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STAT4 and T-bet Are Required for the Plasticity of IFN-γ Expression across Th2 Ontogeny and Influence Changes in \textit{Ifng} Promoter DNA Methylation

Christopher L. Williams,*† Marcia M. Schilling,*† Sung Hoon Cho,*† Keunwook Lee,*† Mei Wei,*† Aditi,*† and Mark Boothby,*†,‡

CD4⁺ T cells developing toward a Th2 fate express IL-4, IL-5, and IL-13 while inhibiting production of cytokines associated with other Th types, such as the Th1 cytokine IFN-γ. IL-4-producing Th2 effector cells give rise to a long-lived memory population committed to reactivation of the Th2 cytokine gene expression program. However, reactivation of these effector-derived cells under Th1-skewing conditions leads to production of IFN-γ along with IL-4 in the same cell. We now show that this flexibility (“plasticity”) of cytokine expression is preceded by a loss of the repressive DNA methylation of the \textit{Ifng} promoter acquired during Th2 polarization yet requires STAT4 along with T-box expressed in T cells. Surprisingly, loss of either STAT4 or T-box expressed in T cells increased \textit{Ifng} promoter CpG methylation in both effector and memory Th2 cells. Taken together, our data suggest a model in which the expression of IFN-γ by Th2-derived memory cells involves attenuation of epigenetic repression in memory Th2 cells, combined with Th1-polarizing signals after their recall activation. \textit{The Journal of Immunology}, 2013, 191: 000–000.

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Abbreviations used in this article: CFDA-SE, 5 (and 6)-carboxyfluorescein diacetate-succinimidyl ester; ChIP, chromatin immunoprecipitation; DNMT, DNA methyltransferase; IRES, internal ribosomal entry sequence; KO, knockout; me-, methyl-; T-box, T-box expressed in T cells; WT, wild-type.

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CpG of the Ifng promoter is a key aspect of repressing expression of this Th1 cytokine in Th2 effector cells.

Immunological memory is a key feature of the adaptive immune system and provides protection against reinfection after a first exposure to a pathogen (30). After an Ag has been cleared, a fraction of responding cells survives as a long-lived population and appears to acquire a memory program differing from the effector state (31). Because cytokine production by memory cells upon Ag exposure can instruct a new generation of immune effectors, the profile of cytokines produced by recall responses of a memory CD4 population can dictate its protective value in repeat exposures to a given pathogen. For example, rapid production of IL-4 by memory-phenotype CD4+ T cells sufficed to guide CD4+ T cells to adopt a Th2 effector program following exposure to Leishmania major, leading to a failure to resolve the infection (32). In contrast, IFN-γ promotes resistance to such pathogens. Th2-derived memory cells arise from IL-4–producing Th2 effectors; after acquisition of a relatively quiescent state, Ag restimulation of these Th2-derived memory cells rapidly leads to IL-4 production (33, 34). After stimulation and growth in Th1-biased conditions in vitro or in vivo, these reactivated memory cells continue to produce IL-4, illustrating that Th2 memory cells retain a commitment to produce IL-4 (35).

Although production of IL-4 remains part of the programming for Th2-derived memory cells, restimulation under Th1 conditions also drove these cells to produce substantial amounts of IFN-γ (35–37). These findings revealed that the nature of gene silencing as part of the Th program could be changed in memory cells, so that IFN-γ and the Th2 cytokine genes can be coexpressed within an individual CD4 lymphocyte. However, almost nothing is known about the molecular mechanisms for this plasticity of programming. Recent work indicated that, in addition to the Th1 master regulator T-bet, IL-12 was required for the induction of Ifng gene expression after reactivation of memory Th2 populations (38). The signal or signals downstream from IL-12 and essential for plasticity of Ifng regulation are not established. Moreover, a key unanswered question regarding the mechanisms permitting IFN-γ production by Th2-derived memory cells is whether repressive epigenetic modifications of the Ifng promoter that occur during Th2 polarization are maintained in the memory phases. In this article, we have tested whether STAT4 affects the ability of memory Th2 cells to express T-bet or IFN-γ in Th1 recall conditions. Further, we analyzed Ifng promoter DNA methylation in naive, Th1-, Th2-, and Th2-derived memory CD4+ T cells, and explored the relationship between promoter methylation and the Th1-determining transcription factors STAT4 and T-bet.

Materials and Methods

Mice

BALB/c I4-IRESC-Gfp (“4get”; internal ribosomal entry sequence [IRES]), DO11.10 mice were bred with BALB/c Tbx21+/- [knockout (KO)] or BALB/c Stat4 KO mice (The Jackson Laboratory). BALB/c-B6J (The Jackson Laboratory) and athymic BALB/c nude (Fossilm/Fossilm) mice were used as recipients for transfer experiments. Recipients were 4–6 wk old at the time of transfer. Mice were maintained in microisolation cages at a Vanderbilt University facility in accordance with Institutional Animal Care and Use Committee guidelines and an approved protocol.

Reagents

Fluorophore-conjugated and purified mAbs were obtained from BD Pharmingen (San Jose, CA) and purified recombinant cytokines from Leinco (St. Louis, MO) unless otherwise indicated. Purified 11B11 anti–IL-12 and recombinant hIL-2 were obtained from the Biological Response Modifiers Program (National Cancer Institute, Frederick MD). Anti–T-bet–fFluor 660 was obtained from eBioscience (San Diego, CA). Oligonucleotides were synthesized by Invitrogen (Grand Island, NY) unless otherwise stated.

Cell culture and purification of GFP+ IL-4–producing effectors and adoptive transfers

DO11.10 cells were activated with OVA323–339 peptide, and all cells were cultured as described (35), with the following modifications. For Th1 culture conditions, cells were plated at 7 × 105 cells per milliliter and received 1 μg/ml OVA peptide, 5 mg/ml IL-12, and 3 μg/ml anti–IL-4 Ab 11B11. For Th2 cell cultures, cells were plated at 3.5 × 105 cells per milliliter and received 0.5 μg/ml GM-CSF, 7.5 μg/ml IL-4, 3 μg/ml anti–IFN-γ Ab, and 2 μg/ml anti–IL-12 Ab. Both Th1 and Th2 cell cultures were supplemented with IL-2 (50 U/ml 24 and 72 h after Ag stimulation). GFP+ Th2 effector cells were purified for transfer as described (35). After 4 d of culture in Th2 conditions, 4get, DO11.10 cells [Tbx21+/−, Stat4−/−, or transcriptionally wild-type (WT)] were stained with APC-conjugated anti-CD4 and PE-conjugated KJ1-26 (anti-DO11.10 TCR) Abs and flow sorted on a FACS Aria (BD, Franklin Lakes, NJ) to purify (>98.5%) viable DO11.10 KJ1-26+ CD4+ Gfp+ cells. Prior to transfer or DNA isolation for methyl-CPG analyses, these cells were cultured in Th2 conditions for 9–10 d after restimulation with APCs (4:1 with T cells) and 0.25 μg/ml OVA323–339 (13–14 d total).

Quantitation of cytokine production

Th cell cultures plated with APCs at a 1:4 ratio, and single-cell suspensions of recipient spleens, were stimulated with 1 μg/ml OVA323–339 Cytokine concentrations in supernatant collected after 36 h were quantified using a flow cytometric kit (Th1/Th2/Th17 cytokine bead array; BD Pharminigen). Intracellular cytokines were quantified by stimulating cells 18 h with OVA323–339 (1 μg/ml) in the presence of APCs, followed by GolgiStop (BD) (2–3 h), and staining in the presence of anti-CD16/32 (Fc Block; BD Pharminigen), as previously described (35). Viable cells were selected based on forward and side scatter characteristics, and fluorescence signals representing intracellular cytokines were determined in cells positive for the DO11.10 TCR and CD4.

Bisulfite analysis of DNA methylation

DNA isolated from flow-purified naive, effector (Th1, Th2, 13 d), and memory CD4 T cells, or from tissues, was digested with BamHI (New England BioLabs, Ipswich MA), bisulfite modified using the Imprint bisulfite modifying kit (Sigma-Aldrich, St. Louis, MO), then used as template in quadruplicate PCR performed using primer pairs specific for each modified strand sequence in the Ifng promoter (Supplemental Table I) (29). The noncoding strand of the Ifng promoter was amplified with a single reaction, whereas the coding strand was amplified using a nested PCR. After pooling four tubes of separate amplification for each sample, specific PCR products were identified on agarose gels, extracted using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), ligated with T Easy Vector (Promega, Madison, WI), and then transformed into JM109 cells (Agilent, Santa Clara, CA). For each of the three independent biological replicate cell samples and for each strand, 8–10 clones derived from each reaction pool were sequenced and scored for the frequency of unmethylated C residues in the CpG dinucleotides; the modification frequency for C residues outside of CpG dyads was verified as >99% for all sample sets.

EMSA

EMSA were performed as reported (29, 39), except that extracts of Th1 cells developing from primary mouse CD4+ T cells were used. Methylated upper-strand oligonucleotides were synthesized by Invitrogen (Grand Island, NY). The upper-strand oligonucleotides were designated as unmethylated, methylated (meC–53) hemimethylated, and trihemimethylated (meC at –53, –45, and –34) (Supplemental Table I). Each was annealed to an unmethylated lower-strand oligonucleotide after radio-labeling with γ[32P]-ATP (Perkin-Elmer, Waltham MA) and T4 poly nucleotid kinase (New England BioLabs). For competition assays, unlabeled competitor was added simultaneously with the labeled probe at molar ratios (competitor:probe) of 100:1, 10:1, and 1:1. Abs used for the supershift assays were CREB1 (sc-186), ATF2 (sc-187), and c-Jun (sc-45) (Santa Cruz Biotechnology, Santa Cruz, CA).

Transient transfection and reporter assays

Nucleofection was carried out via the Amaza T cell kit (Lonza, Basel, Switzerland) using a minimal Ifng promoter reporter P1P2-Luc (40) along with pCMS-Sport6-CREB1 or pCMS-Sport6. All results were normalized to GFP expression from the I-SceI-GFP plasmid (Lonza) measured via flow cytometry. Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer’s protocols.
Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChiP) assays were performed essentially as described previously (15). After cross-linking with formaldehyde, primary Th1 and Th2 cells were sonicated in a Bioruptor (Diagenode, Denville, NJ) to produce an average sheared DNA length of 400 bp. Immunoprecipitation was carried out using anti-AcH3(K9) (acetyl-lysine 9 of histone H3; #DAM1813175; Millipore), or CREB1 (sc-186X; Santa Cruz), and the precipitates were analyzed by PCR using primers shown in Supplemental Table I.

Proliferation in vitro and in vivo

Proliferation studies using CFSE partitioning and BrdU incorporation were carried out as described (39). For CFSE partitioning in vivo, Th2 cells were grown for 5 d, labeled with 5 (and 6)-carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE; Invitrogen) (2.5 μM, 15 min) following manufacturer instructions, and then transferred into BALB/c recipients. Fluorescence was measured on donor-derived cells recovered 12 d after transfer, with gating as described above. For CFSE partitioning assays in vitro, DO11.10 splenocytes were either labeled, Ag-stimulated, and cultured 2 d, or activated with Ag, cultured in Th2 conditions (11 d, with one interim Ag stimulation, as for cells used in adoptive transfers), labeled with CFDA-SE as above, and then cultured 2 d in IL-2–supplemented medium before analysis by flow cytometry. For assays of BrdU incorporation into donor cells in vivo, recipient mice were injected twice (72 h, 24 h before harvest; 3 mg i.p. per injection) with BrdU (Sigma-Aldrich) in sterile saline. Cells harvested 12 d posttransfer were then processed as described (39) to detect Alexa-647 anti-BrdU (Invitrogen) in donor- (KJ1-26+) and recipient-derived CD4+ T cells by flow cytometry. For in vitro assays, BrdU (1 μM) was added to Th2 cultures (days 2 and 13 after Ag activation), followed 4 h later by processing, direct immunofluorescent anti-BrdU staining, and flow cytometry of KJ1-26+ CD4+ cells.

Retroviral transduction

Retroviral transduction was carried out using as previously described (15). In brief, retrovirus-containing supernatants from 293T packaging cells transfected with MSCV-IRES-Thy1.1 (MIT) or MSCV-T-bet-IRES-Thy1.1 (MIT-T-bet) were used to transduce established GFP+ DO11.10 Th2 cells 2 d after restimulation. Cells were then cultured under Th1 or Th2 conditions, followed by measurements of Ag-stimulated cytokine production or intracellular cytokines as above.

Results

Strand-biased acquisition of Ifng promoter methylation in Th2 effector cells

The finding that memory Th2 cells, unlike their effector-phase precursors, can respond to an inflammatory milieu by efficient induction of IFN-γ production (35, 36) raises questions about the fate of repressive DNA methylation observed in Th2 effectors once these cells transition to memory status. Accordingly, we used bisulfite modification of DNA followed by strand-specific PCR to analyze CpG methylation in Ifng promoter DNA of naive CD4+ lymphocytes, Th1, and Th2 T cells. Naive CD4+ and Th1 effector cells showed little methylation of either strand of DNA upstream from the transcription start site (Fig. 1), whereas high methyla-
tion densities were found at two dinucleotides in exon 1, independent of T cell differentiation, as expected (41, 42). In effector Th2 cells (Fig. 1B, 1C), we found increased methylation of the coding strand of the Ifng promoter, with a majority of samples exhibiting modification of the key –53 CpG whose modification appeared to abrogate promoter activity (29). Surprisingly, however, the noncoding strand was reproducibly and significantly less methylated in Th2 effector cells relative to the coding strand (Fig. 1D), particularly at the –53 CpG (Fig. 1E). In light of this unexpected result, we tested samples including DNA from a mouse brain and 3T3 cells, both of which would be expected to have symmetrical hypermethylation of the Ifng promoter, along with thymocytes and naive CD4 lymphocytes. Brain and 3T3 cell DNA demonstrated a high density of symmetrical methylation across the surveyed region and in particular at the crucial –53 CpG, whereas thymocytes, like naive CD4 T cells, exhibited little meC (Table I). The frequency of non-coding strand DNA methylation in Th2 cells was too low simply to represent a lack of modification on one chromosome, e.g., from monoallelicism (43). Separate analyses (Table I and later results) exclude a strand bias in the detection method as the basis for the observation. Accordingly, we infer from these data that Ifng promoters were hemimethylated in these Th2 effector cells. The asymmetry of CpG methylation might in principle be observed because a high proportion of the Th2 population was actively in cell cycle and rapidly undergoing DNA synthesis. However, virtually no divisions were observed in CFSE analyses of the effector Th2 cells in the 2 d prior to the time at which DNA samples were prepared (Fig. 1F). Moreover, only a small fraction of the population was in cell cycle, as indicated by a low frequency of BrdU+ cells at this time (Fig. 1G). From these low frequencies of cycling cells, we conclude that at most a very small minority of asymmetrical methylation observed in Th2 effectors could be due to DNA replication, whereas the remainder is an epigenetic feature of the Ifng locus at this stage in Th2 effectors.

Table I. CpG methylation in naive CD4+ T cells, thymus, brain, and NIH 3T3 cells

<table>
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<th>Strand</th>
<th>CpG position</th>
<th>Total</th>
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<tr>
<td></td>
<td>−205</td>
<td>−190</td>
<td>−170</td>
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<td>Naive CD4</td>
<td>Coding</td>
<td>11.4 (3.6)</td>
<td>11.4 (3.6)</td>
</tr>
<tr>
<td>Thymus</td>
<td>Noncoding</td>
<td>17 (3.6)</td>
<td>10.8 (1.6)</td>
</tr>
<tr>
<td>Brain</td>
<td>Coding</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>Noncoding</td>
<td>100</td>
<td>86.6 (2)</td>
</tr>
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<table>
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<tr>
<th>Sample</th>
<th>Strand</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>−205−170</td>
<td>−53−34</td>
</tr>
<tr>
<td>Naive CD4</td>
<td>Coding</td>
<td>0.9 (0.25)</td>
</tr>
<tr>
<td>Thymus</td>
<td>Noncoding</td>
<td>0.7 (0.34)</td>
</tr>
<tr>
<td>Brain</td>
<td>Coding</td>
<td>0.11 (0.07)</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>Noncoding</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Asymmetrical methylation affects transcription factor binding to the Ifng promoter

On the basis of evidence that the Ifng promoter in many Th2 cells can be in a state of asymmetrical methylation, we investigated whether hemimethylation might affect transcription factor recruitment to the Ifng promoter. EMSA using nuclear extracts of primary Th1 cells were carried out using unmethylated or hemimethylated probes (Fig. 2A). Both hemimethylated probes impaired the formation of the slower migrating complex (indicated by filled arrow, Fig. 2B). Competition assays using unlabelled competitor DNA confirmed that the mobility shift bands represented sequence-specific binding; moreover, 10-fold more cold competitor was needed to attenuate the slower migrating complex to the WT compared with the hemimethylated probe (Fig. 2C). To characterize this complex, we performed Ab blocking/supershift assays with the unmethylated probe and Abs against CREB/ATF family members. The upper band was impacted by anti-CREB1 (Fig. 2D), whereas Abs against ATF2 and e-Jun had no discernible effect, leading us to conclude that the slower migrating complex is predominantly formed by CREB1. Consistent with the hemimethylation observed at the Ifng promoter having an impact on CREB1 recruitment in vivo, ChIPs performed using anti-CREB1 Ab showed greater promoter occupancy in Th1 cells than did their Th2 counterparts (Fig. 2E). The decreased binding of CREB1 in effector-stage Th2 cells, in which the Ifng gene is not active, would be consistent with CREB1 function as a trans activator. To test if CREB1 can increase activity of the Ifng promoter in primary Th1 cells, we performed nucleolectons of developing Th1 cells, using a minimal Ifng promoter reporter construct and either a CREB1 expression vector or an empty vector control (Fig. 2F). We found that CREB1 increased activity of the Ifng reporter construct. In sum, these findings show that upper-strand hemimethylation of the CpG at −53 can impair binding of CREB1, a trans activator of the Ifng promoter.

Loss of Ifng methylation in Th2-derived memory cells

Th2-derived memory cells can produce IFN-γ when exposed to Th1-skewing conditions during recall responses (35, 36). To
investigate the relationship between this capacity and the repressive methylation observed in primary Th2 cells, we prepared DNA from purified effector cells and their memory Th2 descendants (Fig. 3A). As expected, cells in the donor-derived memory pool in each type of recipient underwent homeostatic divisions after transfer (Fig. 3B), and these memory cells produced IFN-γ after reactivation by Ag and growth in Th1 conditions (Fig. 3C). Weeks after transfers into normal or lymphopenic BALB/c mice, donor-derived cells were purified from the recipient lymphoid organs. Strand-specific PCR analyses of bisulfite-modified donor-derived cellular DNA showed that methylation of multiple sites decreased (Fig. 4B) and the −53 CpG of the Ifng promoter coding strand was almost completely unmethylated (Fig. 4A, 4C). These results were independent of whether the recipients had normal endogenous T cells or were lymphopenic (data not shown). These findings provide evidence of dynamic change in Ifng promoter methylation, as the population of Th2 effectors yields a memory Th2 subset.

**STAT4 is required for flexible IFN-γ production**

The development of Th1 effector cells from naive CD4+ T cell precursors is highly dependent on IL-12-induced STAT4 and, in most settings, on T-bet (4, 7, 44). IL-12 is required for the facultative induction of IFN-γ production by memory Th2 cells after recall stimulation in vitro and in vivo (35, 36, 38). However, the IL-12R elicits multiple intracellular signals (45, 46), and which of these is essential for the plasticity of gene expression is not known. Accordingly, we compared the amounts of IFN-γ produced after recall stimulation and cultures of memory Th2 cells from Tbx21−/− and Stat4−/− T cells to that derived from parallel controls with normal transcriptional function (Fig. 5A, Supplemental Fig. 1A). When cytokine production was elicited 1 wk after recall restimulation with peptide Ag and culture under Th1 and Th2 conditions, samples of each transcription factor–deficient memory Th2 population produced substantially less IFN-γ than did matched WT controls (Fig. 5A). IFN-γ production elicited after Th1-skewed recall was higher than background with each type of knockout cell type. To assess the extent to which double-producing (IL-4+ IFN-γ+) cells could be generated from memory Th2 cells, we used intracellular staining for these cytokines (Fig. 5B, 5C). Although subject to the likelihood that the limits of detection are more sensitive for secreted cytokine than intracellular staining, these analyses consistently revealed almost no IFN-γ donor-derived (KJ1-26+ CD4+ T cells) in the absence of either STAT4 or T-bet (Fig. 5C). In sharp contrast, ample IL-4+ IFN-γ− CD4+ T cells were abundant (31% of donor T cells) when controls with normal transcription factor genes were used (Fig. 5C). These data indicate that STAT4 is required in support of the capacity for memory Th2 cells to turn on IFN-γ production to an extent similar to that of T-bet.

**FIGURE 2.** Asymmetrical methylation suffices to inhibit transcription factor binding to the Ifng promoter. (A) Schematic of the hemimethylated probes used. (B) Shown is an autoradiograph representative of EMSA results after using probes that are unmethylated (lane 1), methylated at the coding strand C(−53)pG (lane 2) or methylated at coding strand C(−45)pG, C(−45)pG, and C(−34)pG (lane 3). (C) EMSA using unmethylated probe in competition with oligonucleotides that were unmethylated (lanes 2–4), hemimethylated at C(−53) (lanes 5–7), or hemimethylated at all three upper-strand residues (lanes 8–10), at 1, 10, and 100-fold excess of competitor over probe. Shown is one result representative of three replicate assays. (D) Ab blocking/supershift assay against ATF2, c-Jun, CREB, or control (nonspecific IgG). Shown is one result representative of three replicate assays. (E) ChIPs using Abs specific for CREB1 or AcH3(K9). Eluted precipitates were analyzed for Ifng promoter sequences by PCR (left panel). Signals (mean ± SEM) from three independent biological replicates were quantified (right panel) as product relative to input, as described in Materials and Methods. **p ≤ 0.01; BLD, below limit of detection. (F) After nucleofection of primary Th1 cells with a minimal Ifng promoter–luciferase construct and pSport6 or pSport6-CREB1, luciferase activities were normalized to GFP expression. Shown are mean (± SEM) data from three independent experiments. *p ≤ 0.05.
after 2 wk, gated on CD4+ DO11.10 TCR+ cells. (transferred into WT (shaded) or nude recipients (outline) and recovered indicated. Values in each individual sample were normalized to the fre-
activation and growth (6 d) under Th1- or Th2-polarizing conditions, as indicated. Values in each individual sample were normalized to the frequencies of DO11.10 TCR+ CD4+ cell number to account for differences in cell prevalence. Shown are data from a single experiment representative of four independent replicates and consistent with earlier work (35, 36).

Low rates of S-phase entry were similar for all genotypes (Fig. 6C). Transferred cells almost completely exited the cell cycle and that
transcription factors (Fig. 6B). To compare proliferation of the transcriptationally deficient memory Th2 cells to WT controls long after the transfer, recipient mice received BrdU and its incorporation into DNA was measured. This analysis provided evidence that the transferred cells almost completely exited the cell cycle and that low rates of S-phase entry were similar for all genotypes (Fig. 6C).

**FIGURE 3.** Elicitation of IFN-γ production from memory Th2 cells (A) Schematic overview of the experimental design and generation of Th2-derived memory cells. (B) CFSE partitioning assay for DO11.10 cells transferred into WT (shaded) or nude recipients (outline) and recovered after 2 wk, gated on CD4+ DO11.10 TCR+ cells. (C) IFN-γ secretion data are shown for memory Th2 cells at the time of harvest and after recall activation and growth (6 d) under Th1- or Th2-polarizing conditions, as indicated. Values in each individual sample were normalized to the frequencies of DO11.10 TCR+ CD4+ cell number to account for differences in cell prevalence. Shown are data from a single experiment representative of four independent replicates and consistent with earlier work (35, 36).

**FIGURE 4.** Altered DNA methylation of the Ifng promoter in effector-derived memory Th2 cells. (A) Representative bisulfite sequencing results from freshly isolated memory cells are diagrammed as in Fig. 1A. (B) Mean methylated CpG dinucleotides per clone are shown as in Fig. 1C. The Th2 effector results are taken from Fig. 1 and are shown for comparison. (C) Methylation of the Ifng promoter −53 CpG in effector versus memory Th2 cells is compared as in Fig. 1E. *** p < 0.001.

T-bet induction in developing Th1 cells is driven by STAT1 and NF-κB (47, 48), but STAT4 regulates a later, IL-12–dependent phase of T-bet expression in the development of primary Th1 responses (49, 50). Therefore, we also tested whether STAT4 is required for Ifng plasticity in memory Th2 cells because it is essential for T-bet induction. Consistent with prior work (49, 50), intracellular stains detected T-bet immunofluorescence in STAT4-deficient Th1 effectors at levels equivalent to those observed for Th2 effectors (Fig. 6A, *top panel*). After recall activation and culture under Th1 conditions, however, STAT4-deficient memory Th2 cells displayed more T-bet protein expression, with at least half of the cells exhibiting induction to Th1 levels (Fig. 6A, *middle panel*). This finding indicates that the regulation of T-bet expres-
sion by STAT4 in this memory setting was not sufficient to explain the defect in Ifng induction. Together, the data show that the gene expression plasticity of memory Th2 cells—that is, elicitation of IFN-γ—requires independent input from STAT4 as well as T-bet.

**T-bet and STAT4 alter Ifng promoter methylation pattern but not homeostatic divisions**

Homeostatic divisions of cells in which asymmetrical methyl-CpG marks were present could lead to descendants in which this repressive mark was absent from the Ifng promoter on both chromosomes. Accordingly, we tested whether the rates of division were slower for Tbx21−/− or Stat4−/− Th2 cells in recipient mice. CFSE partitioning assays showed no defect in rates of division for DO11.10 Th2 cells that were T-bet- or STAT4-deficient cells, compared with controls that were WT with respect to the tran-
scription factors (Fig. 6B). To compare proliferation of the transcriptationally deficient memory Th2 cells to WT controls long after the transfer, recipient mice received BrdU and its incorporation into DNA was measured. This analysis provided evidence that the transferred cells almost completely exited the cell cycle and that low rates of S-phase entry were similar for all genotypes (Fig. 6C).

An alternative model is that the absence of T-bet or STAT4 led to a higher or more symmetric density of CpG methylation at the Ifng promoter during the development of Th2 effectors. Significant changes in upper-strand meCpG densities were not observed (Supplemental Fig. 1B), but the methylation frequency of the non-
coding strand was strikingly higher for T-bet–null Th2 cells. In addition, STAT4-deficient cells had essentially symmetrical DNA methylation (Fig. 6D) and increased noncoding strand methyla-
tion, especially at the crucial −53 CpG (Supplemental Fig. 1C). Moreover, meCpG densities in the Ifng promoter DNA from transcription factor–deficient memory Th2 cells, recovered after several weeks in vivo, were higher than those from WT memory controls (Fig. 6E; Supplemental Fig. 1D). Thus, T-bet and STAT4 each influenced Ifng promoter methylation in memory as well as effector Th2 lymphocytes.

Prior studies have supported several potential relationships between T-bet and the capacity to produce IFN-γ after Th2 differ-
etation. In one study, most human CD4 T cells could switch from polarized Th2 cytokine gene expression to turn on their IFNG gene; a subset of the helper cells unable to exhibit such flexibilit was attributed to lack of T-bet expression (51). Parallel work indicated that differentiation progressively reduced the ca-
pacity of such Th2 cells to turn on IFN-γ expression in the presence of forced T-bet expression (52). In light of the failure of T-bet–deficient Th2 cells to exhibit flexibility (Fig. 5A) and their...
cytokine staining was performed after Ag stimulation with OVA323–339 and Th1 or Th2 culture, as indicated (shown for events within the donor-derived (KJ1-26+ CD4+) gate and Stat4 represents consistent observations in three biological replicates.

a T-bet–transduced Tbx21 this finding, restimulation elicited substantial IFN-γ and IFN-γ secretion in its absence. Forcing expression of this transcription factor after Th2 differentiation by memory Th2 cells. CD4+ Th2 cells of DO11.10 4get+ Tbx21−/− or DO11.10 4get+ Stat4−/− mice were generated in parallel with DO11.10 4get− controls, flow purified, and transferred into recipients, as in Fig. 3A. Secreted IFN-γ and IL-4 were measured at the time of recovery and after recall activation and culture (6 d) under Th1 and Th2 conditions (A). Results are displayed as mean (±SEM) IFN-γ production by Tbx21−/− or Stat4−/− cells relative to WT controls after Ag restimulation with OVA323–339 peptide. *p < 0.05, **p < 0.01, ***p < 0.001. Intracellular cytokine staining was performed after Ag stimulation with OVA323–339 peptide at the time of cell recovery (B) and 1 wk after recall stimulation and Th1 or Th2 culture, as indicated (C). Cytokine staining profiles are shown for events within the donor-derived (KJ1-26+ CD4+) gate and represent consistent observations in three biological replicates.

Ifng promoter methylation pattern, we explored the impact of forcing expression of this transcription factor after Th2 differentiation in its absence. Tbx21−/− Th2 cells were transduced with a bicistronic retrovector (“MiT”) directing T-bet expression linked to Thy1.1, and compared with parallel transductions of the retrovector without T-bet cDNA. After culture in Th1 conditions and restimulation with APCs and Ag, intracellular staining for IL-4 and IFN-γ (Fig. 6F) revealed that high-level T-bet expression forced IFN-γ expression in Tbx21−/− Th2 cells. Consistent with this finding, restimulation elicited substantial IFN-γ production by a T-bet–transduced Tbx21−/− Th2 population as compared with controls. We conclude that T-bet at a sufficient level can overcome the block to IFN-γ production by established Tbx21−/− Th2 cells. On the basis of collective findings, we propose (see Discussion) that at least two barriers to Ifng gene expression are present in effector Th2 cells but are reversible once cells have experienced a transition to memory state.

Discussion
The capacity of memory cells derived from Th2 effectors to produce IFN-γ in recall responses represents a naturally occurring form of cellular reprogramming. Apart from a requirement for IL-12, type I IFNs, and the transcription factor T-bet (35, 38), nothing is known about the molecular mechanisms by which this plasticity of gene expression is effected. We have found that the Ifng promoter exhibits asymmetrical expression in committed Th2 effectors. The coding strand DNA preferentially acquires significantly increased methylation relative to the low frequency of meCPG in naive CD4+ T cells and on the noncoding strand. A hemimethylated state created by such asymmetry suffices to impair CREB1 binding to an Ifng promoter sequence that is highly conserved and strongly required for promoter activity. Consistent with these data, nucleofection assays provide evidence that CREB1 is a trans activator of the Ifng promoter, and that this ubiquitously expressed transcription factor preferentially binds to the promoter in Th1 cells as compared with Th2 counterparts. Strikingly, CpG methylation of the Ifng promoter in memory Th2 cells was observed at a frequency little different from that of the naive progenitor. Inasmuch as promoter methylation is a strongly repressive mark, these findings suggest that loss of meCPG marks contributes to the plasticity of Ifng gene expression upon recall activation. In investigating the transcription factor requirements for this facultative production of IFN-γ, we found that the IL-12–induced factor STAT4 is required along with T-bet. Surprisingly, increased densities of CpG methylation were observed in T-bet–deficient Th2 cells relative to WT controls, as well as in memory Th2 cells deficient in either of these essential transcription factors. We suggest that changes in the frequency of this repressive mark at promoters forms one—but not the only—part of the molecular basis for reprogramming of gene expression in memory Th2 cells after recall activation.

Consistent with this component of our overall model, methyl- ation of the coding stand of the Ifng promoter inhibited CREB1 binding, as well as CREB1 trans-activated Ifng promoter activity in primary Th1 cells. Previous work showed that Ifng promoter DNA methylation inhibited mobility shift complexes of the CREB/ATF family, and a more recent study used ChIP of the Th1 clone AE7 to implicate ATF2 as a major factor in this scenario (29, 53). One likely factor in a difference of results is the use of primary Th1 cells as opposed to a clone. The functional impact of CREB1 and other ATF transcription factors has been unclear, perhaps in part because of a paucity of analyses in primary cells. CREB1 appeared to inhibit (53, 54) or increase (55) Ifng transcription in T cells. However, CREB1 occupancy of the Ifng promoter enhanced transcription in human CD4+ T cells (56). Furthermore, exposure to M. tuberculosis induced CREB1 binding to the Ifng promoter in human CD4+ T cells, and RNAi–driven depletion of CREB1 in these cells decreased in IFN-γ production (57). Thus, the balance of evidence indicates that CREB1 promotes Ifng gene expression, so that asymmetrical methylation at the highly conserved CREB/ATF binding site likely contributes to the inhibition of IFN-γ production in the developing Th2 cell. Loss of such hemimethylation—for example—at the −53 site, would facilitate trans activator interactions.
As in previous reports (26, 28, 29), our analyses indicate that thymocytes and naïve CD4 T cells have quite low levels of CpG methylation at the Ifng promoter. Thus, molecular processes that are part of Th2 differentiation include direction of de novo DNA methylation to this site. In general, DNA methyltransferases (DNMTs) of the DNMT3 family (DNMT3a, b) appear to execute the process of adding new marks. T cell activation via TCR engagement increased DNMT3a expression, and a conditional loss-of-function study indicated that DNMT3a was important for repression of inappropriate cytokine genes in Th differentiation (58). Intriguingly, although memory cell generation and maintenance were not analyzed, in vitro analyses of DNMT3a-deficient cells detected plasticity of cytokine production somewhat akin to what memory Th2 cells are able to execute naturally (35, 36). Further analysis of this Dnmt3a model indicated that, similar to the low density of meCpG marking of the Ifng promoter in memory Th2 cells in our analyses, the de-repressed in vitro effectors lacking DNMT3a had levels of Ifng promoter methylation similar to those of naïve CD4 T cells (59). DNA replication naturally creates hemimethylation of CpG dyads, and establishment or enforcement of symmetry is predominantly executed by DNMT1 (60). Deletion of this DNMT early in T cell development led to a CD4 T cell population that produced >10-fold more IFN-γ upon primary ex vivo activation (61), underlining the importance of DNA methylation in the restriction of cytokine expression in CD4+ T cells to a specific effector program. Surprisingly, we found that even among cells under Th2-differentiating conditions, absence of T-bet led to increased methylation of the coding strand and substantially greater symmetry (i.e., increased noncoding strand meCpG). These results suggest that, surprisingly, the low level of T-bet present early after activation of naïve CD4+ lymphocytes under Th2 conditions (6, 7, 44) directly or indirectly impedes access of DNMTs to the Ifng promoter. Beyond this unexpected function, we infer there is an additional aspect of T-bet in the molecular events underlying plasticity of Ifng gene expression. Although the promoter methylation and its symmetry increased in T-bet-deficient Th2 cells, a second impediment to IFN-γ production lies in a block to expression of T-bet when Th2 effectors are switched to Th1-promoting conditions without a period as memory cells. This idea would be consistent with the correlative data from Th1 signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote re-expression. STAT4 is vital for Th1 differentiation, but IL-12 also signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote Ifng gene expression. STAT4 is vital for Th1 differentiation, but IL-12 also signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote Ifng gene expression. STAT4 is vital for Th1 differentiation, but IL-12 also signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote Ifng gene expression. STAT4 is vital for Th1 differentiation, but IL-12 also signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote Ifng gene expression. STAT4 is vital for Th1 differentiation, but IL-12 also signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote Ifng gene expression. STAT4 is vital for Th1 differentiation, but IL-12 also signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote Ifng gene expression. STAT4 is vital for Th1 differentiation, but IL-12 also signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote Ifng gene expression. STAT4 is vital for Th1 differentiation, but IL-12 also signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote Ifng gene expression. STAT4 is vital for Th1 differentiation, but IL-12 also signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote Ifng gene expression. STAT4 is vital for Th1 differentiation, but IL-12 also signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote Ifng gene expression. STAT4 is vital for Th1 differentiation, but IL-12 also signals through both STAT4-dependent (4) and -independent mechanisms (45, 62), each of which can promote Ifng gene expression.
gene encoding T-bet (46). Studies using conditional alleles will be needed to test whether there is an additional contribution from the mTOR–Akt–FoxO pathway, but our data establish that STAT4 is required for the flexible production of IFN-γ by Th2-derived memory cells. Of interest, this mechanism appears distinct from the primary effector phase, as we confirmed a vital role for STAT4 in maintaining T-bet protein levels in late (day 5) effector cells under Th1 conditions (49) but found little such effect in the memory Th2 cells. Thus, the requirement for STAT4 in the plasticity of memory Th2 cells is independent of an effect on T-bet expression.

A key finding of the work presented in this article was the unexpected dynamism in methylation density of promoter DNA as effector Th2 cells became a population with the low frequency of replicating (Brdu) + characters of the memory subset. Previous work on Ifng promoter regulation in memory or memory–phenotype T cells as compared with their naive or effector counterparts has focused mostly on the CD8 lineage. Pioneering work indicated that a low level of CpG methylation present in naive cells was lost upon cell activation (63–65). Surprisingly, resting memory–phenotype (total CD44hiCD8−) Th2 cells had substantial CpG methylation at their Ifng promoters, which was quickly lost upon recall stimulation (65). One potential model, which cannot be rigorously tested for a population of effector cells yielding memory, would involve active demethylation of the meCpG. Although remaining a controversial area, especially from the standpoint of molecular mechanisms, rapid loss of II2 promoter DNA methylation scored by endonuclease sensitivity was found to occur without CD4 cell division (66), and other work also supports the existence of active DNA demethylation (67). Nonetheless, our findings suggest a straightforward passive mechanism by which the observed dynamism can be explained. Although Th2 clones were reported to exhibit almost uniform methylation of CpG at the Ifng promoter (26), under the conditions used for the current study we observed meCpG frequencies well below 100% at every site on the coding strand and an asymmetry in which a high fraction of coding strand meCpG dinucleotides exist in a base quartet in which the coding strand is paired with an unmethylated CpG. This finding indicates that the functional capacity of DNMT1 to establish symmetry after initial deoxycytosine methylation was insufficient.

Memory cell homeostasis is maintained by periodic divisions after DNA replication. As a consequence, the frequency of daughter strands lacking meCpG at each site would, in the setting of hemimethylation, increase as divisions progressed. Although likely not the entire mechanistic explanation, the greater symmetry of Ifng promoter DNA methylation observed in the absence of T-bet or STAT4 in the primary Th2 effectors is associated with higher meCpG densities in the memory population, T-bet–deficient, STAT4–deficient, and WT CD4 T cells had comparable cycling and division rates. Accordingly, we infer that the requirement for these transcription factors in memory Th2 cell plasticity stems not from an impact on rates of memory cell division, but rather in part from their impact on promoter methylation and from requirements for them in trans activation.

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Disclosures

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