The Journal of Immunology
RESEARCH ARTICLE | MARCH 15 2011

Epigenetic Changes at *Il12rb2* and *Tbx21* in Relation to Plasticity Behavior of Th17 Cells FREE

David Bending; ... et. al

J Immunol (2011) 186 (6): 3373–3382.
https://doi.org/10.4049/jimmunol.1003216

Epigenetic Changes at *Il12rb2* and *Tbx21* in Relation to Plasticity Behavior of Th17 Cells

David Bending,* Stephen Newland,* Alena Krejčí,† Jenny M. Phillips,* Sarah Bray,‡ and Anne Cooke*

Plasticity within Th cell populations may play a role in enabling site-specific immune responses to infections while limiting tissue destruction. Epigenetic processes are fundamental to such plasticity; however, to date, most investigations have focused on in vitro-generated T cells. In this study, we have examined the molecular mechanisms underpinning murine Th17 plasticity in vivo by assessing H3K4 and H3K27 trimethylation marks at *Tbx21*, *Rorc*, *Il17a*, *Ifng*, and *Il12rb2* loci in purified ex vivo-isolated and in vitro-generated Th17 cells. Although both populations had largely comparable epigenetic signatures, including bivalent marks at *Tbx21*, freshly isolated ex vivo Th17 cells displayed restricted expression from *Il12rb2* due to the presence of repressive chromatin modifications. This receptor, however, could be upregulated on isolated ex vivo Th17 cells after in vitro activation or by in vivo immunization and was augmented by the presence of IFN-γ. Such activated cells could then be deviated toward a Th1-like profile. We show that IL-12 stimulation removes H3K27 trimethylation modifications at *Tbx21*/T-bet leading to enhanced T-bet expression with in vitro Th17 cells. Our study reveals important potential phenotypic differences between ex vivo- and in vitro-generated Th17 cells and provides mechanistic insight into Th17 cell plasticity. *The Journal of Immunology*, 2011, 186: 3373–3382.

Naive Th cells can differentiate into several effector phenotypes (e.g., Th1, Th2, or Th17) in response to different stimuli (1–4). These effector cells have been categorized into lineages according to the production of signature cytokines and the expression of unique transcription factors. IL-12 drives naive Th cells to differentiate into IFN-γ–producing Th1 cells through the induction of the master transcription factor T-bet (5). The presence of IL-4 results in the induction of Th2 cells in a STAT6-dependent manner (6). The transcription factor GATA-3 is also required for the Th2 program, and these cells secrete the cytokines IL-4, IL-5, and IL-13. A third lineage of effector T cells called Th17 cells has also been described (3, 7, 8). Despite recent debate over the exact differentiation requirements for Th17 cells (9–11), it has been well documented that TGF-β and IL-6 are potent Th17-polarizing factors (12, 13). The Th17 program is dependent on the transcription factors retinoic acid-related orphan receptor (ROR)α and RORγt (14, 15). Additionally, the cytokine IL-23 has been shown to be involved in the Th17 response. In vitro studies have indicated that IL-23 is not involved in the initial polarization of Th17 cells but plays a role in their expansion and survival (12, 13). IL-23 has, furthermore, been demonstrated to be essential for the terminal differentiation of Th17 cells in vivo (16).

*Department of Pathology, University of Cambridge, Cambridge CB2 1QP, United Kingdom; †Biology Centre, University of South Bohemia, 370 05 Ceske Budejovice, Czech Republic; and ‡Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 1QP, United Kingdom

Received for publication September 28, 2010. Accepted for publication January 11, 2011.

A.K. is funded by the Medical Research Council. D.B. is funded by the Biotechnology and Biological Sciences Research Council.

Address correspondence and reprint requests to Anne Cooke, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, United Kingdom. E-mail address: ac@mole.bio.cam.ac.uk

The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; ROR, retinoic acid-related orphan receptor; Treg, regulatory T cell; UTR, untranslated region.

It is believed that Th1 and Th2 cells represent separate Th lineages because long-term cultured Th1 cells are refractory to Th2 deviation by IL-4, and, conversely, Th2 cells are resistant to IL-12–mediated conversion to Th1 (17). However, even Th2 cells appear more plastic than previously thought, and in the presence of strong Th1 conditions (type 1 IFNs and IL-12), they can adopt a mixed Th2/1 phenotype (18). Analysis of chromatin modifications at the *Ifng* (IFN-γ) and *Il4* (IL-4) gene loci in these cells has suggested that epigenetic mechanisms play an important role in mediating lineage commitment (1, 19, 20), and it is widely believed that histone modifications are important in the regulation of gene expression (21). For example, histone three lysine nine acetylation and histone three lysine four trimethylation (H3K4me3) are associated with actively transcribed genes (22, 23), whereas histone three lysine nine and histone three lysine twenty-seven trimethylation (H3K27me3) are associated with gene repression (24). Under certain circumstances, H3K4me3 and H3K27me3 modifications colocalize to the same regions, so-called "bivalent" domains (25), a signature that has been observed at the promoters of some genes in embryonic stem cells and in other examples where cells retain some plasticity (25, 26). These bivalent modifications are usually associated with low levels of transcription (25) and are thought to poise genes for activation or repression during cell differentiation.

A recent study that globally mapped H3K4me3 and H3K27me3 marks in many T cell effector lineages concluded that the promoter of the gene *Tbx21* (T-bet) exhibits bivalent modifications in most in vitro-differentiated T cells, including in vitro-differentiated Th17 cells (27). T-bet is a key transcription factor in Th1 cell development (5), and these results therefore suggest that this master regulator remains poised in Th17 cells implying that they retain plasticity and could reactivate T-bet given the right environmental cues (27). Indeed, in vitro-polarized Th17 cells have now been shown to exhibit plasticity both in vitro (28–31) and in lymphopenic environments in vivo (28, 29, 32). One mechanism proposed for this plasticity was that they retain expression of the IL-12 receptor thus making them capable of responding in vitro to

the Th1-promoting cytokine IL-12 (28, 29). Furthermore, IL-12 stimulation brings about chromatin modifications at the *Ifng* and *Il17a* loci to resemble those present in Th1 cells (33). From these studies, it has been proposed that the *Ifng* locus remains accessible in Th17 cells, enabling rapid upregulation of this cytokine in response to appropriate environmental stimuli.

The challenges in investigating the epigenetic mechanisms underlying T cell differentiation arise first from the fact that plasticity has been studied in cells that have been cultured in vitro using different cytokine environments. In such populations, the fraction of cells that differentiate into a particular subtype varies, making it potentially difficult to distinguish whether there are bivalent modifications in a single cell type versus a mixed population of cells with differing modifications. Second, all studies have been conducted using in vitro-generated cells with the assumption that in vitro or in vivo reprogramming of these cells achieves cell states that are analogous to effector cells in vivo. However, it appears that Th17 cells isolated ex vivo have a stable cytokine secretion pattern upon reculture with IL-12 in vitro (30), raising the question as to whether Th17 plasticity is an artifact of in vitro-generated Th17 cells or whether plasticity in ex vivo Th17 cells requires different conditions. For instance, the expression status of the cytokine receptor *Il12rb2* on ex vivo Th17 cells is currently unknown. Third, because Th17 cell plasticity is both STAT4 and *Tbx21*/T-bet dependent (33, 34), it is important to determine the epigenetic profiles at genes such as *Il12rb2* (whose expression is needed to permit IL-12/Stat4 signaling) and *Tbx21*. This will reveal whether Th17 cells, when reprogrammed via IL-12, acquire a bona fide Th1 epigenetic state or whether they still retain some Th17-associated signatures.

To address these issues, we have profiled H3K4me3 and H3K27me3 modifications at genes whose expression is characteristic of Th1 (*Tbx21*/T-bet, *Ifng*, and *Il12rb2*) or Th17 (*Rorc* and *Il17a*) cells purified ex vivo or generated in vitro. Even in these purified Th17 cells, bivalent modifications are still detected at the promoter of *Tbx21*/T-bet arguing that both ex vivo and in vitro Th17 cells retain the potential for expression of this gene. However, freshly isolated ex vivo Th17 cells had restricted expression of *Il12rb2* with repressive H3K27me3 modifications at this locus—a stark difference compared with in vitro Th17 cells. Notably, in vitro activation of these ex vivo Th17 cells is sufficient to induce chromatin remodeling at the *I12rb2* locus to a permissive H3K4me3 dominant state. This permits comparable phenotype deviation to the Th1 profile as observed with in vitro Th17 cells upon secondary culture of ex vivo Th17 cells with IFN-γ and IL-12. In addition, the repressive H3K27me3 modification at the *Tbx21* (T-bet) promoter is lost rapidly upon IL-12 stimulation suggesting that this is an early event in the phenotypic transition of Th17 cells to Th1-like cells. Additionally, examination of Th17 cells isolated from immunized mice displayed elevated *I12rb2* expression providing supportive evidence of our findings. Taken together, these data provide evidence of phenotype plasticity in ex vivo Th17 cells and emphasize the critical role played by cellular activation and the cytokine milieu in eliciting a change in their program. These results also provide further mechanistic insight into the molecular control of plasticity of Th17 cells and highlight an important difference between in vitro-generated and "natural" Th17 cells.

Materials and Methods

Mice

BDC2.5NOD and NOD mice were maintained under barrier conditions in the Biological Services facility of the Department of Pathology at the University of Cambridge. All animal experiments were done according to institutional guidelines and UK Home Office regulations.

Abs and flow cytometry

Anti–IFN-γ (XMG1.2), anti-CD3 (145.2C11), anti–IL-4 (11B11), anti-CD28 (37.51), and mouse IgG1 (OX-1) were grown from hybridomas in our own laboratory. Commercial Abs used were anti-CD4 Alexa 647 or PerCP conjugates (RM4-5), anti-CD25 PE (PC61), anti-CD44 FITC (IM7), anti–IFN-γ PE, PE-Cy7 or FITC conjugates (XMG1.2), biotinylated anti–IL-17A (TC11-8H4.1), and anti-CCR6 Alexa 647 (140706) (all BD Biosciences). Anti–IL-17A PE, FITC or Alexa 647 conjugates (eBIO17B7), and biotinylated anti-CD45.1 (A20) was obtained from eBioscience. Intracellular staining was performed as described previously (28).

In vitro T cell differentiation

$CD4^+$ T cells were first column purified by magnetic beads (Miltenyi Biotec). Naive CD4 T cells were subsequently sorted by flow cytometry ($CD4^+CD25^-CD44^{lo}$) and cultured in IMDM (Invitrogen) supplemented with 10% heat-inactivated FCS (Biosource), 2×10^{-3} M L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M mercaptoethanol (all Sigma). For generation of Th17 and Th1 polarized cells, naive T cells were cultured on anti-CD3 (2 μg/ml) and anti-CD28 (10 μg/ml) coated 24-well plates in the presence of either 25 ng/ml IL-6 (Peprotech), 10 ng/ml IL-1β (Invitrogen), 2 ng/ml recombinant human TGF-β (R&D Systems), 50 μg/ml anti–IFN-γ (XMG1.2), and 10 μg/ml anti–IL-4 (1B11) blocking Abs (Th17 condition) or with 10 ng/ml IL-12 (Peprotech), 100 U/ml IFN-γ (R&D Systems), and 100 U/ml human recombinant IL-2 for Th1 polarization.

T cells were cultured for 4 d (except in Fig. 8, where the Th1 cells were cultured for 5 d), and then Th17 cells were transferred to fresh plates without stimulus and rested for 4 d. Thereafter, Th17 cells were restimulated on anti-CD3 (2 μg/ml) and anti-CD28 (10 μg/ml) coated plates in the presence of 20 ng/ml IL-23 (R&D Systems) and 1 ng/ml TGF-β for 2 d before purification by the stated cytokine capture protocol.

For in vitro conversion of purified in vitro-generated Th17 cells, 1×10^5 cells were placed on anti-CD3 (2 μg/ml) and anti-CD28 (10 μg/ml) coated 24-well plates in the presence of either 2 ng/ml TGF-β, 20 ng/ml IL-23 (R&D Systems), or 10 ng/ml IL-12 (Peprotech) plus 100 U/ml IFN-γ (R&D Systems) plus 100 U/ml human recombinant IL-2 for 5 d or 24 h (Fig. 9) before harvesting for chromatin immunoprecipitation (ChIP).

For in vitro conversion of purified ex vivo-isolated Th17 cells, the cells were cultured with irradiated (30 cGy), CD4-depleted (by magnetic column separation [Miltenyi Biotec]) BDC2.5NOD splenocytes and 0.4 μg/ml of a BDC2.5 mimotope (acetyl-RTRPLWVRME-NH-2) from Southampton Polypeptides (35) for 5–6 d in the presence of medium alone, 20 ng/ml IL-23 (R&D Systems), or 100 U/ml IFN-γ (R&D Systems) and 10 ng/ml IL-12 (Peprotech). For Fig 5*D*, cultures were set up the same as stated directly above with 20 ng/ml IL-23 with or without 50 μg/ml of anti–IFN-γ Ab (XMG1.2) before cells were harvested, and RNA was extracted after 4 d of culture.

For secondary culture of ex vivo Th17 cells (Fig. 7), primary cultures were set up as above with 20 ng/ml IL-23 for 4 d to expand the Th17 cell population. Cells were then harvested and stained for CD4 before sorting by MoFlo. Th17 cells (2×10^4) were then recultured with 2×10^4 irradiated (30 cGy), CD4-depleted NOD splenocytes and 0.4 μg/ml of a BDC2.5 mimotope (acetyl-RTRPLWVRME-NH-2) for 4 d in the presence of 20 ng/ml IL-23 (R&D Systems) or 100 U/ml IFN-γ plus 10 ng/ml IL-12 (Peprotech).

Th17 cell purification by cytokine capture

The method for tetramer formation and IL-17 capture was adapted from Streeck et al. (36). IL-17A tetramers were made as previously described (28). To obtain purified Th17 cells, naive T cells were differentiated, rested, and restimulated as previously described. Cells were harvested, washed three times in 2% FCS PBS, and 4×10^6 cells were incubated on ice for 15 min with 1 aliquot of IL-17 tetramer. Tubes were then topped up with 50 ml culture medium and placed on a rotator for 1 h at 37°C to allow cytokine capture, followed by washing and staining with anti–IL-17A PE (eBioscience) and subsequent cell sorting (Figs. 8, 9, Supplemental Fig. 2).

Isolation of Th17 cells using commercial cytokine capture

$CD4^+$ T cells were isolated from the spleens of unimmunized, 3- to 6-mo-old BDC2.5NOD mice by column purification (Miltenyi Biotec), and then ex vivo $CD4^+$ T cells or day 4 in vitro-polarized Th17 cells (5×10^6 cells/ml) were stimulated for 1 h 30 min in culture with 500 ng/ml phorbyl 12,13-dibutyrate and ionomycin (both Sigma). The cytokine capture method was then performed according to the manufacturer's instructions (Miltenyi Biotec). Cells were stained with biotin anti–IL-17A followed by PE anti-biotin and anti-CCR6 Alexa 647. Cells were sorted on a MoFlo

cell sorter for $CD4^+CCR6^+IL\text{-}17A^+$ for ex vivo Th17 cells or $CD4^+IL\text{-}17A^+$ for in vitro-generated Th17 cells (Figs. 1–7, Supplemental Fig. 3).

Immunization of BDC2.5NOD mice

BDC2.5NOD mice were immunized with an emulsion containing 3 μg of a BDC2.5 mimotope (acetyl-RTRPLWVRME-NH-2) in CFA (H37 Ra; Difco Laboratories). Mice received 50-μl injections s.c. in each flank plus the base of the tail. Seven days later, inguinal and axillary lymph nodes were harvested and pooled, then $CD4^+$ T cells were isolated before cytokine capture was performed (Fig. 6*B*).

ChIP and DNA quantification

Live cells were sorted on a MoFlo cell sorter using propidium iodide (Sigma) (Figs. 8, 9, Supplemental Fig. 2) or by forward-scatter/side-scatter profiles. Cells [0.5–1 $\times$ 10^6 cells (Figs. 8 , 9, Supplemental Fig. 2); 1 $\times$ 10^5 to 1.5 $\times$ 10^5 cells (Figs. 1, 2, 5); and 4 $\times$ 10^4 cells (Fig. 4)] were cross-linked with 1% formaldehyde for 10 min, the reaction was quenched by 0.125 M glycine for 5 min, and the cells were washed four times with PBS and resuspended in nuclear lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS, 1× Roche complete inhibitor). Lysate was sonicated by Bioruptor (Diagenode) then diluted 10 times in immunoprecipitation dilution buffer (20 mM Tris-HCl pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.01% SDS, 1× Roche complete inhibitor), and precleared with normal rabbit serum (Sigma), mouse IgG1 (OX-1), and protein G agarose (Santa Cruz Biotechnology). Fifty microliters of precleared samples were taken as input controls, then ChIP reactions were performed overnight at 4°C with lysate and 1 μg of anti-H3K4me3 [Abcam ab8580, Millipore 07-473 (Fig. 5*C*)] or 5–10 μg of anti-H3K27me3 (Abcam ab6002). Immunocomplexes were isolated with protein G agarose, washed twice with wash buffer 1 (20 mM Tris-HCl pH 8.1, 50 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS) and twice with wash buffer 2 (10 mM Tris-HCL pH 8.1, 250 mM LiCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholic acid). They were then eluted by twice vortexing for 10 min with 250 μl immunoprecipitation elution buffer (0.1 M NaHCO, 1% SDS), and finally cross-linking was reversed by incubating at 65°C in 0.3 M NaCl. Samples were treated with 1 mg/ml proteinase K and the DNA isolated by extraction with phenol/chloroform with addition of 10 μg carrier tRNA (Roche) and were ethanol-precipitated overnight at −20°C. Pellets were washed twice, air dried, then resuspended in 30 μl water, and 1 μl was used for real-time PCR except for experiments of Fig. 4 where pellets were suspended in 15 μl and 2 μl used per real-time PCR reaction. For quantification of immunoprecipitated DNA from ChIP experiments, Sensimix SYBR Lo-ROX (Qantance) was used according to the manufacturer's instructions. Primers were designed using Primer BLAST National Center for Biotechnology Information for the promoter [arbitrarily defined as within the first kb upstream of the 5′ untranslated region (UTR) of the gene of interest for *Tbx21*, *Ifng*, *Il17a*, and *Il12rb2* or +4585 bp for *Rorc* (37)] plus upstream and downstream of the transcription start site [sequences (Supplemental Fig. 1*A*, 1*B*) and diagram (Supplemental Fig. 1*C*)]. Immunoprecipitated DNA was quantified by creating a line of best fit from a standard curve using serial dilutions of genomic template DNA to allow normalization of primer sets. Data are presented as a fraction of input DNA for each primer, or in Fig. 4*C* as log to the base 2 of the ratio of the immunoprecipitated amounts of DNA of H3K4me3 to H3K27me3 pull-downs.

Purification of mRNA and quantitative PCR analysis

RNA was extracted using RNeasy Mini or Micro kits (Qiagen) following standard manufacturer protocols and reverse transcribed using QuantiTect reverse transcription kit; cDNA was used as template for the amplification of genes of interest and for the reference gene (*Hprt*) by real-time SYBR green PCR. The following primers (all from Qiagen unless stated) for transcription factors, cytokines, and receptors were tested: *Tbx21*, *Ahr*, *Il17a*, *Il17f*, *Ifng*, *Il12rb2*, and *Il23r*. Primers for RORγt encoded by *Rorc* were as follows: forward, 5′-ACCTCCACTGCCAGCTGTGTGCTGTC-3′; reverse, 5′-TCATTTCTGCACTTCTGCATGTAGACTGTCCC-3′ (38). Amplification plots were analyzed using a 7500 Fast Real-Time PCR Detector System (Applied Biosystems) with 7500 Fast Software System version 1.4.6. Gene expression was calculated using the comparative method for relative quantification upon normalization to *Hprt* gene expression.

Statistics

Statistical analysis was performed with GraphPad Prism software using either a two-tailed, unpaired *t* test or one-way ANOVA with Bonferroni's multiple comparison test. Results were deemed significant if $p < 0.05$.

Results

Evidence of Tbx21 *bivalency in purified in vitro-polarized Th17 cells*

Because studies of histone modifications are based on the average profiles across populations of cells, our first goal was to generate a purified Th17 population to minimize the possibility that uncommitted T cells or contaminant Th1 cells could complicate analysis. To achieve this, we used cytokine capture to generate Th17 cells of ∼90% purity based on captured IL-17A. The H3K4me3 and H3K27me3 modifications at key genes involved in differentiation of Th1 (*Tbx21*/T-bet and *Ifng*) or Th17 (*Ror*c and *Il17a*) cells were then analyzed in these purified cells and contrasted with in vitro-polarized Th1 cells (Supplemental Fig. 2*A*). Primer pairs (Supplemental Fig. 1) were designed for the approximate promoter regions for each gene (defined as within the first kb upstream of the 5′ UTR of the gene of interest if unknown), as well as for regions upstream and downstream of the gene of interest (Supplemental Fig. 1*C*). ChIP was then performed with Abs to H3K4me3 and H3K27me3, and immunoprecipitated DNA was then quantified by quantitative PCR. As expected, Th1 cells exhibited predominantly permissive H3K4me3 modifications at the promoter regions of *Tbx21* and *Ifng*, while displaying H3K27me3 modifications at *Rorc* and *Il17a* (Supplemental Fig. 2), in agreement with recently published data (27, 33). Similarly, Th17 cells were enriched for permissive H3K4me3 modifications at *Rorc* and *Il17a*, but there was no notable increase in H3K27me3 modifications at the *Ifng* locus. However, the Th17 cells exhibited both modifications at the *Tbx21* locus, albeit the enrichment for H4K4me3 was significantly lower than that in Th1 polarized cells ($p = 0.0328$, two-tailed unpaired *t* test) (Supplemental Fig. 2*B*). These initial findings provide supportive evidence to the notion that the *Tbx21* locus exhibits bivalent modifications in purified in vitro-generated Th17 cells.

Ex vivo-isolated Th17 cells also exhibit bivalent marks at the Tbx21 *promoter*

Although plasticity of in vitro-polarized Th17 cells has recently been well documented, some controversy remains over whether the same is true for Th17 cells naturally found in vivo. To determine whether ex vivo Th17 cells have identical characteristics to in vitro-generated Th17 cells, we compared the epigenetic profiles of the two populations. To purify these cells, a commercially available cytokine capture assay was used. Because most Th17 cells in vivo express the chemokine receptor CCR6 (Supplemental Fig. 3*A*, *left panel*), whereas most IFN-γ–secreting cells do not (Supplemental Fig. 3*A*, *right panel*), cells were gated for $CD4^+$ and $CCR6^+$ $IL\text{-}17A^+$ cells sorted (Supplemental Fig. 3*B*). This typically yielded cells of a high purity (>90%, Supplemental Fig. 3*C*, 3*D*) and a near absence of any IFN-γ–secreting cells detectable by intracellular staining (Supplemental Fig. 3*D*). As CCR6 does not provide a good selection marker for in vitro-generated Th17 cells, this step was not included in the purification of the latter (Fig. 1*A*). ChIP was then performed to assess H3K4me3 and H3K27me3 modifications at *Tbx21*, *Rorc*, *Ifng*, and *Il17a* (Fig. 1*B*). The two cell types had similar patterns of H3K4me3 modification at the *Rorc* and *Il17a* loci, although enrichment downstream at *Il17a* was greater in the ex vivo cells. Similarly, H3K27me3 was present at *Tbx21* and *Ifng* with similar profiles in both populations of Th17 cells. In addition, both exhibited bivalent marks in the promoter region of *Tbx21*. Thus, ex vivo Th17 cells are likely to have similar potential for T-bet expression as in vitro Th17 cells, suggesting that they retain potential for plasticity despite the limitations in the ability to repolarize such ex vivo cells (30).

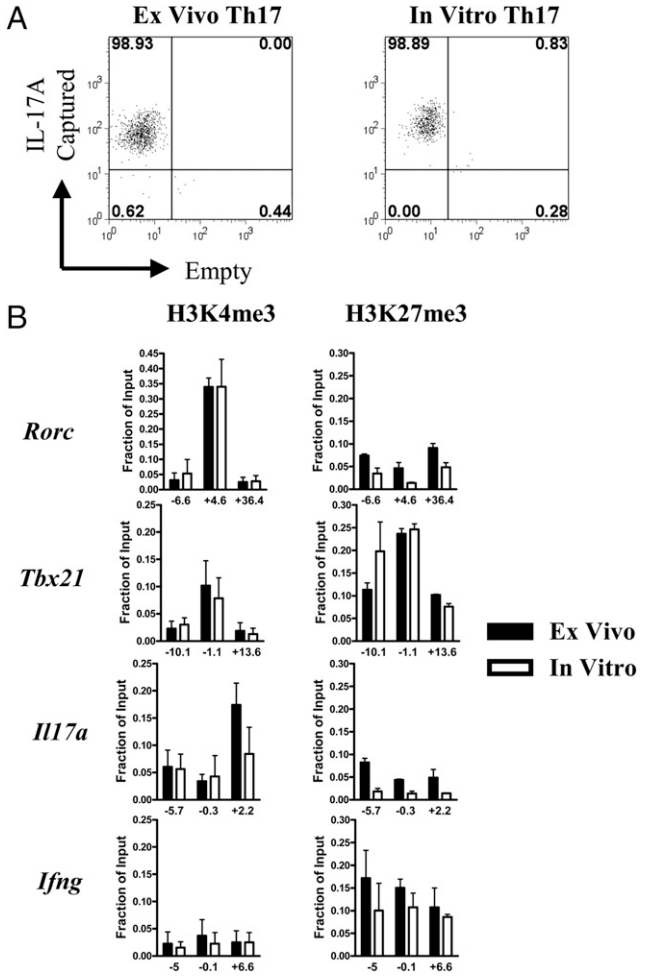

FIGURE 1. Ex vivo-isolated Th17 cells also exhibit bivalent modifications at *Tbx21*. Ex vivo Th17 cells ($CD4^+$ $CCR6^+IL\text{-}17A^+$) were purified as described in Supplemental Fig. 3, and day 4 in vitro-polarized Th17 cells ($CD4^+$ $IL\text{-}17A^+$) were also purified as described in *Materials and Methods*. *A*, The purities based on captured cytokine are shown for ex vivo Th17 cells (*left panel*) and in vitro Th17 cells (*right panel*). After sorting, ChIP was performed with Abs to H3K4me3 and H3K27me3 as described previously. *B*, Levels of immunoprecipitated DNA with H3K4me3 modifications (*left panels*, data from two independent experiments) or H3K27me3 modifications (*right panels*, data from two independent experiments) normalized to input controls (±SEM) at the loci of the genes *Rorc*, *Tbx21*, *Il17a*, and *Ifng* are shown for ex vivo-isolated (black) or in vitro-generated (white) Th17 cells. The *x*-axis details the approximate location of the primers relative to 5′ UTR of gene of interest in kb.

Th17 cells isolated ex vivo have repressive histone modifications at the Il12rb2 *locus*

Although in vitro-polarized Th17 cells are responsive to IL-12 stimulation (28, 29), it has been reported that Th17 cells isolated ex vivo could not be "converted" by one round of IL-12 stimulation in vitro (30). The expression status of *Il12rb2* on ex vivo Th17 cells is, however, currently unknown. RNA was therefore extracted from both ex vivo Th17 and in vitro Th17 cells to compare the expression status *of Il12rb2* and *Il23r* [because this receptor is proposed to be required for the terminal differentiation of Th17 cells in vivo (16)] (Fig. 2*A*, 2*B*). Although both cell types expressed *Il23r*, there was strikingly a near absence of detectable message for the *Il12rb2* in ex vivo-isolated Th17 cells (Fig. 2*B*) (but not in the purified in vitro-generated cells). This could explain why such cells could not be deviated by IL-12 in vitro (30).

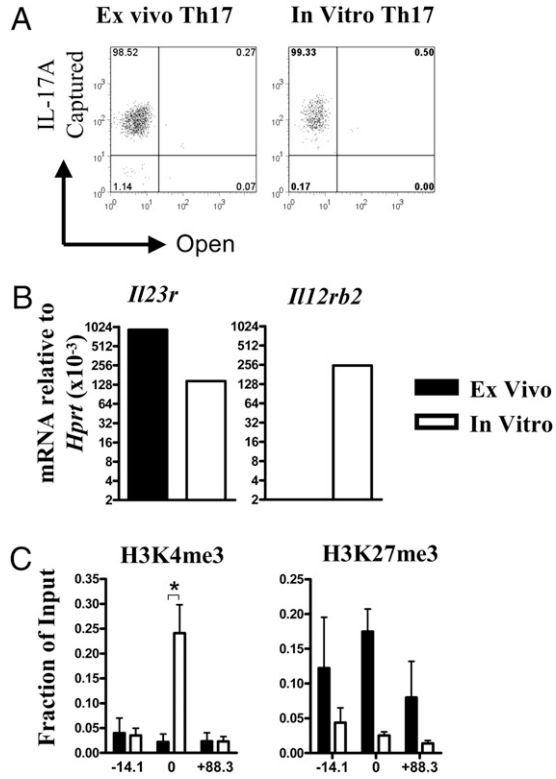

FIGURE 2. Nonpermissive modifications at the *Il12rb2* locus in ex vivo-isolated Th17 cells. $CD4^+CCR6^+IL\text{-}17A^+$ ex vivo-isolated Th17 cells and $CD4^+$ $IL\text{-}17A^+$ in vitro-generated Th17 were purified as described in Fig. 1. RNA was extracted from the two populations. *A*, FACS plots showing the purities of captured IL-17A for the purified T cell populations. *B*, mRNA levels were then analyzed by RT-PCR for the expression levels of *Il23r* and *Il12rb2* relative to *Hprt*. *C*, Immunoprecipitated DNA from Fig. 1 was analyzed for levels of H3K4me3 (*left panel*) and H3K27me3 (*right panel*) modifications for the approximate promoter regions of the *Il12rb2* plus primers upstream and downstream of the 5′ UTR of this gene (Supplemental Fig. 1*C*). The *x*-axis details the approximate location of the primers relative to 5′ UTR of *Il12rb2* gene in kb. For each locus, the amount of DNA immunoprecipitated was quantified and normalized to the input controls. Black bars, ex vivo Th17; white bars, in vitro Th17. Data from three independent experiments (H3K4me3) or two independent experiments (H3K27me3); error bars represent ± SEM. $*p < 0.05$ (two-tailed unpaired *t* test).

As well as transcriptional analysis, H3K4me3 and H3K27me3 modifications at the *Il12rb2* locus were examined by ChIP on similarly isolated cells (Fig. 2*C*). Here there were also striking differences between ex vivo- and in vitro-generated cells. The latter had significantly ($p = 0.0206$) higher levels of H3K4me3 at the promoter of *Il12rb2*, but in contrast, ex vivo-isolated Th17 cells had high levels of H3K27me3 in this region (Fig. 2*C*). These modifications suggest that expression from *Il12rb2* is likely to be more restricted in ex vivo-isolated Th17 cells and correlates well with our observations from mRNA levels. Taken together, these data suggest that as yet unknown mechanisms exist to control *Il12rb2* expression in differentiated Th17 cells in vivo.

Ex vivo Th17 cells express Il12rb2 *after in vitro stimulation*

To investigate further the mechanisms underlying ex vivo Th17 cell stability, the effects of different cytokines on gene expression and histone modifications were analyzed. Ex vivo purified Th17 cells from BDC2.5NOD mice (Fig. 3*A*) were cultured with irradiated CD4-depleted splenocytes and a BDC2.5 mimotope under conditions of medium alone, IL-23, or IFN-γ plus IL-12 and then

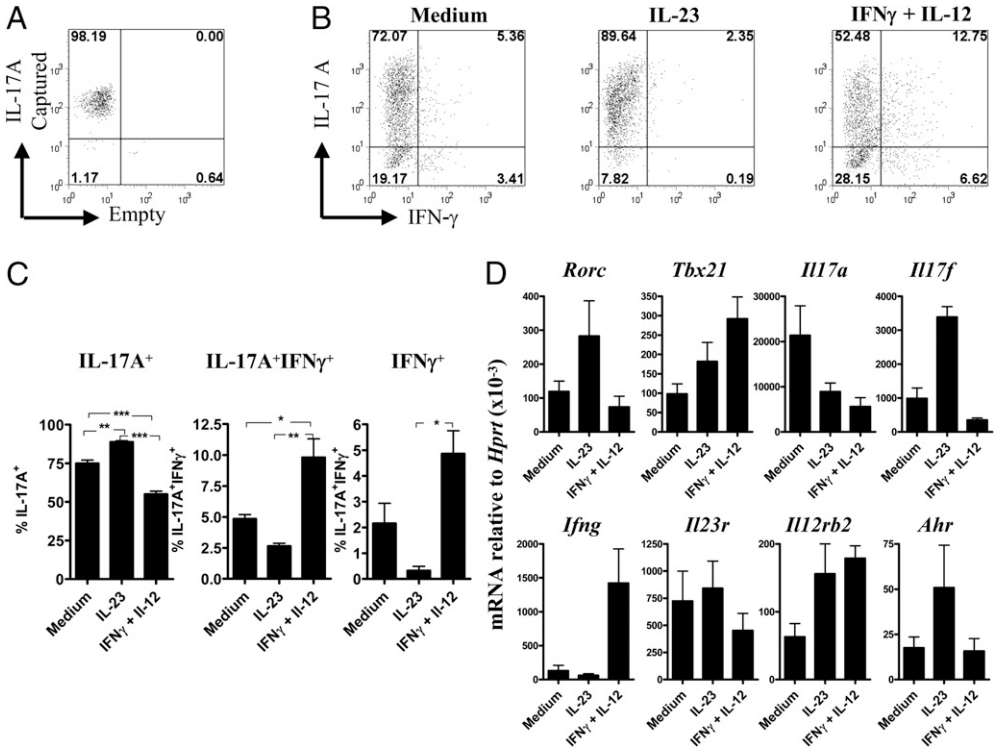

FIGURE 3. Ex vivo Th17 cells upregulate message for *Il12rb2* after activation. Ex vivo Th17 cells were isolated as described in Supplemental Fig. 3 then cultured with irradiated splenocytes and a BDC2.5 mimotope for 5–6 d in the presence of either medium alone, 20 ng/ml IL-23, or 10 ng/ml IL-12 plus 100 U/ml IFN-γ. *A*, Purity of Th17 cells based on captured IL-17A after ex vivo isolation. *B*, Intracellular staining at the end of the culture for IL-17A and IFN-γ in the different T cell cultures. *C*, Data from three independent experiments comparing the percentage of single IL-17A producers (*left panel*), IL-17A$^+$ IFN-γ$^+$ double producers (*middle panel*), and IFN-γ$^+$ single producers (*right panel*) based on intracellular staining analyses of the different T cell cultures. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (one-way ANOVA with Bonferroni's multiple comparison test). *D*, mRNA levels relative to *Hprt* in the different T cell cultures for stated transcription factors, cytokines, and receptors ($n = 3$). Error bars represent ± SEM.

analyzed for intracellular IFN-γ (indicative of reprogramming to Th1) or intracellular IL-17A (indicative of maintaining Th17) (Fig. 3*B*), as well as other characteristics (Fig. 3*D*). IL-23 best maintained the Th17 phenotype with an average of 88.83 ± 0.84% of cells positive for IL-17A (Fig. 3*B*, *middle panel*, Fig. 3*C*, *left panel*) compared with 74.89 ± 2.17% of cells that retained a Th17 phenotype in the absence of exogenous cytokines (Fig. 3*B*, *left panel*). In contrast, in the presence of IFN-γ plus IL-12, there was a significant reduction in the percentage of IL-17A–producing cells and a significant increase in the percentage of IL-17A$^+$IFN-γ$^+$ double producing cells compared with medium alone ($p < 0.05$) or IL-23 stimulation ($p < 0.01$) (Fig. 3*C*, *middle panel*). There was also a significant difference in IFN-γ–only producers between IL-23 maintained and IFN-γ plus IL-12 cultured Th17 cells (Fig. 3*C*, *right panel*). These findings were echoed by an increase in transcription of *Tbx21* and *Ifng* with IFN-γ plus IL-12 (Fig. 3*D*) and a reduction in mRNAs for *Il17a*, *Il17f*, and *Rorc*. However, the most striking finding was that *Il12rb2 mRNA* was detectable under all culture conditions, indicating that *Il12rb2* was upregulated as a result of in vitro activation. Next, H3K4me3 and H3K27me3 modifications at the *Il12rb2* locus were also reassessed after activation and culture [cells were isolated (Fig. 4*A*) then cultured for 2 d in the presence of IL-23 (Fig. 4*B*)]. As suggested by the mRNA expression profiles, the *Il12rb2* locus had undergone a change in histone methylation status, exhibiting an increase in enrichment for H3K4me3 between $t = 0$ and $t = 48$ h, with a modest reduction in H3K27me3 over this time period (Fig. 4*B*). The ratio of H3K4me3 to H3K27me3 correlated with an increased activation state of the gene over the 48-h period based on these epigenetic marks (Fig. 4*C*). Further assessment of these modifications at a later time point of 5 d (Fig. 5) suggested that by now the region exhibited predominately H3K4me3 modifications (Fig. 5*C*). It is possible that irradiated splenocytes themselves may make cytokines. One notable cytokine, IFN-γ, has been postulated to have a role in the modulation of *Il12rb2* expression on developing T cells (39). To investigate if IFN-γ was contributing to *Il12rb2* expression, ex vivo Th17 cells were cultured for 4 d in the presence of IL-23 with or without an anti–IFN-γ Ab (Fig. 5*D*). As can be seen from Fig. 5*D*, whereas message for *Il23r* was not notably changed between the two groups, there was a significant 2-fold reduction in message for *Il12rb2* when the Th17 cells were cultured with anti–IFN-γ ($p = 0.0318$). This suggests that unaccounted for sources of IFN-γ were augmenting expression of *Il12rb2* on the Th17 cells. These findings show a similarity to previously published work on developing Th cells, which suggested that *Il12rb2* can be expressed on naive T cells via activation through the TCR and that IFN-γ acts to modulate the level of expression of this receptor (39).

Thus, although ex vivo Th17 cells appear to have restricted expression from the *Il12rb2* locus, they can upregulate expression upon activation, and the receptor appears to be modulated by the cytokine IFN-γ. Because in vitro-differentiated Th17 cells already express the IL-12R, this may provide an explanation for their apparent greater plasticity (after one round of in vitro culture) compared with ex vivo-isolated Th17 cells.

In vivo immunization augments expression of Il12rb2 *on Th17 cells in vivo*

To investigate the effects of activation on Th17 cells in vivo, BDC2.5NOD mice were immunized with a mimotope peptide

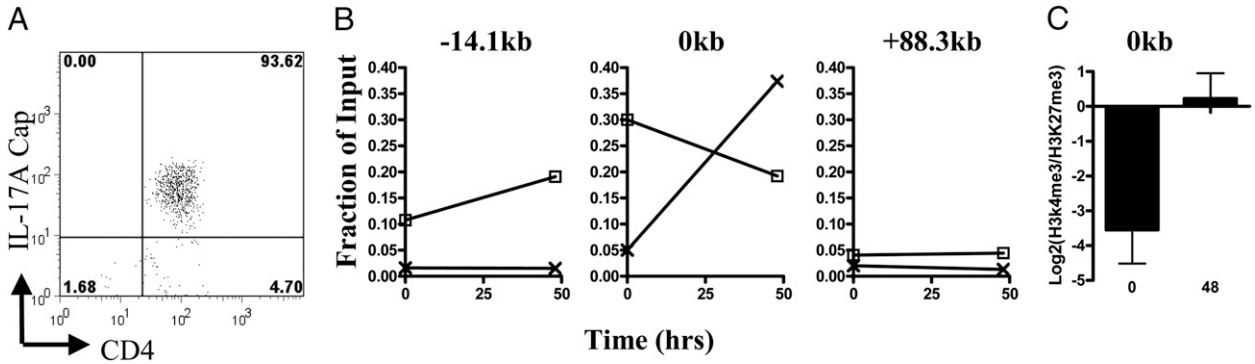

FIGURE 4. Chromatin remodeling occurs at the *Il12rb2* locus after in vitro culture. Ex vivo Th17 cells were isolated by cytokine capture from the spleens of BDC2.5NOD mice as described in Supplemental Fig. 3. *A*, Purity of ex vivo-isolated Th17 cells based on CD4 and captured IL-17A staining. *B*, Th17 cells (4×10^4) were taken and processed for ChIP with Abs to H3K4me3 and H3K27me3 ($t = 0$ h). The remaining 4×10^4 cells were cultured with CD4-depleted, irradiated splenocytes plus 0.4 μg/ml mimotope in the presence of 20 ng/ml IL-23 for 2 d. $CD4^+$ cells were isolated by MoFlo sorting from cultures then processed for ChIP ($t = 48$ h). Open squares, levels of H3K27me3 modifications; black crosses, levels of H3K4me3 modifications normalized to input controls across the *Il12rb2* locus at the indicated time points and genomic positions. *C*, Log_2 of the ratio of relative amount of DNA precipitated with H3K4me3 or H3K27me3 Abs at 0 kb from the *Il12b2* gene at the indicated time points ($n = 2$). Error bars represent mean ± SEM.

emulsified in CFA (Fig. 6). The aim was to induce activation or induction of Th17 cells in vivo and allow comparison with splenic-derived Th17 cells from unimmunized mice (Fig. 6*A*, 6*B*). Immunization increased the proportional representation of IL-$17A^+$ $CCR6^-$ cells compared with IL-$17A^+CCR6^+$ cells (Fig. 6*A*, 6*B*, *left* and *middle panels*). Therefore for immunized mice, all IL-$17A^+$ cells were sorted regardless of CCR6 status (Fig. 6*B*) and RNA extracted and compared with RNA isolated from IL-$17A^+$ $CCR6^+$ cells from unimmunized mice (Fig. 6*C*). All Th17-associated genes were more highly expressed in unimmunized Th17 cells compared with immunized cells, whereas message for *Tbx21* and *Ifng* was comparable. However, of note was the 4-fold increase in *Il12rb2* in the Th17 cells derived from immunized mice. This would fit with the notion that *Il12rb2* can be upregulated by the activation of these cells both in vitro and in vivo. Although message for *Il12rb2* was detected in the ex vivo-isolated Th17 cells from unimmunized mice, it was very low in these experiments with Ct values greater than 36, suggesting levels of detection similar to background.

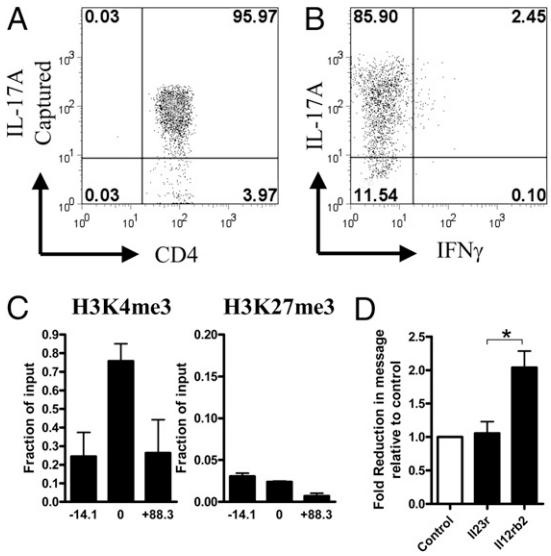

FIGURE 5. In vitro stimulation and IFN-γ act to modulate expression from *Il12rb2*. Ex vivo Th17 cells were purified as described in Supplemental Fig. 3 then cultured with irradiated splenocytes and a BDC2.5 mimotope for 5 d in the presence of 20 ng/ml IL-23. *A* and *B*, FACS plots of purified Th17 cells showing (*A*) captured IL-17A versus surface CD4 and (*B*) on day 5 of culture showing intracellular IL-17A versus IFN-γ. Cells were harvested after 5 d and then live $CD4^+$ cells sorted by MoFlo and ChIP performed with Abs to H3K4me3 and H3K27me3. *C*, Levels of H3K4me3 (*left panel*) and H3K27me3 (*right panel*) normalized to input controls at the *Il12rb2* locus of 5-d-cultured ex vivo Th17 cells. Data are from two independent experiments. *D*, Ex vivo Th17 cells were purified as described in Supplemental Fig. 3 and cultured with irradiated CD4-depleted splenocytes and a BDC2.5 mimotope for 4 d in the presence of 20 ng/ml IL-23 with or without 50 μg/ml anti–IFN-γ (XMG1.2). Cells were harvested and RNA extracted and processed for RT-PCR. Graph shows fold reduction in message compared with control (no anti–IFN-γ) for the genes *Il23r* and *Il12rb2* when anti–IFN-γ Ab is present in culture ($n = 3$). Error bars represent ± SEM. *$p < 0.05$ (two-tailed unpaired t test).

Prominent conversion of ex vivo Th17 cells to Th1-like cells after secondary culture with IFN-γ and IL-12

Because *Il12rb2* was upregulated on ex vivo Th17 cells after one round of culture, the cells should be more ready to respond to the actions of IL-12 in a subsequent secondary culture. To test this theory, ex vivo Th17 cells were isolated from BDC2.5NOD mice and cultured for 4 d with irradiated, CD4-depleted splenocytes, a BDC2.5 mimotope, and IL-23 (to maintain the population). $CD4^+$ cells were then sorted from culture wells and restimulated with fresh, irradiated, CD4-depleted splenocytes and peptide, either in the presence of IL-23 or IFN-γ plus IL-12 for a further 4 d (Fig. 7*A*). As can be seen after secondary culture, there was a greater proportional representation of double IFN-γ^+IL-$17A^+$ and single IFN-γ^+ cells, with typically >35% of all cells (Fig. 7*B*) now making detectable IFN-γ (compared with ~15% after one round) (Fig. 3*B*). Analysis of mRNA levels mirrored a similar transition with reductions in message for *Rorc*, *Il17a*, *Il17f*, *Il23r*, and *Ahr* for IFN-γ plus IL-12 cultures relative to IL-23 and an increase in transcription for *Tbx21* and *Ifng* (Fig. 7*C*). Notably, there was no observable increase in *Il12rb2* expression.

Th17 cells undergo rapid chromatin remodeling at the Tbx21 *locus in response to IL-12 stimulation*

The results suggest that the ability to respond to IL-12 is a critical step in the reprogramming of Th17 to Th1. In agreement, a detailed assessment of chromatin modifications at the *Ifng* and *Il17a* cytokine loci suggested that rapid epigenetic remodeling at these loci occurs in response to IL-12 (33). It has been proposed that this is dependent on T-bet and STAT4 (IL-12 transducer). We therefore investigated the effects of IL-12 on modifications at *Tbx21* (T-bet)

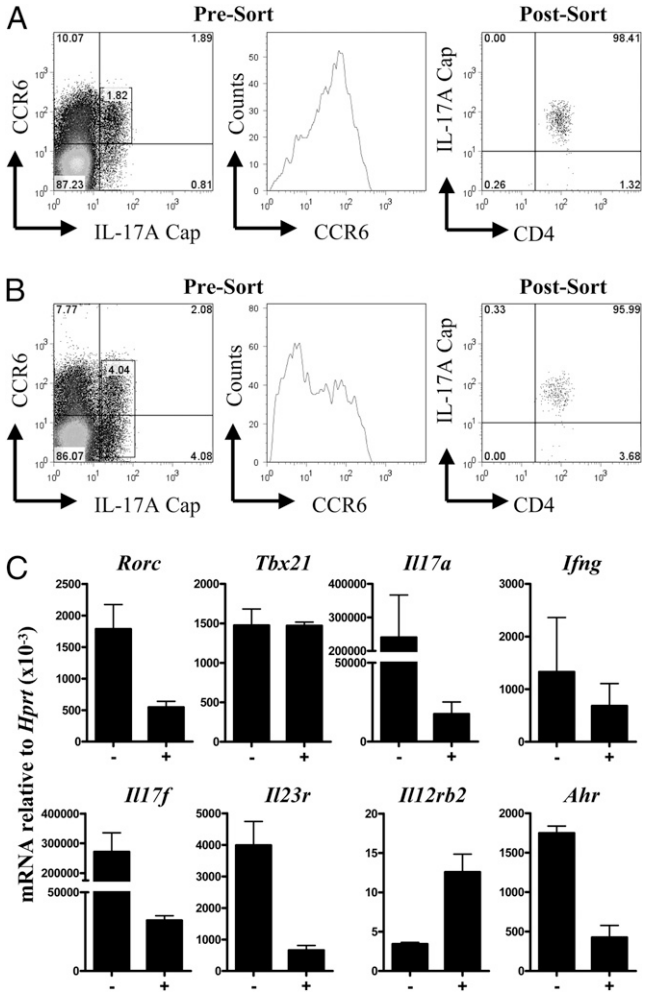

FIGURE 6. In vivo immunization with CFA and peptide induces upregulation of *Il12rb2* on Th17 cells in vivo. *A*, In the *left panel*, the FACS plot shows CCR6 versus IL-17A capture staining gated on $CD4^+$ cells isolated from the spleens of unimmunized BDC2.5NOD mice according to Supplemental Fig. 3. In the *middle panel*, the histogram displays levels of CCR6 on $CD4^+$ $IL\text{-}17A^+$ cells. In the *right panel*, $CCR6^+$ $IL\text{-}17A^+$ cells were sorted, and the purity of captured IL-17A versus CD4 on the population is shown. *B*, BDC2.5 NOD mice were immunized s.c. in the right and left flanks with 3 μg of a BDC2.5 mimotope emulsified in CFA. Seven days later, inguinal and axillary lymph nodes were harvested and pooled, and $CD4^+$ T cells were isolated and cytokine capture performed. In the *left panel*, the FACS plot shows CCR6 versus IL-17A capture staining gated on $CD4^+$ cells before sorting. In the *middle panel*, the histogram displays levels of CCR6 on $CD4^+$ $IL\text{-}17A^+$ cells. In the *right panel*, all $IL\text{-}17A^+$ cells were sorted, and purity of captured IL-17A versus CD4 is displayed. Note that cells in *A* and *B* were isolated and sorted on different days. *C*, RNA was extracted from the two populations, reverse transcribed, then stated cytokines, transcription factors, and receptors were analyzed by real-time PCR. Message levels are displayed relative to the housekeeping gene *Hprt*. −, Th17 cells from unimmunized mice; +, Th17 cells from immunized mice. Data is pooled from two independent experiments. Error bars represent mean ± SEM.

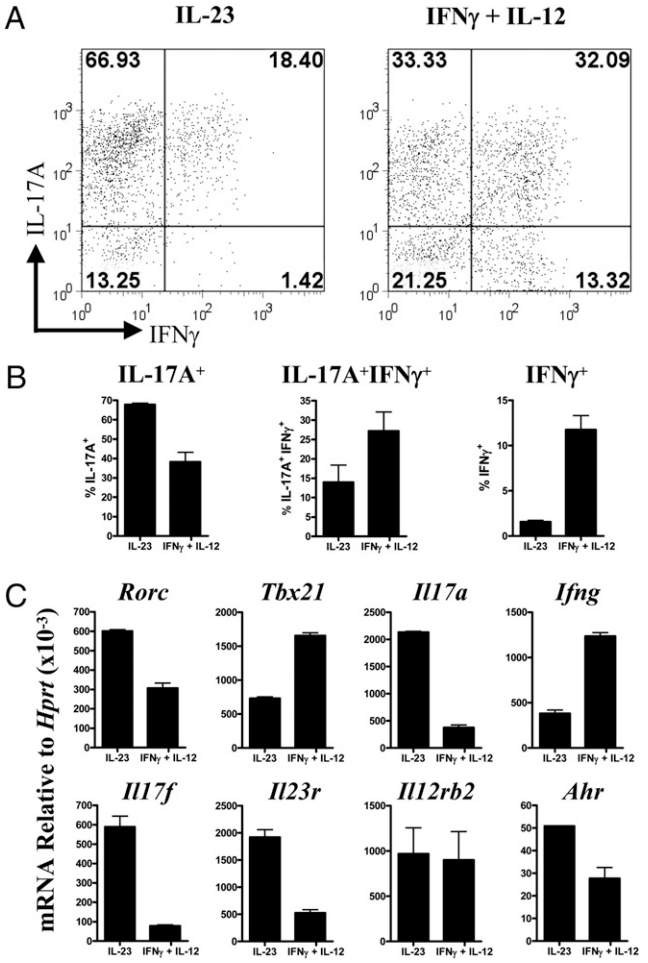

FIGURE 7. Secondary culture of ex vivo-isolated Th17 cells permits a more pronounced conversion to Th1-like cells. Ex vivo Th17 cells were purified as described in Supplemental Fig. 3 then cultured with irradiated splenocytes and a BDC2.5 mimotope for 4 d in the presence of 20 ng/ml IL-23. Cells were harvested and $CD4^+$ cells sorted from cultures by MoFlo. Secondary cultures were then set up with irradiated splenocytes and a BDC2.5 mimotope for a further 4 d in the presence of either 20 ng/ml IL-23 or 10 ng/ml IL-12 plus 100 U/ml IFN-γ. *A*, Representative FACS plots showing intracellular staining of IL-17A versus IFN-γ after 4 d of reculture with either IL-23 (*left panel*) or IL-12 plus IFN-γ (*right panel*). *B*, Data from two independent experiments comparing the percentage of single IL-17A producers (*left panel*), $IL\text{-}17A^+$ $IFN\text{-}\gamma^+$ double producers (*middle panel*), and $IFN\text{-}\gamma^+$ single producers (*right panel*) based on intracellular staining analyses of the different T cell cultures. *C*, mRNA expression levels relative to *Hprt* of the stated transcription factors, cytokines, and receptors for the different T cell cultures. Error bars represent ± SEM, $n = 2$.

in comparison with other conditions [e.g., TGF-β, which maintains Th17 (29)] using in vitro-polarized Th17 cells.

After purification by cytokine capture, Th17 cells generated in vitro were recultured for 5 d in the presence of a Th1-polarizing mixture (IL-12, IFN-γ, and IL-2) or maintained by culture with either TGF-β or IL-23 (Fig. 8*A*, Supplemental Fig. 4). ChIP was performed with Abs to H3K4me3 and H3K27me3 as before and immunoprecipitated DNA quantified by quantitative PCR (Fig. 8*B*). Consistent with their effects on the phenotypes of the cells, the Th1 mixture elicited a drastic reduction ($p = 0.0385$) in H3K27me3 modifications around the *Tbx21* promoter, and TGF-β and IL-23 maintained this region in a bivalent state (Fig. 8*B*).

The Th1-polarizing conditions in this experimental system, however, were not sufficient to fully elicit the pattern of histone modifications characteristic for Th1 cells. Thus, although the Th1 mixture elicited a significant increase in H3K4me3 modifications at the *Ifng* gene ($p = 0.0433$) to approach that in Th1 cells, it failed to recapitulate the modifications at *Rorc*. H3K4me3 remained elevated at the promoter of *Rorc* in IL-12–treated cells, and there was no increase in H3K27me3 modifications. In addition, IL-12 elicited a reduction in H3K4me3 at *Il17a* without a concomitant increase in H3K27me3 (Fig. 8*B*). Thus, one round of Th1-

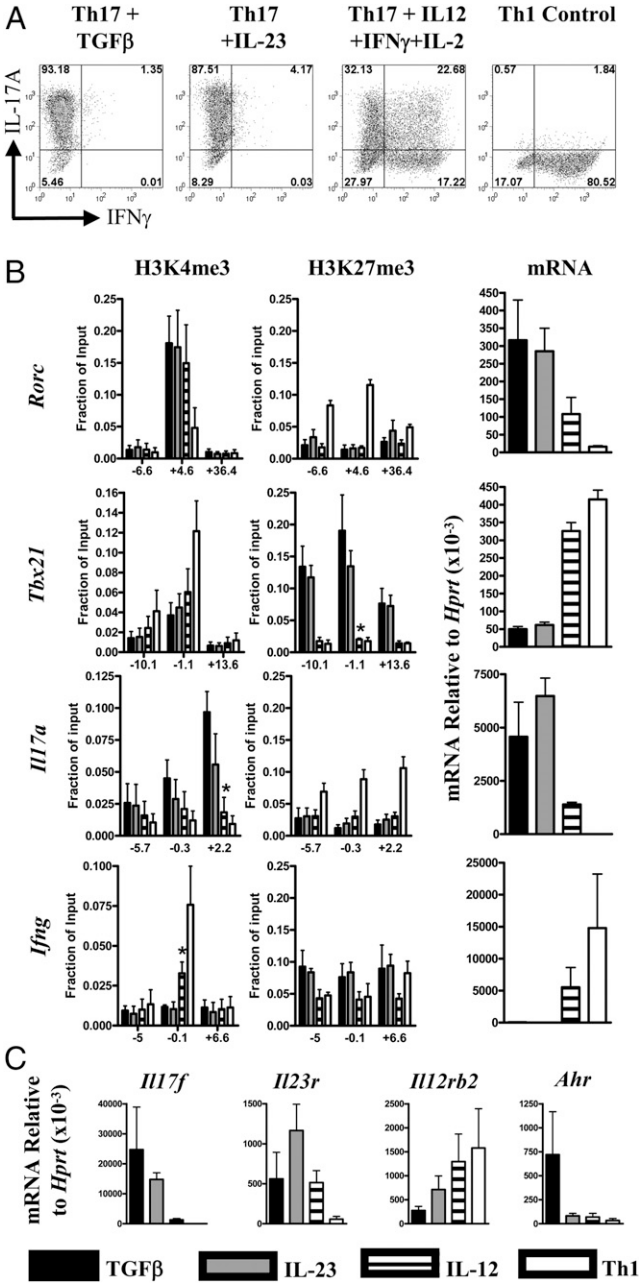

FIGURE 8. IL-12 resolves bivalent domain at *Tbx21* to an H3K4me3 dominant state. Naive $CD4^+$ T cells were cultured for 4 d under Th17-polarizing conditions. Cells were rested for 4 d in medium alone then restimulated with IL-23 and TGF-β for 2 d before sorting IL-$17A^+$ cells by cytokine capture. These purified Th17 cells were then restimulated on anti-CD3 (2 μg/ml) and anti-CD28 (10 μg/ml) coated plates for 5 d in the presence of either 2 ng/ml TGF-β or 20 ng/ml IL-23 or cultured under Th1-polarizing conditions (100 U/ml IFN-γ plus 10 ng/ml IL-12 plus 100 U/ml IL-2). At the same time, naive $CD4^+$ T cells were sorted and cultured for 5 d under Th1-polarizing conditions as a control. Live cells were sorted from day 5 cultures, and ChIP was then performed with Abs to H3K4me3 and H3K27me3. *A*, Intracellular staining for IL-17A and IFN-γ at day 5 from the different T cell cultures (see also Supplemental Fig. 4). *B*, Levels of immunoprecipitated DNA with H3K4me3 (*left panels*; $n = 3$) or H3K27me3 (*middle panels*; $n = 3$) modifications normalized to input controls (presented as a fraction of input controls ± SEM) at the loci of the genes *Rorc*, *Tbx21*, *Il-17a*, and *Ifng*. Th17 cells after 5 d of culture in TGF-β (black), IL-23 (gray), IL-12 plus IFN-γ plus IL-2 (horizontal stripes), or Th1-polarized control cells (white). The *x*-axis details the approximate location of the primers relative to 5′ UTR of the gene of interest in kb. mRNA expression levels for these genes are shown in the *right panels* ($n = 2$). mRNA expression value not visible for Th1 on the graph at *Il17a* is 0.0586 ± 0.016, and values for TGF-β and IL-23 at *Ifng* are 61.23 ± 23.19 and 16.75 ± 8.411. *$p < 0.05$ from unpaired *t* test comparing TGF-β versus IL-12 stimulated Th17 cells. *C*, mRNA expression levels relative to *Hprt* of stated transcription factors, cytokines, and receptors for the different T cell cultures. mRNA expression value not visible for Th1 on the graph at *Il17f* is 1.368 ± 0.8227.

polarizing conditions is capable of mimicking some, but not all, of the patterns of chromatin modifications seen in Th1 cells. In contrast, the effects of TGF-β matched those predicted by the cell phenotypes. Thus, the promoter regions of *Rorc* and *Il17a* were strongly enriched for H3K4me3 modifications in TGF-β–stimulated Th17 cells as previously reported (33).

Levels of mRNA (Fig. 8*B*, 8*C*) mirrored the epigenetic modifications with a progressive reduction in *Rorc* and *Il17a* mRNA in IL-12–stimulated Th17 cells relative to TGF-β maintained and with a concurrent increase in *Tbx21* and *Ifng* mRNA in IL-12–stimulated Th17 cells relative to TGF-β maintained (Fig. 8*B*). Notably, TGF-β also elicited the highest expression from *Ahr*, a gene that encodes a receptor whose biology is indicated to be important for some Th17 functions (40). Taken together, these data suggest that maintenance of the Th17 lineage by TGF-β is accompanied by retention of the H3K27me3 modifications at the *Tbx21* locus. However, this bivalent domain resolves to lose the H3K27 modification upon treatment with IL-12.

To gauge the kinetics of the changes occurring at the *Tbx21* locus, H3K27me3 levels were quantified at 0 and 24 h postculture with the Th1-polarizing mixture (IL-12 plus IFN-γ plus IL-2) or with TGF-β. Already, after 24 h, there was a noted reduction in H3K27me3 across the *Tbx21* locus in the presence of IL-12, whereas there was no change in modifications in the presence of TGF-β (Fig. 9). This implies that changes in *Tbx21* are an early event in the response of cells to IL-12. In the ex vivo cells, the initial low levels of the IL-12 receptor would compromise this initial change, and responsiveness could only be achieved under conditions leading to receptor expression.

Discussion

The number of proposed Th subsets has grown considerably in recent years and includes Th17 cells, regulatory T cells (Tregs), T follicular helper cells, and Th9 and Th22 cells (41–43) in addition to the well-documented Th1 and Th2 lineages. To what extent most of these proposed subsets represent discrete lineages is both highly topical and of some considerable debate (44), further complicated by the apparent plasticity of many Th subsets (34, 44, 45). Here we have focused on the question of Th17 regulation, investigating the extent to which cells with defined Th17 characteristics are identical and what governs their potential to be deviated to Th1 fates. We have identified clear differences between in vivo-isolated and in vitro-generated Th17 cells that explain previously identified differences in their repolarization capabilities. We also demonstrate that epigenetic changes are an early manifestation of reprogramming to Th1 fates.

Our comparison between the chromatin modifications in ex vivo-isolated Th17 cells and those generated in vitro reveals many similarities. Most notably, both have bivalent modifications at the gene encoding T-bet. The presence of bivalent modifications at *Tbx21* in the ex vivo cells argues that the potential for plasticity exists in Th17 cells arising naturally in vivo. Furthermore, the fact that bivalency is detected even in these purified populations (we have used cytokine capture to obtain >90% purity of both in vitro-

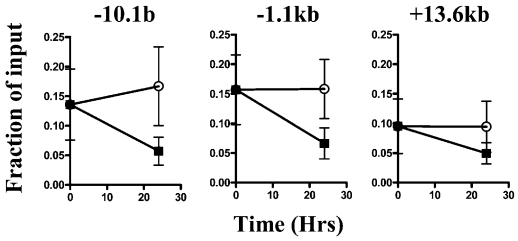

FIGURE 9. Chromatin remodeling begins within 24 h at the *Tbx21* locus. Naive $CD4^+$ T cells were cultured for 4 d under Th17-polarizing conditions. Cells were rested for 4 d in medium alone then restimulated with IL-23 and TGF-β for 2 d before sorting $IL\text{-}17A^+$ cells by cytokine capture. These purified Th17 cells were then restimulated on anti-CD3 (2 μg/ml) and anti-CD28 (10 μg/ml) coated plates for 24 h in the presence of either 2 ng/ml TGF-β or 10 ng/ml IL-12 plus 100 U/ml IFN-γ plus 100 U/ml IL-2. Samples were taken at $t = 0$ h and $t = 24$ h and ChIP performed with an H3K27me3 Ab. Levels of immunoprecipitated DNA normalized to input controls (shown as fraction of input ± SEM) from three independent experiments are shown at different genomic positions (in kb) at the *Tbx21* locus. Open circles represent TGF-β–cultured Th17 cells; closed squares represent IL-12/IFN-γ/IL-2–cultured Th17 cells.

generated and ex vivo cells) goes some way to assert that bivalency at *Tbx21* is a bona fide property of these cells and not a result of mixed cell populations. However, it is still possible that even these IL-17A–producing cells are a heterogeneous population. For instance, in vitro-generated Th1 cells had significantly higher levels of K4 at the gene encoding T-bet compared with those of Th17 cells (Supplemental Fig. 2). Performing sequential ChIP would be a possible way of confirming the presence of both domains in these cells; however, this would be technically very difficult due to the comparably low number of ex vivo Th17 cells that can be isolated. Nevertheless, these data build on the earlier studies by Wei et al. (27), who first proposed the existence of bivalent domains at key transcription factors involved in T cell differentiation by demonstrating that such bivalent properties are present in cells naturally occurring in vivo.

One notable feature unique to the ex vivo cells was restricted *Il12rb2* expression. Recent data have shown that in vitro-generated Th17 cells express the *Il12rb2* chain, the unique chain of the IL-12R. It has been proposed that this enables them to respond to IL-12 in vitro (28, 29). Our observation that ex vivo-isolated Th17 cells did not express or expressed very low levels of *Il12rb2* message was therefore of great surprise. This correlated with H3K27me3 modifications at the promoter region of *Il12rb2* indicative of repression at the locus and provides an explanation for the inability of one round of IL-12 stimulation to convert these cells to a Th1 phenotype (30). Our data also demonstrate the importance of studying ex vivo T cells; a similar difference was noted for Tregs where in vitro-generated cells showed reduced demethylation at CpG motifs in the *Foxp3* locus and less stable Foxp3 expression compared with those of natural Tregs (46).

The ex vivo Th17 cells could be induced to upregulate expression of *Il12rb2* by in vitro stimulation with peptide-loaded, irradiated splenocytes. This was detected even in the absence of any additional exogenous cytokines, where ~74% of cells retained expression of IL-17A. Upon closer investigation, we found a role for IFN-γ in modulating the expression level of *Il12rb2* on Th17 cells, with a significant 2-fold reduction in *Il12rb2* message on Th17 cells stimulated with IL-23 in the presence of a blocking anti–IFN-γ Ab. This would suggest that IFN-γ acts to enhance or stabilize *Il12rb2* expression and would fit with the concept previously published for Th2 cells that the presence of IFN can create an environment conducive to Th1 deviation (18). We also provide evidence that immunization with CFA can induce higher levels of *Il12rb2* expression in Th17 cells in vivo.

Because chromatin remodeling occurs at *Il12rb2* to permit expression during in vitro activation, it was not a surprise that ex vivo Th17 cells showed higher levels of conversion similar to in vitro Th17 cells when deviation was attempted on a secondary culture (Fig. 7*A* versus Fig. 9*A*). Thus, control of *Il12rb2* expression may be an important mechanism of dictating Th17 cell fate in vivo.

It is plausible that Th17 cells isolated ex vivo represent a memory phenotype, whereas in vitro-generated Th17 cells more closely mirror effector cells. Reactivation of the memory cells via TCR and/or costimulatory signaling could induce the upregulation of receptors such as *Il12rb2* that have been observed on in vitro-generated Th17 cells. Phenotypically, the ex vivo-isolated Th17 cells began to appear much more like their in vitro counterparts after one round of in vitro culture. Despite the fact that *Il12rb2* increased around 4-fold on Th17 cells that had been isolated from immunized mice, the exact conditions functioning in vivo remain to be clarified. The use of an IL-17A fate reporter would enable this to be tested during the natural course of a Th17 response.

In the presence of the IL-12 receptor, IL-12 treatment resolves the bivalency at *Tbx21* (T-bet), resulting in a rapid decrease in H3K27me3, which correlates with increased transcription of *Tbx21*. These changes can be detected within 24 h suggesting that they are an important part of the change in fate. Conversely, TGF-β maintains H3K27me3 modifications at this locus. This correlates with a recent study suggesting that TGF-β may act indirectly to promote Th17 responses by inhibiting Th1 differentiation, in part by inhibiting T-bet expression (10). It may also explain why TGF-β can maintain the Th17 phenotype, but not achieve full lineage commitment. Our observation that Th17 cells had relatively low levels of H3K27me3 at the gene encoding IFN-γ also fits with recently published work suggesting that the *Ifng* locus may be more poised for expression than previously thought in activated Th17 cells (33).

The exact role for IL-23 in Th17 differentiation has been unclear. One study suggested an absolute requirement for IL-23 signaling for terminal differentiation of Th17 cells in vivo (16). Others suggested that IL-23 acts to deviate cells to a Th1 phenotype in vitro (30, 34). Our data suggested that IL-23 did not bring about any conversion of Th17 to Th1 after one round of stimulation (Fig. 3, Supplemental Fig. 4). Epigenetic modifications were similar between TGF-β– and IL-23–maintained in vitro-generated Th17 cells, further arguing that it is not a primary signal for deviation to Th1. Ex vivo-isolated Th17 cells were also well maintained over at least 5 d of primary culture in vitro in the presence of IL-23, although there was evidence of an increased proportion of $IL\text{-}17A^+IFN\text{-}\gamma^+$ cells in secondary cultures (Fig. 7*A*, 7*B*). This increased proportion of double producers could be due to the fact that the ex vivo Th17 cells were not cultured in an APC-free environment (unlike the in vitro-generated Th17 cells), and the possibility of APC-derived signals such as IFNs and IL-12 cannot be ruled out. Therefore, although repeated rounds of IL-23 stimulation may enable Th1 phenotype conversion, it does not appear sufficient to elicit any drastic change in any of the conditions used here.

In summary, our study demonstrates that ex vivo-isolated Th17 cells retain the potential for plasticity, evident in the bivalency at *Tbx21* and the relatively low levels of H3K27me3 at *Ifng*. We additionally demonstrate that ex vivo Th17 cells can be converted to Th1-like cells in vitro by acquiring expression of *Il12rb2*. Our findings are extended to propose how different cytokines govern lineage maintenance (TGF-β or IL-23) or lineage diversion (IFN-

γ plus IL-12) at least in part by controlling *Tbx21* and *Il12rb2* expression. Our novel finding that ex vivo-isolated Th17 cells have restricted expression of the IL-12R suggests that regulation of *Il12rb2* expression on Th17 cells may be an important in vivo mechanism that limits plasticity of Th17 cells. It is possible that under conditions of infection, signals from cells of the innate immune system could control the functional fate of Th17 cells thus enabling a rapid switch to a Th1 profile when required.

Acknowledgments

We thank Dr. Nigel Miller for assistance with cell sorting and Dr. N. Holmes for critical reading of this manuscript.

Disclosures

The authors have no financial conflicts of interest.

References

1. Ansel, K. M., I. Djuretic, B. Tanasa, and A. Rao. 2006. Regulation of Th2 differentiation and Il4 locus accessibility. *Annu. Rev. Immunol.* 24: 607–656.
2. Murphy, K. M., and S. L. Reiner. 2002. The lineage decisions of helper T cells. *Nat. Rev. Immunol.* 2: 933–944.
3. Weaver, C. T., R. D. Hatton, P. R. Mangan, and L. E. Harrington. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol.* 25: 821–852.
4. Szabo, S. J., B. M. Sullivan, S. L. Peng, and L. H. Glimcher. 2003. Molecular mechanisms regulating Th1 immune responses. *Annu. Rev. Immunol.* 21: 713–758.
5. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655–669.
6. Kaplan, M. H., U. Schindler, S. T. Smiley, and M. J. Grusby. 1996. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4: 313–319.
7. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201: 233–240.
8. Harrington, L. E., P. R. Mangan, and C. T. Weaver. 2006. Expanding the effector CD4 T-cell repertoire: the Th17 lineage. *Curr. Opin. Immunol.* 18: 349–356.
9. Santarlasci, V., L. Maggi, M. Capone, F. Frosali, V. Querci, R. De Palma, F. Liotta, L. Cosmi, E. Maggi, S. Romagnani, and F. Annunziato. 2009. TGF-beta indirectly favors the development of human Th17 cells by inhibiting Th1 cells. *Eur. J. Immunol.* 39: 207–215.
10. Das, J., G. Ren, L. Zhang, A. I. Roberts, X. Zhao, A. L. Bothwell, L. Van Kaer, Y. Shi, and G. Das. 2009. Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation. *J. Exp. Med.* 206: 2407–2416.
11. O'Garra, A., B. Stockinger, and M. Veldhoen. 2008. Differentiation of human T (H)-17 cells does require TGF-beta! *Nat. Immunol.* 9: 588–590.
12. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24: 179–189.
13. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235–238.
14. Ivanov, I. I., B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua, and D. R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126: 1121–1133.
15. Yang, X. O., B. P. Pappu, R. Nurieva, A. Akimzhanov, H. S. Kang, Y. Chung, L. Ma, B. Shah, A. D. Panopoulos, K. S. Schluns, et al. 2008. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* 28: 29–39.
16. McGeachy, M. J., Y. Chen, C. M. Tato, A. Laurence, B. Joyce-Shaikh, W. M. Blumenschein, T. K. McClanahan, J. J. O'Shea, and D. J. Cua. 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat. Immunol.* 10: 314–324.
17. Murphy, E., K. Shibuya, N. Hosken, P. Openshaw, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1996. Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. *J. Exp. Med.* 183: 901–913.
18. Hegazy, A. N., M. Peine, C. Helmstetter, I. Panse, A. Fröhlich, A. Bergthaler, L. Flatz, D. D. Pinschewer, A. Radbruch, and M. Löhning. 2010. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity* 32: 116–128.
19. Djuretic, I. M., D. Levanon, V. Negreanu, Y. Groner, A. Rao, and K. M. Ansel. 2007. Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. *Nat. Immunol.* 8: 145–153.
20. Ansel, K. M., D. U. Lee, and A. Rao. 2003. An epigenetic view of helper T cell differentiation. *Nat. Immunol.* 4: 616–623.
21. Kouzarides, T. 2007. Chromatin modifications and their function. *Cell* 128: 693–705.
22. Santos-Rosa, H., R. Schneider, B. E. Bernstein, N. Karabetsou, A. Morillon, C. Weise, S. L. Schreiber, J. Mellor, and T. Kouzarides. 2003. Methylation of histone H3 K4 mediates association of the Isw1p ATPase with chromatin. *Mol. Cell* 12: 1325–1332.
23. Schneider, R., A. J. Bannister, F. A. Myers, A. W. Thorne, C. Crane-Robinson, and T. Kouzarides. 2004. Histone H3 lysine 4 methylation patterns in higher eukaryotic genes. *Nat. Cell Biol.* 6: 73–77.
24. Ringrose, L., H. Ehret, and R. Paro. 2004. Distinct contributions of histone H3 lysine 9 and 27 methylation to locus-specific stability of polycomb complexes. *Mol. Cell* 16: 641–653.
25. Bernstein, B. E., T. S. Mikkelsen, X. Xie, M. Kamal, D. J. Huebert, J. Cuff, B. Fry, A. Meissner, M. Wernig, K. Plath, et al. 2006. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 125: 315–326.
26. Azuara, V., P. Perry, S. Sauer, M. Spivakov, H. F. Jørgensen, R. M. John, M. Gouti, M. Casanova, G. Warnes, M. Merkenschlager, and A. G. Fisher. 2006. Chromatin signatures of pluripotent cell lines. *Nat. Cell Biol.* 8: 532–538.
27. Wei, G., L. Wei, J. Zhu, C. Zang, J. Hu-Li, Z. Yao, K. Cui, Y. Kanno, T. Y. Roh, W. T. Watford, et al. 2009. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* 30: 155–167.
28. Bending, D., H. De La Pena, M. Veldhoen, J. M. Phillips, C. Uyttenhove, B. Stockinger, and A. Cooke. 2009. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *J. Clin. Invest.* 119: 565–572.
29. Lee, Y. K., H. Turner, C. L. Maynard, J. R. Oliver, D. Chen, C. O. Elson, and C. T. Weaver. 2009. Late developmental plasticity in the T helper 17 lineage. *Immunity* 30: 92–107.
30. Lexberg, M. H., A. Taubner, A. Förster, I. Albrecht, A. Richter, T. Kamradt, A. Radbruch, and H. D. Chang. 2008. Th memory for interleukin-17 expression is stable in vivo. *Eur. J. Immunol.* 38: 2654–2664.
31. Stritesky, G. L., N. Yeh, and M. H. Kaplan. 2008. IL-23 promotes maintenance but not commitment to the Th17 lineage. *J. Immunol.* 181: 5948–5955.
32. Martin-Orozco, N., Y. Chung, S. H. Chang, Y. H. Wang, and C. Dong. 2009. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. *Eur. J. Immunol.* 39: 216–224.
33. Mukasa, R., A. Balasubramani, Y. K. Lee, S. K. Whitley, B. T. Weaver, Y. Shibata, G. E. Crawford, R. D. Hatton, and C. T. Weaver. 2010. Epigenetic instability of cytokine and transcription factor gene loci underlies plasticity of the T helper 17 cell lineage. *Immunity* 32: 616–627.
34. Lee, Y. K., R. Mukasa, R. D. Hatton, and C. T. Weaver. 2009. Developmental plasticity of Th17 and Treg cells. *Curr. Opin. Immunol.* 21: 274–280.
35. Judkowski, V., C. Pinilla, K. Schroder, L. Tucker, N. Sarvetnick, and D. B. Wilson. 2001. Identification of MHC class II-restricted peptide ligands, including a glutamic acid decarboxylase 65 sequence, that stimulate diabetogenic T cells from transgenic BDC2.5 nonobese diabetic mice. *J. Immunol.* 166: 908–917.
36. Streeck, H., K. W. Cohen, J. S. Jolin, M. A. Brockman, A. Meier, K. A. Power, M. T. Waring, G. Alter, and M. Altfeld. 2008. Rapid ex vivo isolation and long-term culture of human Th17 cells. *J. Immunol. Methods* 333: 115–125.
37. Eberl, G., and D. R. Littman. 2003. The role of the nuclear hormone receptor RORgammat in the development of lymph nodes and Peyer's patches. *Immunol. Rev.* 195: 81–90.
38. He, Y. W., M. L. Deftos, E. W. Ojala, and M. J. Bevan. 1998. RORgamma t, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. *Immunity* 9: 797–806.
39. Szabo, S. J., A. S. Dighe, U. Gubler, and K. M. Murphy. 1997. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J. Exp. Med.* 185: 817–824.
40. Veldhoen, M., K. Hirota, A. M. Westendorf, J. Buer, L. Dumoutier, J. C. Renauld, and B. Stockinger. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453: 106–109.
41. Breitfeld, D., L. Ohl, E. Kremmer, J. Ellwart, F. Sallusto, M. Lipp, and R. Förster. 2000. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* 192: 1545–1552.
42. Veldhoen, M., C. Uyttenhove, J. van Snick, H. Helmby, A. Westendorf, J. Buer, B. Martin, C. Wilhelm, and B. Stockinger. 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat. Immunol.* 9: 1341–1346.
43. Eyerich, S., K. Eyerich, D. Pennino, T. Carbone, F. Nasorri, S. Pallotta, F. Cianfarani, T. Odorisio, C. Traidl-Hoffmann, H. Behrendt, et al. 2009. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J. Clin. Invest.* 119: 3573–3585
44. Bluestone, J. A., C. R. Mackay, J. J. O'Shea, and B. Stockinger. 2009. The functional plasticity of T cell subsets. *Nat. Rev. Immunol.* 9: 811–816.
45. Zhou, L., M. M. Chong, and D. R. Littman. 2009. Plasticity of CD4+ T cell lineage differentiation. *Immunity* 30: 646–655.
46. Floess, S., J. Freyer, C. Siewert, U. Baron, S. Olek, J. Polansky, K. Schlawe, H. D. Chang, T. Bopp, E. Schmitt, et al. 2007. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol.* 5: e38.