g by polarized Th17 cells. Based on these data, and unpublished studies that have examined transcription factor binding to *cis* elements in the *Ifng* locus (Balasubramani et al., manuscript in preparation), we chose to analyze eight CNSs and the promoter shown in **Figure 1A** in subsequent analyses.

The *Il17a* and *Il17f* genes are closely linked on opposite strands of mouse chromosome 1. Based on a DNase I scan of ~2.5 megabases of DNA flanking these genes in the indicated T cell subsets, we opted to focus on an ~185 kb of DNA region flanked by the *Pkhd1* and *Mcm3* genes for further study due to the presence of sites unique to Th17 cells in this region (**Figure 1B**). Prominent DNase I HS peaks were

primarily limited to Th17 cells, consistent with their unique capacity to produce IL-17A and IL-17F. Several sites (eg, HS sites -5 and +23 kb relative to the predicted transcription start site of the *Il17a* gene) were only detected following activation of Th17 cells. With few exceptions, HS sites correlated well with CNSs. In striking contrast to the *Ifng* locus in Th17 cells, the *Il17a/f* locus in Th1 cells was essentially devoid of DNase I HS sites, consistent with the resistance of Th1 cells to express IL-17A or IL-17F even under Th17 re-polarizing conditions (Lee et al., 2009). Based on these DNase I HS data, we chose 12 sites, including the *Il17a* and *Il17f* promoters, for further analysis.

Rapid epigenetic remodeling of the *Ifng* locus in Th17 cells stimulated with IL-12

To determine whether the plasticity in cytokine expression phenotype that accompanies the divergence of Th17 cell precursors is reflected in corresponding epigenetic changes, we analyzed histone modifications across the extended *Ifng* and *Il17a/f* loci. Specifically, H3K4 methylation (H3K4me) and H3K27me3 were examined by chromatin immunoprecipitation (ChIP) as marks of permissive and repressive chromatin modifications, respectively, at the key *cis*-elements identified above (Wei et al., 2009). Th1 and Th17 cells were derived from naïve $CD4^+$ T cells under polarizing cytokines conditions, and Th17 cells were further stimulated with the antigen in the presence of Th1 (IL-12)– or Th17 (TGF-b)–polarizing conditions for an additional 6 d. Consistent

with our previous study (Lee et al., 2009), the frequency of IL-17A-expressing cells was sustained and increased in cultures of Th17 cells maintained in the presence of TGF-β (**Figure 2**, Th17/TGF-β). In contrast, a large majority of Th17 cells restimulated with IL-12 (**Figure 2**, Th17/IL-12) produced IFN-γ, and IL-17A expression was markedly decreased by IL-12 stimulation.

Concordant with their capacity for high *Ifng* expression, Th1 cells showed substantially increased permissive H3K4me modifications across the *Ifng* locus compared to naïve $CD4^+$ T cells, with the notable exceptions of CNSs -34 and -22, where no significant changes in permissive H3K4me marks were evident above the substantial permissive marks already present at those sites in naïve T cells (**Figure 3A and supplemental Figure S1A**) (Hatton et al., 2006; Schoenborn et al., 2007). Th17 cells showed less, but appreciable, levels of permissive H3K4me modification compared to Th1 cells at several distal CNSs of the *Ifng* locus, but the levels at CNS-6 and the *Ifng* promoter were negligible in Th17 cells, consistent with the DNase I HS data (**Figure 1A**). Remarkably, Th17/IL-12 cells demonstrated up-regulated permissive modifications across the locus comparably to Th1 cells, concordant with their high *Ifng* expression upon restimulation. Th17/TGF-β cells, whose frequency of IFN-g^+ cells (22%) was much lower than Th1 (86%) or Th17/IL-12 (87%) cells, but appreciably higher than Th17 cells (3%) (**Figure 2B**), showed higher H3K4me levels than Th17 in most of the sites at the

Ifng locus. However, Th17/TGF-β cells had very low levels of H3K4me at CNS-6, which was comparable to that of Th17 precursors and much lower than that of Th1 or Th17/IL-12 cells. Th17 and Th17/TGF-β cells showed slightly more repressive histone modification than Th1 or Th17/IL-12 cells, although the difference among subsets was less pronounced than the differential H3K4me changes. The IL-12-induced changes in histone methylation were reflected in long-range DNase I hypersensitivity of the *Ifng* locus in Th17/IL-12 and Th17/TGF-β cells (**Figure S2**). Thus, Th17/IL-12 cells demonstrated more prominent DNase I hypersensitivity peaks at several CNSs in the *Ifng* locus compared to Th17 precursors or control Th17/TGF-β cells, most notably at the *Ifng* promoter, which, at rest, was DNaseI hypersensistive only in the IL-12-transtitioned Th17 progeny, analogous to conventional Th1 cells (**Figure 1**).

Because in the foregoing experiments an appreciable fraction of T cells generated under Th17-polarizing conditions did not express either IL-17A or IFN-g, we repeated the analysis using *Il17f*$^{Thy1.1/Thy1.1}$ mice to identify and isolate committed Th17 cells as a starting population for divergence of late development and assessment of epigenetic changes (Lee et al., 2009). The results were comparable to those when OTII mice-derived CD4$^+$ T cells were used to polarize Th17 cells (**Figure S1B**, supplementary data). Thus, pure IL-17F$^+$ cells showed similar permissive H3K4me modification at key CNSs of the *Ifng* locus, but not at CNS-6 or the promoter as compared with naïve CD4$^+$ T cells

(**Figure 3A**). When IL-17F$^+$ cells were restimulated with IL-12, permissive H3K4me modification increased across the *Ifng* locus but most notably at CNS-6 and the promoter (6.5 and 5.9 fold increase, respectively). Concordant with an increase in permissive H3K4me modification, repressive H3K27me3 modification decreased across the *Ifng* locus following IL-12 stimulation of IL-17F$^+$ cells. When IL-17F$^+$ cells were restimulated in the presence of TGF-β, permissive H3K4me modification slightly increased across the locus, consistent with modest increases in IFN-g production by this population (data not shown), but it remained at low levels at CNS-6. Together with the DNaseI HS data, these data indicate that the chromatin organization of the *Ifng* locus in Th17 precursor cells shares remarkable similarity to conventional Th1 cells, and is highly poised for transition to high-level transcription of *Ifng* expression following IL-12-induced signaling. The DNase I hypersensitivity and histone modification data also collectively suggest that the *Ifng* locus of Th17 cells has a highly poised chromatin structure but lacks epigenetic remodeling at some critical distal regulatory elements – particularly the proximal promoter and CNS-6. Based on these results, we mainly focused on permissive H3K4me modification in CNS-6 and the promoter for further analyses.

To define the temporal relationship between induction of enhanced *Ifng* expression and epigenetic remodeling at the *Ifng* locus, we next compared kinetics of IFN-g

production and that of histone modifications at the *Ifng* locus of Th17 cells following IL-12 stimulation. Permissive H3K4me modifications at the *Ifng* locus increased by more than two-fold at CNS-6 and the promoter as early as 24 h after IL-12 stimulation, and further increased at the promoter at 48 h (**Figure 3C**). The increase in H3K4 methylation correlated well with the kinetics of increased IFN-γ production (**Figure 3B**). In contrast, changes in repressive H3K27 methylation were modest at these sites (**Figure 3A**). Analysis of H4 acetylation (H4Ac), another permissive histone modification, showed similar kinetics to that of the observed H3K4me changes (data not shown). Correlation of the kinetics of histone modifications at key *cis*-elements *Ifng* locus with markedly increased IFN-γ production suggests that rapid chromatin remodeling underlies this transition in Th17 cells following IL-12 stimulation.

Epigenetic remodeling of *Ifng* locus in transitioning Th17 cells is STAT4- and T-bet-dependent

STAT4 and T-bet are important transcription factors in the IL-12-dependent development of Th1 cells (Murphy and Reiner, 2002; Thieu et al., 2008; Usui et al., 2003; Wilson et al., 2009). We previously showed that in the IL-12-induced transition of Th17 cells to Th1-like cells, induction of IFN-γ required both STAT4 and T-bet, while the extinction of IL-17 was largely dependent on STAT4 but only partially on T-bet (Lee et al., 2009).

We therefore examined whether STAT4 directly binds to the *Ifng* locus. As shown in **Figure 4A**, ChIP analysis demonstrated prominent binding of STAT4 to CNS-34, CNS-22, and CNS+46 at 24 h, consistent with findings in conventional Th1 cells (Balasubramani et al., manuscript in preparation). STAT4 binding to these sites was also observed when pure Th17 precursors derived from *Il17f* $^{Thy1.1/Thy1.1}$ mice were restimulated for 24 h in the presence of IL-12, but not in the presence of TGF-β (**Figure S3**). There was no detectable STAT4 binding to sites in the *Ifng* locus in Th17/IL-12 cells derived from *Stat4*$^{-/-}$ mice (data not shown). STAT4 binding was negligible at 6 h and reached a peak at 24 h (Figure 4b) and remained detectable only at CNS+46 on Day 6. These results suggest that STAT4 directly induces IFN-γ by binding to the *Ifng* locus downstream of IL-12 signaling. The kinetics of STAT4 binding further suggests that STAT4 might induce histone modifications in the *Ifng* locus following IL-12 stimulation of Th17 cells.

To determine whether STAT4 is required for the epigenetic changes observed following IL-12 stimulation, the H3K4me status of the *Ifng* promoter and CNS-6 was analyzed in Th17 cells derived from wild-type (WT) and *Stat4*$^{-/-}$ mice, before and after IL-12 stimulation. Since T-bet has been shown to direct chromatin remodeling at the *Ifng* locus in developing Th1 cells (Mullen et al., 2001; Wilson et al., 2009), we also analyzed cells derived from T-bet-deficient (*Tbx21*$^{-/-}$) mice. Consistent with previous studies

(Harrington et al., 2005; Lee et al., 2009), Th17 cells derived from WT, *Stat4*$^{-/-}$, and *Tbx21*$^{-/-}$ mice showed comparable frequencies of IL-17A^{+} cells, and induction of IFN-g in Th17 cells following restimulation with IL-12 was strongly inhibited in cells derived from *Stat4*$^{-/-}$ and *Tbx21*$^{-/-}$ mice compared to WT controls (**Figure S4**). ChIP analyses of these cells revealed that permissive H3K4me modifications were markedly increased at CNS-6 in WT cells following IL-12 stimulation (**Figure 4C**), consistent with previous results (**Figures 3A** and **S1**). In contrast, the induction of H3K4 methylation was significantly inhibited in cells derived from *Stat4*$^{-/-}$ and *Tbx21*$^{-/-}$ mice. Induction of H3K4me was similarly STAT4- and T-bet-dependent at the *Ifng* promoter following IL-12 stimulation. In contrast, the modest increases in H3K4me at CNS+17-19 induced by IL-12 signaling were similar in Th17 cells derived from WT, *Stat4*$^{-/-}$ and *Tbx21*$^{-/-}$ mice. Collectively, these data indicate that STAT4 and T-bet play a critical role in the epigenetic remodeling of key *cis*-elements in the *Ifng* locus, and further highlight the importance of epigenetic changes at CNS-6 and the promoter in controlling high-level expression of *Ifng* in Th17 cells deviated by IL-12 stimulation.

IL-12-induced down-regulation of *Il17a* and *Il17f* genes in Th17 cells is accompanied by extensive epigenetic remodeling

In concert with up-regulated of expression IFN-g, IL-12-stimulated Th17 precursors down-regulated *Il17a* and *Il17f* expression. We therefore examined whether the chromatin structure of the *Il17a/f* locus was stable or not during the transition. Naïve, Th1, Th17, Th17/IL-12, Th17/TGF-β cells were prepared and permissive H3K4me and repressive H3K27me3 modifications of the *Il17a/f* locus were analyzed. In comparison with naïve $CD4^+$ T cells, Th17 cells demonstrated markedly increased permissive H3K4me modifications at HS sites across the *Il17a/f* locus, while repressive H3K27me3 modifications were minimally altered (**Figures 5A** and **S5A**). Th1 cells, in contrast, showed negligible induction of permissive H3K4me modifications, whereas repressive H3K27me3 modifications were substantially increased. Thus, Th1 and Th17 cells show differential repressive and permissive histone modifications across the extended the *Il17a/f* locus, respectively, reflecting their differential production of IL-17A and IL-17F.

Remarkably, IL-12-treated Th17 cells (Th17/IL-12), which rapidly down-regulated IL-17A andIL-17F expression, demonstrated substantial increases in repressive H3K27me3 modifications at most hypersensitive sites across the *Il17a/f* locus. At several sites, repressive marks surpassed those found in conventional Th1 cells. Although, H3K4 methylation of these sites appeared relatively unchanged in Th17/IL-12 cells derived Th17 precursors of normal mice (**Figure 5A**), this might to be due to contamination of this population with cells derived from uncommitted precursors, as use of pure Th17 cells

derived from *Il17f* $^{Thy1.1/Thy1.1}$ mice as precursors demonstrated significant decreases in permissive histone marks at many of these sites (**Figure S5**), particularly at the *Il17a* and *Il17f* promoters and their most proximal upstream elements (HS sites *Il17a*-5 and *Il17f*-7). Notably, Th17 precursors restimulated in the presence of TGF-β and absence of IL-12 (Th17/ TGF-β cells) demonstrated increased levels of permissive H3K4 methylation of nearly all sites examined, consistent with the higher frequency of IL-17A+ and IL-17F^{+} cells of this population relative to Th17 precursors (Lee et al., 2009). While this was accompanied by increased repressive marks at some sites in Th17 cells derived from normal mice (**Figure 5A**), again, this appeared to be due to use of incompletely committed precursors; Th17 precursors derived from *Il17f* $^{Thy1.1/Thy1.1}$ mice demonstrated no increases in the repressive histone methylation at these sites (**Figure S5B**). Similarly, the repressive H3K27me3 marks found in IL-12-stimulated progeny of Th17 precursors from IL-17F reporter mice were substantially increased across the *Il17a/f* locus compared to those observed in Th17/IL-12 cells derived from non-enriched Th17 precursors. Collectively, these data establish that chromatin structure is remodeled over a considerable distance flanking the *Il17a/f* locus in developing Th17 cells, particularly upstream of the *Il17a* gene, but is remarkably unstable and undergoes rapid and extensive remodeling contingent upon the dominant cytokines present under restimulation conditions.

In accordance with the observed transitions in histone methylations, DNase I HS analyses of the *Il17a/f* locus of Th17/IL-12 and Th17/TGF-β cells demonstrated substantial changes compared to Th17 precursors. Most notably, hypersensitivity sites at the promoters of the *Il17a and Il17f* genes were reversed in Th17/IL-12 cells, as were key intergenic HS sites at CNS *Il17a*+10 and *Il17a*+23, the latter of which were markedly amplified in Th17/TGF-b cells that expressed greater IL-17A and IL-17F (**Figure S6**). Thus, the rapid modifications of repressive and permissive histone methylations that were associated with the suppression of *Il17a* and *Il17f* expression following IL-12 signaling or enhanced expression of both genes upon continued culture in TGF-b, were reflected in extensive chromatin remodeling that resulted in the reversal or enhancement, respectively, of DNase I HS sites across the locus.

To examine the temporal relationship between down-regulation of IL-17A and the observed histone methylation changes, we compared their kinetics (**Figure 5B**, **C**). Because IL-12-induced changes in histone modifications in Th17 cells were observed at multiple sites across the *Il17a/f* locus, we focused our analysis on permissive (H3K4me) and repressive (H3K27me3) modification at the *Il17a* and *Il17f* promoters and their nearest upstream *cis*-elements, where prominent changes in both permissive and repressive histone methylations distinguished IL-12 and TGF-b treated Th17 precursors. As shown in **Figure 5C**, repressive H3K27 trimethylation at the *Il17a* promoter (*Il17a*P)

and HS *Il17a*-5 were not significantly increased by 48 h, whereas IL-17A expression was decreased by more than half (**Figure 5B**). In contrast, H3K27me3 at the *Il17f* promoter (*Il17f*P) and HS *Il17f*-7 increased by 3-fold or more at 48 h. Permissive H3K4me modifications were also limited during the first 48 h of IL-12 stimulation, indicating an absence of absolute correlation between the histone modifications at these sites and the observed decreases in cytokine expression, and suggesting that other mechanisms of suppression of *Il17a* (and *Il17f*, data not shown) were contributory.

STAT4 and T-bet play a role in epigenetic remodeling at the *Il17a/f* locus

We reported previously that STAT4 was essential for extinction of the expression of IL-17A and IL-17F by Th17 cells restimulated in the presence of IL-12 (Lee et al., 2009). To determine whether the STAT4-dependent extinction of *Il17a* and *Il17f* resulted from direct effects of STAT4 on the *Il17a/f* locus, ChIP analysis for STAT4 binding to the extended locus was performed on Th17 cells restimulated in the presence of IL-12 or TGF-β (**Figure S7**). STAT4 binding was detected at CNSs *Il17a*+10 and *Il17a*+28 at the peak of STAT4 DNA binding (24 h; **Figure 4**, and data not shown), but was undetectable at other sites and was substantially and consistently weaker than that measured in the *Ifng* locus (**Figure 4A**). STAT4 binding was not detected in Th17/TGF-β cells **(Figure S7)** or Th17/IL-12 cells derived from *Stat4*$^{-/-}$ mice (data not shown).

In accordance with the finding that deficiencies of both STAT4 and T-bet were associated with impaired silencing of the *Il17a/f* locus, we found that in comparison to WT Th17 cells, in which increased repressive H3K27me3 marks were significantly induced by IL-12 at d 6 at each of the four sites examined (**Fig. 5C,** and data not shown), these modifications were reduced in Th17 cells from *Stat4*$^{-/-}$ and *Tbx21*$^{-/-}$ mice, consistent with a role for both STAT4 and T-bet in optimal acquisition of repressive H3K27me3 modifications of the *Il17a/f* locus in Th17/IL-12 cells.

IL-12-induced extinction of the *Il17a/f* locus in Th17 cells is associated with reciprocal up- and down-regulation of T-bet and RORgt, respectively

The relatively limited binding of STAT4 to the *Il17a/f* locus found in Th17/IL-12 cells in the foregoing experiments, suggested that STAT4 might silence the *Il17a/f* locus by an indirect mechanism. The down-regulation of several key Th17 lineage transcription factors in Th17/IL-12 cells observed previously (Lee et al., 2009), suggested that IL-12-induced STAT4 activation might induce repressive chromatin changes at the *Il17a/f* locus through suppression of Th17 lineage-associated transcription factors. We analyzed the kinetics of mRNA expression of several Th17 lineage-associated transcription factors, as well as the Th1 lineage-associated transcription factor T-bet, that followed IL-12-mediated deviation of Th17 precursors (**Figure 6**). Committed Th17 cells derived from

Il17f$^{Thy1.1/Thy1.1}$ mice were restimulated in the presence of IL-12 or TGF-β and analyzed at various time points post-stimulation for mRNA expression the indicated transcription factors. Compared to Th17 cells restimulated with TGF-b, which retained high levels of transcripts for each of the Th17-lineage factors, expression of *Rora* and *Ahr* transcripts were markedly reduced by 24 h following IL-12 stimulation, and remained low over the 5 d period of analysis. While IL-12 also induced a substantial decline in *Rorc* (RORγt) transcript by 24 h, it continued to decline with time, approaching background levels by day 5 (**Figure 6A**). Notably, no decrement in the expression of *Irf4* transcripts was observed, remaining equivalent to the TGF-b-stimulated control population throughout. In contrast, the *Tbx21* transcript, which remained at background levels in Th17/TGF-β cells, was rapidly and progressively increased in Th17/IL-12 cells, demonstrating a reciprocal pattern of expression to that of *Rorc*.

Given the requirement for STAT4 and, to a lesser extent, T-bet, for IL-12-induced suppression of *Il17a and Il17f* expression (**Figure S4,** and (Lee et al., 2009)), we examined whether the IL-12-driven down-regulation of Th17-related transcription factors is STAT4- or T-bet-dependent. Th17 cells derived from WT, *Stat4*$^{-/-}$, and *Tbx21*$^{-/-}$ mice were restimulated in the presence of IL-12 for 6 d and analyzed for expression of *Rorc*, *Rora* and *Ahr* transcripts. Remarkably, while the IL-12-driven decrease in *Rorc* transcripts was largely prevented in the absence of STAT4 or T-bet, transcripts of *Rora*

and *Ahr* were not, such that Th17 cells derived from WT, STAT4-deficient, and T-bet-deficient cells demonstrated comparable decreases in *Rora* and *Ahr* transcripts (**Figure 7B**). These data indicate STAT4 and T-bet play an important role in IL-12-induced down-regulation of ROR γ t in Th17 cells, whereas ROR α and Ahr are suppressed by a mechanism that is STAT4- and T-bet-independent.

Extinction of the *Il17a/f* locus is linked to STAT4- and T-bet-induced epigenetic repression of the *Rorc* gene

The correlation of STAT4- and T-bet-dependent extinction of the *Il17a/f* locus with suppression of *Rorc* expression suggested that ROR γ t plays a principal role in maintaining *Il17a* and *Il17f* expression in committed Th17 cells, such that down-regulation of ROR γ t by IL-12-induced signaling might initiate remodeling of the locus with attendant extinction of *Il17a* and *Il17f*. To test this possibility, the effect of ectopic ROR γ t expression on the IL-12-induced transition of Th17 cells to Th1-like cells was evaluated. $CD4^+$ T cells from $Il17f^{Thy1./Thy1.1}$ mice were polarized under Th17 conditions and were transduced during their differentiation with a retrovirus that directed expression of ROR γ t (ROR γ t-GFP) or a control retrovirus (GFP). Committed ($Thy1.1^+$/$IL\text{-}17F^+$) Th17 cells were then isolated by magnetic sorting and restimulated in the presence of IL-12 or TGF- β (**Figure 7A**).

IL-12 stimulation of Thy1.1^{+} Th17 precursors transduced with the control retrovirus resulted in induction of IFN-γ, and extinction of IL-17A (**Figure 7A**) and IL-17F (Thy1.1) (**Figure S8**), consistent with previous results (**Figure 2A**) (Lee et al., 2009). In contrast, retrovirally expressed RORγt, which was resistant to IL-12-mediated suppression, completely inhibited extinction of IL-17A expression, whereas extinction of IL-17F expression was partially inhibited (**Figure S8**). TGF-β restimulation Th17 precursors transduced with either the control or RORgt-expressing retrovirus retained comparable, high expression of IL-17A and IL-17F (Thy1.1) (**Figure S8,** and data not shown). Surprisingly, retroviral expression of RORγt nearly completely inhibited IL-12-mediated IFN-γ induction in Th17 cells (**Figure 7A**).

The primary role for RORγt in maintaining *Il17a* expression that was evident from the foregoing results prompted us to examine potential IL-12-induced epigenetic remodeling at the *Rorc* locus in Th17 cells. It was previously demonstrated that Th17 cells have permissive histone methylation marks (H3K4me3) around the RORγt isoform-specific exon of the *Rorc* locus, whereas Th1 cells have repressive marks (H3K27me3) across the coding region and the immediate upstream non-coding region of the *Rorc* locus (Wei et al., 2009). We therefore examined permissive and repressive histone modifications near the RORγt isoform-specific exon of the *Rorc* locus (*Rorc*+5.0) and at a upstream site near the second exon of the *Rorc* gene (*Rorc*+2.7),

before and after the IL-12-induced transition of Th17 cells to Th1-like cells. Th17 cells derived from *Il17f*$^{Thy1.1/Thy1.1}$ mice were restimulated with IL-12 or TGF-β as before, and were recovered 6 d later for histone methylation analyses. As shown in **Figure 7B**, IL-12 stimulation of Th17 cells specifically induced a decrease in H3K4me both at *Rorc*+2.7 and *Rorc*+5.0, while Th17 cells restimulated with TGF-β retained H3K4 methylation at both sites. Concordant with decreased permissive modifications, IL-12 induced reciprocal increases in H3K27 trimethylation at both sites, more prominently at the site near the RORγt isoform-specific exon (*Rorc*+5.0), which has been implicated as a RORγt-specific promoter (Eberl and Littman, 2003). In contrast, neither permissive nor repressive modifications at these sites were significantly altered in Th17/TGF-β cells, suggesting that IL-12 signaling suppresses *Rorc* expression in Th17 cells at least in part through repressive epigenetic remodeling of the *Rorc* locus.

We next determined whether IL-12-induced epigenetic remodeling at the *Rorc* locus in Th17 cells was dependent on STAT4 or T-bet, focusing on H3K27me3 changes at *Rorc*+5.0 where the greatest disparity between Th17 and Th17/IL-12 cells was observed (**Figure 7B**). Th17 cells derived from WT, *Stat4*$^{-/-}$, and *Tbx21*$^{-/-}$ mice were restimulated with IL-12, and ChIP assays were performed to assess the status of repressive H3K27me3 marks at *Rorc*+5.0 before and after the IL-12-induced phenotype transition (**Figure 7C**). The induction of repressive H3K27 trimethylation at this site was almost completely

inhibited in STAT4- or T-bet-deficient cells, consistent with STAT4- and T-bet-dependent down-regulation of *Rorc* transcripts (**Figure 6B**). Thus, IL-12-induced STAT4 and T-bet play a critical role in reversing permissive epigenetic modifications of the *Rorc* locus in Th17 cells, which is linked with the down-regulation of *Rorc* expression and extinction of gene expression in the *Il17a/f* locus.

DISCUSSION

Epigenetic modifications of key cytokine gene loci accompany T helper cell differentiation and have been considered to provide a basis for the heritability of gene expression patterns acquired by each T helper cell lineage (Ansel et al., 2003; Wilson et al., 2009). In this study, we find that the chromatin structure of the *Il17a/f*, *Ifng* and *Rorc* loci in Th17 cells is not stable, but changes rapidly in response to cell-extrinsic factors as a basis for the late developmental plasticity of Th17 cells. While lineage-committed Th17 cells maintained with TGF-b demonstrated enhanced epigenetic modifications across the *Il17a/f* locus in accordance with their enhanced production of IL-17A and IL-17F (Lee et al., 2009), the same precursors deviated towards highly induced IFN-γ expression and IL-17A and IL-17F extinction by IL-12 demonstrated marked reciprocal H3 methylation changes at CNS/HS elements across the *Il17a/f* locus that mirrored STAT4- and T-bet-dependent epigenetic remodeling of the *Rorc* locus and its loss of expression. This is consistent with a critical role for persistent RORgt expression in the maintenance of transcriptional competence of the *Il17a/f* gene cluster in Th17 cells. Thus, epigenetic modifications of key cytokine and transcription factor gene loci that accompany Th17 differentiation are subject to substantial, and rapid, reversibility, which corresponds to, and is likely the basis for, their phenotypic plasticity (Lee et al., 2009; Lexberg et al., 2008).

Although histone modifications of cytokine genes in Th17 cells have been reported previously (Akimzhanov et al., 2007; Wei et al., 2009), to our knowledge, this is the first report that describes extensive DNase I hypersensitivity mapping at the *Il17a/f* and *Ifng* loci of Th17 cells. A notable feature of the DNase I HS map of the *Ifng* locus of Th17 cells is its similarity to Th1 cells. Remarkably, activated Th17 cells especially demonstrated a DNase I hypersensitivity pattern quite similar to that of activated Th1 cells, despite their development in the absence of Th1-specifying cytokines (eg, IFN-g or IL-12), or expression of canonical Th1 transcription factor, T-bet. Resting Th17 cells had prominent hypersensitivity peaks at downstream CNSs and CNS-22, but lacked hypersensitivity at CNS-6 and the *Ifng* promoter. These DNase I hypersensitivity data are consistent with histone modification data, where Th17 cells (resting) showed little permissive H3K4me modification at CNS-6 and the *Ifng* promoter, while they showed increased H3K4me at several distal CNSs, especially downstream of the *Ifng* gene. Previous reports showed an absence of permissive H3Ac and H3K4me3 modifications, but substantial repressive H3K27me3 modifications within the promoter of the *Ifng* gene in Th17 cells (Akimzhanov et al., 2007; Wei et al., 2009). Our study confirms these findings at the *Ifng* promoter, but also finds that many of the distal *Ifng* regulatory elements, with the notable exception of CNS-6, have permissive histone modifications in addition to DNase I accessibility and become more favorable for transcription upon cell

activation. Accordingly, Th17 cells have extensive chromatin remodeling of the *Ifng* locus, consistent with a far more highly "poised" state than previously appreciated, and which occurs to a remarkable extent in the absence of STAT4 or T-bet signaling. This likely provides a basis for the rapid up-regulation of IFN-γ production by Th17 cells in response to IL-12, but also their capacity to express lower amounts of IFN-g in the absence IL-12 (or IL-23) and presence of TGF-b. Thus, while *Tbx21* was reported to have bivalent histone methylation marks at its promoter (Wei et al., 2009), and undergoes rapid IL-12-induced up-regulation that is important for enhancement of *Ifng* expression in transitioning Th17 cells (Lee et al., 2004), STAT4- and T-bet-independent mechanisms that are yet to be defined enable extensive remodeling of the *Ifng* locus in committed Th17 cells, and permit expression of *Ifng* in the absence of these factors. This is in stark contrast to virtual absence of DNase I HS sites or permissive histone modifications of the *Il17a/f* locus in Th1 cells, and is in accord with Th1 cells' resistance to the developmental plasticity that characterizes Th17 cells (Harrington et al., 2005; Lee et al., 2009).

The comparative findings of DNase I HS and histone modifications at CNS-6 in the *Ifng* locus point to a key role for this element in controlling high-level transcription of *Ifng*. Although activated Th17 cells demonstrated a global DNase I HS pattern highly similar to Th1 cells including accessibility at the promoter, CNS-6 was hypersensitive to DNase I only in Th1 cells. Furthermore, the balance of permissive and repressive H3

methylations at CNS-6 in Th1, Th17, Th17/IL-12, and Th17/TGF-β cells correlated well with their capability to produce IFN-γ upon activation, and enhanced permissive histone methylation at CNS-6 was totally abolished in IL-12-stimulated Th17 cells derived from mice deficient for STAT4 or T-bet, concordant with diminished *Ifng* induction. CNS-6 has been shown previously to function as an enhancer for *Ifng* expression (Lee et al., 2004; Schoenborn et al., 2007) and to be a site of T-bet binding in the *Ifng* locus (Hatton et al., 2006; Shnyreva et al., 2004). Further, CNS-6 contains consensus binding sites for NFAT and AP-1 and has been shown to bind NFAT-1 in Th1 cells following stimulation (Lee et al., 2004). Collectively, these findings suggest that IL-12-induced increases in permissive histone modifications of CNS-6 enable acute transcription factors activated by TCR signaling, such as NFAT or AP-1 (Lee et al., 2004), to efficiently bind CNS-6 in Th17 cells deviated to a Th1-like phenotype and induce high-level transcription of *Ifng*.

Rapid binding of STAT4 to several distal CNSs in the *Ifng* locus of Th17 cells following IL-12 stimulation occurred at pre-existent DNase I HS sites (eg, CNSs -34, -22 and +46), but appeared insufficient for high-level induction of IFN-g and epigenetic remodeling in the absence of T-bet. In view of the recently described role for T-bet in cooperating with the CCCTC-binding factor (CTCF) to induce conformational changes in the *Ifng* locus that juxtapose distal enhancers with the *Ifng* promoter (Sekimata et al., 2009), the function of STAT4 to enhance *Ifng* transcription following binding of distal

CNS elements could well be due to its ability to initially recruit T-bet to these sites, which in turn recruits distal *cis*-regulatory elements to the promoter in concert with CTCF-containing complexes. This is supported by the finding that ectopic T-bet expression in Th17 cells transduced with retrovirus encoding *Tbx21* was inefficient in the induction of *Ifng* compared to IL-12 signaling (Supplemental data, **Figure S10**), and is consistent with a model of *Ifng* locus regulation in which IL-12-induced STAT4 acts both to enhance *Tbx21* expression and target its interactions with distal *cis*-regulatory elements to enable juxtaposition of these elements to the *Ifng* proximal promoter in cooperation with CTCF-containing architectural complexes.

Long-range mapping of DNase I HS across over 2.5 megabases of sequence flanking the *Il17a and I17f genes* in Th17 cells defined the extent of lineage-specific accessibility at over a dozen sites contained within ~185 kb of the genome, including, but not limited to, elements previously identified as binding sites for the Th17-associated transcription factors Runx1 and RORgt (*Il17a* promoter and *Il17a*HS-5) (Zhang et al., 2008), STAT3 (*Il17a* promoter) (Chen et al., 2006) and BATF (*Il17a* and *Il17f* promoters, and intergenic sites CNS *Il17a*+10 and CNS *Il17a*+28) (Schraml et al., 2009). This is consistent with the histone modification data herein, which establishes permissive Th17 cell-specific modifications at most these sites and identifies novel candidates for *cis*-regulation of the *Il17a/f* locus, substantially extending previous studies that reported epigenetic signatures

limited to the gene-proximal regions (Akimzhanov et al., 2007; Wei et al., 2009). In contrast to the IL-12-induced extinction of *Il17a* and *Il17f* expression, which was characterized by rapid and extensive reciprocal increases in H3K27me3 and decreases in H3K4me across the *Il17a/f* locus, TGF-b-induced maintenance of *Il17a* and *Il17f* expression reflected enhanced H3K4me. Notably, the rapid inversion of H3 methylations in IL-12-stimulated Th17 cells resulted in a pattern of epigenetic modifications that closely resembled that of Th1 cells, and like the extensive H3K27 trimethylation of Il17a/f locus *cis*-elements in Th1 cells, was associated with irreversible silencing of the *Il17a/f* locus ((Lee et al., 2009), and unpublished observations). In essence, once the locus became epigenetically modified by a preponderance of repressive histone methylation commensurate with the loss of *Rorc* expression, permissive remodeling of the locus appeared to be difficult or impossible. H3K27me3 has been shown to recruit histone-binding proteins that contain chromodomains, including Polycomb group proteins that are components of multiprotein complexes implicated in gene silencing (Ansel et al., 2006; Kouzarides, 2007; Schuettengruber et al., 2007). The inability to induce *Il17a* or *Il17f* in Th1 and Th17/IL-12 cells might therefore be attributable, at least in part, to the transcriptionally unfavorable chromatin structure resulting from extensive recruitment of Polycomb group proteins to the *Il17a/f* locus that ensues in the absence of persistent RORgt expression.

The rapid kinetics of IL-12-induced down-regulation of *Rorc*, *Rora*, and *Ahr* transcripts, but not that of *Irf4*, implicated loss of the former three transcription factors in the extinction of the *Il17a/f locus*. However, only transcripts encoding ROR γ t were inhibited by STAT4 and T-bet in Th17/IL-12 cells, whereas transcripts encoding ROR α and Ahr were inhibited independently of STAT4 or T-bet. These findings, coupled with the requirement for STAT4 and T-bet for optimal extinction of the *Il17a/f* locus, suggest a dominant role for ROR γ t in maintenance of the *Il17a/f* locus in Th17 cells, despite the fact that each of the other Th17 lineage factors have been implicated in the initial development of Th17 cells from naïve precursors (Huber et al., 2008; Ivanov et al., 2006; Kimura et al., 2008; Quintana et al., 2008; Veldhoen et al., 2008; Yang et al., 2008). This is supported by the observation that constitutive ROR γ t expression strongly inhibited the IL-12-induced down-regulation of IL-17A. The finding that retention of *Il17a/f* locus competency was more dependent on STAT4 than on T-bet could reflect a role for direct STAT4 binding to two intergenic sites in locus silencing, although this appeared to be modest, if contributory at all. Interestingly, in other studies we have found that these same two intergenic sites bind STAT3 in Th17 cells, suggesting that STAT4 might bind weakly to the same *cis*-elements important for STAT3-mediated control of *Il17a* and *Il17f* expression (unpublished findings). Notably, however, sustained expression of ROR γ t by retroviral transduction only partially rescued suppression of IL-17F by IL-12

signaling, consistent with differential regulation of IL-17A and IL-17F expression and a greater role for RORγ in *Il17f* expression. Compared to the reductions in transcription factor expression and down-regulation of IL-17A production, epigenetic remodeling at the *Il17a/f* locus following IL-12 stimulation of Th17 cells was relatively delayed, especially in the vicinity of the *Il17a* promoter. This suggests that changes in chromatin structure might not be causally related to the rapid down-regulation of IL-17A expression, rather they might stabilize the repressed state through Polycomb group protein recruitment (Ringrose and Paro, 2004). The data from Th17 cells deficient in STAT4 or T-bet support this.

An important finding to emerge from this study concerns the rapid extinction of *Rorc* in committed Th17 cells by IL-12-induced STAT4 and T-bet. This was linked to reversal of permissive histone modifications of two lineage-specific DNase I HS sites that reside just upstream of the alternative transcription start site for the RORgt isoform in the second intron of the *Rorc* gene (*Rorc*+2.7 and *Rorc*+5.0), and correlates with loss of DNase I accessibility at both of these sites, as well as a cluster of two prominent HS sites in *Rorc* intron 4, following the IL-12-induced transition of Th17 cells to Th1-like cells. Interestingly, in addition to its contributions to the maintenance of *Il17a* and *Il17f* expression, retroviral expression of RORγt also blocked IL-12-mediated IFN-γ induction in Th17 cells, which was linked to its blockade of T-bet induction (**Figure 7**,

and data not shown). Given the requirement for both STAT4 and T-bet in reversing permissive histone modifications of the *Rorc* locus, this indicates an antagonism between the Th17- and Th1-specifying lineage factors, in which the STAT4 and T-bet appear dominant through their capacity to suppress *Rorc* expression. In this regard it is notable that the previously reported "bivalency" of H3 methylation of *Tbx21* gene in Th17 cells (Wei et al., 2009) is consistent with our finding of up-regulation of *Tbx21* in IL-12-stimulated Th17 cells, whereas the *Rorc* gene in Th17 cells lacked repressive histone marks in that study and our own. Thus, if histone methylation bivalency is associated with induction of expression of key transcription factors in the context of lineage transitions, it does not appear to be a prerequisite for termination of transcription factor expression associated with lineage transitions, and the chromatin structure in the *Rorc* locus appears to be particularly susceptible to epigenetic remodeling in Th17 cells.

In summary, our findings substantially extend the long-range mapping of the *Il17a/f* and *Ifng* gene loci in distinct T cell subsets. They further establish that the epigenetic modifications that underlie changes in cytokine and transcription factor gene expression early in Th17 lineage specification are particularly unstable, and provide a mechanism whereby the developmental plasticity that appears to be characteristic of the Th17 lineage is enabled by reversal and remodeling of chromatin structure in response to local environmental cues.

METHODS

Mice. The following mice were purchased from the Jackson Laboratories and/or bred at our facility: BALB/cByJ (BALB/c), C57BL/6J (B6), B6.OT-II TCR transgenic mice (OT-II), B6.129S6-*Tbx21*^{tm1Glm}/J (T-bet-deficient), and B6.129S1-*Il12b*$^{tm/Jm}$/J (IL-12p40-deficient). The generation of *Il17f*$^{Thy1.1/Thy1.1}$ reporter mice was described previously (Lee et al., 2009). B6.*Stat4*$^{-/-}$ mice used in some studies were a kind gift from Mark H. Kaplan (Indiana University School of Medicine). All animals were bred and maintained in accordance with Institutional Animal Care and Use Committee regulations.

CD4$^+$ T cell preparation and culture. CD4$^+$ T cells were purified from pooled spleen and lymph nodes by mouse CD4 Dynabeads followed by DETACHaBEAD mouse CD4, according to the manufacture's directions (Invitrogen) and used as CD4$^+$ T cells for DNase hypersensitivity mapping and chromatin immunoprecipitation. Unless otherwise indicated, CD4$^+$ T cells were cultured at a ratio of 1:5 with irradiated (3000 rads) splenic feeder cells for 6 d in RPMI containing 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 μM sodium pyruvate, 1x non-essential amino acids, 2.5 μM β-mercaptoethanol, 2 μM L-glutamine, 10 mM HEPES (R-10). OT-II TCR transgenic CD4$^+$ cells were activated with 5 μg/ml OVA peptide (OVAp), whereas non-transgenic

cells were stimulated with 2.5 μg/ml anti-CD3 (clone 145-2C11), supplemented with 2.5 or 5 ng/ml rhTGF-β1 (R&D Systems), 20 ng/ml rmIL-6 (R&D Systems), 10 μg/ml anti-IFN-g (clone XMG1.2) and 10 μg/ml anti-IL-4 (clone 11B11) to obtain Th17 cells. Th1 cells were generated as described previously (Hatton et al., 2006). Thy1.1^{+} cells of *Il17f*$^{Thy1./Thy1.1}$ reporter mice were isolated on d6 of Th17 cultures by magnetic sorting according to the manufacture's instruction (Miltenyi). In restimulation cultures, cells were recovered on d 6 of previous cultures and activated with fresh irradiated splenocytes and antibody mixtures as indicated and 10 ng/ml rmIL-12 (R&D Systems) or 5 ng/ml rhTGF-β1 (R&D Systems). For some experiments, cells were restimulated with plate-bound anti-CD3 (clone 145-2C11, 10 μg/ml) and 2.5 μg/ml soluble anti-CD28 (clone 37.51, eBioscience) or anti-CD3/CD28 coated beads (Invitrogen, according to the manufacturer's instructions) instead of using irradiated splenocytes.

Flow cytometric analyses. CD4^{+} T cells were collected and where indicated, stimulated with PMA (50 ng/ml; Sigma) and ionomycin (750 ng/ml; Calbiochem) for 4 h in the presence of Golgi Plug (BD). Intracellular staining was performed as previously described (Maynard et al., 2007). LIVE/DEAD Fixable Green or near IR Dead Cell Stain (Invitrogen) was used to exclude dead cells in flow cytometric analyses. Phycoerythrin (PE)-conjugated anti-CD90.1 (OX-7), anti-IL-17 (TC11-18H10) and peridinin

chlorophyll protein (PerCP)-conjugated anti-CD90.1 (OX-7) were purchased from BD Biosciences; allophycocyanin (APC)-conjugated anti-IFNγ (XMG1.2), anti-CD4 (GK1.5 and RM4-5) and PE-Cy7-conjugated anti-CD4 (GK1.5) were purchased from eBioscience. Samples were acquired on an LSRII instrument (BD Biosciences) and data was analyzed using FlowJo software (Tree Star Inc.).

DNase hypersensitivity mapping. DNase I hypersensitivity sites were mapped as described (Crawford et al., 2006; Shibata and Crawford, 2009). Briefly, nuclei from $CD4^+$ T cells were digested with DNase I, and DNase-digested ends were ligated to biotinylated linkers, followed by sonication to shear DNA into smaller fragments. Randomly sheared, non-DNase-digested genomic DNA was also ligated to biotinylated linkers and sonicated. After enrichment using streptavidin beads, DNase-digested and randomly sheared captured ends were amplified by PCR following ligation of a second set of linkers. DNase-digested and randomly sheared ends were labeled with Cy5 and Cy3 dyes and cohybridized to NimbleGen tiled microarrays. Regions of the genome with DNase I hypersensitivity were identified using the tiled array peak calling software ACME.

Chromatin Immunoprecipitation (ChIP). ChIP was performed as previously described (Hatton et al., 2006). Briefly, chromatin was prepared from $1x10^7$ cells for each reaction using the Chromatin Immunoprecipitation (ChIP) Assay Kit from Millipore according to the manufacturer's instructions. Immunoprecipitations were performed with either an anti-mono/di/trimethyl-Histone H3K4 antibody (H3K4me, Millipore 05-791), an anti-trimethyl-Histone H3K27 antibody (H3K27me3, Millipore 17-622), or an anti-STAT4 antibody (SantaCruz sc-486X). Immunoprecipitated DNA released from cross-linked proteins was quantitated by real-time PCR using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) or SYBR GreenER qPCR SuperMix (Invitrogen) on a BioRad iQ system. PCR primers and probes are included in the Supplemental Data. For H3K4me ChIP samples, PCR reactions for regions of interest (ROI) were normalized to the amplification of the 16S ribosomal protein promoter. The relative H3K4me values were calculated using the $\Delta\,\Delta$Ct method as follows: [naïve $CD4^+$ T (ROI Ct – 16S ribosomal promoter Ct)] – [each cell sample (ROI Ct - 16S ribosomal promoter Ct)] = n with 2^n representing the relative values. For H3K27me3 and STAT4 ChIP samples, PCR reactions for regions of interest were normalized to the amplification of diluted input DNA samples; and the relative values were expressed as % input.

Gene expression analyses. RNA was extracted from T cells using TRIZOL (Invitrogen) and then treated with DNA-free (Ambion). cDNA synthesis was performed using Superscript III first-strand synthesis system (Invitogen). Real-time PCR was performed on a Bio-Rad iCycler with primer pairs specific for cDNAs of each mRNA transcript using SYBR GreenER qPCR SuperMix (Invitrogen). Primer sequences for *Rora* were as described previously (Lee et al., 2009). Other primer sequences used were: Rorc (ROR γ t-isoform specific), sense primer, 5'-GGAGGACAGGGAGCCAAGTT-3', antisense primer, 5'-TTGTCCCCACAGATCTTGCA -3'; Ahr, sense primer, 5'-CAGCAGATGCCTTGGTCTTCT-3', antisense primer, 5'-ATACGCTCTGATGGATGACATCA-3'; Irf4, sense primer, 5'-CCACGACGAGCCCTGAAG-3', antisense primer, 5'-AGGACCTGGTCCAGGTTGCT-3' Tbx21, sense primer, 5'-ACCAGAGCGGCAAGTGGG-3', antisense primer, 5'-TGGACATATAAGCGGTTCCCTG-3'; 18S rRNA, sense primer, 5'-GCCGCTAGAGGTGAAATTCTTG-3', antisense primer, 5'-CATTCTTGGCAAATGCTTTCG-3'; β 2-microglobulin, sense primer, 5'-CCTGCAGAGTTAAGCATGCCAG-3', antisense primer, 5'-TGCTTGATCACATGTCTCGATCC-3'. Reactions were run in duplicate and normalized to 18S rRNA or β 2-microglobulin, which gave comparable results.

Retroviral transduction. The ROR γ t cDNA was PCR amplified and cloned into the bicistronic retroviral vector pMIB (Cotta et al., 2003; Thal et al., 2009), which contains IRES-regulated GFP and was kindly provided by Dr. Christopher A. Klug (University of Alabama at Birmingham). Phoenix cells were transfected with the plasmids encoding for either the control GFP or ROR γ t retroviruses by calcium-phosphate transfection and the supernatant was harvested after 48 h. For infection, $CD4^+$ T cells ($2x10^5$ cells/well in round-bottomed 96-well plates) were plated under Th17-polarizing conditions as described above using anti-CD3/CD28 coated beads on day 0. On day 1, the retrovirus supernatant containing 5 μ g/mL polybrene (Sigma) was added and the cells were spun at 2,500 rpm for 2 h at 30°C. After spin infection, the cells were cultured under Th17-polarizing conditions in the presence of 30 U/mL IL-2 and harvested on day 6 for intracellular cytokine staining and restimulation cultures.

Statistical analysis. Statistical significance was calculated by unpaired Student's *t* test. All p values ≤ 0.05 are considered significant, and are referred to as such in the text.

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FIGURE LEGENDS

Figure 1. Mapping of DNase I hypersensitivity sites in the *Ifng* and *Il17a/f* loci of naive, Th1 and Th17 CD4$^+$ T cells. CD4$^+$ T cells from OT-II TCR transgenic mice were isolated and cultured with irradiated splenic feeder cells from *Il12b*$^{-/-}$ (IL-12p40-deficient) mice and 5 μg/mL OVAp for 6 d under Th1- or Th17-polarizing conditions. Th17 cells were recovered and cultured with fresh irradiated splenocytes, cytokines and antibody mixtures for another 6 d. Cells were harvested without (rest) or with (stim) 4 h of PMA/ionomycin stimulation and subjected to DNase digestion and DNaseI-chip analysis. Top and bottom panels show results of the *Ifng* **(A)** and *Il17a/f* **(B)** loci, respectively, aligned with a corresponding VISTA plot (Frazer et al., 2004), where mouse sequence is shown on the x axis and percentage similarity to human on the y axis.

Figure 2. IL-12 and TGF-β induce differential cytokine expression phenotype in Th17 precursors. (A) CD4$^+$ T cells from OT-II TCR transgenic mice were cultured with irradiated *Il12b*$^{-/-}$ (IL-12p40-deficient) splenic feeder cells and 5 μg/mL OVAp for 7 d under Th17-polarizing conditions. Cells were harvested and restimulated with irradiated splenic feeder cells in the presence of TGF-β or IL-12, anti-IL-4 and OVAp for an additional 6 d. Th1 cell controls were obtained by culturing CD4$^+$ T cells from OT-II

TCR transgenic mice with irradiated B6 splenic feeder cells and 5 μg/mL OVAp for 5 d under Th1-polarizing conditions. Cells were stained intracellularly for IL-17A and IFN-γ after PMA/ionomycin activation. Data in the quadrants are the frequencies of $CD4^+$ cells. **(B)** Cumulative data for frequencies of cytokine-positive $CD4^+$ T cells analyzed as **(A)**. Th1 and Th17 cells were harvested on d 5 for ChIP analysis. For Th17/IL-12 and Th17/TGF-β cells, Th17 cells were harvested on d 6 or 7 of the first polarization and restimulated in the presence of IL-12 or TGF-β for another 6 d. Data represent the averages and SD of three separate cell samples that were used for ChIP analysis in Figures 3A and 5A.

Figure 3. Th17 cells undergo rapid epigenetic remodeling and acquire Th1-like histone modifications across the *Ifng* locus following restimulation with IL-12. (A) Th1, Th17, Th17/IL-12 and Th17/TGF-β cells were derived from $CD4^+$ T cells of OT-II TCR transgenic mice as in Figure 2. The cells were then processed for ChIP analysis using antibodies specific for permissive H3K4me or repressive H3K27me3 histone modifications. Data for H3K4me and H3K27me3 were normalized against the value for naïve $CD4^+$ T cells and input DNA, respectively. Data represent the averages and SD of three separate cell samples. **(B)** $CD4^+$ T cells from OT-II TCR transgenic mice were cultured with irradiated *Il12b*$^{-/-}$ (IL-12p40-deficient) splenic feeder cells and 5 μg/mL

OVAp for 6 d under Th17-polarizing conditions. Th17 cells were harvested and restimulated with anti-CD3 and anti-CD28 in the presence of anti-IL-4 and TGF-β or IL-12 for 24 or 48 h and stained intracellularly for IFN-γ after 4 h of PMA/ionomycin activation. Data represent the averages ± the ranges of two separate cell samples that were used for the ChIP assays in **(C)**. **(C)** Th17 cells were polarized and restimulated with anti-CD3 and anti-CD28 in the presence of IL-12 and anti-IL-4 for 6, 24, or 48 h as in **(B)**. Cells were then processed for ChIP analysis with antibodies specific for H3K4me or H3K27me3. Data were normalized against those without restimulation (0 h) and represent the averages ± the ranges of two separate cell samples.

Figure 4. STAT4 and T-bet are necessary for epigenetic remodeling of the *Ifng* locus following IL-12 stimulation in Th17. (A) $CD4^+$ T cells from OT-II TCR transgenic mice were cultured with irradiated *Il12b*$^{-/-}$ (IL-12p40-deficient) splenic feeder cells and 5 μg/mL OVAp for 6 d under Th17-polarizing conditions. Th17 cells were harvested and restimulated with anti-CD3 and anti-CD28 in the presence of IL-12 and anti-IL-4 for 24 h and processed for ChIP analysis using an antibody specific for STAT4. Data were normalized against input DNA and represent the averages and the ranges of two separate cell samples. **(B)** Th17 cells were polarized and restimulated with anti-CD3 and anti-CD28 in the presence of IL-12 and anti-IL-4 for 6, 24, or 48 h and cells were

processed for STAT4 ChIP analysis as in **(A)**. Data were normalized against input DNA and represent the averages ± SD of pooled data from two to four separate experiments. **(C)** $CD4^+$ T cells from wild type (WT), *Stat4*$^{-/-}$, and *Tbx21*$^{-/-}$ mice were cultured with irradiated splenic feeder cells and anti-CD3 for 6 d under Th17-polarizing conditions. Th17 cells were harvested and restimulated with irradiated splenic feeder cells in the presence of IL-12, anti-CD3, anti-IL-4, and anti-IFN-γ for additional 6 d. Th17 cells and cells after 6 d stimulation with IL-12 (Th17/IL-12) were processed for ChIP assay with an antibody specific for H3K4me. Data were normalized against naïve $CD4^+$ T cells and represent the averages and SD of three separate ChIP experiments except for Th17 from *Stat4*$^{-/-}$, where data of two experiments. (*) indicates $p<0.05$ and (**) indicates $p<0.01$ vs. WT group.

Figure 5. Th17 cells acquire extensive repressive histone modification at the *Il17a/f* locus following restimulation with IL-12. **(A)** Naïve, Th1, Th17, Th17/IL-12, Th17/TGF-β cells were derived from $CD4^+$ T cells of OT-II TCR transgenic mice as in Figure 3A. The cells were then processed for ChIP assay with an antibody specific for permissive H3K4me or repressive H3K27me3 histone modifications. Data for H3K4me and H3K27me3 were normalized against naïve $CD4^+$ T cells and input DNA, respectively. Data represent the averages and SD of three separate cell samples. Scales

are kept constant among all the cell types. **(B)** $CD4^+$ T cells from OT-II TCR transgenic mice were cultured with irradiated *Il12b*$^{-/-}$ (IL-12p40-deficient) splenic feeder cells and 5 μ g/mL OVAp for 6 d under Th17-polarizing conditions. Th17 cells were harvested and restimulated with anti-CD3 and anti-CD28 in the presence of anti-IL-4 and TGF-β or IL-12 for 24 or 48 h and stained intracellularly for IL-17A after 4 h of activation with PMA/ionomycin. Data represent the averages ± the ranges of two separate cell samples which were used for the ChIP assays in **(C)**. **(C)** Th17 cells were polarized and restimulated with anti-CD3 and anti-CD28 in the presence of IL-12 and anti-IL-4 for 6, 24, or 48 h as **(B)**. Th17 cells were then processed for ChIP assay with an antibody specific for H3K4me or H3K27me3. Data were normalized against cells without restimulation (0 h) and represent the averages ± the ranges of two separate cell samples.

Figure 6. IL-12-induced extinction of the *Il17a/f* locus in Th17 cells is associated with rapid modulation of expression of Th17- and Th1-lineage transcription factors. **(A)** $CD4^+$ T cells isolated from *Il17f*$^{Thy1.1/Thy1.1}$ mice were cultured with irradiated *Il12b*$^{-/-}$ splenic feeder cells under Th17-polarizing conditions for 6 d. $Thy1.1^+$ ($IL\text{-}17F^+$) cells were isolated by magnetic sorting and were restimulated with anti-CD3 and anti-CD28 in the presence of anti-IL-4, anti-IFN-γ, and IL-12 or TGF-β for 1, 3, or 5 d and processed for mRNA quantification of the indicated genes by RT-PCR. All data were

normalized to 18S rRNA, are expressed as relative values to Thy1.1^{+} cells used before restimulation. The horizontal dotted lines indicate the level of each mRNA in naïve CD4^{+} T cells from *Il17f*$^{Thy1.1/Thy1.1}$ mice. Data are representative of two independent experiments. **(B)** CD4^{+} T cells from wild type (WT), *Stat4*$^{-/-}$, and *Tbx21*$^{-/-}$ mice were cultured with irradiated splenic feeder cells and anti-CD3 for 6 d under Th17-polarizing conditions. Th17 cells were harvested and restimulated with irradiated splenic feeder cells in the presence of IL-12, anti-CD3, anti-IL-4, and anti-IFN-γ for additional 6 d. Th17 precursors and T cells isolated stimulation with IL-12 for 6 d (Th17/IL-12) were processed for mRNA quantification of the indicated genes by RT-PCR. All data were normalized to β2-microglobulin and are expressed as relative values to WT Th17 cells. Data represent the averages and SD of replicates and are representative of two similar experiments.

Figure 7. Extinction of the *Il17a/f* locus is linked to STAT4- and T-bet-induced epigenetic repression of the *Rorc* gene. **(A)** CD4^{+} T cells isolated from *Il17f*$^{Thy1.1/Thy1.1}$ mice were plated with anti-CD3/CD28 coated beads under Th17-polarizing conditions on day 0 and were transduced with the retroviral vectors encoding IRES-GFP (GFP (control)) or RORγt-IRES-GFP (RORγt-GFP) on day 1. Th17 cells were harvested on day 6 and a fraction of cells were stained for Thy1.1 and intracellular IL-17A and IFN-γ

after PMA/ionomycin activation. A second fraction of collected cells were used to isolate Thy1.1$^+$ (IL-17F$^+$) cells by magnetic sorting and isolated Thy1.1$^+$ cells were restimulated with anti-CD3 and anti-CD28 in the presence of anti-IL-4, anti-IFN-γ, and IL-12 for 6 d. The resulting Th17/IL-12 cells were stained intracellularly for IL-17A and IFN-γ after PMA/ionomycin activation. Thy1.1$^+$GFP$^+$ cells and GFP$^+$ cells were gated for analysis of Th17 and Th17/IL-12 cells, respectively. **(B)** CD4$^+$ T cells of *Il17f*$^{Thy1.1/Thy1.1}$ mice were cultured with irradiated splenic feeder cells under Th17-polarizing conditions in the presence of an anti-IL-12p40 antibody for 6 d. Thy1.1$^+$ (IL-17F$^+$) cells were isolated by magnetic sorting. A fraction of the isolated Thy1.1$^+$ cells were processed for ChIP assay with an antibody specific for permissive H3K4me or repressive H3K27me3 histone modifications. A second fraction of isolated Thy1.1$^+$ cells were restimulated with irradiated splenic feeder cells in the presence of anti-CD3, anti-IL-4, anti-IFN-γ, and IL-12 or TGF-β for 6 d. These cells were also processed for ChIP assay with an antibody specific for permissive H3K4me or repressive H3K27me3 histone modifications. Data for H3K4me and H3K27me3 were normalized against Thy1.1$^+$ cells and input DNA, respectively. Data represent the averages and the ranges of duplicate determinations. **(C)** CD4$^+$ T cells from wild type (WT), *Stat4*$^{-/-}$, and *Tbx21*$^{-/-}$ mice were cultured with irradiated splenic feeder cells and anti-CD3 for 6 d under Th17-polarizing conditions. Th17 cells were harvested and restimulated with irradiated splenic

feeder cells in the presence of IL-12, anti-CD3, anti-IL-4, and anti-IFN-γ for additional 6 d. Th17 cells and cells after 6 d stimulation with IL-12 (Th17/IL-12) were processed for ChIP assay with an antibody specific for H3K27me3. Data were normalized against input DNA and represent the averages and SD of three separate ChIP experiments except for Th17 from *Stat4*$^{-/-}$, where data of two experiments. (*) indicates $p<0.05$ and (**) indicates $p<0.01$ vs. WT group.

LEGEND FOR SUPPLEMENTAL FIGURES

Figure S1. Committed IL-17F$^+$ (Thy1.1$^+$) Th17 cells undergo epigenetic remodeling and acquire Th1-like histone modifications at the *Ifng* locus following restimulation with IL-12. CD4$^+$ T cells from *Il17f*$^{Thy1.1/Thy1.1}$ mice were cultured with irradiated splenic feeder cells under Th17-polarizing conditions in the presence of an anti-IL-12p40 antibody for 6 d. Cells were collected and Thy1.1$^+$ (IL-17F$^+$) cells were isolated by magnetic sorting (Lee et al., 2009). A fraction of the isolated Thy1.1$^+$ cells were processed for ChIP assay with an antibody specific for permissive H3K4me or repressive H3K27me3 histone modifications prior to secondary stimulation, and assessed at the indicated *Ifng* CNS sites and *Ifng* promoter (P) (**A**). A second fraction of isolated Thy1.1$^+$ cells were restimulated with irradiated splenic feeder cells in the presence of anti-CD3, anti-IL-4, anti-IFN-γ, and IL-12 or TGF-β for 6 d (**B**). These cells were then recovered and processed for ChIP assay as in (**A**). Data for H3K4me and H3K27me3 were normalized against naïve CD4$^+$ T cells and input DNA, respectively. Data represent the averages and the ranges of duplicate determinations.

Figure S2. Comparative analysis of DNase I hypersensitivity of the extended *Ifng* locus in Th17 cells before and after propagation under maintenance (TGF-b) or Th1-deviating (IL-12) cytokine conditions. CD4$^+$ T cells from OT-II TCR transgenic

mice were cultured with irradiated *Il12b*$^{-/-}$ (IL-12p40-deficient) splenic feeder cells and 5 μg/mL OVAp for 6 d under Th17-polarizing conditions. Cells were recovered and cultured with fresh irradiated *Il12b*$^{-/-}$ splenocytes and 5 μg/mL OVAp for another 6 d under Th17-polarizing conditions. The resulting Th17 cells were restimulated with irradiated B6 (Th17/IL-12) or *Il12b*$^{-/-}$ (Th17/TGF-β) splenic feeder cells, 5 μg/mL OVAp, anti-IL-4, anti-IFN-γ, and IL-12 (Th17/IL-12) or TGF-β (Th17/TGF-β) for 6 d and then subjected to DNase I-chip analysis (Boyle et al., 2008). Data are representative of two independent experiments.

Figure S3. STAT4 binds to selected sites in the *Ifng* locus of IL-12-stimulated Th17 cells. CD4$^+$ T cells from *Il17f*$^{Thy1.1/Thy1.1}$ mice were cultured with irradiated *Il12b*$^{-/-}$ (IL-12p40-deficient) splenic feeder cells under Th17-polarizing conditions for 6 d. Cells were collected and Thy1.1$^+$ (IL-17F$^+$) cells were isolated by magnetic sorting. Isolated Th17 cells were restimulated with anti-CD3 and anti-CD28 in the presence of anti-IL-4, anti-IFN-γ, and IL-12 or TGF-β for 24 h and processed for ChIP assay at the indicated *Ifng* CNS sites and *Ifng* promoter (P) with an antibody specific for STAT4. Data were normalized against input DNA and represent the averages and the ranges of duplicate determinations.

Figure S4. Transition of Th17 cells to Th1-like cells is STAT4 and T-bet dependent. $CD4^+$ T cells from wild type (WT), *Stat4*$^{-/-}$, and *Tbx21*$^{-/-}$ mice were cultured with irradiated splenic feeder cells and anti-CD3 for 6 d under Th17-polarizing conditions, and a fraction of recovered cells was analyzed for intracellular IL-17A and IFN-γ after PMA-ionomycin stimulation for 4 h. The remaining fraction of cells was restimulated with irradiated splenic feeder cells in the presence of IL-12, anti-IL-4, anti-IFN-γ, and anti-CD3 for an additional 6 d (Th17/IL-12) and analyzed for intracellular IL-17A and IFN-γ after PMA-ionomycin stimulation for 4 h. Numbers in quadrants indicate percentages of total CD4 T cells in each.

Figure S5. **Committed Th17 cells acquire extensive repressive and diminished permissive histone modifications at the *Il17a/f* locus following restimulation with IL-12.** $CD4^+$ T cells from *Il17f*$^{Thy1.1/Thy1.1}$ mice were cultured with irradiated splenic feeder cells under Th17-polarizing conditions in the presence of an anti-IL-12p40 antibody for 6 d. $Thy1.1^+$ ($IL\text{-}17F^+$) cells were isolated by magnetic sorting. A fraction of the isolated $Thy1.1^+$ Th17 cells were processed for ChIP assay with an antibody specific for permissive H3K4me or repressive H3K27me3 histone modifications, assessed at the indicated *Il17a/f* distal sites and the *Il17a* and *Il17f* promoters (P) (**A**). The remaining $Thy1.1^+$ cells were divided into two fractions and restimulated with irradiated splenic

feeder cells in the presence of anti-CD3, anti-IL-4, anti-IFN-γ, plus IL-12 (Th17/IL-12) or TGF-β (Th17/TGF-β) for 6 d (**B**). These cells were then recovered and processed for ChIP assay as in (**A**). Data for H3K4me and H3K27me3 were normalized against naïve $CD4^+$ T cells and input DNA, respectively. Data represent the average and the ranges of duplicate determinations.

Figure S6. Transition of Th17 cells to Th1-like cells is associated with decreased DNase I hypersensitivity across the *Il17a/f* loci. $CD4^+$ T cells from OT-II TCR transgenic mice were cultured with irradiated *Il12b*$^{-/-}$ (IL-12p40-deficient) splenic feeder cells and 5 μg/mL OVAp for 6 d under Th17-polarizing conditions. Cells were recovered and cultured with fresh irradiated *Il12b*$^{-/-}$ splenocytes and 5 μg/mL OVAp for another 6 d under Th17-polarizing conditions. The resulting Th17 cells were restimulated with irradiated B6 (for Th17/IL-12) or *Il12b*$^{-/-}$ (for Th17/TGF-β) splenic feeder cells, 5 μg/mL OVAp, anti-IL-4, anti-IFN-γ, and IL-12 (for Th17/IL-12) or TGF-β (for Th17/TGF-β) for 6 d and subjected to DNase I-chip analysis. Data are representative of two independent experiments.

Figure S7. **STAT4 binds to select sites in the *Il17a/f* locus of IL-12-stimulated Th17 cells.** $CD4^+$ T cells from OT-II TCR transgenic mice were cultured with irradiated

splenic feeder cells and 5 μ g/mL OVAp for 6 d under Th17-polarizing conditions. Th17 cells were harvested and restimulated with anti-CD3 and anti-CD28 in the presence of IL-12 or TGF-β and anti-IL-4 for 24 h and processed for ChIP analysis at the indicated *Il17a/f* locus sites and *Il17a* and *Il17f* promoters (P) with an antibody specific for STAT4. Data were normalized against input DNA and represent the averages and SD of pooled data from three separate experiments.

Figure S8. **Retrovirally induced expression of RORgt partially inhibits IL-12-induced down-regulation of IL-17F in Th17 cells.** $CD4^+$ T cells isolated from *Il17f*$^{Thy1.1/Thy1.1}$ mice were plated with anti-CD3/CD28 coated beads under Th17-polarizing conditions on day 0 and were transduced with retroviral vectors encoding GFP (control) or RORγt and GFP (RORγt-GFP) on day 1. Cells were collected on day 6 and Thy1.1$^+$ (IL-17F$^+$) cells were isolated by magnetic sorting. Isolated Thy1.1$^+$ cells were restimulated with anti-CD3 and anti-CD28 in the presence of anti-IL-4, anti-IFN-γ, and IL-12 (Th17/IL-12) or TGF-β (Th17/TGF-b) for 6 d. Cells were then stained for Thy1.1 and CD4 after PMA/ionomycin activation. GFP^+ cells were gated for analysis.

Figure S9. Ectopic expression of T-bet in Th17 cells is insufficient for transition into Th1-like cells. $CD4^+$ T cells isolated from *Il17f*$^{Thy1.1/Thy1.1}$ mice were cultured under Th17-polarizing conditions for 6 d. $Thy1.1^+$ ($IL\text{-}17F^+$) cells were isolated by magnetic sorting and stained for CD4 and Thy1.1 post-isolation (left panel, **B**), or were stimulated with PMA/ionomycin and stained for IL-17A and IFN-γ (left panel, **C**), to verify isolation purity and Th17 phenotype (note that the $IL\text{-}17A^+$ cells represent a subpopulation of the IL-17F positive cells, as previously reported (Lee et al., 2009)). Th17 ($Thy1.1^+$) cells were restimulated with plate-bound anti-CD3 and anti-CD28 in the presence of anti-IL-4 and anti-IFN-γ without exogenous cytokine addition ("Neutral"), or with IL-12 ("IL-12") or TGF-β ("TGF-β") and were transduced with retrovirus encoding control GFP (GFP-RV) or T-bet and GFP (T-bet-RV) on d 1 following restimulation. Cells were recovered on d 3 and a fraction of the cells was stained for CD4 and intracellular T-bet (**A**) (CD4-gated)**.** The remaining cells were stimulated with PMA/ionomycin and stained for Thy1.1 (IL-17F) (**B**) and IL-17A and IFN-γ (**C**). $CD4^+$ and $CD4^+GFP^+$ cells were gated for analysis in (**B**) and (**C**), respectively. Note that ectopic T-bet expressed at levels comparable to that of cells restimulated with IL-12 (right panel, **A**) failed to induce substantial IFN-g in comparison to IL-12-stimulated controls, despite inducing moderate suppression of *Il17a,* independently of IL-12

signaling. Thus, T-bet expression alone is insufficient to drive the Th1-like conversion of Th17 precursors.

Figure 1

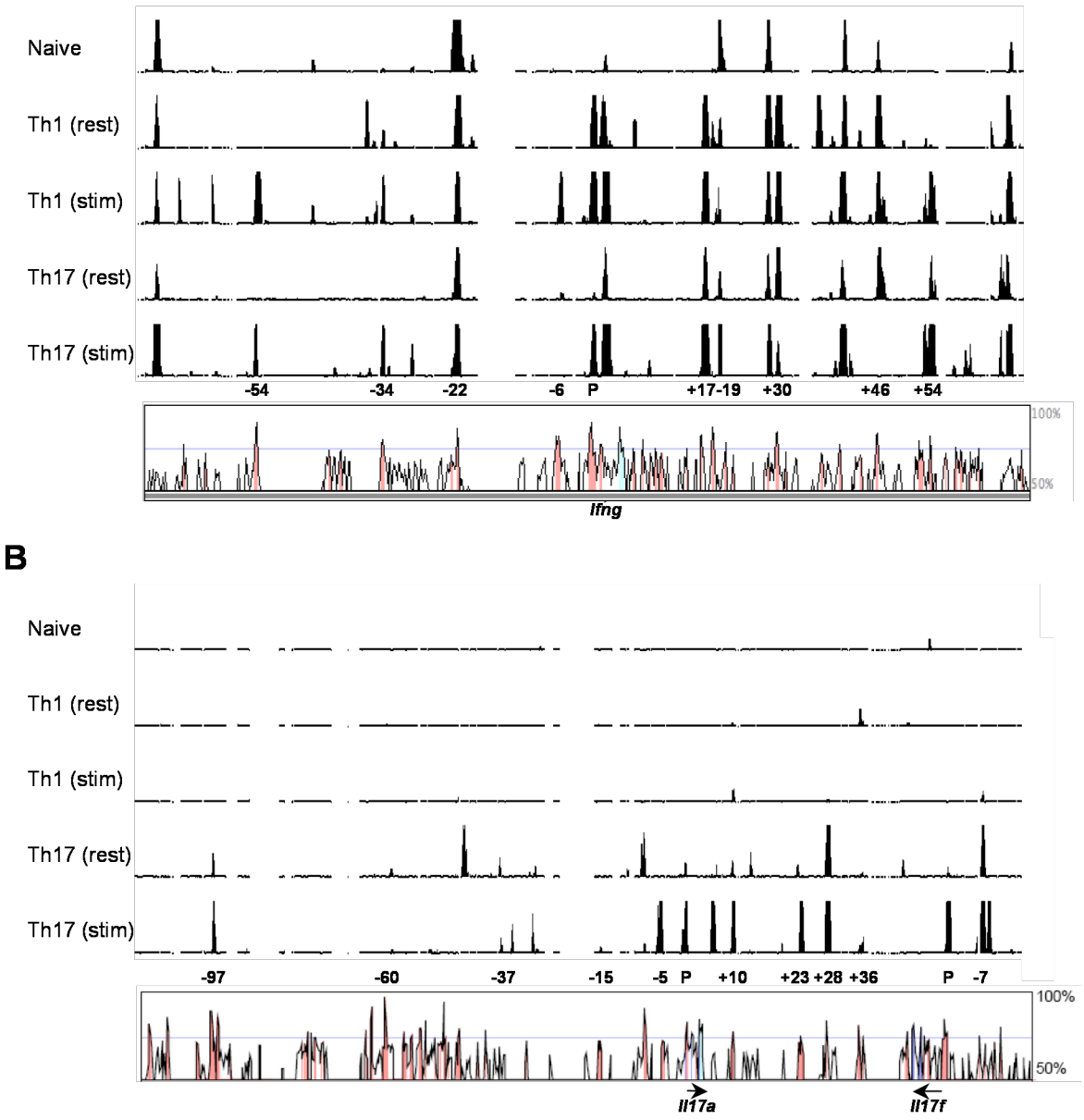

A
Naive
Th1 (rest)
Th1 (stim)
Th17 (rest)
Th17 (stim)
-54
-34
-22
-6
P
+17-19
+30
+46
+54
100%
50%
Ifng
B
Naive
Th1 (rest)
Th1 (stim)
Th17 (rest)
Th17 (stim)
-97
-60
-37
-15
-5
P
+10
+23
+28
+36
P
-7
100%
50%
Il17a
Il17f

Figure 2

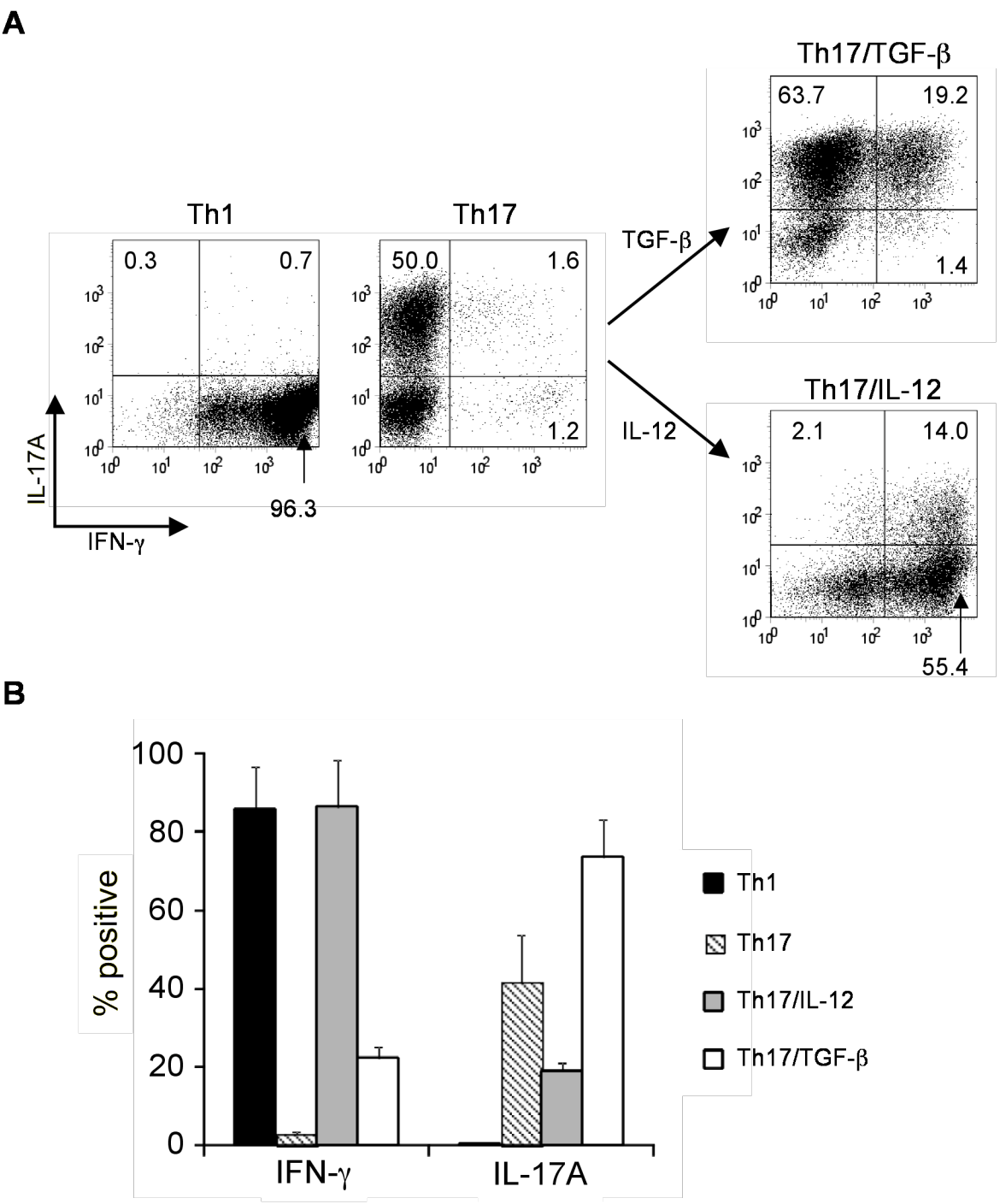
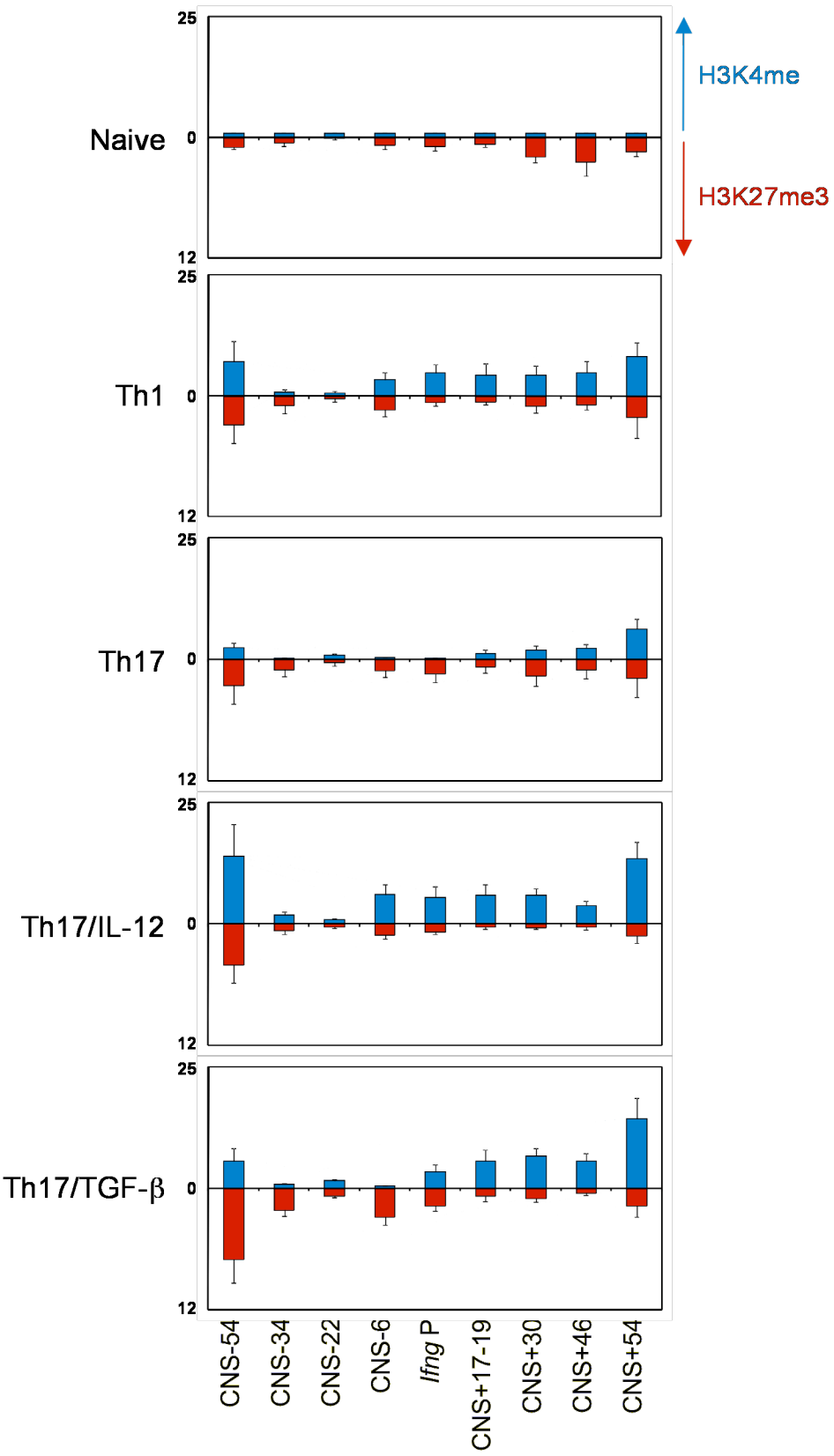
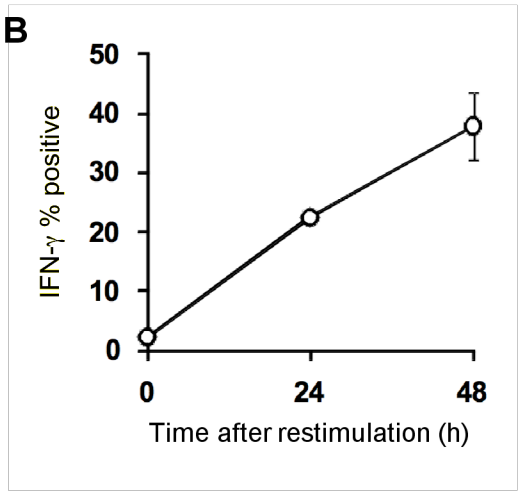

A
Th1
Th17
0.3
0.7
50.0
1.6
1.2
96.3
IL-17A
IFN-γ
TGF-β
IL-12
Th17/TGF-β
63.7
19.2
1.4
Th17/IL-12
2.1
14.0
55.4
B
% positive
100
80
60
40
20
0
IFN-γ
IL-17A
Th1
Th17
Th17/IL-12
Th17/TGF-β

Figure 3


A
Naive
Th1
Th17
Th17/IL-12
Th17/TGF-β
25
0
12
H3K4me
H3K27me3
CNS-54
CNS-34
CNS-22
CNS-6
Ifng P
CNS+17-19
CNS+30
CNS+46
CNS+54


B
IFN-γ % positive
50
40
30
20
10
0
0
24
48
Time after restimulation (h)

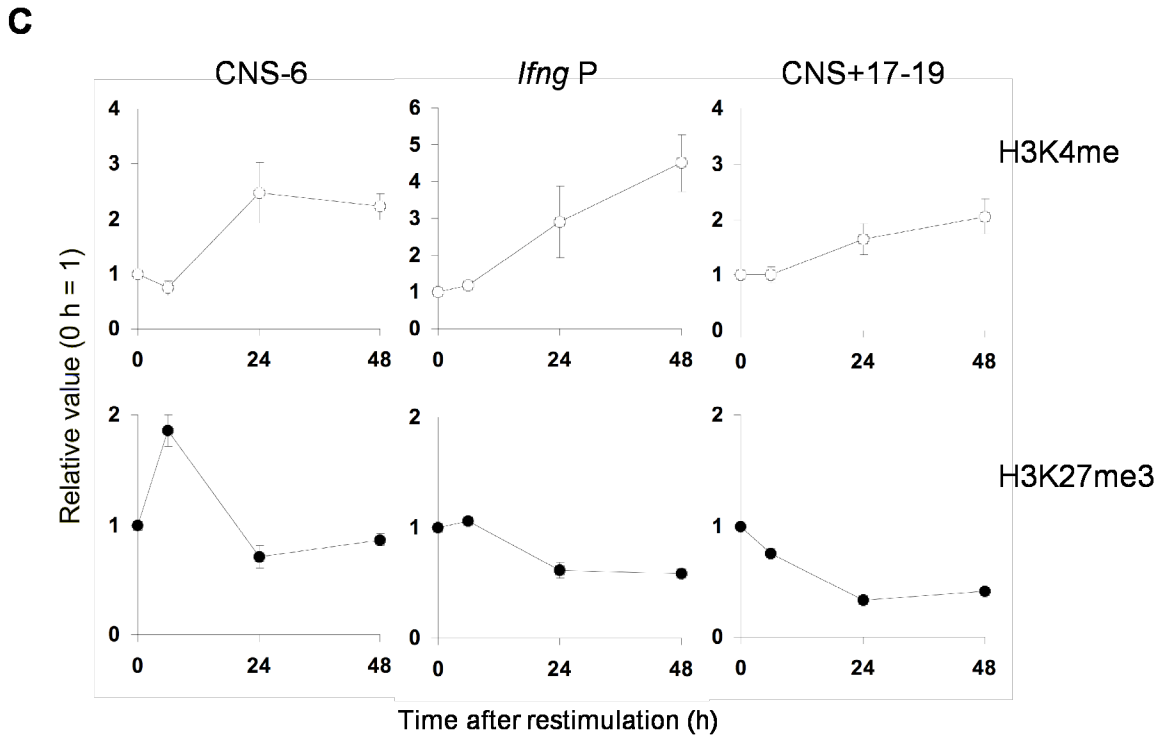
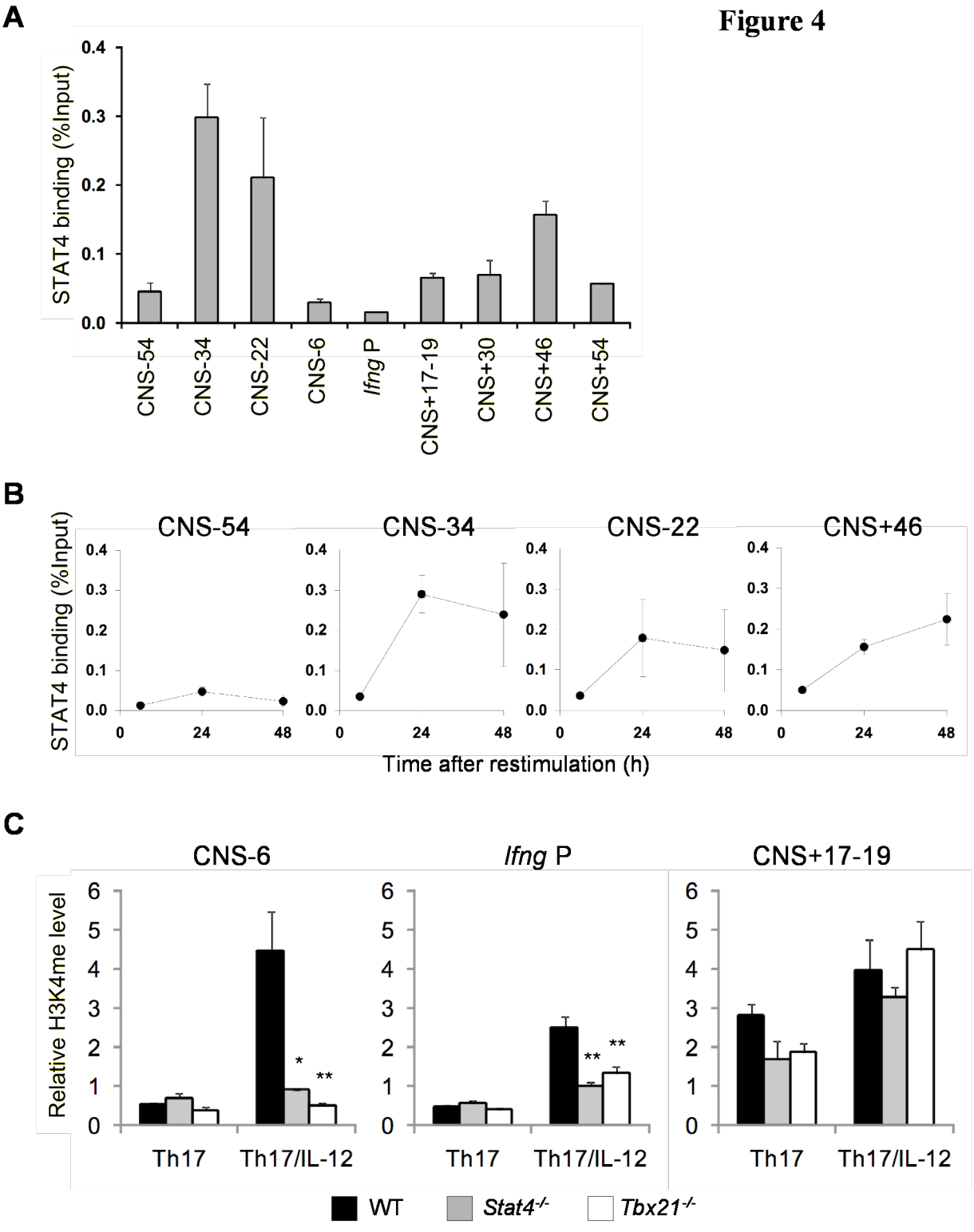

C
CNS-6
Ifng P
CNS+17-19
H3K4me
H3K27me3
Relative value (0 h = 1)
Time after restimulation (h)

Figure 4


A
STAT4 binding (%Input)
0.4
0.3
0.2
0.1
0.0
CNS-54
CNS-34
CNS-22
CNS-6
Ifng P
CNS+17-19
CNS+30
CNS+46
CNS+54
B
CNS-54
CNS-34
CNS-22
CNS+46
STAT4 binding (%Input)
0
24
48
Time after restimulation (h)
C
CNS-6
Ifng P
CNS+17-19
Relative H3K4me level
Th17
Th17/IL-12
*
**
WT
Stat4-/-
Tbx21-/-

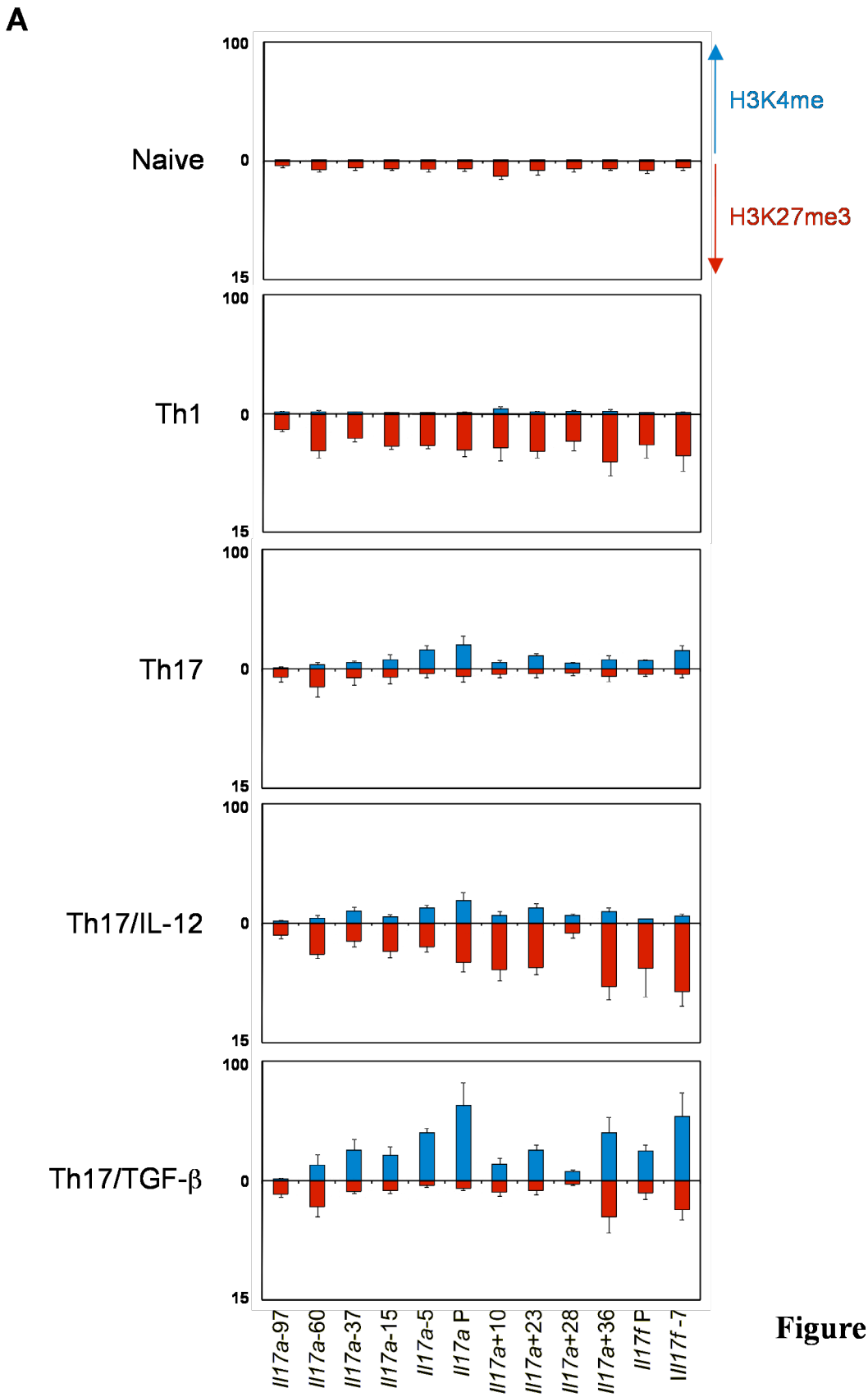
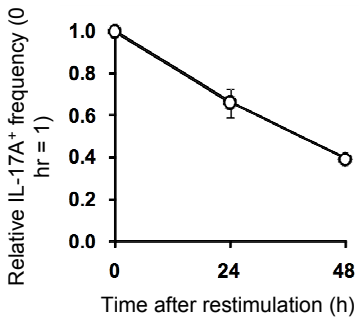

A
Naive
Th1
Th17
Th17/IL-12
Th17/TGF-β
100
0
15
H3K4me
H3K27me3
Il17a-97
Il17a-60
Il17a-37
Il17a-15
Il17a-5
Il17a P
Il17a+10
Il17a+23
Il17a+28
Il17a+36
Il17f P
Il17f-7

Figure 5

B


Relative IL-17A+ frequency (0 hr = 1)
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
Time after restimulation (h)

C

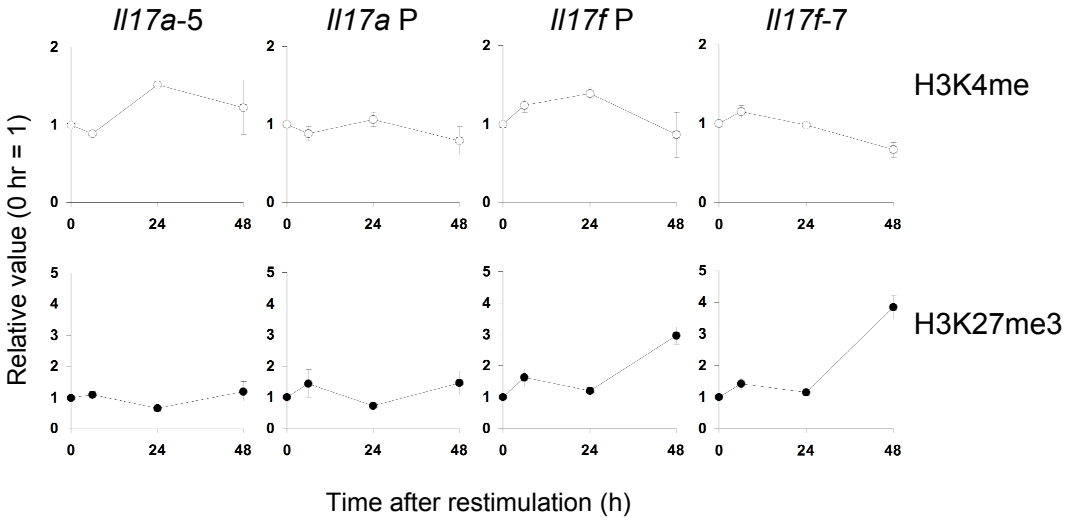
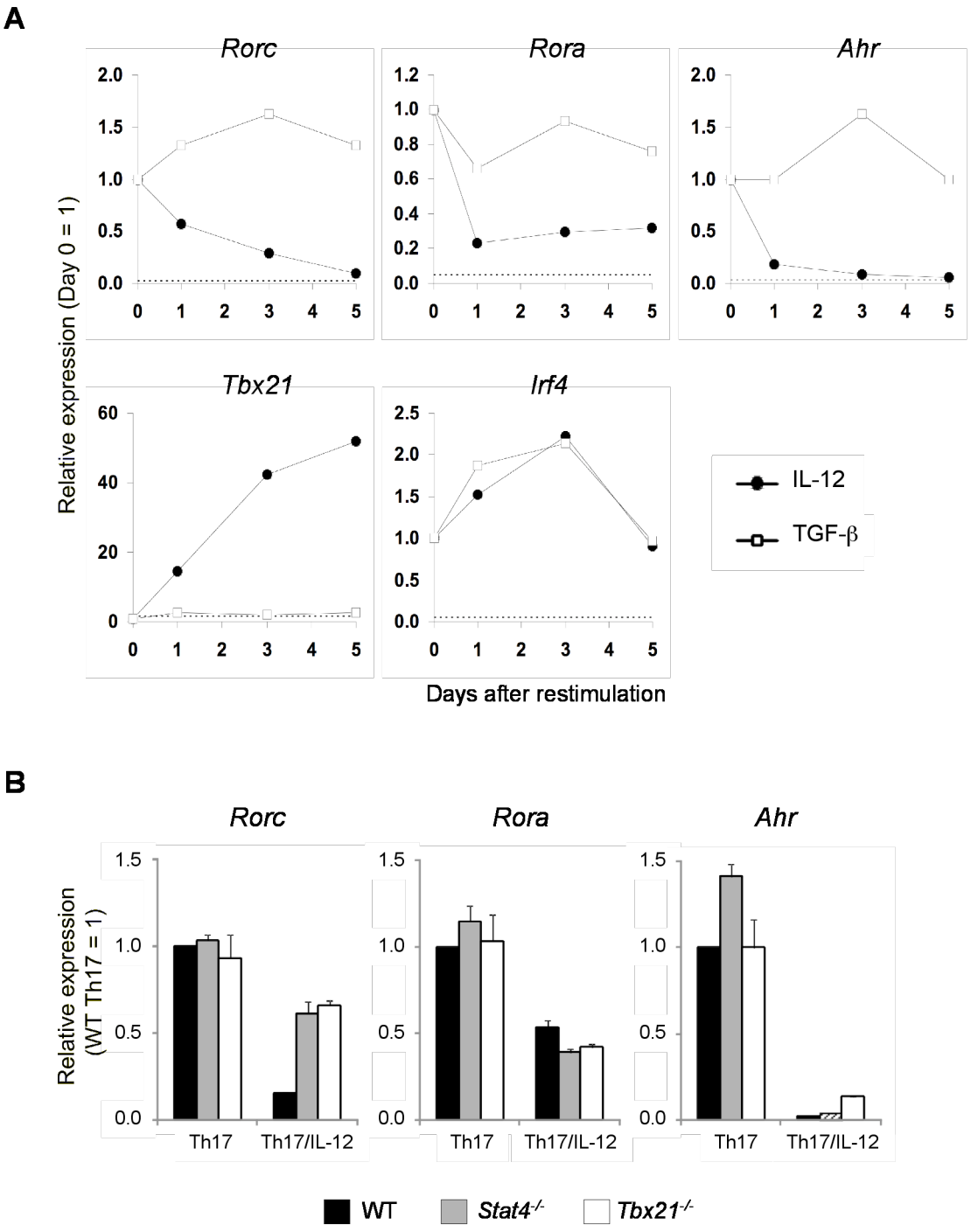

Il17a-5
Il17a P
Il17f P
Il17f-7
H3K4me
H3K27me3
Relative value (0 hr = 1)
Time after restimulation (h)

Figure 6


A
Rorc
Rora
Ahr
Tbx21
Irf4
Relative expression (Day 0 = 1)
Days after restimulation
IL-12
TGF-β
B
Rorc
Rora
Ahr
Relative expression (WT Th17 = 1)
Th17
Th17/IL-12
WT
Stat4-/-
Tbx21-/-

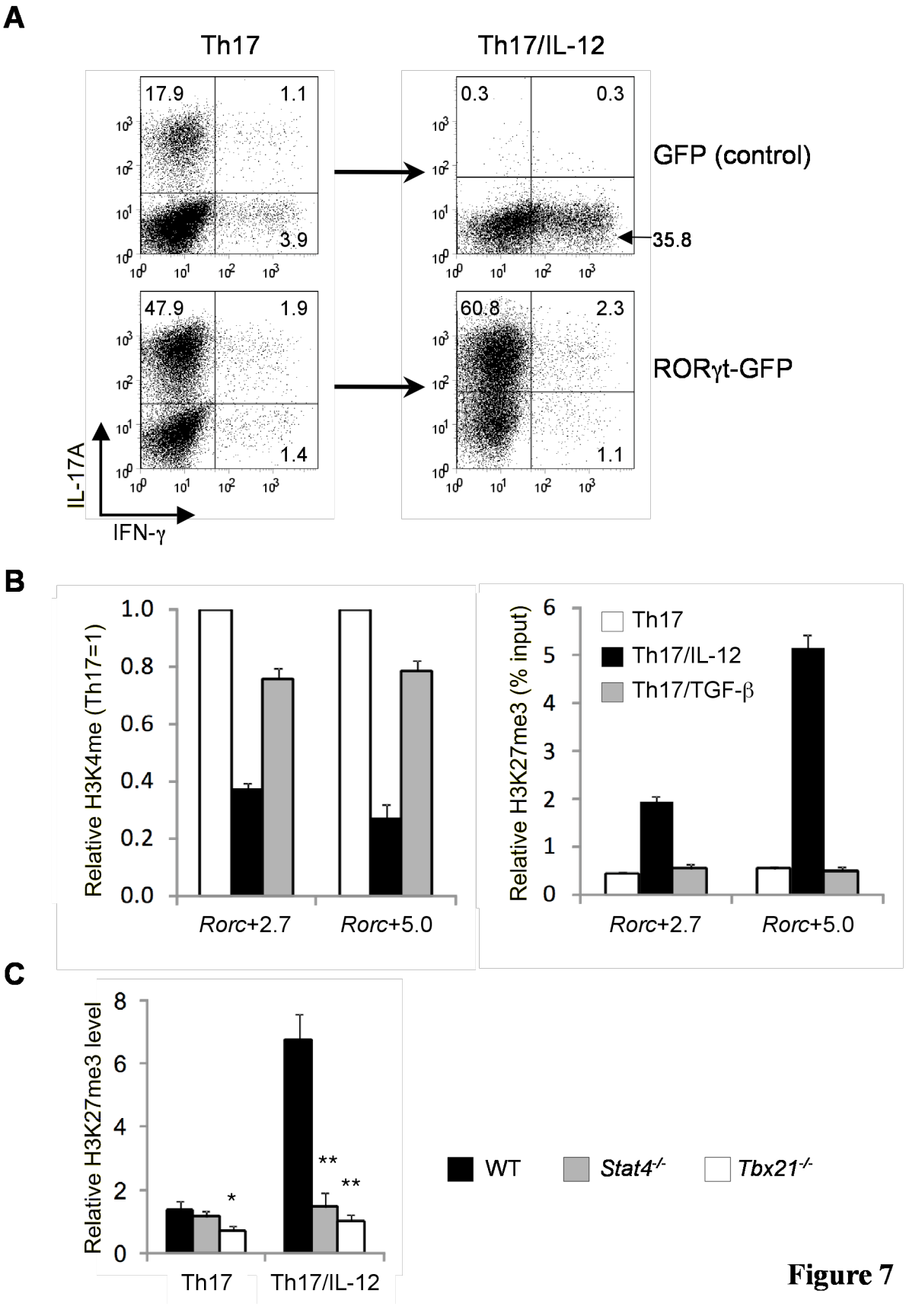

A
Th17
Th17/IL-12
17.9
1.1
3.9
0.3
0.3
GFP (control)
35.8
47.9
1.9
1.4
60.8
2.3
1.1
RORγt-GFP
IL-17A
IFN-γ
B
Relative H3K4me (Th17=1)
1.0
0.8
0.6
0.4
0.2
0.0
Rorc+2.7
Rorc+5.0
Relative H3K27me3 (% input)
6
5
4
3
2
1
0
Th17
Th17/IL-12
Th17/TGF-β
Rorc+2.7
Rorc+5.0
C
Relative H3K27me3 level
8
6
4
2
0
*
**
**
Th17
Th17/IL-12
WT
Stat4-/-
Tbx21-/-

Figure 7

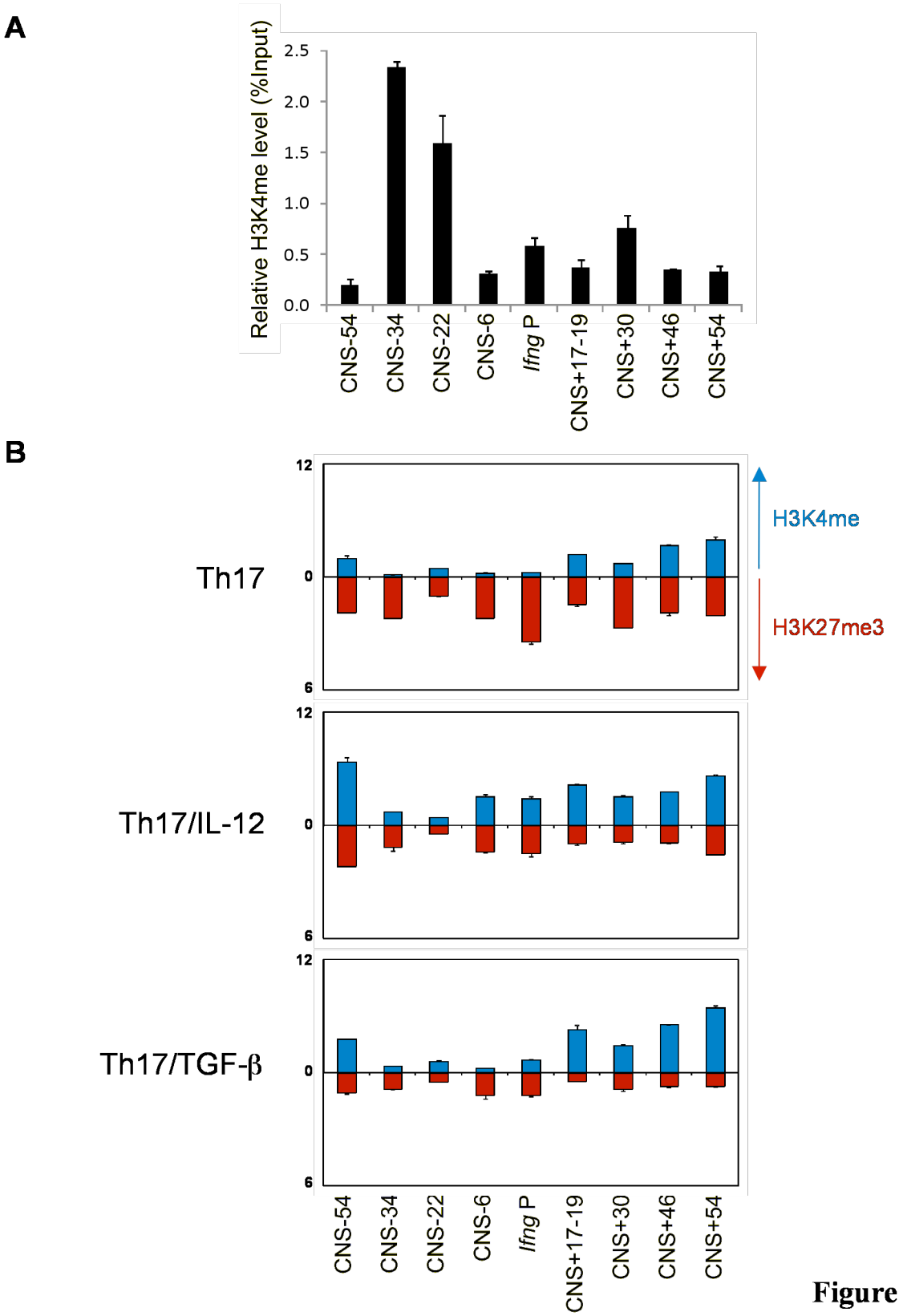

A
Relative H3K4me level (%Input)
2.5
2.0
1.5
1.0
0.5
0.0
CNS-54
CNS-34
CNS-22
CNS-6
Ifng P
CNS+17-19
CNS+30
CNS+46
CNS+54
B
Th17
Th17/IL-12
Th17/TGF-β
12
0
6
H3K4me
H3K27me3
CNS-54
CNS-34
CNS-22
CNS-6
Ifng P
CNS+17-19
CNS+30
CNS+46
CNS+54

Figure S1

Figure S2

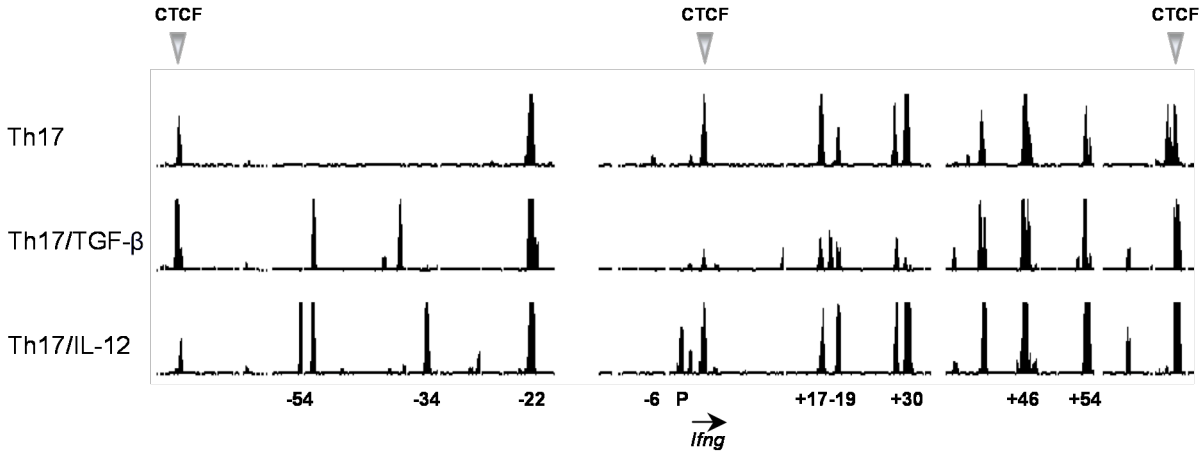

CTCF
CTCF
CTCF
Th17
Th17/TGF-β
Th17/IL-12
-54
-34
-22
-6
P
+17-19
+30
+46
+54
Ifng

Figure S3

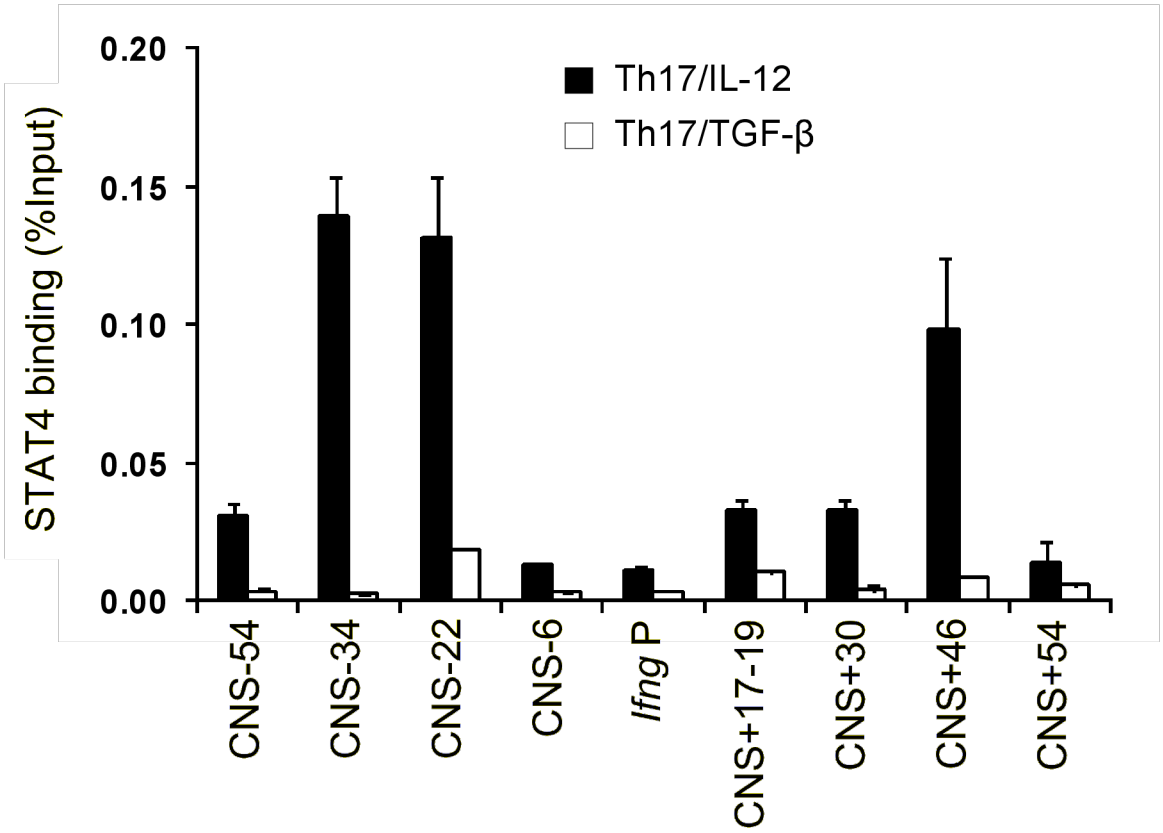

STAT4 binding (%Input)
0.20
0.15
0.10
0.05
0.00
Th17/IL-12
Th17/TGF-β
CNS-54
CNS-34
CNS-22
CNS-6
Ifng P
CNS+17-19
CNS+30
CNS+46
CNS+54

Figure S4

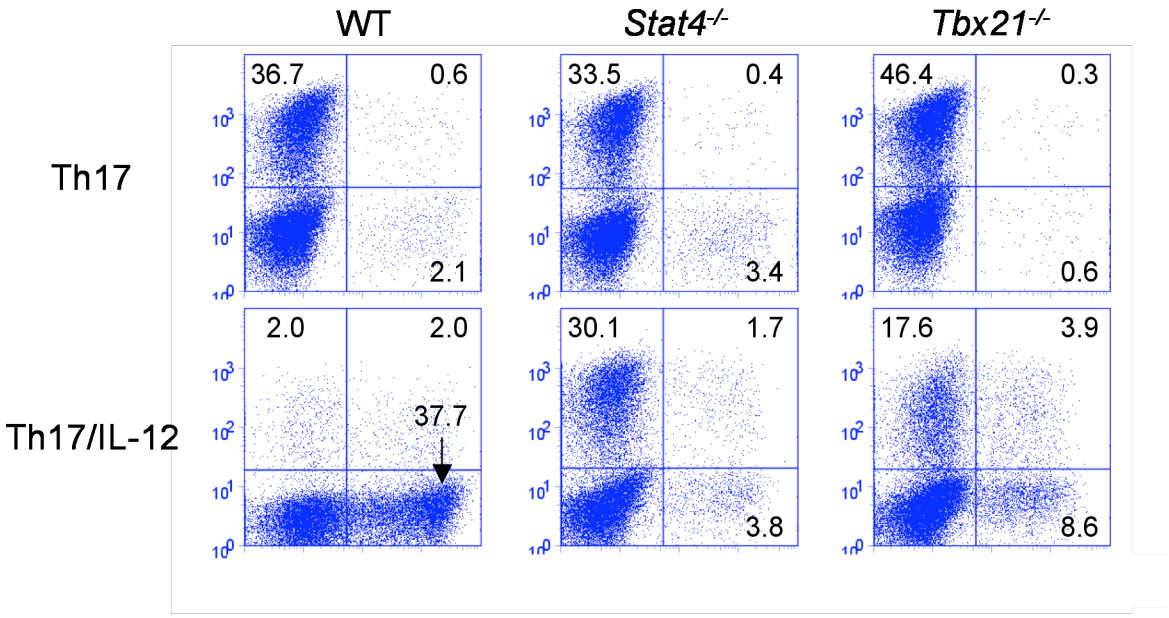

WT
Stat4-/-
Tbx21-/-
Th17
Th17/IL-12
36.7
0.6
2.1
33.5
0.4
3.4
46.4
0.3
0.6
2.0
2.0
37.7
30.1
1.7
3.8
17.6
3.9
8.6

Figure S5

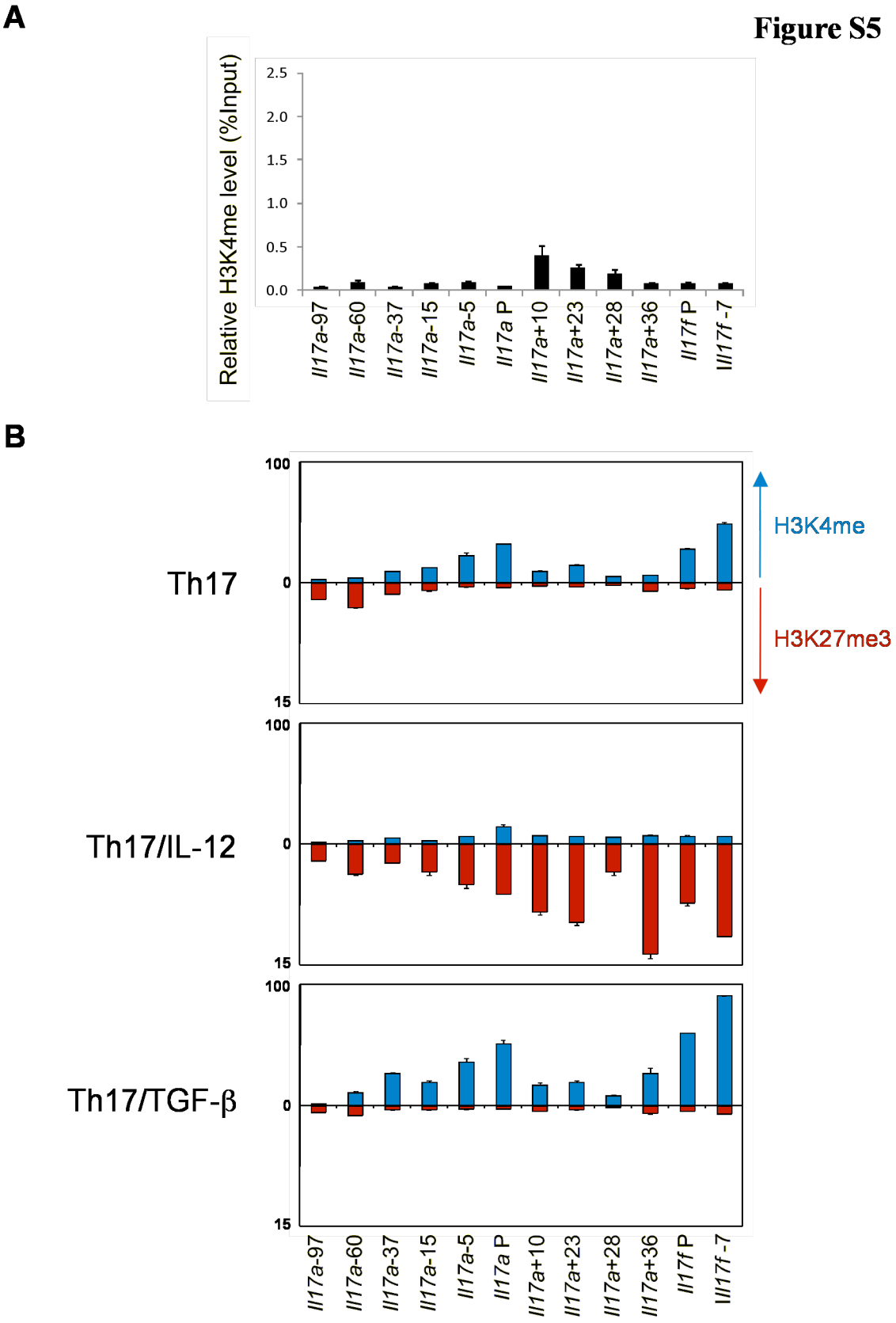

A
Relative H3K4me level (%Input)
2.5
2.0
1.5
1.0
0.5
0.0
Il17a-97
Il17a-60
Il17a-37
Il17a-15
Il17a-5
Il17a P
Il17a+10
Il17a+23
Il17a+28
Il17a+36
Il17f P
Il17f -7
B
Th17
Th17/IL-12
Th17/TGF-β
100
0
15
H3K4me
H3K27me3

Figure S6

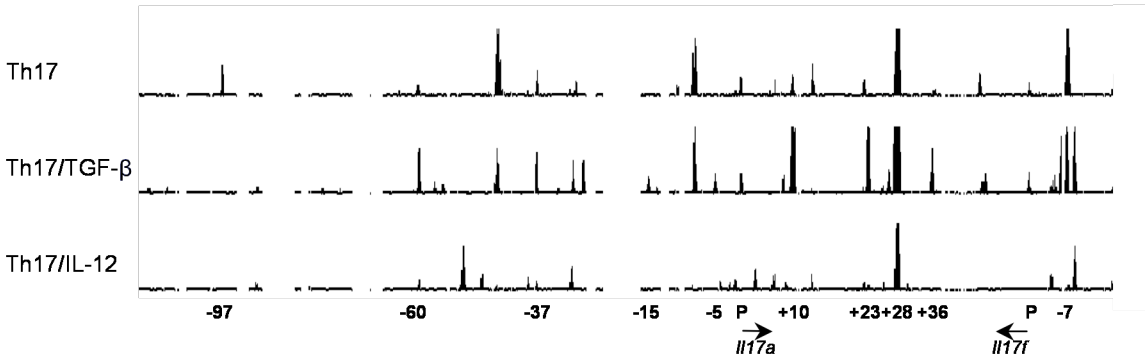

Th17
Th17/TGF-β
Th17/IL-12
-97
-60
-37
-15
-5
P
+10
+23+28
+36
P
-7
Il17a
Il17f

Figure S7

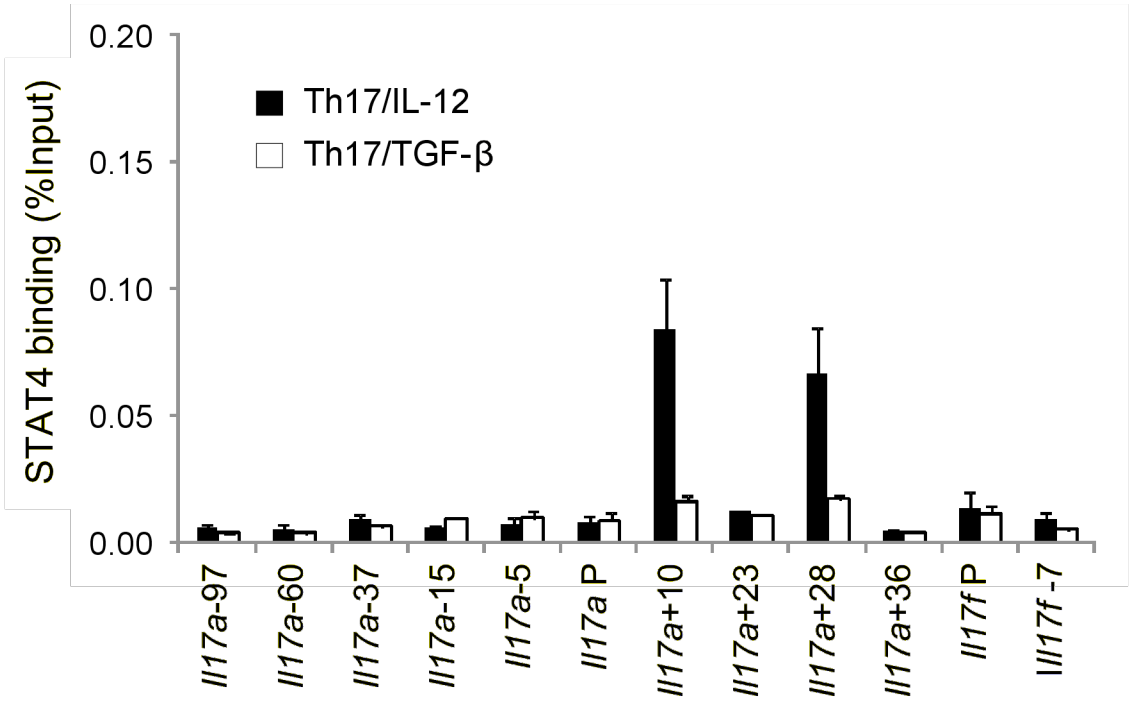
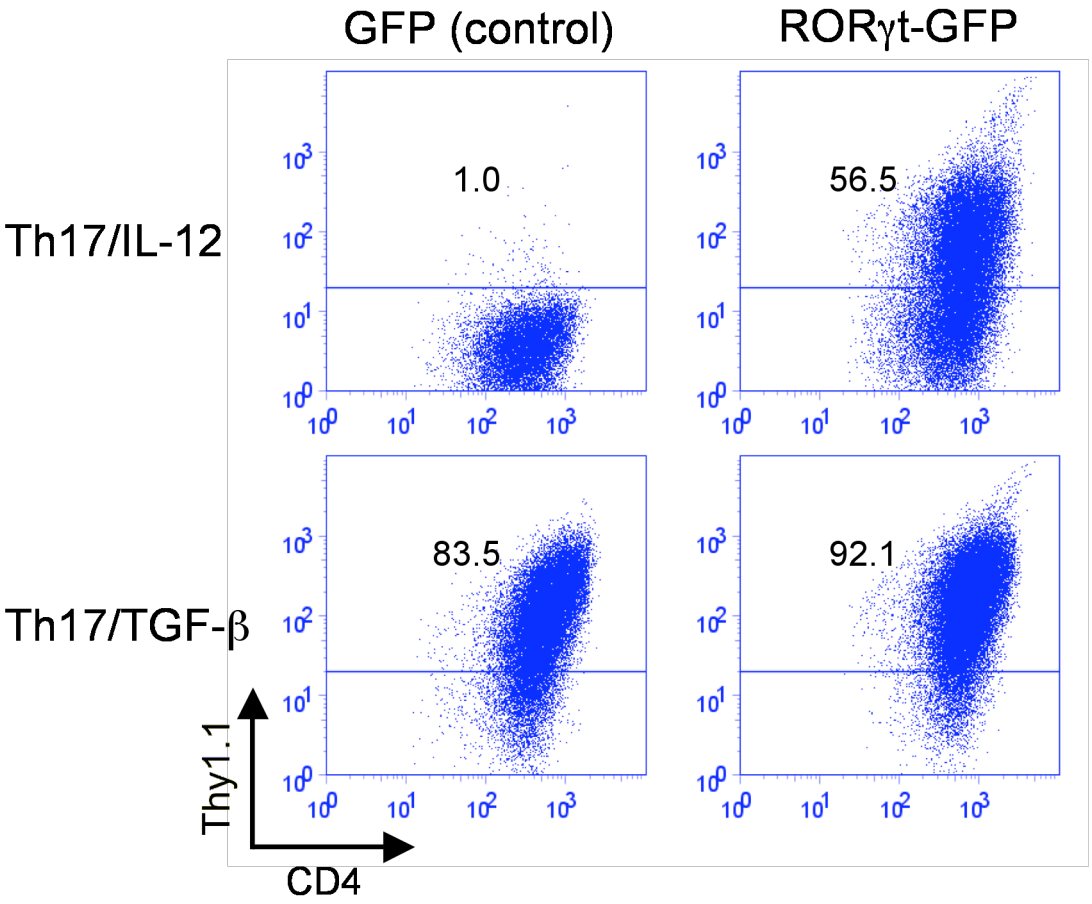

STAT4 binding (%Input)
0.20
0.15
0.10
0.05
0.00
Th17/IL-12
Th17/TGF-β
Il17a-97
Il17a-60
Il17a-37
Il17a-15
Il17a-5
Il17a P
Il17a+10
Il17a+23
Il17a+28
Il17a+36
Il17f P
Il17f -7

Figure S8


GFP (control)
RORγt-GFP
Th17/IL-12
Th17/TGF-β
1.0
56.5
83.5
92.1
Thy1.1
CD4

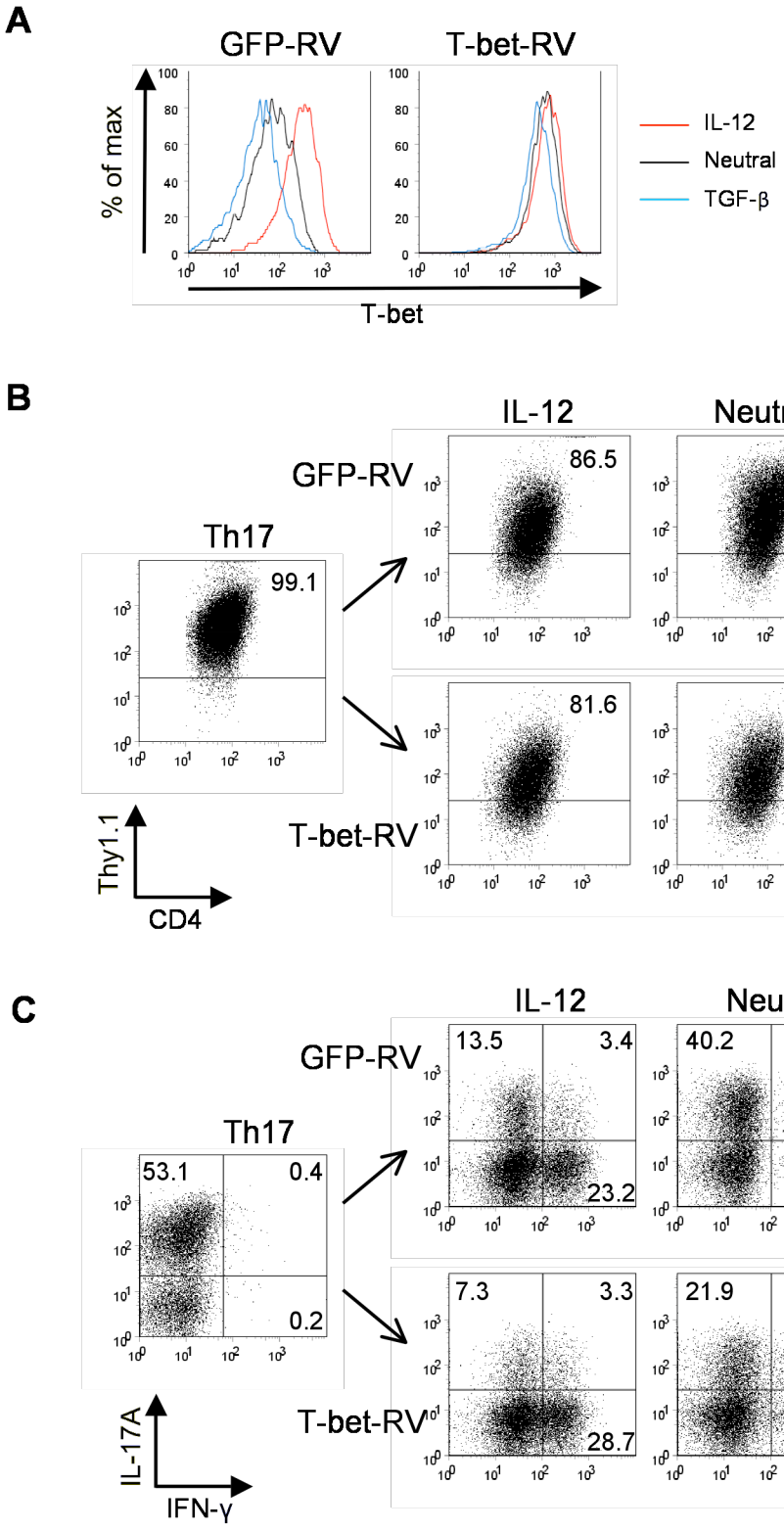

A
GFP-RV
T-bet-RV
% of max
T-bet
IL-12
Neutral
TGF-β
B
IL-12
Neutral
TGF-β
GFP-RV
T-bet-RV
Th17
99.1
86.5
91.6
95.1
81.6
81.9
86.1
Thy1.1
CD4
C
IL-12
Neutral
TGF-β
GFP-RV
T-bet-RV
Th17
53.1
0.4
0.2
13.5
3.4
23.2
40.2
0.8
0.9
56.9
0.9
0.4
7.3
3.3
28.7
21.9
2.3
4.9
39.7
3.2
2.5
IL-17A
IFN-γ

Figure S9

SUMMARY

The differentiation of naïve CD4+ T cells into effector cells poised to produce cytokine underlies an effective adaptive immune response. Functional specialization by discrete lineages of T helper cells protects the host from a range of pathogens. The T helper 17 lineage has emerged as an important arm of adaptive immunity which is aimed at defending mucosal barriers from extracellular bacterial pathogens[3]. We have described interactions between genomic *cis*-elements and the transcription factors and epigenetic mediators they partner with to modify gene expression. In the course of our studies we identified multiple conserved sites within the *Il17a/f* locus that exhibit permissive histone modifications[15], forming the basis for heritability of *Il17* transcriptional competence. We discuss how under some circumstances, e.g. limited TGF-β, Th17 cells diminish their IL-17 expression and transition into IFN-γ producing cells[14]. This reflects an unstable epigenetic conformation at the *Il17* locus that allows T helper cells to alter their cytokine expression in response to changing cytokine signals in their local environment. These data undermine the assertion that T helper cells represent irreversible differentiation products and suggest that the Th17 developmental program, and perhaps those of other T helper lineages or phenotypes exhibit substantial plasticity.

Cytokines, co-stimulation, transcriptional circuitry, clonality, and chromatin states all influence T cell stability and plasticity[38]. Work by Curtis et al[36]. demonstrated that infection with *Listeria monocytogenes* induced a polyclonal T cell response in which the majority of cells heritably committed to the Th1 lineage, but 10-20% retained plasticity to produce IL-17[36]. The authors determined that cell proliferation and continuity of instructive cytokine signals were the key factors contributing to this plasticity. How cell

division and pathogen-induced cytokines contribute to heterogeneity in expanding CD4 T cell populations will require further study. Transcriptional circuits that stabilize T helper phenotypes are universally self-reinforcing. In Th2 cells autoactivation of Gata3 occurs in a positive feedback loop to strengthen lineage commitment[23]. Likewise, T-bet induces its own expression by both direct and indirect mechanisms[21]. In contrast, there is no evidence to date for transcriptional autoactivation of RORγt, suggesting that Th17 cells may require continuous IL-6 signaling to maintain RORγt expression[38]. It is not yet clear how other Th17-associated transcription factors feed into pathways that reinforce lineage commitment and stability, and this will require examination.

In vitro polarization never yields a 100% pure cell phenotype, so in many circumstances plasticity may simply arise from uncommitted cells present within a heterogeneous T helper population[38]. To what extent evidence of plasticity truly reflects individual CD4+ T cell flexibility versus population-based heterogeneity is unresolved. The field will have to continue to examine the existing and emerging CD4+ effector 'subsets' to determine whether their transcriptional capacities reflect individual lineages or alternative pathways or cell activation. Such findings will contribute to knowledge about what constitutes an effective vaccine for a given disease. Vaccines must induce stable, long-lasting memory Th responses and the correlates of protection for individual pathogens are profoundly influenced by plasticity/stability of T helper cells.

The T helper cell cytokine loci have emerged as important models for understanding gene regulation[21-23]. To that end, it is possible that research aimed at mapping *cis*-elements important for Th cytokine transcription, and transcription factors

that interact with them under different conditions, will further knowledge of mechanisms that regulate lineage- and activation-dependent eukaryotic transcription in general.

In the studies described herein we identified six enhancer elements for the linked *Il17a/f* genes, a subset of which were STAT-dependent. We demonstrated that these four CNSs recruit the Th17-lineage specifying transcription factor STAT3, and the Th17-inducing cytokine IL-1β facilitates this action of STAT3. IL-1β promotes complex interactions between *cis*-elements and the transcription factors that bind them to promote *Il17a/f* transcription. We speculate that IL-1β impacts chromatin conformation in a way that supports greater accessibility to the transcription factor STAT3, and IL-1-induced *trans* factors that cooperate with it, such as RelA and c-Rel.

RelA has a critical role in inducing IFN-γ production by Th1 cells[26], and we show that it has a similar function in regulating acute transcription *Il17a/f*. We observed that CD4 cells deficient in RelA or c-Rel had diminished IL-17 production and reduced expression of a number of Th17-associated genes. Thus, in addition to their roles in assisting cytokine-induced cytokine production by effector T cells, these NF-κB proteins impact early events in Th17 differentiation.

Taken together, our data have functionally mapped multiple interactions between histone modification factors, cytokine-induced transcription factors, and their genomic targets within the *Il17a/f* locus which converge to regulate *Il17* transcription and promote Th17 responses.

These discoveries may be contrasted to existing data on *Ifng* transcriptional regulation. The finding that multiple *cis*-elements regulating *Ifng* exhibit STAT4-dependent RelA binding[26] is incongruent with our observation that IL-1 signaling

enhances STAT3 recruitment to multiple CNSs within the *Il17a/f* locus. However, a similar dichotomy exists in that there is modular utilization of *cis*-elements in TCR- versus cytokine-induced transcription of the *Ifng* and *Il17a/f* cytokine genes.

The discovery of cryopyrin (NALP3) and the caspase-1 inflammasome revealed a pivotal role for IL-1 in autoimmune/inflammatory diseases. NALP3, a member of the nucleotide-binding oligomerization domain (NOD) protein family, associates with other intracellular proteins to form a complex known as the inflammasome[39,40]. This complex functions to convert inactive procaspase-1 to active caspase-1, which cleaves the inactive IL-1β precursor to a secreted, active cytokine. Caspase-1 also cleaves the precursors of IL-18, IL-33 and IL-1F7 to produce functional cytokines.[40]

Anticytokine agents have been effective drugs for treatment of a variety of autoimmune inflammatory diseases[5]. Anti-TNFα therapy is a mainstay of treatment for inflammatory bowel disease (IBD). However, anti-TNFα is ineffective in approximately 50% of Crohn's disease patients over time, so a need for additional anticytokine therapies is clear[5]. Patients suffering from familial cold autoinflammatory syndrome (FCAS) as well as rheumatoid arthritis, juvenile idiopathic arthritis, refractory adult Still's disease, and several other inflammatory disorders benefit from treatment with IL-1R antagonist (IL-1Ra), or anakinra[39]. It is possible that anakinra could be useful in the treatment of Th17-mediated inflammatory diseases, given mounting evidence of IL-1's importance in Th17 development and production of the cytokines IL-17A/F. Likewise, our data suggest that interference with IL-18- and IL-33-receptor binding might prove beneficial in dampening Th1 and Th2-mediated inflammation due to the effects of these cytokines on Th cell STAT activation. Future studies will have to continue to examine how cytokines

derived from each T helper lineage contribute to autoimmune inflammation to identify suitable proximal targets for intervention.

GENERAL REFERNCES

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4. Coghill, J.M.*, et al.* Effector CD4+ T cells, the cytokines they generate, and GVHD: something old and something new. *Blood* **117**, 3268-3276 (2011).
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APPENDIX A

IUCUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

Notice of Approval for Protocol Modification

DATE: July 22, 2011

TO: CASEY T WEAVER, M.D.
BBRB-870 2170
FAX: (205) 975-8310

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Control of Effector Cytokine Gene Induction in Immunopathogenic T Cells
Sponsor: Crohn's and Colitis Foundation
Animal Project Number: 110508746

On July 22, 2011, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the modification as described: Additional Personnel to protocol: Sarah Whitley The sponsor for this project may require notification of modification(s) approved by the IACUC but not included in the original grant proposal/experimental plan; please inform the sponsor if necessary. The following species and numbers of animals reflect this modification.

Species	Use Category	Number in Category
Mice	A	Zero - Procedural modification only
Mice	B	Zero - Procedural modification only
Mice	C	Zero - Procedural modification only

The IACUC is required to conduct continuing review of approved studies. This study is scheduled for annual review on or before May 4, 2012. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files.

Refer to Animal Protocol Number (APN) 110508746 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

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