Conserved Intergenic Elements and DNA Methylation Cooperate to Regulate Transcription at the il17 Locus*▲

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Background: IL-17 is an inflammatory cytokine that mediates immunopathology in autoimmune disease.

Results: DNA methylation at the il17 locus is lineage-restricted and blocks STAT3 binding.

Conclusion: Expression of the il17 genes is regulated by promoter methylation and a novel intergenic enhancer.

Significance: Understanding the mechanisms regulating IL-17 production will facilitate therapeutic control of inflammatory immune responses.

Naive CD4+ T cells can differentiate into distinct lineages with unique immune functions. The cytokines TGFβ and IL-6 promote the development of Th17 cells that produce IL-17, an inflammatory cytokine not expressed by other T helper lineages. To further understand how IL-17 production is controlled, we studied an ~120-kb genomic region containing the murine il17a and il17f genes and seven evolutionarily conserved, intergenic noncoding sequences. We show that the +28-kb noncoding sequence cooperates with STAT3, RORyt, and Runx1 to enhance transcription from both il17a and il17f promoters. This enhancer and both promoters exhibited Th17 lineage-specific DNA demethylation, accompanied by demethylation of lysine 27 of histone H3 (H3K27) and increased H3K4 methylation. Loss of DNA methylation tended to occur at STAT3 consensus elements, and we show that methylation of one of these elements in the il17a promoter directly inhibits STAT3 binding and transcriptional activity. These results demonstrate that TGFβ and IL-6 synergize to epigenetically poise the il17 loci for expression in Th17 cells, and suggest a general mechanism by which active STAT3 may be epigenetically excluded from STAT3-responsive genes in non-Th17 lineages.

Naive CD4+ T cells activated in the presence of TGFβ and IL-6, a physiologic stimulus provided by antigen-presenting cells that phagocytose apoptotic debris from pathogen-infected cells (1), gain the capacity to secrete inflammatory cytokines IL-17A and IL-17F. IL-17F is involved in host defense at mucosal surfaces, whereas IL17A mediates immunity to fungi and bacteria and is involved in autoimmune disease processes such as multiple sclerosis and inflammatory bowel disease (reviewed in Ref. 2). IL-17-producing Th17-2 cells behave as a distinct lineage from cells of the Th1, Th2, and regulatory T cell lineages, which do not produce IL-17, suggesting that distinct transcriptional mechanisms may control il17 gene expression. Genetic studies have established that a combination of STAT3, Runx1, Batf, and IRF4 and the nuclear receptors RORyt, RORα, and Ahr is involved in the induction of IL-17 during Th17 differentiation (reviewed in Ref. 3), but why so many factors are required and how they interact with the genomic architecture of the closely linked il17a and il17f genes is not known.

We show that the il17a promoter is primarily responsive to STAT3 or Runx1, whereas the il17f promoter is largely RORyt-responsive, and neither promoter is capable of supporting significant transcriptional cooperativity among these three factors. Cytokine genes are commonly regulated by long distance cis regulatory elements (4); therefore, we interrogated seven noncoding sequences (CNS) across an ~120-kb region of the mouse genome encompassing the il17a and il17f loci and identified two intergenic regions capable of enhancing transcription from the il17a and il17f promoters. One of these elements is located 5 kb upstream of the il17a gene and corresponds to a previously identified enhancer (5). We now show that this enhancer is particularly responsive to RORyt and does not synergize with STAT3 or Runx1. The second element is located between the two il17 genes, 28 kb downstream of il17a and 24 kb downstream of il17f. This is a previously unidentified enhancer, and we show that it synergizes with STAT3, RORyt, and Runx1 to drive transcription from both il17a and il17f promoters.

Like Th17 differentiation from naive CD4+ precursors, astrocyte differentiation from neuroepithelial precursors in the developing brain is driven by the combination of a TGFβ family member bone morphogenetic protein 2 (BMP2) and an IL-6 family member leukemia inhibitory factor (LIF) (6). LIF/BMP2 cooperativity in this system results in demethylation of STAT3 cis elements in astrocyte-specific gene promoters, an epigenetic process required for the astrocyte cell fate decision. We therefore hypothesized that lineage-specific induction of the il17 kinase-like orphan receptor; BMP2, bone morphogenetic protein 2; LIF, leukemia inhibitory factor; PMA, phorbol 12-myristate 13-acetate; NFAT, nuclear factor of activated T-cells; Ab, antibodies; TSS, transcription start site.

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▲This article contains supplemental Table 1.
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2 The abbreviations used are: Th, T helper; CNS, conserved noncoding sequence(s); H3K27me3, trimethylation of lysine 27 of histone H3; H3K4me3, trimethylation of lysine 4 of histone H3; ROR, receptor tyrosine kinase-like orphan receptor; BMP2, bone morphogenetic protein 2; LIF, leukemia inhibitory factor; PMA, phorbol 12-myristate 13-acetate; NFAT, nuclear factor of activated T-cells; Ab, antibodies; TSS, transcription start site.
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gene by TGFβ and IL-6 in Th17 cells may be regulated by an analogous mechanism. We demonstrate that CpG dinucleotides in the il17a promoter, the +28 kb enhancer, and the il17f promoter are preferentially demethylated during Th17 lineage commitment and that DNA methylation directly blocks STAT3 binding to a CpG dinucleotide sequence required for full il17a promoter activity. These studies demonstrate that the il17a and il17f genes are regulated transcriptionally by at least two intergenic enhancers, and epigenetically by DNA methylation, which acts as a biochemical switch for STAT3 binding.

EXPERIMENTAL PROCEDURES

Mice—C57BL/6 mice, 4–6 weeks old, were obtained from The Jackson Laboratory and maintained at the laboratory animal facility of The Children’s Hospital of Philadelphia. All animal experiments were conducted according to approved institutional protocols and guidelines.

Monoclonal Antibodies—Monoclonal antibodies against CD3ε (2C11) and CD28 (37.51) Ab were purchased from Bio-Express. Mouse recombinant IL-4 and monoclonal Ab against IL-4 (11B11), IFNγ (XMG1), and IL-12 (17.8) were purchased from BD Biosciences. Recombinant IL-12, IL-6, and IL-23 were purchased from eBioscience. TGFβ was purchased from Promega. All molecular biology reagents were analytical grade and purchased from Sigma-Aldrich.

Vectors—Vectors pGL4-il17a containing the 0.6-kb promoter (5), pRC/CMV-STAT3C (7), pTATA-TK-Luc containing four STAT3 binding elements, and retroviral MIGR-RORγt were obtained from Addgene. MIGR1-Runx1 was a obtained from S. Sakaguchi (Kyoto University, Japan). Control Renilla-luciferase vector was obtained from Promega. Mutations were introduced in the STAT3 binding site in the il17a 0.6-kb promoter vector using a mutagenesis kit (Stratagene). Conserved noncoding sequence elements surrounding the il17a–il17f loci were cloned into the il17a promoter vector at the XhoI site following the protocol provided by the vendor, bound protein complexes bound to the DNA probes. Beads were washed following the protocol provided by the vendor, bound protein complexes were eluted with Laemmli buffer, and STAT3 was visualized by immunoblot using antibody (C-20) purchased from Santa Cruz Biotechnologies.

Cell Culture—Single cell suspensions of spleen and lymph node were prepared, CD8+ cells were depleted using Miltenyi CD8 microbeads and columns, and the remaining CD4+ T cells and antigen presenting cell were cultured with soluble anti-CD3 and anti-CD28 (1 μg/ml each) for 4 days in the presence of IL-12 (10 ng/ml) and anti-IL-4 Ab (10 μg/ml) for Th1, with IL-4 (40 ng/ml), anti-IFNγ (10 μg/ml), and anti-IL-12 (10 μg/ml) for Th2, with TGFβ (5 ng/ml) and IL-2 (20 units/ml) for iTreg, or with anti-IL-4 (20 μg/ml), anti-IFNγ (20 μg/ml), TGFβ (1 ng/ml), and IL-6 (10 ng/ml) for Th17. In a separate batch of Th17 cultures, IL-23 (10 ng/ml) was also added. After 4 days, cells were harvested, washed, and restimulated for 48 h with plate-bound anti-CD3 and anti-CD28 (1 μg/ml each). For intracellular staining, restimulated cells were boosted with PMA (10 ng/ml) and ionomycin (1 μM) for 5 h in the presence of Golgi stop reagent (BD Biosciences). For histone and DNA methylation analysis, CD4+ cells were purified from the restimulated cultures (96% purity) using Miltenyi columns.

DNA Methylation—DNA methylation at the il17a–il17f multilocus region was assessed by sodium bisulfite conversion and cloning as described previously (8). Genomic DNA isolated from T helper cells was treated with sodium bisulfite to convert cytosines to uracil. The cis regulatory elements described were PCR-amplified (supplemental Table 1) and cloned into PGEM-T easy vector (Promega), and plasmid DNA isolated from 25–30 clones was sequenced.

Chromatin Immunoprecipitation (ChIP) Analysis—T helper cultures were formaldehyde-fixed, and chromatin was sheared and solubilized by sonication. Chromatin containing histone 3 lysine 4 trimethylation (H3K4me3) and histone 3 lysine 27 trimethylation (H3K27me3) was enriched using specific antibodies (Millipore, Cell Signaling Technologies) and a ChIP assay kit (Millipore), and enriched DNA was purified. cis regulatory elements at the il17 and ifng loci were detected in pre-ChIP versus post-ChIP fractions and quantified by quantitative PCR (supplemental Table 1) as described (9).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA of STAT3 binding to [γ-32P]ATP-labeled double-stranded oligonucleotides representing sequences in the il17a promoter containing a STAT3 consensus element was determined using nuclear extracts prepared from Th17 cells. To check the methylation effect on STAT3 binding, a methylated probe was synthesized by incorporating the cytosine methylation at a CpG site located within the STAT3 binding element. EMSA reactions were performed as described previously (10).

Oligonucleotide Pulldown Assay—Oligonucleotide probes used for EMSA analysis were 5’-end-labeled with biotin and incubated (0.5 μM) with total cell lysate (400 μg) prepared from stimulated Th17 cells under the same ionic conditions used in EMSA reactions. Magnetic streptavidin–conjugated agarose beads (MagSelect, Sigma-Aldrich) were used to collect protein complexes bound to the DNA probes. Beads were washed following the protocol provided by the vendor, bound protein complexes were eluted with Laemmli buffer, and STAT3 was visualized by immunoblot using antibody (C-20) purchased from Santa Cruz Biotechnologies.

Transient Promoter-Reporter Assays—293T or EL4 cells were transfected with wild-type or mutant 0.6-kb il17a promoter-luciferase vectors for 48 h and stimulated with PMA (30 ng/ml) and ionomycin (1 μM) for 5 h before harvesting. Renilla luciferase driven by a constitutive vector was used to normalize the transfection. Transfection experiments were performed with cells co-transfected with STAT3C (constitutively active form), Runx1, or RORγt. STAT3-negative and STAT3-positive reporter luciferase vectors were used as positive and negative controls for STAT3 transfections. Bioluminescence produced by firefly versus Renilla luciferase was estimated using cell lysate prepared from the transfected cells by a Dual-Luciferase assay kit purchased from Promega.

RESULTS

Lineage-specific Histone Methylation across the il17a and il17f Loci in Th17 Cells—To study the epigenetic processes associated with induction versus silencing of il17 gene expres-
In differentiating CD4+ helper T cells, we generated Th1, Th2, and Th17 cells in vitro. As expected, when restimulated under neutral conditions, Th1 cells produced IFNγ but not IL-4, IL-17A, or IL-17F (Fig. 1, A–C), Th2 cells produced IL-4 but not IFNγ, IL-17A, or IL-17F (Fig. 1, D–F), and Th17 cells produced IL-17A and IL-17F but not IFNγ or IL-4 (Fig. 1, G–I). The cytokine IL-23 has been reported to enhance Th17 differentiation and is required for pathogenicity in vivo (11, 12). The addition of IL-23 to Th17 cultures did not influence IL-17A production, but augmented the secretion of IL-17F (Fig. 1, J–L).

To identify potential cis regulatory elements for the il17a and il17f loci, we measured methylation of lysine 4 of histone H3 (H3K4me3), a chromatin mark associated with gene activity or expression potential, and methylation of lysine 27 of histone H3 (H3K27me3), a chromatin mark associated with active gene silencing (reviewed in Ref. 13), at several defined (14, 15) evolutionarily conserved CNS surrounding the il17a and il17f genes (Fig. 2A). Naive CD4+ T cells exhibited significant H3K27 trimethylation at the il17a and il17f promoter regions and at CNS regions −60, −5, −2.5, +10, +23, +28, and +36 kb from the il17a TSS (Fig. 2B, left panel). The levels of H3K27me3 at the il17 loci were as high or higher than that observed at the ifnγ locus in naive cells (Fig. 2B, right panel). Although a small amount of H3K4 trimethylation could be detected near the ifnγ gene, the il17 multilocus regions was completely devoid of this mark. The chromatin at the il17a and il17f loci was maintained in this same configuration in differentiated Th1 cells (Fig. 2C), whereas the ifnγ locus exhibited the expected increase in H3K4me3 and a loss of H3K27me3. Th2 cells strongly silenced both of these genomic regions (Fig. 2D). Similar to previous studies (14, 16), we observed strong trimethylation of H3K4 at the il17a and il17f promoters, as well as at the −5, +10, +23, and +28 CNS, but not at the −60 CNS (Fig. 2E). The increase in H3K4 methylation was accompanied by a significant loss of the opposing H3K27me3 mark, except at −60 and −2.5 CNS, which maintained naive levels of the silencing H3K27me3
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A

B

C

D

E

F

naive
Th1
Th2
Th17
Th17 +IL-23
mark. Conversely, the ifnγ locus in these cells did not accumulate H3K4me3, but instead was subjected to increased methylation at lysine 27. Th17 cells cultured with IL-23, which led to increased IL-17F secretion (Fig. 1L), retained strong H3K4me3 levels at the il17f promoter, but actually reduced H3K4 trimethylation at the il17a locus (Fig. 2F). These results highlight intergenic regions that may be involved in the transcriptional control of the il17a and il17f loci and indicate that these genes are subject to epigenetic regulation at the level of chromatin remodeling.

The +28 Intergenic CNS Is a STAT3-, RORγt-, and Runx1-responsive Enhancer for il17 Transcription—To determine whether the CNS regions analyzed in Fig. 2 are directly involved in the regulation of il17 gene transcription, we cloned each CNS (Fig. 3A) in front of the il17a promoter or the il17f promoter and tested the capacity of these DNA elements to drive luciferase activity upon transient transfection in 293T cells. As reported previously (5, 17), the 0.6-kb il17a promoter was moderately active by itself in unmanipulated cells (Fig. 3D, yellow bars), and none of the CNS regions were able to augment transcription under these conditions (Fig. 3D, green to blue bars). IL-6 induces il17a gene expression through the activation of STAT3 (18, 19); therefore, we tested whether the CNS regions surrounding the il17a and il17f genes were responsive to the activity of this transcription factor. Co-transfection with a constitutively active form of STAT3 (STAT3C (Ref. 7)) resulted in increased promoter activity (Fig. 3D, yellow bars), and the +28 CNS was the only one of the seven conserved regions able to further enhance il17a promoter-driven transcription under these circumstances (Fig. 3D, blue bars). Consistent with this, in silico analysis of the +28 CNS sequence identified a strong STAT3 consensus element (Fig. 3C), and a previous genome-wide ChIP analysis indicates that STAT3 is bound to this region in Th17 cells (20). The transcription factor RORγt is specifically induced during Th17 differentiation and is required for il17 gene expression (21); therefore, we tested whether the CNS regions surrounding the il17a and il17f genes were responsive to the activity of this transcription factor. The −5 CNS was recently shown to contain an RORγt consensus element (Fig. 3F) and to enhance il17a promoter activity in response to RORγt (5). Consistent with this study, we found that co-transfection with RORγt did not affect minimal promoter activity (Fig. 3D, yellow bars), but the promoter became RORγt-responsive when linked to −5 CNS (Fig. 3D, green bars). However, in addition to the −5 CNS, we find that the +28 CNS is also able to cooperate with RORγt to drive transcription from the il17a promoter (Fig. 3D, blue bars). Consistent with the fact that the +28 CNS, but not the −5 CNS, contains consensus elements for both STAT3 and RORγt (Fig. 3, B and C), the +28 CNS was the only region capable of enhancing transcription over that of the minimal promoter when co-transfected with both STAT3C and RORγt (Fig. 3E, blue bars). The +28 enhancer also contains multiple putative binding sites for Runx1/AML1 (Fig. 3C), another factor known to transactivate the il17a promoter (5); therefore, we tested whether this enhancer is responsive to Runx1. The promoter itself was moderately responsive to Runx1 when expressed alone (Fig. 3E, yellow bars in empty vector versus Runx1), but neither the −5 CNS nor the +28 CNS was able to significantly enhance transcription under these conditions (Fig. 3E, green and blue bars). Likewise, when Runx1 was co-expressed with RORγt, it did not drive transcription from any of the promoter-enhancer constructs significantly more than RORγt did by itself (Fig. 3E, RORγt versus Runx1 + RORγt). However, Runx1 was able to cooperate strongly with STAT3 at both the promoter (Fig. 3E, yellow bars in Runx1 + STAT3) and the +28 enhancer (Fig. 3E, blue bars in Runx1 + STAT3) to drive il17a promoter activity to levels 4–5-fold higher than in the presence of either Runx1 or STAT3 alone.

Like the il17a promoter, the il17f promoter was also moderately responsive to STAT3 (Fig. 3F, yellow bars). However, unlike il17a, none of the intergenic CNS elements, including the +28 element, was able to further enhance il17f promoter activity under these conditions (Fig. 3F, STAT3C). The il17f promoter was likewise unresponsive to Runx1 (Fig. 3G, yellow bars in Runx1), and neither the −5 nor the +28 CNS elements were able to enhance il17f transcription in the presence of this transcription factor (Fig. 3G, green and blue bars in Runx1). The il17f promoter was responsive to RORγt (Fig. 3, F and G, yellow bars in RORγt), and both the −5 and the +28 CNS were able to further enhance il17f promoter activity in the presence of this transcription factor (Fig. 3, F and G, green and blue bars in RORγt). Unlike at the il17a promoter, RORγt was unable to synergize with STAT3 or Runx1 to drive transcription from the il17f promoter (Fig. 3G). These data indicate that STAT3, RORγt, and Runx1 synergize to transactivate the il17a/−5/+28 promoter-enhancer unit, whereas the il17f/−5/+28 promoter-enhancer unit is largely responsive to RORγt. These data establish the +28 CNS as an intergenic cis regulatory element that undergoes lineage-specific chromatin remodeling in Th17 cells and is capable of coordinating the activities of STAT3, RORγt, and Runx1 to enhance transcription from the il17a and il17f promoters.

Lineage-specific Demethylation of DNA at the +28 Enhancer and the il17a and il17f Promoters in Th17 Cells—Another important mode of epigenetic regulation occurs at the level of DNA methylation at CpG dinucleotides. CpG islands, defined as a >200-bp region with >55% GC content, are often found at cis regulatory regions and are used by cells to nucleate DNA methylation machinery at silenced genes (22). The il17 multi-locus region is devoid of CpG islands; however, the il17a promoter, the +28 enhancer, and the il17f promoter regions each contain approximately a dozen CpG dinucleotides (Fig. 4). Therefore, we measured DNA methylation at these elements to
determine whether this epigenetic mark is regulated in a lineage-specific manner in T helper cells.

CpG dinucleotides within the ~650-bp region upstream of the il17a TSS (Fig. 4A), an ~300-bp region including the +28 enhancer (Fig. 4B), and the region ~200–500 bp upstream of the il17f TSS (Fig. 4C) were all highly methylated (at frequencies of 80–100%) in naive CD4+ T cells (Fig. 4, first row). During Th1 and Th2 differentiation, CpG at the il17a and il17f promoters remained highly methylated, with the exception of the −33 dinucleotide adjacent to the TATA box (Fig. 4A, second and third rows) and the −245 dinucleotide in the il17f promoter (Fig. 4C, second and third rows), which were 60–70% methylated in these lineages. Th1 cells exhibited significant loss of methylation at the +28 enhancer (Fig. 4B, second row), whereas methylation was largely maintained in Th2 cells (Fig. 4B, third row). Conversely, differentiating Th17 cells exhibited a 80–90% loss of DNA methylation across an ~175-bp region of the il17a promoter (Fig. 4A, fourth row) and across an ~250-bp region upstream of the il17f gene (Fig. 4C, fourth row). The +28 enhancer also exhibited a marked loss of DNA methylation at CpG dinucleotides in Th17 cells, to a much larger extent than that observed in Th1 or Th2 cells (Fig. 4B, fourth row).
addition of IL-23, which selectively enhanced the secretion of IL-17F by Th17 cells (Fig. 1), caused preferential demethylation of the il17f promoter, particularly at the −326 CpG (Fig. 4C, fifth row). These data suggest that DNA demethylation at the il17 loci facilitates il17 gene expression.

In this in vitro culture model, Th17 differentiation is driven by the combination of TGFβ and IL-6 (in the presence of neutralizing Ab to IFNγ and IL-4). To determine the contribution of TGFβ receptor-coupled signaling versus IL-6 receptor-coupled signaling to the demethylation of DNA observed at the il17 loci in the Th17 lineage, we stimulated naive CD4+ T cells under the same Th17 culture conditions described above, except that either IL-6 was omitted (Fig. 4, sixth row) or TGFβ was omitted (Fig. 4, seventh row). Neither signals from IL-6 in the absence of TGFβ nor signals from TGFβ in the absence of IL-6 were able to induce full DNA demethylation at the il17a or il17f promoters (Fig. 4, A and C). However, TGFβ, which in the absence of IL-6 promotes regulatory T cell differentiation, was able to mediate partial demethylation of the il17a −144 CpG (Fig. 4A, sixth row). Furthermore, demethylation at the +28 CNS occurred in cells cultured in either TGFβ alone (Fig. 4B) or cells cultured with IL-6 alone (Fig. 4B). These data suggest that TGFβ by itself may be capable of partial epigenetic priming of the il17 loci, but that full demethylation of the il17a–f multilocus region and commitment to the Th17 lineage require the action of both cytokines.

**STAT3 Binding to the il17a Promoter Is Lineage-restricted and Is Inhibited by DNA Methylation**—Interestingly, the most drastic drop in DNA methylation in the Th17 lineage tended to occur at CpG dinucleotides within consensus elements for STAT3, e.g. −144 in the il17a promoter and −309 and −326 in the il17f promoter (Fig. 4, A and C, red asterisks). This led us to hypothesize that DNA methylation at STAT3 cis elements in non-Th17 cells inhibits STAT3 binding at the il17 genes. The minimal il17a promoter contains a putative STAT3 binding site at −144 (CCGTCG), and our results in Fig. 3 show that this promoter is STAT3-responsive. To determine whether this element directly contributes to the transcriptional activity of the il17a promoter, we mutated the second cytosine of this element to thymidine (CGTTCG), a substitution (indicated by bold) that would be expected to reduce the affinity of this sequence for STAT3 (23). When transiently transfected into EL-4 cells, the wild-type promoter was moderately active and was responsive to stimulation with PMA, ionomycin, and IL-6 (Fig. 5A, dark gray bars). The C → T mutation in the −144 element resulted in a 50–70% reduction in promoter activity under both resting and stimulated conditions (Fig. 5A, light gray bars). To specifically determine the effect of this mutation on STAT3 activity, we utilized constitutively active STAT3C. The wild-type il17a promoter showed moderate activity in 293T cells and was increased 2-fold when co-transfected with STAT3C (Fig. 5B, dark gray bars). However, when co-transfected with a promoter containing the mutated −144 element, the stimulatory activity of STAT3 was abrogated (Fig. 5B, light gray bar). These data demonstrate that the STAT3 consensus element at −144 is required for full il17a promoter activity; therefore, we tested whether DNA methylation of this element influences STAT3 binding.

To examine STAT3 binding in this region, we first measured STAT3 binding to the endogenous il17a promoter in primary T helper subsets by chromatin immunoprecipitation analysis. Consistent with a previous study (24), we detected strong binding of native STAT3 to the il17a promoter in activated Th17 cells (Fig. 5C). However, to test whether STAT3 occupancy at
The *il17a* promoter is lineage-restricted, we also examined STAT3 binding in naive, Th2, and Th1 cells. As expected, no STAT3 occupancy was detected in naive CD4+ T cells or activated Th2 cells (Fig. 5C). However, we also failed to detect STAT3 binding at the *il17a* promoter in activated Th1 cells (Fig. 5C) despite the fact that IL-12 activates STAT3 in addition to STAT4 in murine Th1 cells (25). These data suggest that a gene-proximal checkpoint restricts STAT3 binding to the *il17a* promoter in non-Th17 cells.

To directly test whether methylation of the *il17a* promoter opposes STAT3 DNA binding, we probed nuclear extracts of primary Th17 cells with a radiolabeled dsDNA oligonucleotide representing the putative −144 STAT3 element (CCpGTCA) from the *il17a* promoter using an electrophoretic mobility shift approach. This sequence, which simulates the unmethylated −144 site as it would exist in Th17 cells (i.e. Fig. 4A), formed two protein-DNA complexes (Fig. 5D, lanes 4 and 5). These data demonstrate a sequence-specific, STAT3-dependent binding activity toward the −144 *il17a* promoter element in Th17 cells. To simulate the −144 STAT3 site as it would exist in non-Th17 cells (i.e. Fig. 4A), we used an oligonucleotide that was synthetically methylated at the CpG dinucleotide (CC(CH3)pGTCA) to probe Th17 nuclear extracts. This methylated STAT3 oligonucleotide completely failed to form complex II and exhibited a reduced capacity to form complex I (Fig. 5D, lanes 6–8). Indeed, although the unmethylated −144 element competed very effectively with its own binding (Fig. 5D, lanes 11 and 12), the methylated form was a much less efficient competitor of the unmethylated element binding activity (Fig. 5D, lane 9), comparable with a control NFAT/AP-1 site in the ARRE-2 element from the *il2* promoter (Fig. 5D, lane 13), and only showed partial inhibition of −144 binding at greater excess (Fig. 5D, lane 10). These EMSA data show that methylation of the −144 STAT3 element in the *il17a* promoter greatly reduces its binding affinity.

In a separate approach, we coupled the unmethylated CCpGTCA or methylated CC(CH3)pGTCA oligonucleotides to streptavidin beads. These beads were then incubated with extracts from Th17 cells, washed, and subjected to SDS-PAGE followed by immunoblot analysis with an antibody specific for STAT3. These experiments showed that native STAT3 from Th17 cells binds to the unmethylated −144 *il17a* promoter element (Fig. 5E, lane 3) with an affinity comparable with the binding of STAT3 to a defined STAT3 element from the *c-fos* promoter (Fig. 5E, lane 2). This binding was sequence-specific as a control NFAT/AP-1 site in the ARRE-2 element from the *il2* promoter was unable to precipitate STAT3 (Fig. 5E, lane 2). However, STAT3 exhibited greatly reduced binding to the methylated −144 *il17a* promoter element (Fig. 5E, lane 4). Furthermore, although the *c-fos* STAT3 element and the unmethylated −144 element efficiently competed with −144 for binding to STAT3 (Fig. 5E, lanes 6 and 8), the methylated −144 was a less efficient competitor of STAT3 binding to the unmethylated element.
lated −144 element (Fig. 5E, lane 7). These data demonstrate that the −144 CpG, which is demethylated specifically in differentiating Th17 cells, resides in a bona fide STAT3 binding element that is required for full il17a promoter activity. Binding of STAT3 to this element is inhibited by DNA methylation, establishing this process as an epigenetic switch that regulates il17 gene expression during T helper differentiation.

DISCUSSION

In this study, we show that the closely linked il17a and il17f loci exhibit Th17 lineage-specific epigenetic modification of histones and DNA. In particular, marked DNA demethylation occurred at the il17a promoter, at the il17f promoter, and at an intergenic conserved noncoding sequence +28 kb from the il17a gene in CD4+ T cells stimulated in the presence of both TGFβ and IL-6. One of the CpG dinucleotides preferentially demethylated in Th17 cells is located within a STAT3 consen-
sus element in the il17a promoter. We show that this element is a bona fide STAT3 binding site, that this element is required for full il17a promoter activity, and that DNA methylation at this site abrogates STAT3 binding.

These data suggest that CD4+ T cells use methylation of STAT3 DNA elements in cell type-specific promoters as an epigenetic switch for regulation of cell fate decisions during an immune response. An analogous mechanism has been shown to regulate the astrocyte versus neuronal cell fate decision during brain development (6). Similar to Th17 differentiation from naive CD4+ T cells, astrocyte differentiation from neuroepithelial precursors is driven by the combined action of the IL-6 family member LIF and the TGFβ family member BMP2. Combined LIF-BMP2 signaling leads to demethylation of a STAT3 element in the promoter of the gene encoding the glial fibrillary acidic protein (GFAP), a STAT3-dependent factor required for astrocyte differentiation. We therefore hypothesized that TGFβ receptor-coupled morphogenic signals during T cell activation might lead to the initial demethylation of STAT3 elements in the il17a and il17f promoters, whereas IL-6 would induce the STAT3 activity required to transactivate these and other Th17-specific genes. We tested this and found that TGFβ in the absence of IL-6 was able to mediate full demethylation of the +28-kb enhancer and partial demethylation of the STAT3 element in the il17a promoter (Fig. 4). This partial priming of the il17 locus provides an explanation for previous results in which CD4+ T cells expressing constitutively active STAT3 exhibited IL-17 production when stimulated with TGFβ in the absence of IL-6 (18, 19). However, IL-17 production under these circumstances was still ~10-fold lower than that produced by cells stimulated with TGFβ and IL-6, suggesting that STAT3-independent IL-6 receptor signaling may be involved and/or that the residual DNA methylation at the il17a−f loci under these conditions can oppose STAT3 activity.

The cytokine IL-23 is required for in vivo Th17 function, but IL-6 can substitute for its activity in vitro (19). Consistent with this, we found that IL-23 did not influence IL-17A production in TGFβ plus IL-6 cultures, but did augment IL-17F secretion in a manner associated with reduced DNA methylation at the −326 STAT3 consensus element in the il17f promoter. IL-23 has also been shown to promote the appearance of IFN-γ-pro-
ducing Th17 cells in vivo during the development of experimental autoimmune encephalitis (26). However, we found no effect of IL-23 on either histone or DNA methylation at the ifng locus in our short term in vitro Th17 cultures,3 suggesting that IL-23 acts indirectly to promote IFN-γ production by Th17 cells in vivo or that factors in addition to IL-23 are required for this phenomenon.

A combination of several transcription factors including STAT3, RORγt, and Runx1 is required to induce il17 expression. Deletional analysis of the il17a promoter by Ichiyama et al. (17) indicated that the core promoter is contained within the first ~300 bp of the region upstream of the TSS, and Zhang et al. (5) found that inclusion of sequences up to −2 kb increased transcriptional activity. Whether the −2-kb region is a bona fide part of the promoter versus a proximal enhancer (e.g. like the il2 promoter/enhancer) was not determined; therefore, we chose to use the −0.6-kb region as the promoter in our studies. We find that the −0.6-kb il17a promoter contains a bona fide STAT3 cis element and is highly STAT3-responsive, whereas it is not responsive to RORγt and is only mildly responsive to Runx1. The il17f promoter has not been previously characterized to our knowledge. We determined that the region 0.6 kb upstream of the il17f coding region contains a well defined TATA box and multiple ROR, AP-1, NFAT, and Runx1 consensus elements and was demethylated specifically in Th17 cells. Therefore, we used this region as the promoter in our study. Our results demonstrate that although the il17a promoter is responsive to STAT3 and Runx1, these factors exhibit no cooperativity at this promoter. Likewise, we show that the il17f promoter is moderately STAT3-responsive, highly RORγt-responsive, but by itself is also incapable of supporting synergy between STAT3, RORγt, and Runx1.

The −5-kb CNS and the +28-kb CNS exhibit permissive epigenetic marks and DNase hypersensitivity in activated Th17 cells (our studies and Ref. 14) and were able to enhance transcription from both promoters in transient reporter assays, indicating that these enhancers are part of the transcriptional architecture of the il17a and il17f multilocus region. The −5-kb CNS was previously shown to enhance transcription from a −2-kb il17a upstream construct in response to RORγt and Runx1 (5). Our studies confirm that the −5-kb enhancer is highly RORγt-responsive; however, this CNS was unable to enhance transcription from the minimal −0.6-kb promoter in response to either STAT3 or Runx1. The −5-kb CNS was also able to enhance transcription from the already highly RORγt-responsive il17f promoter, but did not respond to Runx1 or STAT3, individually or in combination. Therefore, we conclude that the primary role for the −5-kb enhancer is to coordinate RORγt activity and that another element must provide cooperativity to the il17a−il17f transcriptional unit.

It is possible that the action of multiple cis regulatory elements, although these elements are not capable of organizing cooperativity between STAT3, Runx1, and RORγt as a single element, might synergize to drive transcription when brought together in space. However, the combinatorial possibilities for

3 R. M. Thomas, H. Sai, and A. D. Wells, unpublished data.
such cooperativity in cis within the ~120-kb il17a–il17f multilocus region appears to be limited as our studies indicate that the ~5-kb CNS and the promoters do not synergize under conditions of forced proximity and that the other intergenic CNS fail to exhibit enhancer activity in the context of either promoter. This could be explained by the fact that the −2.5-kb CNS retains a significant level of H3K27me3 mark in the Th17 lineage and contains no STAT3 or ROR consensus elements, and the +36-kb CNS exhibits low levels of H3K4me3 and DNase hypersensitivity in Th17 cells (our studies and Ref. 14), contains only a weak match for a STAT3 consensus element, and does not contain an ROR site. The +23 CNS is primed in Th17 cells (i.e. accumulates high levels of the H3K4me3 mark and loses H3K27me3), but contains no STAT3 nor ROR consensus elements. The −60 CNS contains a single, weak match for the STAT3 consensus element and a single weak match for the ROR consensus, but this region remains epigenetically silent (i.e. high H3K27me3) in the Th17 lineage. The +10 CNS exhibits a strong change in H3K4 and H3K27 trimethylation and DNase hypersensitivity in Th17 cells (our studies and Ref. 14), contains a strong STAT consensus element, and binds STAT3 in vivo in activated Th17 cells (20). Why this element does not enhance in our system is not clear, but as with all these CNS, it is possible that additional factors not expressed in 293T cells may be required.

However, an excellent candidate for an element that per se supports cooperativity among the various factors required for IL-17 production is the +28-kb CNS, which we show is a strong enhancer of transcription from both the il17a and the il17f promoters. Although many of the CNS examined here exhibited DNase hypersensitivity in activated Th17 cells in the study by Mukasa et al. (14), the +28-kb CNS was the only CNS that showed constitutive hypersensitivity in resting Th17 cells, indicating the presence of a unique chromatin structure in this region. This enhancer shows strong STAT3 binding in vivo (20) and is highly responsive to STAT3 in our transient reporter assays. This enhancer is also responsive to RORγt and Runx1, but importantly, this enhancer is able to support transcriptional cooperativity between all three factors when expressed together. This CNS may also cooperate with additional DNA-binding proteins as this region also contains putative binding sites for SP1 and NFAT. Interestingly, one of these NFAT sites contains a CpG dinucleotide that we show is methylated in non-Th17 cells and unmethylated in Th17 cells. Methylation of an NFAT element has been shown to block NFAT binding and transactivation of the human il2 promoter (27), suggesting that DNA methylation could act in an analogous manner at the il17 loci to oppose NFAT-mediated transactivation. The +28-kb enhancer also contains multiple AP-1 consensus elements, suggesting that Fos/Jun heterodimers may act at this region. Alternatively, these sites may recruit Batf, an alternative Jun partner that was recently shown to be required for il17a gene expression (28). Indeed, this previous study demonstrated Batf binding to the endogenous +28 CNS region in Th17 cells, further indicating that this factor transactivates via interacting with the +28-kb enhancer. Therefore, we propose that the +28-kb enhancer is an integral component of the il17a–il17f transcriptional architecture that organizes the activity of multiple transcription factors around the il17a and il17f promoters. Although this work does not strictly establish whether the +28-kb CNS is required for Th17 development, these studies do establish the element as a classically defined enhancer that can be targeted by homologous recombination in future studies.

In summary, we show that a conserved intergenic element can coordinate the activities of STAT3, RORγt, and Runx1 to enhance transcription from the il17a and il17f promoters and that Th17 cells utilize an evolutionarily conserved mechanism involving methylation at DNA binding elements to restrict STAT3 activity at the il17a gene in non-Th17 lineages.

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