Identification of DNA Methyltransferase 3a as a T Cell Receptor-Induced Regulator of Th1 and Th2 Differentiation

Christopher J. Gamper, Agoston T. Agoston, William G. Nelson and Jonathan D. Powell

*J Immunol* 2009; 183:2267-2276; Prepublished online 22 July 2009; doi: 10.4049/jimmunol.0802960

http://www.jimmunol.org/content/183/4/2267

References

This article cites 40 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/183/4/2267.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Identification of DNA Methyltransferase 3a as a T Cell Receptor-Induced Regulator of Th1 and Th2 Differentiation

Christopher J. Gamper,* Agoston T. Agoston,† William G. Nelson,* and Jonathan D. Powell2*

Ag-specific T cell cytokine expression is dictated by the context in which TCR engagement occurs. Recently it has become clear that epigenetic changes play a role in this process. DNA methyltransferase 3a (DNMT3a) is a de novo methyltransferase important to the epigenetic control of cell fate. We have determined that DNMT3a expression is increased following TCR engagement and that costimulation mitigates DNMT3a protein expression. T cells lacking DNMT3a simultaneously express IFN-γ and IL-4 after expansion under nonbiasing conditions. While global methylation of DNA from wild-type and knockout T cells is similar, DNMT3a-null T cells demonstrate selective hypomethylation of both the Ifng and Il4 loci after activation. Such hypomethylated knockout Th2 cells retain a greater capacity to express IFN-γ protein when they are subsequently exposed to Th1-biasing conditions. Based on these findings we propose that DNMT3a is a key participant in regulating T cell polarization at the molecular level by promoting stable selection of a context-specific cell fate through methylation of selective targets in T cells. The Journal of Immunology, 2009, 183: 2267–2276.

The fate of a T cell after encounter with Ag is critically dependent on the context in which activation occurs. The particular cytokines present during activation promote expansion and differentiation of T cells with restricted and stable patterns of selective cytokine expression. Such specification of cell fate is strongly associated with induction of particular transcription factors by local cytokines; for example, T-bet, GATA-3, ROR-γt, and FOXP3 are critical to Th1, Th2, Th17, and regulatory T cell differentiation, respectively (1–4). However, epigenetic changes through chromatin remodeling are also important to the stability of the differentiated phenotype (5).

Epigenetic regulation is in part controlled by DNA methylation at CpG dinucleotides near transcription initiation sites, resulting in a chromatin structure termed heterochromatin that represses transcription. DNA methylation is catalyzed by DNA methyltransferases (DNMTs), including the maintenance methyltransferase DNMT1 that acts on hemimethylated substrates to maintain methylation patterns after DNA replication and the de novo methyltransferases DNMT3a and DNMT3b that catalyze methylation of unmethylated DNA (6). The structurally related DNMT3L lacks intrinsic catalytic activity; however, it can directly bind to DNMT3a and DNMT3b, increasing their catalytic activity (7). Interestingly, DNMT3a also appears to play a role in the active de

methylion of DNA at some promoters during cyclical demethylation and remethylation that occurs surrounding transcription of these genes (8).

Alterations in DNMT expression that correlate with changes in genomic DNA methylation are well described in many cancers, including leukemias. Mizuno et al. reported that DNMT1, DNMT3a, and DNMT3b transcripts were all overexpressed in acute myelogenous leukemia and acute phase chronic myelogenous leukemia compared with normal bone marrow or chronic phase chronic myelogenous leukemia. Furthermore, the cell cycle regulator p15(INK4B) was methylated to a greater extent in cells with higher DNMT1 or DNMT3B expression (9). More recently, several reports have examined genomic methylation and DNMT expression in autoimmune disease. In immune thrombocytopenic purpura patients, Tao et al. found decreased DNMT3a and DNMT3b transcripts in PBMC compared with healthy controls (10). Luo et al. specifically examined T cell methylation and DNMT expression in patients with lupus and found that CD4 T cells had hypomethylation that was associated with lower DNMT1 and DNMT3a transcript levels (11). While Balada et al. also observed genomic hypomethylation in CD4 T cells in their cohort of lupus patients, they did not find any consistent changes in DNMT transcript levels (12). Taken together, these studies suggest that altering genomic methylation through modulation of DNMT expression may be a factor contributing to multiple diseases.

Detailed examination of the DNA methylation patterns in T cells have been reported for the prototypical Th1 cytokine gene Ifng and the Th2 cytokine gene cluster consisting of Il4, Il13, and Il5. In naive CD4 T cells, the Ifng locus is largely demethylated and remains so following activation under Th1-biasing conditions. However, under Th2-biasing conditions, it acquires de novo methylation as the locus is silenced (13–17). In contrast, the Il4 locus is largely methylated in naive CD4 T cells and remains so under Th1-biasing conditions. It is only under Th2-biasing conditions that the Il4 locus becomes progressively demethylated, favoring enhanced expression of IL-4, IL-5, and IL-13 (18–21). Such an inhibitory effect of genomic methylation on cytokine expression is supported by the observation that 5-aza-2-deoxycytidine, which inactivates DNMT1 and leads to progressive loss of DNA methylation, enhances expression of both IFN-γ and IL-4 in cultured T

*Department of Oncology, Johns Hopkins University, School of Medicine, Baltimore, MD 21231; and †Department of Pathology, Brigham and Women’s Hospital, Boston, MA 02115

Received for publication September 9, 2008. Accepted for publication June 9, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This research was supported by National Institutes of Health Grant T32CA60441 (to C.G.), a Hyundai Scholar Award (to C.G.), and American Cancer Society Grant RSG0005301-LB (to J.D.P.)

2 Address correspondence and reprint requests to Dr. Jonathan D. Powell, Department of Oncology, Johns Hopkins University, School of Medicine, 1650 Orleans Street, Cancer Research Building 1, Room 443, Baltimore, MD 21231. E-mail address: powello@jhmi.edu

3 Abbreviations used in this paper: DNMT, DNA methyltransferase; 5C.C7, B10.A/2-RAG2 mouse; ICS, intracellular cytokine staining; KO, knockout; WT, wild type.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802960
cells (19). Furthermore, T cell-specific deletion of DNMT1, which results in profound global genomic DNA hypomethylation, leads to increased and promiscuous T cell cytokine expression (22). Mice that lack MBD2 (methyl-CpG-binding domain protein 2), whose gene product binds to methylated DNA and helps partition methylated genes into silent chromatin, share a similar phenotype with T cells that coexpress IFN-γ and IL-4 (23).

A potential role for the de novo DNMTs, DNMT3a and DNMT3b, in regulation of T cell cytokine expression remains incompletely defined. DNMT3a is reported to localize at the Ifng and Il11R1 promoters in T cells (13, 24). In other cell types it is reported to localize at the Fgf-1, Sxxcl2, Hmgal1, and Wmhp genes (25, 26). Association at these sites is correlated with increased methylation and decreased target gene expression. Mice globally deficient in DNMT3a, DNMT3b, or both have been reported; DNMT3a knockout (KO) mice are runted after birth and do not survive past 4 wk of age, while the DNMT3b and double KO mice are embryonic lethal (27). Hematopoietic stem cell precursors from such mice are capable of generating T cells in lethally irradiated recipients; however, functional characterization of such T cells has not been described (28).

Given the dynamic changes in methylation identified at T cell cytokine gene loci and the apparent correlation between methylation and decreased gene expression, it was of interest when we observed that expression of one DNMT family member is regulated by TCR signaling. Our laboratory previously reported a microarray analysis that compared unstimulated T cells with cells stimulated with anti-CD3 alone or in combination with a variety of TCR signaling inhibitors to better understand regulation of T cell fate after activation (29). These experiments demonstrated a selective 38-fold up-regulation of DNMT3a transcription after TCR signaling. This pattern of TCR-regulated de novo methyltransferase activity made DNMT3a a strong candidate for participation in the acquired methylation described in activated and differentiated T cells (5). By utilizing T cells lacking DNMT3a expression, we demonstrate that TCR-induced DNMT3a is required for establishing normal patterns of DNA methylation at the Ifng locus under Th2-biasing conditions. Such methylation appears dispensable for directing the appropriate patterns of Ifng and Il4 expression in Th1- and Th2-skewed cells. However, when DNMT3a−/− Th2 cells are placed into Th1-biasing conditions, they are able to produce increased amounts of IFN-γ compared with wild-type (WT) Th2 cells. These data support DNMT3a as a critical participant in the stability of T cell gene expression via DNA methylation.

Materials and Methods

Mice

B10.A mice and B10.A/AlTac-[Tg]TcRcyt5CC7-1-[KO]Rag2 mice were purchased from Taconic. TcRcyt5CC7-1 [KO]Rag2 mice were provided by E. Li (Novartis Pharmaceuticals). DNMT3a−/− mice were bred from C57.B6/129 onto 6.5 TCR-transgenic Thy-1.1+ B10.D2 mice, a gift from C. Drake (Johns Hopkins University, Baltimore, MD). DNMT3a−/− mice were bred from 2loxP or 2loxP;CD4Cre mice were cultured with 5 μg/ml plate-bound anti-CD3 and 10 μg/ml soluble anti-CD28 in 2 ml of media. Th1 cultures included 10 ng/ml IL-12 (eBioscience) and 10 μg/ml anti-IL-4 (11B11; National Cancer Institute Repository, Frederick, MD). Th2 cultures included 10 ng/ml IL-4 (eBioscience) and 10 μg/ml anti-IFN-γ (BD Biosciences). After 48 h of stimulation, cells were collected and added to 8 ml of fresh media supplemented with 1 ng/ml IL-2 and cultured an additional 5 days. For subsequent restimulation, 2 × 106 cells were recultured with 1 μg/ml plate-bound anti-CD3 and 10 μg/ml soluble anti-CD28 plus biasing cytokines and Abs as above for 48 h and then collected and added to 8 ml of media with 1 ng/ml IL-2 and cultured an additional 5 days. Anti-DNMT3a (H-295; Santa Cruz Biotechnology), anti-actin (Sigma-Aldrich), and anti-rabbit IgG HRP (GE Healthcare) were used for Western blots. Anti-CD4-PE, anti-CD4-PerCP, anti-CD8-FITC, anti-CD8-allophycocyanin, anti-IL-4-allophycocyanin, and anti-IFN-γ-PE (BD Biosciences) were used for FACS.

Software

Statistical analysis and graphing was performed with Prism v5.0b (GraphPad Software).

Microarray analysis

Generation and analysis of the microarray data set has been described (29).

Real-time RT-PCR

Total RNA was isolated from cultured T cells using TRIzol reagent (Invitrogen). Reverse transcriptase was performed on 1 μg of total RNA with the SuperScript III reverse transcriptase kit (Invitrogen). Real-time PCR was performed using ready-made primer/probe sets for DNMT3a1, DNMT3a2, 18S endogenous control, and 2 × PCR Master Mix (Applied Biosystems) on ABI 7900 and 7500 machines. Data were analyzed using SDS 2.3 and SDS 1.5 software (Applied Biosystems). RNA abundance was calculated using the ΔCt method normalized to control 18S RNA and is plotted as 2−ΔΔCt unless otherwise indicated.

Immunoblot analysis

Whole-cell extracts were made with RIPA lysis buffer containing 1 μM orthovanadate, 1 mM sodium fluoride, 5 μg/ml PMSF, and 1× protease inhibitor mixture (Sigma-Aldrich), separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Invitrogen). Western blot by standard methods was detected with the ECL Plus detection kit (GE Healthcare).

ELISA, flow cytometry, and intracellular cytokine staining (ICS)

IL-2, IFN-γ, and IL-4 were measured in culture supernatants by ELISA (eBioscience). ICS was performed on 2 × 106 cells stimulated for 4 h with PMA and ionomycin in the presence of GolgiStop. Cells were stained using the Cytofix/Cytoperm kit (BD Biosciences). Samples were run on a FACSCalibur cytometer and data analyzed using CellQuest software (BD Biosciences).

5-Methylcytosine level determination by mass spectrometry

Total 5-methylcytosine content in genomic DNA, as a percentage of total cytosine content, was determined by a HPLC-mass spectrometry as described previously (31).

Methylation-sensitive PCR

Genomic DNA prepared with the DNeasy purification kit (Qiagen) was quantified and diluted to 10 ng/μl in water. For Il4 methylation analysis, 200 ng of DNA was incubated in enzyme buffer alone or enzyme buffer
plus HpaII (NEB) for IL-4 PCR and digested for 1 h followed by heat inactivation for 20 min at 80°C. Mock-digested DNA and HpaII-digested DNA were amplified using primers targeting DNase hypersensitive site V flanking an HpaII site (methylation primers) (forward, 5'-GAGAGGAG AGCAATTGGCTAAATCC-3', reverse, 5'-CGTCTTTTCCGTTGAC TCTCAA-3') or control primers lacking an HpaII site from hypersensitive site Va (forward, 5'-GAGAGGAGAGCAATT GGCTAAATCC-3', reverse, 5'-TGCACACATGCTCTAAATATACAGAT-3') that lie 3' of the IL-4 gene locus. PCR products were detected by ethidium bromide staining after agarose gel electrophoresis. For fng methylation analysis, 84 ng of DNA was incubated in enzyme buffer with or without 5 U of SnuBI (NEB), digested for 1 h, and heat inactivated. Mock- and SnuBI-digested DNA were amplified using 2× SYBR Green Master Mix (Applied Biosystems) and primers from the promoter (forward, 5'-CCACCTATCTGCTACCA TCTTAA-3', reverse, 5'-GCCAGGCTGGCCCTTCTTCTTATAAC-3') that flank a SnuBI site at ~35 bp from the transcription start site (methylation primers) or control primers from exon 1 (forward, 5'-GCCAGGCTGGCCCTTCTTCTTATAAC-3').

Quantitative real-time PCR was performed under standard conditions using a standard curve of undigested genomic DNA. Fractional methylation is calculated as: amount of 2loxP DNA amplified)/(amount of 2loxP DNA amplified + amount of 1loxP DNA amplified).

Real-time PCR to detect nondeleted 2loxP alleles

Primer sequences to detect 2loxP (142 forward, 5'-CTGTGGGATCTTACG GGTGATGAGC-3', 144 reverse, 5'-AAGGCTCGAAGCCTCTAGG C AAGAT-3') and 1loxP (142 forward shown above, 145 reverse, 5'-TGGTGGAGCCCGACGTATC-3') were provided by Dr. E. Li. PCR was performed on 5 ng of input genomic DNA per well in triplicate wells with plate-bound anti-CD3. Reactions were quantified against a standard curve of unamplified DNA and expressed as fold induction. Percentage nondeleted was calculated as: 100(amount of 2loxP DNA amplified + amount of 1loxP DNA amplified)/(amount of 2loxP DNA amplified) of 2loxP DNA amplified.

Results

DNMT3a is up-regulated by TCR stimulation

Prior work from our laboratory used microarray analysis to identify genes up-regulated by TCR signaling using the murine CD4 T cell clone A.E7 (29). We noted that one member of the DNMT family, DNMT3a, was selectively up-regulated 38-fold by TCR stimulation (Fig. 1A). This was confirmed by real-time PCR of samples from both A.E7 cells (unpublished data) as well as primary CD4 T cells from 5C.C7 TCR transgenic Rag2−/− mice that were previously activated by Ag/ APC and rested to model Ag-experienced cells. DNMT3a has two alternative promoters that result in a longer DNMT3a1 and a shorter DNMT3a2 transcript (32), and both are regulated by TCR signaling (Fig. 1B). DNMT3a1 transcript levels progressively increase in 5C.C7 T cells during 24 h of stimulation to 15-fold above baseline at 24 h. DNMT3a2 transcripts peak at 8-fold induction after 6 h of stimulation, and almost return to baseline by 24 h.

Having demonstrated TCR-regulated DNMT3a3a transcription in primary CD4 T cells, we wanted to determine the effects of TCR stimulation with and without costimulation in previously activated (CD62L−, CD44+) and in naive CD4 T cells (CD62L+, CD44+) at the RNA level. Previously activated T cells show low basal levels of DNMT3a message with 3- to 4-fold induction after overnight CD3 stimulation that was only slightly increased by CD28 costimulation (Fig. 1C, left panel). Unstimulated, naive 5C.C7 T cells demonstrate similar basal DNMT3a1 transcript expression to previously activated cells; however, induction with anti-CD3 was a more modest 1.5-fold, with CD28 costimulation enhancing this to a 2-fold increase from baseline (Fig. 1D, left panel). DNMT3a2 transcription (Fig. 1E) was ~2 orders of magnitude lower than DNMT3a1 transcription. However, it was similarly induced ~4- to 5-fold in previously activated cells and ~2-fold in naive cells. CD28 costimulation did not affect DNMT3a2 transcript levels.

Interestingly, regulation of DNMT3a protein expression was found to differ from mRNA. DNMT3a amino-terminal-specific polyclonal antiserum preferentially binds DNMT3a1 protein due to the greater amount of the Ag present in the larger polypeptide (our unpublished observation). This antiserum detected constitutive expression of DNMT3a1 protein in resting previously activated 5C.C7 T cells with dramatic induction by anti-CD3, ~12-fold.
Up-regulation of DNMT3a protein expression in previously activated cells is consistently attenuated by the presence of CD28 costimulation, to about half that of cells activated with anti-CD3 alone (Fig. 1C, right panel). This is despite a modest augmentation in RNA level by CD28 costimulation. DNMT3a1 expression in resting naïve cells is similar to resting previously activated cells, but induction by anti-CD3 is more modest (1.6-fold). As in previously activated cells, induction of DNMT3a was attenuated by CD28 costimulation (Fig. 1D, right panel). Similar results were obtained using either whole-cell lysate with RIPA buffer (shown) or fractionated nuclear extracts (unpublished data). Thus, DNMT3a1 protein expression in T cells differs from that predicted by mRNA expression, in that CD28 costimulation appears to exert a negative posttranscriptional effect on DNMT3a1 protein levels.

**Similar T cell phenotype with reduced cellularity in DNMT3a KO mice**

DNMT3a KO mice have abnormal postnatal development with severe runting and death by 4 wk of age (27). While these authors noted thymic expression of DNMT3a, they did not characterize the immune phenotype of the runted KO mice. Therefore, we obtained DNMT3a KO mice and examined the number and phenotype of T cells isolated from runted 2.5 to 3.5-wk-old KO animals in comparison to their littermates. As expected, DNMT3a1 protein was readily detected in thymocytes of WT littermates, but not in DNMT3a KO thymocytes (Fig. 2A). The immunophenotype of thymocytes from DNMT3a KO mice (Fig. 2B) demonstrated a variable increase in the percentage of double-negative cells (mean ± SD) from 4.3 ± 1.1% in WT to 19.8 ± 19.5% in KO and a corresponding decrease in the percentage of double-positive cells from 78.2 ± 2.5% in WT to 52.5 ± 14.7% in KO, with a modestly increased percentage of CD4 single-positive cells (WT, 14.2 ± 2.6%; KO, 22.8 ± 8.6%) and similar percentage of CD8 single-positive cells (WT, 3.4 ± 1.4%; KO, 4.9 ± 3.8%) (Fig. 2C). Despite these differences in thymocyte subset percentages, in the spleen, percentages of CD4 and CD8 T cells were similar between WT and KO mice (Fig. 2D). Consistent with their overall runted phenotype, the total cellularity of thymocytes and splenocytes in KO mice is variably decreased with average cellularity in ~10% of their WT littermates (Fig. 2, E and F). There was considerable variation noted in cellularity between individual animals, with lower cellularity appearing to correlate with animals that are more severely runted (our unpublished observation). Lower T cell numbers in KO mice do not appear to be due to an intrinsic defect in proliferation, as DNMT3a KO splenocytes proliferate similarly to WT in vitro to anti-CD3 (WT, 37,350 ± 6,956 cpm; KO, 42,060 ± 1,059 cpm at 24 h; n = 2 mice per group, p = 0.5722 by unpaired two-tailed t test). Thus, T cells lacking DNMT3a expression are able to mature through all stages of thymic development and circulate in the periphery of KO mice in similar proportions to WT T cells. Furthermore, their proliferative capacity in vitro remains similar to DNMT3a-expressing T cells. Overall, the decreases in T cell numbers are consistent with the decrease in size of the runted KO mice.

**Simultaneous IFN-γ and IL-4 expression in DNMT3a KO T cells**

Differentiation of naïve CD4 T cells into Th1 or Th2 cells is associated with well-described changes in DNA methylation at the Ifng and Il4 genes (5, 13–21). In naïve T cells, the Ifng locus is hypomethylated and the Il4 locus is hypermethylated. With Th1 differentiation, this is maintained. Under Th2 differentiation If4 becomes progressively hypomethylated and Ifng becomes methylated de novo. To determine whether TCR-induced DNMT3a plays a role in Th1 vs Th2 differentiation, we isolated DNMT3a WT and KO CD4 T cells purified by negative selection with magnetic beads and stimulated them using anti-CD3, irradiated syngeneic WT splenocytes, and IL-2 supplementation. KO T cells underwent similar expansion to WT cells, and both could be cultured for several weeks by restimulation with anti-CD3 and fresh irradiated splenocytes once weekly. Secretion of IL-2 is similar between DNMT3a WT and KO T cells and remained stable following
WT T cells cultured for 5 wk demonstrated expression of IFN-γ and IL-4 in nonselected culture in vitro. While cultured for 3–5 wk and expressing both cytokines by ELISA. While in nonselected culture in vitro.

Thus, stimulation of CD4 T cells that lack DNMT3a with anti-CD3, APC, and IL-2 results in the loss of normally mutually exclusive patterns of IFN-γ and IL-4 expression.

DNMT3a KO T cells express T-bet and GATA-3

The normal pattern of exclusive IFN-γ expression in Th1 cells and IL-4 expression in Th2 cells is regulated both at the transcriptional level by cross-regulation of T-bet and GATA-3 and by changes in DNA methylation. To determine whether transcriptional regulation of cytokine expression was disrupted in DNMT3a KO T cells, we performed real-time PCR to measure T-bet and GATA-3 expression. RNA isolated from control freshly harvested (naive) 5C.C7 CD4 T cells, control Th1-differentiated 5C.C7 T cells, and control Th2-differentiated 5C.C7 T cells demonstrated the expected pattern of low but detectable levels of each transcription factor in naive cells, with up-regulation of T-bet alone in Th1 cells and GATA-3 alone in Th2 cells (Fig. 4). WT T cells cultured for 5 wk that express only IFN-γ under our culture conditions also express only T-bet. DNMT3a KO T cells cultured for 5 wk that express both IFN-γ and IL-4 express high levels of both T-bet and GATA-3 mRNA (Fig. 4). While these findings suggest possible coexpression of both GATA-3 and T-bet in KO T cells, formal determination awaits analysis of T-bet and GATA-3 expression at the single cell level.

Coexpression of IFN-γ and IL-4 by DNMT3a KO T cells is associated with simultaneous demethylation of both genes

To better understand the abnormal regulation of IFN-γ and IL-4, we examined the methylation status of the Ifng and Il4 genes and the effects of exogenous cytokines on Th1 and Th2 skewing of KO T cells. We hypothesized that changes in methylation at the Ifng and Il4 loci in DNMT3a KO T cells would be associated with the abnormal cytokine expression patterns observed. We began by assessing DNMT3a KO T cells for a global derangement of methylation by measuring total methylcytosine content of liver and lymphocyte DNA. We observed that total methylcytosine levels were similar in liver, freshly isolated CD4 T cells, or T cells expanded for 5 wk from WT and DNMT3a KO mice (Fig. 5). Thus, overall levels of methylation in T cells do not appear to be altered by the absence of DNMT3a. This stands in contrast to the profound global hypomethylation noted by the Wilson Laboratory in DNMT1 KO T cells (22).

We next looked for selective changes in methylation of Il4 and Ifng in regions previously noted to be marked by methylation weekly restimulation through 5 wk (Fig. 3A, top left panel). T cells from WT mice on a B10.D2 background spontaneously acquire a Th1 cytokine expression profile in such cultures that only include IL-2 supplementation, with production of increasing amounts of IFN-γ but not IL-4 over time (Fig. 3A, top right and bottom panels). Initially, T cells from KO mice have slightly decreased IFN-γ production compared with WT littermates, but like WT counterparts, they do not secrete IL-4 (Fig. 3A, top right and bottom panels). Following at least two restimulations and increasing with each subsequent restimulation, however, we observed that KO T cells increase their IFN-γ secretion but also begin to spontaneously secrete IL-4 (Fig. 3A, bottom panel), in contrast to the Th1 phenotype acquired by WT T cells under these conditions.

The presence of both IFN-γ and IL-4 in the culture supernatants of DNMT3a KO T cells suggested that they were able to simultaneously differentiate into Th1 and Th2 cells in culture, a pattern normally mitigated by the inhibitory effect of IFN-γ on Th2 differentiation and IL-4 on Th1 differentiation. Therefore, we measured IFN-γ and IL-4 production by ICS performed on cells cultured for 3–5 wk and expressing both cytokines by ELISA. While WT T cells cultured 5 wk demonstrated expression of IFN-γ without IL-4 (Fig. 3B), DNMT3a KO T cells have a population of Th2 phenotype IL-4+IFN-γ− cells (17.7% KO vs 0% WT), a very limited number of Th1 phenotype IFN-γ+IL-4− cells (2.4% KO vs 85.8% WT), and a unique population that secrete both IFN-γ and IL-4 simultaneously (11.3% KO vs 0.5% WT). Thus, stimulation of CD4 T cells with anti-CD3, APC, and IL-2 resulted in the loss of normally mutually exclusive patterns of IFN-γ and IL-4 expression.

FIGURE 3. DNMT3a KO T cells spontaneously secrete IFN-γ and IL-4 in nonselected culture in vitro. A, ELISA for IL-2 (top left), IFN-γ (top right), and IL-4 (bottom) of cell culture supernatant harvested from primary stimulation or after the indicated number of weeks of culture with weekly stimulation with anti-CD3, irradiated splenocytes, and IL-2 as described in Materials and Methods. Results are representative of two experiments. B, Flow cytometry depicting IL-4 vs IFN-γ intracellular staining of CD4 T cells from DNMT3a WT (top) or KO (bottom) mice. The panel depicts cells harvested after 5 wk of culture. Results are representative of three experiments.

FIGURE 4. T-bet and GATA-3 are expressed in DNMT3a KO T cells. Real-time PCR was performed on cDNA prepared from the indicated samples. DNMT3a WT and KO cells had been cultured for 5 wk, resulting in cytokine expression as shown in Fig. 3B. Relative expression is plotted with that of naive T cells set to 1. The figure depicts mean ± SD of triplicate samples and is representative of two experiments.
during Th1 and Th2 differentiation. Several of the described methylation sites are recognized by methyltransferase-sensitive restriction enzymes (13, 20), and therefore we used restriction digestion followed by PCR flanking these sites to assess their methylation status in WT and KO T cells. Semiquantitative PCR of the Ifng locus after restriction digestion with HpaII found that DNA from both DNMT3a WT and KO livers is resistant to digestion, indicating methylation at Ifng (Fig. 5B, top panel). DNA from freshly isolated lymphocytes from both WT and KO mice is likewise resistant to digestion (Fig. 5B, top panel at right, bottom panel at left), consistent with basal Ifng methylation in both liver and T cells arising independently of DNMT3a. DNA from cultured DNMT3a WT T cells, displaying a Th1 phenotype and not secreting IL-4, remains resistant to digestion, indicating methylation. DNA from KO T cells that spontaneously secrete IL-4 becomes sensitive to restriction digestion, indicating loss of methylation (Fig. 5B, bottom panel center and right). Thus, Ifng remains methylated in the liver, freshly isolated T cells, and cultured T cells from WT mice and in the liver and freshly isolated T cells from KO mice; however, cultured KO T cells that gain IL-4 expression demonstrate loss of Ifng methylation.

In contrast to Ifng, the Ifng promoter is hypomethylated in naive T cells but becomes methylated in Th2 cells (13–15). Previously, it has been shown that methylation at a Cpg dinucleotide −53 bp from the transcription start site is well correlated with silencing of IFN-γ expression in Th2 cells. This region of the Ifng promoter is bound by DNMT3a under Th2 but not Th1 conditions, as measured by chromatin immunoprecipitation (13). This site corresponds to a SnaBI methylation-sensitive restriction site; thus, this enzyme was used to interrogate the methylation status of the promoter by amplifying with primers flanking the SnaBI site (methylation primers) or an adjacent region lacking any SnaBI site (control primers). The ratio of DNA amplified from a SnaBI-digested sample divided by that amplified from a mock-digested sample should be unity for control primers. The same ratio of signal resulting from amplification with methylation primers represents the fraction of alleles methylated at the SnaBI site. When we examined the Ifng promoter, DNMT3a is not required for development of methylation of Ifng in the liver, as both WT and KO samples were hypermethylated (Fig. 5C). Freshly isolated WT, control Th1-differentiated WT, and spontaneously generated WT IFN-γ-secreting cells are sensitive to restriction digestion (hypermethylated), but control Th2-differentiated WT cells acquire resistance to digestion (methylation) (Fig. 5C). In contrast, DNMT3a KO T cells that spontaneously acquire the ability to produce IL-4 do not have de novo methylation of the IFN-γ promoter after expansion in vitro (Fig. 5C).

**DNMT3a KO T cells correctly bias into Th1 and Th2 subsets in vitro**

Given the unusual pattern of IFN-γ and IL-4 coexpression and the hypomethylation of both genes that we observed in KO CD4 T cells, we wanted to determine whether KO CD4 T cells were able to correctly bias into Th1 or Th2 cells under strong skewing culture conditions with exogenous cytokines and neutralizing Abs. If methylation was essential to the initiation of Th1/Th2 skewing, then KO cells might produce IFN-γ/IL-4 dual-producing cells under either Th1 or Th2 culture conditions; that is, the intrinsic capacity of naive cells to express low levels of both IFN-γ and IL-4 might simply turn both cytokine genes on, regardless of the local cytokine milieu. If, however, signaling and transcriptional activators were taking advantage of a permissive chromatin structure in KO T cells, then the inclusion of neutralizing Abs in the biasing conditions might be expected to abolish expression of the neutralized cytokine and allow normal skewing to occur.

Due to the limited number of naive T cells that we could obtain from individual DNMT3a KO mice and the challenges associated with their perinatal mortality, we obtained mice bearing a floxed allele of DNMT3a that allow conditional deletion of DNMT3a (33) so that we might collect large enough numbers of naive CD4 T cells to permit isolation after sorting by flow cytometry. We crossed these mice with CD4Cre transgenic mice and obtained animals with no detectable DNMT3a protein in total CD4 T cells before or after TCR stimulation/costimulation (Fig. 6A). However, unlike runted KO mice globally deficient in DNMT3a, the T cell-specific KO offspring were healthy with normal body size and cellularity. The immunophenotype of the thymocytes (Fig. 6B) and pooled spleen and lymph node cells (Fig. 6C) from conditional KO mice was similar to their WT Cre− littermates, and cellularity of the thymus (Fig. 6D) and peripheral T cells (Fig. 6E) were not statistically different (WT, n = 3; KO, n = 4, unpaired t test p = 0.09 for thymus and p = 0.53 for spleen/lymph node). Naive CD4+CD62L+CD44− T cells were isolated by FACS sorting

**FIGURE 5.** Genomic DNA methylation in DNMT3a WT and KO T cells. A, Total genomic DNA 5-methylcytosine content determined after hydrolysis using gas chromatography/mass spectrometry. The fraction of methylated cytosine is depicted as the mean ± SE of triplicate samples. Differences in means between WT and KO samples of each tissue assessed by an unpaired two-tailed t test (liver, p = 0.1388; naive, p = 0.7440; cultured, p = 0.5652) were not significant. B, Ifng gene methylation as detected by methylation-sensitive restriction digestion and semiquantitative PCR. Indicated genomic DNA samples were incubated without (∓), or with (+) HpaII. Semiquantitative PCR was performed in three reactions on serial-diluted input (left to right equals low to high input). WT cultured and KO cultured samples are from cells cultured 5 wk with cytokine expression as depicted in Fig. 3B. Results are representative of three experiments. C, Ifng promoter methylation determined by methylation-sensitive restriction digestion and quantified by real-time PCR. WT CD4 and KO CD4 cells were cultured for 5 wk with cytokine expression as depicted in Fig. 3B. Bars depict mean ± SD of triplicate samples and are representative of three experiments.
from 2loxP;Cre<sup>−</sup> (WT) and 2loxP;Cre<sup>+</sup> (KO) mice and cultured under nonbiasing conditions with anti-CD3 plus irradiated APC or in APC-free Th1 or Th2 culture conditions. As we observed with CD4 T cells from mice constitutively deficient in DNMT3a, conditional KO CD4 T cells spontaneously secrete both IFN-γ and IL-4 after expansion with anti-CD3 plus irradiated APC, while WT cells maintain distinct populations of either IFN-γ- or IL-4-secret- 

ing cells (Fig. 6F). Thus, this second, independently generated mouse line confirms our observation of dysregulated IFN-γ and IL-4 coexpression earlier in T cells from mice deficient in DNMT3a in all tissues. However, under biasing culture conditions, DNMT3a conditional KO CD4 T cells show appropriate mutually exclusive expression of either IFN-γ or IL-4 that is indistinguishable from WT cells (Fig. 6F). IL-13 expression was similarly restricted to Th2-biased WT and KO T cells (our unpublished observation). CD4Cre-mediated deletion of floxed genes has
been reported to be incomplete (22); however, this phenomenon does not explain the similar cytokine expression pattern in our biasing WT and KO cultures. Nondeleted 2loxP alleles were undetectable in 14 of 18 2loxP:CD4Cre samples tested using quantitative real-time PCR as described in Materials and Methods, corresponding to $\geq$98% deletion based on the sensitivity of the PCR assay. This includes the KO Th1 and Th2 samples depicted in Fig. 6C. In the four KO samples where 2loxP alleles were detectable, the fraction present varied: 31% (anti-CD3 plus APC in Fig. 6C), 7% (Th1 biased), 11% (Th2 biased), and 4% (Th1 biased). Most importantly, regardless of the precise efficiency of 2loxP deletion, the “gain of function” IFN-γ IL-4+ dual-expressing KO T cells that recapitulate the global KO mice were consistently suppressed under biasing culture conditions. Thus, this normal patterning of cytokine expression in biased culture suggests that proximal cytokine signaling and critical transcriptional events necessary for Th1 and Th2 differentiation remain intact in the conditional KO T cells, despite the absence of DNMT3a expression. In these experiments, WT Th2 cells had significant de novo methylation of the Ifng promoter, but Ifng methylation was virtually absent in conditional KO cells despite the successful Th2 biasing based on their pattern of intracellular cytokine staining (Fig. 6G). Thus, DNMT3a expression is not required to establish normal patterns of cytokine expression under Th1 and Th2-biasing culture conditions, but it is essential for the de novo methylation of the Ifng promoter observed in Th2 cells.

**DNMT3a conditional KO Th2 cells are able to express higher amounts of IFN-γ after culture under Th1-biasing conditions**

Since DNMT3a conditional KO T cells are able to correctly differentiate into Th2 cells, but the Ifng promoter of these cells is devoid of methylation, we hypothesized that such cells would possess an increased ability to express IFN-γ after transfer to culture in Th1-biasing conditions. It has been reported that CD4 Th1 and Th2 cells possess some plasticity in their cytokine phenotype after up to 1 wk in biasing cultures, but that this plasticity is extinguished by 3 wk in culture under biasing conditions (34). Therefore, we cultured WT or conditional DNMT3a KO CD4 T cells under Th2-skewing conditions for 2 wk, followed by culture for 1 wk under either Th2- or Th1-skewing conditions. Although the percentage of IFN-γ producing cells after culture of Th2 cells in Th1-biasing conditions varied between individual experiments, percentages of conditional KO Th2 cells expressing IFN-γ were consistently higher than paired WT samples (representative individual experiment in Fig. 7A, pooled data from five experiments with six pairs of mice in Fig. 7B). When we examined samples used for the reversal experiment depicted in Fig. 7A, we were unable to detect nondeleted 2loxP alleles. Thus, the selective loss of methylation marks at the Ifng promoter of DNMT3a KO Th2 cells correlates with an increased ability to recover IFN-γ expression after culture of Th2 cells under Th1-skewing conditions.

**Discussion**

The acquired restriction of cytokine expression that is observed as naïve T cells are recruited to an immune response is controlled both by the selective expression of specific transcriptional activators and also by the accumulation of histone modifications and dynamic changes in DNA methylation at many effector genes. It was therefore of great interest when we observed that expression of a luciferase reporter with the DNMT3a 3′ untranslated region and reduced cellular DNA methylation levels. The consequences of enhanced DNMT3a expression in the absence of costimulation remain under investigation, but the basis of our original microarray screen that identified DNMT3a induction by TCR signaling was, in fact, to identify candidate genes that participate in T cell anergy (29). A recent report of increased genomic methylation at the Il2 locus in anergic cells provides support for such a hypothesis (36). Characterization of the response of DNMT3a KO T cells to anergizing stimuli may help clarify the potential role of de novo methylation in the stability of the anergic phenotype.

DNMT3a is a de novo methyltransferase, that is, it is able to add new methylation marks to unmethylated cytosine bases during differentiation (6). This distinguishes it from DNMT1, which acts to

![FIGURE 7. DNMT3a KO Th2 cells are able to express more IFN-γ when cultured under Th1-biasing conditions. A. Intracellular cytokine staining of CD4 T cells from a pair of DNMT3a WT and conditional KO mice cultured for 3 wk under Th2 conditions or 2 wk under Th2 conditions followed by 1 wk under Th1 conditions. Histograms depict isotype control (filled gray) or IFN-γ (solid line). B. Aggregate data depicting IFN-γ secretion for six pairs of DNMT3a WT and KO CD4 T cells cultured for 2 wk under Th2 conditions followed by 1 wk under Th1 conditions demonstrating percentage of IFN-γ expression measured by ICS as in top panel. Bars depict mean value, n = 6 mice/group; *, p = 0.0145 by paired, two-tailed Student’s t test.](http://www.jimmunol.org/)

Downloaded from http://www.jimmunol.org/ by guest on April 30, 2017
copy existing methylation marks on hemimethylated DNA from parental to daughter strands following DNA replication. These differences in substrate specificity are apparent in the phenotypic differences reported between our two models of T cells lacking DNMT3a and earlier reports from the Wilson Laboratory describing the phenotype of T cells deficient in DNMT1 (22, 37). While the DNMT1-deficient T cells have global defects in DNA methylation owing to the dilution of DNA methylation marks following each round of proliferation, our mice demonstrated preservation of total DNA methylation but defective addition of DNA new methylation marks at the Ifng locus that we examined. Cytokine expression of the DNMT1-deficient T cells, while modulated by local cytokines, is promiscuous even under skewing Th culture conditions (37). In contrast, we find that DNMT3a-deficient T cells are able to maintain faithful Th cytokine gene expression under skewing conditions but fail to maintain this pattern when skewing conditions are subsequently changed. Such plasticity in the form of increased IFN-γ expression in the DNMT3a KO T cells is associated with decreased de novo promoter methylation at the Ifng locus under Th2 culture conditions.

Although the observed methylation data support that changes in cytokine expression are due to defective silencing at the level of the cytokines themselves, they cannot exclude additional defects in methylation that affect more proximal elements regulating cytokine expression. For example, GATA-3 induction during Th2 differentiation typically interferes with the ability of IL-12 to promote Th1 differentiation, T-bet expression, and IFN-γ expression due to the down-regulation of STAT4 (38). Precise roles for DNA methylation in this process have not been defined, but it has been reported that culture of human T cells with a DNMT inhibitor is able to increase STAT4 expression (39). Thus, KO T cells may remain responsive to IL-12 even after they begin IL-4-mediated GATA-3 up-regulation if such cells fail to methylate the STAT4 locus. Alternatively, the promoter of the Tbx21 gene encoding T-bet has also been reported to acquire limited de novo methylation in Th2 cells (40). Either of these processes would be predicted to promote GATA-3 and T-bet coexpression and potentially lead to the dual Th2 helper phenotypes by analysis of the methylation state of the Ifng gene. J. Immunol. 153: 3603–3610.


34. Induction of the murine Ifng promoter is hypermethylated from early T cell development through mature naïve T cells and de novo differentiation, but is hypomethylated in Th2 cells. J. Immunol. 173: 7377–7384.


