De Novo DNA Methylation Is Required to Restrict T Helper Lineage Plasticity

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Background: T cells undergo lineage commitment during an immune response and "remember" their lineage choice after the instructive signals cease.

Results: T cells lacking a de novo DNA methyltransferase fail to silence the Ifn gene.

Conclusion: DNMT3a opposes T cell trans-differentiation by epigenetically silencing "off-lineage" genes.

Significance: The proper control of inflammatory cytokine gene expression is crucial for immune homeostasis.

Naïve CD4+ T cells can differentiate into T helper 1 (Th1) cells that produce IFNγ, a cytokine required for the clearance of intracellular pathogens. Alternatively, naïve cells can differentiate into Th2 cells, a stable lineage that does not produce IFNγ, even if they subsequently encounter their antigen under Th1-promoting conditions (1, 2). Naïve CD4+ T cells can also become inflammatory Th17 cells or regulatory T cells (iTreg), which also do not secrete IFNγ, however, the stability of these lineages when faced with a Th1-promoting environment is less clear. The molecular basis for silencing off-lineage cytokine genes during T helper differentiation is not fully understood, but previous work has suggested that epigenetic mechanisms are involved (reviewed in Refs. 3 and 4).

DNA at the enhancer regions of the Ifn and il4 loci is hypermethylated in naïve T cells that exit the thymus (5), and the chromatin in these regions is methylated on lysine 27 of histone H3 (H3K27me3) (6). Both of these epigenetic marks act to restrict chromatin accessibility and inhibit gene transcription. These regions remain methylated in lineages that do not express the cytokine, but become demethylated in the cytokine-expressing lineages. Deletion of the gene encoding the major maintenance methyltransferase DNMT1 in the thymus results in global, genome-wide hypomethylation in naïve precursors, including those regions normally hypermethylated at cytokine loci (5). Naïve T cells from dnm1-deficient mice are able to produce differentiated cytokines such as IFNγ and IL-4 shortly after activation, indicating that maintenance of highly methylated regions of the genome during T cell development is important to oppose cytokine gene expression by mature, undifferentiated T cells (5, 7).

However, some gene regulatory regions show the opposite pattern, being hypomethylated in naïve T cells and becoming hypermethylated upon differentiation. For instance, CpG dinucleotides in the Ifn promoter are largely unmethylated in naïve CD4+ T cells. Upon Th1 differentiation, the promoter remains hypomethylated, whereas cells differentiating toward

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The abbreviations used are: Th1, T helper 1; DNMT, DNA methyltransferase; Treg, regulatory T cell.
the non–IFNγ-producing Th2 lineage exhibit substantial *de novo* DNA methylation at the *ifnγ* promoter (8, 9). This type of methylation is not catalyzed by DNMT1 but rather by the methyltransferases DNMT3a or DNMT3b (10). Unlike the global genomic effects of maintenance methylation, *de novo* DNA methylation affects <10% of the genome (11) but is crucial for silencing mobile genetic elements, maintaining chromosomal integrity at repeat regions and establishing tissue-specific gene expression patterns during mammalian development (12). In mature T cells, DNMT3a is induced upon T cell receptor ligation (13) and is recruited to the *ifnγ* promoter concomitant with its *de novo* methylation in Th2 cells (14), but whether this process is required to silence off-lineage cytokine gene transcription and to limit lineage plasticity in non-Th1 cells such as Th17 or Treg is not known.

We find that T cell–specific deletion of DNMT3a results in a complete failure of Th2, Th17, and iTreg lineage cells to *de novo* methylate DNA at the *ifnγ* promoter, which in turn results in a decreased capacity of these non-Th1 lineages to maintain the *ifnγ* gene in a silent state. Dysregulated *de novo* DNA methylation led to decreased levels of the silencing H3K27me3 mark, and increased levels of the transcriptionally permissive H3K4me3 mark upon restimulation in the presence of IL-12. These results establish a causal link between DNA methylation and histone H3K4 methylation at an endogenous locus, and demonstrate that established Th1, Th17, and iTreg use *de novo* DNA methylation to actively repress the production of IFNγ when subsequently faced with a Th1 environment.

**Experimental Procedures**

**Mice**—DNMT3a2loxP mice on a B6/129 background were provided generously by Dr. En Li (Novartis). These mice were back-crossed with B10.D2 CD4-Cre mice and B10.D2 Thy1.1 mice (Dr. Charles Drake, Johns Hopkins University) and then back-crossed with B10.D2 mice (Taconic) for 10 generations prior to intercrossing to generate DNMT3a2loxP;CD4-Cre+ or CD4-Cre- mice used in experiments.

**Antibodies, Immunoblotting, and Flow Cytometry**—Flow cytometric staining was performed using CD44-FITC, CD4-PE, CD4-PerCP, IFNγ-PE, IL-4-APC, IL-17-PE, and Tbet-PE from BD Biosciences. CD62L-APC, FoxP3-FITC, FoxP3-PE, and GATA3-APC were obtained from eBiosciences. Intracellular staining of cytokines was performed following stimulation of cells in culture with phorbol myristate acetate (50 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) in the presence of 1:1000 GolgiStop (BD Biosciences). Cells were fixed using the eBioscience FoxP3 Fix/Perm Kit to detect FoxP3, Tbet, or GATA3 along with cytokines or the BD Biosciences Cytofix/Cytoperm kit for detection of cytokines alone. Immunoblotting was performed using rabbit anti-DNMT3a (Santa Cruz Bio-technology, H-295) and rabbit anti-actin (Sigma). For cell culture, anti-CD3ε clone 145–2C11, anti-CD28 clone 37.51, and anti-IFNγ clone XMG1.2 were generously provided by Dr. Drew Pardoll, and mAbs were isolated from hybridoma culture supernatant by protein G chromatography. Anti-IL-4 (11B11) was obtained from the National Cancer Institute Biological Repository.

**Cell Culture**—Pooled spleen and lymph nodes from DNMT3a2loxP+/++;CD4−Cre+ or CD4−Cre− mice were mechanically dissociated, and CD4 T cells were enriched using the CD4 negative selection kit (Miltenyi Biotech). Cells were stained for CD4, CD44, and CD62L, and naïve CD4 T cells were isolated by flow cytometric sorting of CD4+CD62L+CD44low cells using a FACS Aria II (BD Biosciences). The post sort purity was >98%. Six-well dishes (Falcon) were coated with 5 μg/ml of anti-CD3ε in 1 ml of PBS for 2 h, and then plates were washed twice with PBS to remove unbound antibody prior to addition of medium and cells. One to two million cells were cultured in a mixture of 4 ml equal parts of RPMI and Eagle’s Ham’s Amino Acids (Invitrogen) supplemented with 10% heat-inactivated FBS (Denville), 1 mm glutamine (Invitrogen), 50 μM 2-mercaptoethanol (Sigma-Aldrich), and antibiotic/antimycotic (Invitrogen). Costimulation was provided by soluble anti-CD28 (2 μg/ml). Murine IL-2, IL-4, IL-12, IL-23, and human TGFβ were obtained from Peprotech. For Th1 cultures, medium was supplemented with IL-12 (10 ng/ml), IL-2 (1 ng/ml), and anti-IL-4 (2 μg/ml). Th2 cultures were supplemented with IL-4 (10 ng/ml), IL-2 (1 ng/ml), and anti-IFNγ (2 μg/ml). Th17 cultures were supplemented with IL-6 (20 ng/ml), TGFβ (2.5 μg/ml), IL-23 (10 ng/ml), anti-IFNγ (2 μg/ml), and anti-IL-4 (2 μg/ml). iTreg cultures were supplemented with TGFβ (5 ng/ml), IL-2 (5 ng/ml), anti-IFNγ (2 μg/ml), and anti-IL-4 (2 μg/ml). After 72 h of stimulation, cells were collected. Th1 and Th2 cells were expanded into an additional 9 volumes of fresh media supplemented with IL-2 (1 ng/ml). Th17 cells were pelleted and resuspended at 2 million per ml in fresh Th17 culture media and replated onto fresh anti-CD3ε-coated wells. iTreg cells were expanded into an additional 9 volumes of fresh media supplemented with IL-2 (5 ng/ml). After 4 additional days of culture, cells were collected and used for experiments or recultured as above for an additional week of stimulation. In some experiments, cells previously cultured under Th2, Th17, or iTreg conditions were subsequently cultured under Th1 conditions, as described above.

**DNA Methylation Analysis**—CpG methylation mapping of *ifnγ* promoter and intron I enhancer was performed by sodium bisulfite method as described previously (15). Briefly, 2 μg of genomic DNA was digested with BamHI and HindIII. DNA fragments were purified, denatured with 0.3 M NaOH, followed by conversion of cytosine to uracil by sodium bisulfite treatment. The *ifnγ* promoter and enhancer was PCR-amplified from converted DNA using previously described primers (15) designed specifically for converted DNA. PCR product was cloned into PGEM-T-easy vector (Promega), and plasmid DNA from 25 to 30 individual clones were sequenced.

**Chromatin Immunoprecipitation (ChIP) Analysis**—ChIP analysis for H3K4me3 and H3K27me3 was performed on formaldehyde fixed cells using a ChIP assay kit (Millipore) and following the protocols provided by the vendor. Trimethyl K4 and K27-specific antibodies were purchased from Cell Signaling Technologies. DNMT3a ChIP was performed in 1.5-ml tubes using polyclonal anti-DNMT3a or control anti-CD3ε Ab (Santa Cruz Biotechnology). The DNA purified from ChIP samples was analyzed for relative enrichment of *ifnγ* regulatory regions by real-time quantitative PCR with SYBR Green.
Deletion of dnmt3a at Double-Positive Stage Has Minimal Impact on Methylation Status of ifnγ Locus in Mature CD4+ T Cells—To determine the role of de novo DNA methylation in silencing of the ifnγ gene, we utilized CD4+ T cells from mice that contain a functional DNMT3a gene (DNMT3a2loxP), or from mice in which the dnmt3a gene is deleted specifically in double-positive T lymphocytes (DNMT3a2loxPxCd4-Cre). Deletion of dnmt3a at this stage does not significantly impact T cell development (13). Consistent with previous analyses (9, 15, 16), the promoter region of the ifnγ locus is hypomethylated in CD4+CD62L+CD44++ T cells from DNMT3a2loxP (wild-type) mice (Fig. 2A, left panel), whereas the enhancer region in intron I is hypermethylated (Fig. 2A, right panel). More specifically, the majority of wild-type alleles carried only one methylated CpG in the ifnγ promoter (supplemental Fig. 1A), located 17 bp downstream of the transcriptional start site (Fig. 2A, left panel). However, roughly 20% of alleles exhibited additional methylation at −53, −171, or −205 (Fig. 2A and supplemental Fig. 1A). Conversely, >90% of alleles were methylated at 8/9 or 9/9 CpG dinucleotides located in the intronic enhancer (supplemental Fig. 1A). Naive CD4+ T cells in which dnmt3a was deleted late in thymic development (DNMT3a2loxP-Cd4-Cre) exhibited a normal pattern and frequency of DNA methylation at the ifnγ locus (Fig. 2A), the only difference being an increase in the frequency of alleles with no methylated CpG dinucleotides in the promoter and a loss of alleles with two or more methylated alleles (supplemental Fig. 1A).

Loss of DNA Methylation at ifnγ Enhancer in Th1 Cells Is Dampered by DNMT3a—Next, we cultured naive, wild-type, or dnmt3a-deficient CD4+ T cells under Th1-, Th2-, Th17- or iTreg-promoting conditions in vitro through one to two rounds of stimulation. Differentiation of both wild-type and dnmt3a-deficient precursors into Th1 cells was associated with progressive demethylation of the already hypomethylated ifnγ promoter (Fig. 2B, left panels), consistent with the lack of DNMT3a recruitment to the promoter in these cells (Fig. 1D). Indeed, following the second round of polarization the majority of alleles contained no methylated CpG dinucleotides at all (supplemental Fig. 1B). However, although the dynamics of ifnγ promoter methylation occurred independently of DNMT3a in Th1 cells, DNMT1 appeared to oppose demethylation at the intronic enhancer, particularly during the second round of polarization. Wild-type Th1 cells also exhibited a roughly 50% loss of DNA methylation across the entire intronic enhancer after one round of stimulation (Fig. 2B, right panels). This population exhibited a heterogeneous, trimodal allelic distribution, with 20% of the alleles being fully methylated at all 10 CpG dinucleotides, 50% of the alleles methylated at 5, 6, or 7 CpG, whereas 30% of the alleles exhibiting no methylation (supplemental Fig. 1B). Dnmt3a-deficient Th1 cells also showed a loss of methylation at the intronic enhancer during the first round of differentiation; however, this population exhibited a bimodal allelic distribution shifted toward fewer methylated alleles as compared with wild-type Th1 cells. In the absence of DNMT3a, 60% of Th1 alleles contained only 2, 3, or 4 methylated CpG in the intron, 40% of the alleles were methylated at 8 or 9 CpG, and there were no fully methylated alleles. During the second round of Th1 polarization, wild-type cells exhibited de novo DNA
methylation at the intron, particularly at the CpG dinucleotides located at +264 and +419 (Fig. 2B). This was associated with a decrease in the frequency of fully demethylated alleles and a more even “spreading” pattern typified by a roughly equal distribution of alleles with 0, 2, 4, 5, 6, 7, 8, or 10 methylated CpG dinucleotides (supplemental Fig. 1B). However, 85% of the alleles from dnmt3a-deficient Th1 cells contained four or fewer methylated CpG, with those CpG located at +264, +304, +344, and +607 exhibiting the greatest differential methylation compared with wild-type. These data indicate that de novo methylation mediated by DNMT3a balances loss of maintenance methylation at the intronic enhancer.

De Novo DNA Methylation of ifnγ Promoter in Non-Th1 Cells Depends upon DNMT3a—Consistent with the observed DNMT3a occupancy at the ifnγ promoter (Fig. 1D), a marked increase in DNA methylation was observed at the promoter in wild-type Th2, Th17, and iTreg lineages (Fig. 2, C–E, left panels). This de novo methylation was particularly pronounced at the −53, −205, and −375 CpG sites. A distinct bimodal distribution was established in differentiating Th2 cells, with ~40% of the alleles exhibiting only 1 or 2 methylated CpG, whereas 60% of the alleles had accumulated up to 7 methylated CpG (supplemental Fig. 1C). This type of monoallelic behavior has been observed at the il4 locus in Th2 cells (17) and is reminiscent of imprinting of maternal or paternal alleles in the fertilized egg, a process known to be dependent upon DNMT3a (12). Indeed, Th2, Th17, and iTreg cells lacking DNMT3a completely failed to methylate the ifnγ promoter (Fig. 2, C–E), whereas loss of DNMT3a did not influence the intronic enhancer, which remained hypermethylated in all non-Th1 lineages (Fig. 2, C–E, right panels). Retroviral transduction of dnmt3a-deficient Th2 cells with wild-type DNMT3a but not with a catalytically inactive mutant of DNMT3a that is able to bind to chromatin was able to restore de novo methylation of the ifnγ promoter (supplemental Fig. 2). These results indicate that dysregulation of DNA methylation in dnmt3a-deficient CD4+ T cells is not due to a broader developmental defect and together demonstrate that DNMT3a is necessary and sufficient for the de novo DNA methylation observed at the ifnγ locus in non-Th1 lineages.
Dnmt3a Silences ifnγ Gene During T Helper Differentiation

To test whether de novo DNA methylation regulates other epigenetic processes at the ifnγ locus, we measured the levels of two opposing chromatin modifications, trimethylation (me3) of lysine 4 versus lysine 27 of histone H3. In wild-type and dnmt3a-deficient naïve CD4+ T cells, the ifnγ locus exhibits very low levels of H3K4me3 (Fig. 3A, dark red bars), a mark of active transcription, and low to moderate levels of H3K27me3 (Fig. 3A, dark red bars), a mark associated with transcriptionally silent genes, at the promoter, and three distinct enhancer regions. Differentiation of wild-type and dnmt3a-deficient CD4+ T cells toward the Th1 lineage was associated with a loss of H3K27 methylation and a strong increase in H3K4 methylation at the ifnγ locus (Fig. 3B). Conversely, differentiation of wild-type CD4+ T cells toward the Th2, Th17, and iTreg lineages was associated with a strong increase in the H3K27me3 silencing mark, with little or no increase in the active H3K4me3 mark (Fig. 3, C–E). Th2, Th17, and iTreg differentiated in the absence of DNMT3a exhibited a general decrease in H3K27me3 at the promoter and enhancer regions (Fig. 3, C–E, light red bars). Loss of de novo DNA methylation in Th17 cells did not result in an increase in H3K4 methylation (Fig. 3D, light green bars). However, the CNS-22 enhancer accumulated a significant degree of H3K4 methylation in dnmt3a-deficient Th2 cells (Fig. 3C, light green bars), and differentiation of dnmt3a-deficient CD4 cells into iTreg was accompanied by moderate H3K4me3 methylation at both the promoter and the intronic enhancer (Fig. 3E, light green bars). These data demonstrate that de novo DNA methylation influences histone methylation at the ifnγ locus during T helper differentiation.

Failed de Novo Methylation Is Not Sufficient to Permit ifnγ Expression under Non-Th1 Conditions—Methylation of CpG dinucleotides in the ifnγ promoter has been shown to inhibit transcription (8, 14, 18), and loss of de novo DNA methylation at the ifnγ locus led to reduced accumulation of the repressive H3K27me3 chromatin mark and, in some cases, an increase in the permissive H2K4me3 mark (Fig. 3). Therefore, we predicted that dnmt3a-deficient T cells might exhibit increased expression of the ifnγ gene upon Th2, Th17, and iTreg differentiation. To test this, we restimulated primary cultures of wild-type and dnmt3a-deficient Th1, Th2, Th17, and iTreg with PMA and ionomycin and assessed cytokine production by intracellular staining and ELISA. Interestingly, non-Th1 helper cultures lacking DNMT3a did not produce more IFNγ than wild-type cultures (Fig. 4, B–E), consistent with the lack of H3K4 histone methylation in these populations (Fig. 3). Also, the absence of DNMT3a did not significantly influence the production of IFNγ by Th1 cultures (Fig. 4, A and E), IL-4 or IL-13 production by Th2 cultures (Fig. 4, B, F, and G), IL-17-producing cells in Th17 cultures (Fig. 4, C and H), or the frequency of Foxp3+ cells in iTreg cultures (Fig. 4D). The similar pattern of cytokine expression from polarized wild-type and dnmt3a-de-
Dnmt3a Silences ifnγ Gene During T Helper Differentiation

FIGURE 4. Cytokine production by primary, wild-type, and dnmt3a-deficient T helper cells. Sorted CD4+CD62LhighCD44low naïve T cells from DNMT3a2loxP (WT, top) or DNMT3a2loxPxCD4-Cre (ko, bottom) mice were differentiated into Th1 (A and E), Th2 (B, F, and G), Th17 (C and H), or iTreg (D) in vitro. Cultures were restimulated with PMA/ionomycin, and IFNγ, IL-4, IL-17, and Foxp3 expression were measured by intracellular staining at 4 h (A–D) or by ELISA at 24 h (E–H). Results are representative of two to four separate experiments.

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...icient T cells also corresponded with similar patterns of Tbet and GATA3 expression by intracellular staining with the exception of dnmt3a-deficient iTreg, which exhibited increased expression of both transcription factors compared with wild-type iTreg (supplemental Fig. 3). These data indicate that DNMT3a is not required to establish lineage-specific patterns of cytokine production during primary CD4+ T helper differentiation and that promoter hypomethylation per se is not sufficient to drive ifnγ expression in the absence of Th1-promoting signals from IL-12.

DNMT3a Is Required to Maintain Stable Epigenetic Memory of Initial T Helper Lineage Choice at ifnγ Locus

Normal CD4+ T cells accumulate DNA and histone H3K27 methylation at the ifnγ locus and other Th1 effector genes upon Th2 and Th17, an iTreg differentiation (Figs. 2 and 3) (6). However, our data show that in the absence of DNMT3a, the ifnγ locus remains in an epigenetically naïve state in non-Th1 lineages (Figs. 2 and 3) and therefore may be more susceptible to transcriptional activation when subsequently faced with a Th1-promoting environment. To test this, we subjected the same primary wild-type and dnmt3a-deficient T helper cultures from Fig. 3 to restimulation in the presence of IL-12 and assessed H3K4, H3K27, and DNA methylation at the ifnγ locus. The ifnγ locus in established, wild-type Th2 and Th17 cells retained its initial pattern of high H3K27me3 (Fig. 5, B and C, light red bars), low H3K4me3 (Fig. 5, B and C, dark green bars), and high DNA methylation of the promoter (Fig. 5, D and E) following restimulation in the presence of IL-12. Conversely, when faced with Th1 conditions, dnmt3a-deficient Th2 and Th17 cells experienced a loss in H3K27 methylation at the ifnγ promoter and enhancer regions (Fig. 5, B and C, light red bars) and were unable to oppose the accumulation of the transcriptionally permissive H3K4me3 mark at the promoter and the intronic enhancer (Fig. 5, B and C, light green bars). Indeed, the levels of...
H3K4me3 in repolarized dnmt3a-deficient Th2 and Th17 cells were comparable with the H3K4me3 levels in primary Th1 cells (Fig. 5A). Dnmt3a-deficient Th2 and Th17 cells subjected to Th1 repolarization not only continued to exhibit a lack of DNA methylation at the ifn locus but also began to lose methylation at the intronic enhancer (Fig. 5, D and E), indicating that de novo methylation of the promoter is required to protect nearby regulatory elements from DNA demethylation. Together, these data show that active, DNMT3a-dependent mechanisms operate in differentiated Th2 and Th17 cells to oppose H3K4 histone methylation and further DNA demethylation at the ifn locus.

DNMT3a-mediated DNA Methylation Is Required to Silence ifn Gene During T Helper Differentiation—The data in Fig. 5 above indicate that the absence of de novo promoter methylation can precipitate further epigenetic changes that may poise the ifn gene for expression. To test this, we subjected established T helper lines from wild-type and dnmt3a-deficient mice to 1 week of Th1 repolarization in the presence of IL-12, restimulated them under neutral conditions, and measured production of IFNγ by ELISA and intracellular cytokine staining. Repolarization toward Th1 resulted in very little IFNγ secretion by established, wild-type Th2 cells or Foxp3+ iTreg cells, as measured by intracellular staining (Fig. 6, A and B, top histograms) or ELISA (Fig. 6, D and E, open symbols). In contrast, IL-12 signaling was able to elicit significant production of IFNγ by dnmt3a-deficient Th2 and iTreg cells (Fig. 6, A and B, bottom histograms, and D and E, filled symbols). Importantly, loss of de novo methylation led to the appearance of bipotential cells capable of producing both IL-4 and IFNγ or IL-13 and IFNγ (Fig. 6A, lower plots), as well as...
FoxP3+ cells capable of expressing IFNγ (Fig. 6B, lower plots). Wild-type Th17 cells restimulated under Th1 conditions tended to lose IL-17 expression and gain the capacity to secrete IFNγ (Fig. 6C, top histograms), consistent with the known plasticity of the Th17 lineage compared with the Th1 or Th2 lineages. In some experiments, co-expression of IL-17 and IFNγ was observed by repolarized dnmt3a-deficient Th17 cells, but this result was not consistent (data not shown). However, Th17 cultures generated from dnmt3a-deficient CD4 cells consistently exhibited a 2- to 3-fold increase in the frequency of IFNγ producers (Fig. 6C, lower plot) and a >5-fold increase in IFNγ secretion into the supernatant (Fig. 6F, filled symbols). Together, these findings show that DNMT3a is required to keep the ifnγ locus stably silenced in Th2, Th17, and iTreg cells.

**DISCUSSION**

The fertilized egg undergoes global DNA and histone demethylation that erases epigenomic information inherited from each parent, leaving the blastocyst genome largely hypomethylated (reviewed in Ref. 19). Shortly after implantation, a wave of methylation is initiated on lysine 4 of histone H3 by Trithorax-associated methyltransferases, particularly at cis-regulatory regions of highly expressed genes. Many of these genes encode factors required for self-renewal, including components of the Polycomb complex. This complex contains H3K27 methyltransferases that silence genes involved in terminal differentiation (20). Embryonic stem cell differentiation is then accompanied by a wave of de novo DNA methylation that establishes new methylation patterns in the developing tissues. This de novo methylation is concentrated at retro elements, repeat sequences, and genes involved in terminal differentiation and is achieved through the combined action of two de novo DNA methyltransferases, DNMT3a and DNMT3b (12).

Recent genome-wide surveys of histone modifications in helper T cells (6) have indicated that immunity recapitulates ontogeny, in that peripheral T cell immune responses utilize similar mechanisms as the developing embryo to enforce lineage-specific patterns of gene expression. We show in this current study that the promoter of the highly lineage-specific ifnγ gene is methylated on H3K4 and is protected from DNMT3a-
mediated de novo DNA methylation when undifferentiated CD4+ T cell precursors develop into Th1 cells. However, in non-IFNγ-producing lineages such as Th2, Th17, and Treg, chromatin at the ifnγ locus remains methylated at H3K27, H3K4 is not methylated, and the promoter is subjected to DNMT3a-mediated de novo DNA methylation. In the absence of DNMT3a, the pattern of DNA methylation at the ifnγ locus in non-Th1 cells resembles that of naive cells, and histone methylation adopts a bivalent pattern (K4me3/K27me3) that has been observed at lineage-specific genes in ES cells (23). This pattern is thought to poise a locus for either rapid expression or rapid silencing. Our studies demonstrate that patterns of low H3K4 and high H3K27 methylation are actively stabilized by de novo DNA methylation and that DNA methylation actively inhibits de novo H3K4 trimethylation at an endogenous, “off-lineage” gene in differentiated T cells.

Two previous studies, which employed in vitro-methylated DNA reporter plasmids or DNMT1-deficient fibroblasts with a genome-wide loss of DNA methylation, similarly found that DNA methylation can oppose H3K4 methylation (24, 25), but the molecular basis for the antagonism between these two marks was not established. Insight into the mechanism may come from recent work showing that H3K4 methyltransferases use CXXC domains to localize to CpG islands (26). CXXC domain-containing proteins, such as the MLL H3K4 methyltransferase, and CFP1, a crucial component of the SET1 H3K4 methyltransferase complex, bind to unmethylated CpG dinucleotides, in turn leading to K4 methylation of local nucleosomes. Methylation of CpG dinucleotides by DNMT inhibits CXXC domain binding and thus opposes the ability of histone methyltransferases to modify lysine 4. In this way, de novo DNA methylation at the ifnγ promoter actively opposes H3K4 methylation in this region, and may explain why dnmt3a-deficient Th2, Th17, and Treg cells, which are unable to de novo methylate the DNA at the ifnγ locus, fail to protect the ifnγ promoter from H3K4 methylation upon secondary Th1 repolarization.

Our results also show that proper epigenetic regulation of the ifnγ gene at the level of de novo DNA methylation is required for normal expression versus silencing. DNMT3a was not required for initial T helper differentiation, as CD4+ T cells lacking DNMT3a exhibited normal, mutually exclusive patterns of lineage-specific cytokine expression during primary polarization. Although naive CD4+ T cells are capable of adopting a wide variety of gene expression patterns, differentiated T helper cells maintain a stable memory of their initial transcriptional program that cannot be altered readily, even under changing cytokine environments. For instance, established Th2 and iTreg cells produce virtually no IFNγ if restimulated in the presence of IL-12, the major cytokine that drives Th1 differentiation. However, in the absence of DNMT3a, we show that non-Th1 lineage cells retain the ability to trans-differentiate from Th2, Th17, and Treg lineages into IFNγ-producing cells. In response to restimulation with IL-12, dnmt3a-deficient Th2 and Treg cultures contain cells that continue to produce only the factor characteristic of the initial lineage (e.g. IL-4 for Th2, Foxp3 for Treg) but also cells that produce both IFNγ and their initial lineage factor and some cells that produce only IFNγ. This means that DNMT3a operates to restrict T helper plasticity and is specifically involved in the establishment of repressive transcriptional memory.

Recent studies have shown that T helper plasticity can be augmented under some circumstances. For instance, type I interferons can prime established Th2 cells to up-regulate Tbet and produce IFNγ when subsequently faced with a Th1-inducing environment (27), and IL-23 and IL-12 can synergize in vivo to induce IFNγ production by established Th17 cells (28–30). Similarly, we observe that upon in vitro culture with IL-12, wild-type Th17 cells switch from producing IL-17 to IFNγ (Fig. 6), yet maintain strong methylation of DNA and H3K27 at the ifnγ promoter (Fig. 5). These data are consistent with a recent study indicating that human Th17 cells stimulated under Th1 conditions “remember” their initial lineage choice and switch from producing IFNγ back to IL-17 upon neutral stimulation (31). These results have several implications. First, we can conclude that, although DNMT3a clearly contributes to ifnγ silencing, methylation of the promoter is not sufficient to block trans-activation of the ifnγ gene. Second, these data indicate that Th17 cells have the capacity to bypass the epigenetic silencing marks that are initiated by DNMT3a. For instance, Tbet, the major trans-activator of ifnγ, can bind to its promoter element even when the CpG dinucleotide is methylated, displace co-repressor complexes, and reverse silencing marks at the ifnγ locus (32, 33). Why this or other mechanisms appear to be operating in Th17 cells, but not Th2 or iTreg, is not clear. In summary, our results show that de novo DNA methylation is required for establishing an epigenetically silent chromatin structure at the ifnγ locus, thereby contributing to the control of plasticity in differentiated CD4+ helper T cells.

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