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

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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, 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.
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 of STAT4 and Tbx21/T-bet 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 Ifnγ and Il17a 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.

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-CD45RA+ and cultured in IMDM (Invitrogen) supplemented with 10% heat-inactivated FCS (Biosource), 2 × 10^{-5} 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-Ifnγ (XMGI1.2), 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.

In vitro conversion of purified in vitro-generated Th17 cells, 1 × 10^6 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. 13) 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-RTPLWVRME-NH2) 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) plus 100 U/ml human recombinant 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 (1 × 10^5) were then cultured with 2 × 10^5 irradiated (30 cGy), CD4-depleted NOD splenocytes and 0.4 μg/ml of a BDC2.5 mimotope (acetyl-RTPLWVRME-NH2) 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% PBS, and 4 × 10^5 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).

Isolation of Th17 cells using commercial cytokine capture

CD4+ T cells were isolated from the spleens of immunized, 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 × 10^5 cells/ml) were stimulated for 1 h 30 min in culture with 500 ng/ml phorbol 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 MoFlo.
cell sorter for CD4⁺CCR6⁺IL-17A⁺ for ex vivo Th17 cells or CD4⁺IL-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 5 μg of a BDC2.5 mimotope (acetyl-RTTPPLWVRME-NH2) in CFA (H37 Ra, Difco Laboratories). Mice received 50 μl intramuscularly in each flank plus the base of the tail. Seven days later, inguinal and axillary lymph node cell suspensions were harvested and pooled, then CD4⁺ T cells were isolated before cytokine capture was performed (Fig. 6B).

**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 × 10⁶ cells (Figs. 8, 9, Supplemental Fig. 2); 1 × 10⁶ to 1.5 × 10⁶ cells (Figs. 1, 2, 3); and 4 × 10⁵ 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-HCI pH 8.1, 10 mM EDTA, 1% SDS, 1X Roche complete inhibitor). Lysate was sonicated by Bioruptor (Diagenode) then diluted 10 times in immunoprecipitation dilution buffer (20 mM Tris-HCI pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.01% SDS, 1X 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 experiments were performed overnight at 4°C with lysate and 1 μg of anti-H3K4me3 [Abcam ab8580, Millipore 07-473 (Fig. 5C)] 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-HCI pH 8.1, 50 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS) and twice with wash buffer 2 (20 mM Tris-HCI 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 NaHCO₃, 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 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 (Qantace) 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 Th2x1, Ifng, Il17a, and Il12rb2 or +4585 bp for Rorc (37)] plus upstream and downstream of the transcription start site (sequences [Supplemental Fig. 1A, 1B] and diagram (Supplemental Fig. 1C)). 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. 4C 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: Th2x1, Ahr, Il17a, Il17f, Ifng, Il12rb2, and I23r. Primers for RORγt encoded by Rorc were as follows: forward, 5’-ACCTCCACTGCAGCTGTGCTGTC-3’, reverse, 5’-TCAATTCTGACTCAGTGTAAGCTGCC-3’ (38). Amplifications were performed 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 quantitation following 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 Thb21 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 (Rorc and Il17a) cells were then analyzed in these purified cells and contrasted with those of purified Th1 cells (Supplemental Fig. 2A). 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. 1C). 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. 2B). 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. 3A, left panel), whereas most IFN-γ-secreting cells do not (Supplemental Fig. 3A, right panel), cells were gated for CD4⁺ and CCR6⁺ IL-17A⁺ cells sorted (Supplemental Fig. 3B). This typically yielded cells of a high purity (>90%), Supplemental Fig. 3C, 3D) and a near absence of any IFN-γ-secreting cells detectable by intracellular staining (Supplemental Fig. 3D). As CCR6 does not provide a good selection marker for 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. 3A, left panel), whereas most IFN-γ-secreting cells do not (Supplemental Fig. 3A, right panel), cells were gated for CD4⁺ and CCR6⁺ IL-17A⁺ cells sorted (Supplemental Fig. 3B). This typically yielded cells of a high purity (>90%), Supplemental Fig. 3C, 3D) and a near absence of any IFN-γ-secreting cells detectable by intracellular staining (Supplemental Fig. 3D). 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. 1A). ChiP was then performed to assess H3K4me3 and H3K27me3 modifications at Tbx21, Rorc, Ifng, and Il17a (Fig. 1B). The two cell types had similar patterns of H3K4me3 modification at the Rorc and Il17a loci, although enrichment downstream of 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 Tbx21 and Ifng with similar profiles in both populations of 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).
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. 2A, 2B). 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. 2B) (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).

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. 3A) 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
analyzed for intracellular IFN-γ (indicative of reprogramming to Th1) or intracellular IL-17A (indicative of maintaining Th17) (Fig. 3B), as well as other characteristics (Fig. 3D). IL-23 best maintained the Th17 phenotype with an average of 88.83 ± 0.84% of cells positive for IL-17A (Fig. 3B, middle panel, Fig. 3C, left panel) compared with 74.89 ± 2.17% of cells that retained a Th17 phenotype in the absence of exogenous cytokines (Fig. 3B, 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. 3C, 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. 3C, right panel). These findings were echoed by an increase in transcription of Thx21 and Ifng with IFN-γ plus IL-12 (Fig. 3D) 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 assessed after activation and culture [cells were isolated (Fig. 4A) then cultured for 2 d in the presence of IL-23 (Fig. 4B)]. 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. 4B). 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. 4C). 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. 5C). 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. 5D). As can be seen from Fig. 5D, 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 naïve 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
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. 6A, 6B). Immunization increased the proportional representation of IL-17A+ CCR6− cells compared with IL-17A+CCR6+ cells (Fig. 6A, 6B, left and middle panels). Therefore for immunized mice, all IL-17A+ cells were sorted regardless of CCR6 status (Fig. 6B) and RNA extracted and compared with RNA isolated from IL-17A+ CCR6+ cells from unimmunized mice (Fig. 6C). 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.

**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. 7A). 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. 7B) now making detectable IFN-γ (compared with ~15% after one round) (Fig. 3B). Analysis of miRNA 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. 7C). 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 5.** In vitro stimulation and IFN-γ act to modulate expression of 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).

**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 × 10⁷) were taken and processed for ChIP with Abs to H3K4me3 and H3K27me3 (t = 0 h). The remaining 4 × 10⁷ 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, Log2 of the ratio of relative amount of DNA precipitated with H3K4me3 or H3K27me3 Abs at 0 kb from the Il12rb2 gene at the indicated time points (n = 2). Error bars represent mean ± SEM.
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. 8A, Supplemental Fig. 4). ChIP was performed with Abs to H3K4me3 and H3K27me3 as before and immunoprecipitated DNA quantified by quantitative PCR (Fig. 8B). 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. 8B).

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. 8B). Thus, one round of Th1-
Th1-polarizing conditions (100 U/ml IFN-γ presence of either 2 ng/ml TGF-β) or 20 ng/ml IL-23 or cultured under Th1-polarizing conditions. Cells were rested for 4 d in medium alone then restimulated with IL-23 and 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. 8B). 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 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 domain retention of the H3K27 modification upon treatment with IL-12.

FIGURE 8. IL-12 resolves bivalent domain at Tbx21 to an H3K4me3 dominant state. Naïve CD4+ T cells were cultured for 4 d under Th1-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, naïve 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, Il17a, 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 y-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 = 6). mRNA expression value not visible for Th1 on the graph at Il17a is 0.0986 ± 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.
generated and ex vivo cells) goes some way to assert that biva-

cency 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 pop-

culation. 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). Perforating 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-gen-

erated 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 indica-
tive 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 ex-

pression 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. 7A versus Fig. 9A). 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-17A*IFN-γ* cells in secondary cultures (Fig. 7A, 7B). 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 Th2 and Th17 cell differentiation. 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.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure Legends

Fig. S1. Primer Sequences and locations

(A) Sequences of primers used for quantification of DNA from ChIP assays, and their genomic locations (B) relative to the 5’ UTR of the stated gene of interest. (C) Cartoon diagram depicting genomic architecture in assayed regions. NB. Not to scale.

Fig. S2. Evidence of Tbx21 Bivalency in purified in vitro polarised Th17 cells

Naïve CD4+ T cells were cultured for 4 days under Th17 polarising conditions. Cells were rested for 4 days in medium alone then cultured for 2 days with TGFβ and IL-23 before sorting for IL-17A+ cells by cytokine capture. On day 6 (the second day of Th17 resting) naïve T cells were sorted and polarised to Th1 for 4 days. (A) Histogram (left) depicting the purity of the sorted Th17 cell population; grey dotted line shows isotype staining; black bold line displays captured IL-17A staining on purified Th17 cells, (Right) FACS plot of Th1 cells displaying intracellular staining for IL-17A and IFNγ. (B) ChIP was then performed on the purified Th17 and Th1 cells with antibodies to H3K4me3 (left) and H3K27me3 (right). Immunoprecipitated DNA was then quantified by RT-PCR using primers for the approximate promoter (P) regions of Rorc, Tbx21, Il17a and Ifng, plus primers upstream and downstream of the 5’ UTR of the gene of interest (Figs. S1). X axis details the approximate location of the primers relative to 5’ UTR of the gene of interest in kilobases (kbs). For each locus the amount of DNA immunoprecipitated was quantified and normalised to the input controls. Black bars (Th17), white bars (Th1). Data are from three independent experiments, presented as a fraction of input controls ±SEM. * = p<0.05 two tailed, unpaired t test.
Fig. S3. Purification of *ex vivo* Th17 cells

(A) Staining of CCR6 vs intracellular IL-17A (left) or IFNγ (right) on splenic CD4⁺ cells demonstrating that the majority of Th17 cells are CCR6 positive, whilst Th1 cells are not. (B) CD4⁺ splenocytes were isolated by separation with magnetic beads then stimulated for 1h 30 minutes with PdBU and Ionomycin to induce IL-17A secretion. Cells were then incubated with cytokine capture reagents and rotated for 40 minutes to allow cytokine release and capture. After this they were stained for IL-17A and CCR6 and a representative FACS plot, gated on CD4⁺ cells, of IL-17A capture staining versus CCR6 before sorting is shown. (C) An example of typical purity of *ex vivo* isolated Th17 cells based on captured IL-17A staining after sorting. (D) Purified *ex vivo* Th17 cells from (C) were restimulated for a further 3 hours with PdBu and Ionomycin in the presence of Brefeldin A then stained for IL-17A and IFNγ (left plot). To confirm specificity of the IL-17A capture assay staining for intracellular IL-17A using a different fluorochrome was performed in the presence (black line) or absence (grey dotted line) of saponin (right plot).

Fig. S4. Percentage of cells positive for IFNγ or IL-17A used in Fig 8.

Graph showing % of cells positive for stated cytokines under the different culture conditions used in Fig. 8. Error bars represent mean ±SEM, n=3.
### A

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R: 5' cctctcggaacacctgctac 3' | F: 5' cggggatgctttttatttca 3'  
R: 5' cacctgctctgatggagaca 3' | F: 5' cttgcgtctctcctctgc 3'  
R: 5' cccctgtgttggtgagctc 3' |
| Rorc | F: 5' ggaagaatggctggagtca 3'  
R: 5' ccattaccctgcgtagaca 3' | F: 5' gcagcagcttcagatatgtcc 3'  
R: 5' atcaggaagagctgcaaagc 3' | F: 5' tcttggctcttgctcct 3'  
R: 5' atggagcctccattcatc 3' |
| Il17a | F: 5' gggggatgctttttatttca 3'  
R: 5' cacctgctctgatggagaca 3' | F: 5' gcagcagcttcagatatgtcc 3'  
R: 5' atcaggaagagctgcaaagc 3' | F: 5' tcttggctcttgctcct 3'  
R: 5' atggagcctccattcatc 3' |
| Ifng | F: 5' ggaagaatggctggagtca 3'  
R: 5' ccattaccctgcgtagaca 3' | F: 5' gcagcagcttcagatatgtcc 3'  
R: 5' atcaggaagagctgcaaagc 3' | F: 5' tcttggctcttgctcct 3'  
R: 5' atggagcctccattcatc 3' |
| Il12rb2 | F: 5' ggaagaatggctggagtca 3'  
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### B

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Fig. S2

A

Th17

IL-17A Cap

Th1

IFNγ

B

H3K4me3

Rorc

H3K27me3

Tbx21

Il17a

Ifng
Fig. S3.