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DNA Methylation Is a Nonredundant Repressor of the Th2 Effector Program[1]

Karen W. Makar[2] and Christopher B. Wilson[3]

The extent to which DNA methylation contributes to proper regulation of murine T cell effector function is unclear. In this study, we show that in the absence of the maintenance DNA methyltransferase Dnmt1, silencing of IL-4, IL-5, IL-13, and IL-10 in CD8 T cells was abolished, and expression of these Th2 cytokines increased as much as 1000-fold compared with that of control CD8 T cells. Th2 cytokine expression also increased in Dnmt1$^{-/-}$ CD4 T cells, but the increase (~20–40-fold for IL-4 and IL-10, ≤5-fold for IL-5 and IL-13) was less than for CD8 T cells. As a result, both Dnmt1$^{-/-}$ CD4 and CD8 T cells expressed high and comparable amounts of Th2 cytokines. Loss of Dnmt1 had more subtle effects on IL-2 (≤5-fold increase) and IFN-γ (~5–10-fold increase) expression and did not affect the normal bias for greater IL-2 expression by CD4 T cells and greater IFN-γ expression by CD8 T cells, nor the exclusive expression of perforin and granzyme B by the CD8 T cells. These results indicate that Dnmt1 and DNA methylation are necessary to prevent cell autonomous Th2 cytokine expression in CD8 T cells but are not essential for maintaining proper T cell subset-specific expression of Th1 or CTL effectors. We also found that the expression of Th2 cytokines by Dnmt1$^{-/-}$ T cells was appropriately up-regulated in Th2 conditions and down-regulated in Th1 conditions, indicating that transcription factors and DNA methylation are complementary and nonredundant mechanisms by which the Th2 effector program is regulated. ***The Journal of Immunology*****, 2004, 173: 4402–4406.**

Heritable programs of tissue-specific gene expression are controlled in part through epigenetic mechanisms, and several studies suggest a role for DNA methylation in the epigenetic regulation of cytokine gene expression. Analysis of DNA methylation in the *Il4-Il13* locus has demonstrated that the locus is highly methylated in naive CD4 and CD8 T cells and fully polarized Th1 cell lines (1, 2). As CD4 T cells differentiate into Th2 effectors, DNA methylation is slowly lost, and fully polarized Th2 cell lines have almost no DNA methylation at this locus (1, 2). IL-4 expression is up-regulated in CD4 and CD8 T cells with reduced DNA methylation, either due to a lack of the maintenance DNA methyltransferase Dnmt1 (2, 3) or following treatment with the DNA methylation inhibitor 5-azacytidine (1, 4, 5). DNA methylation has also been implicated in the regulation of other cytokines, such as IFN-γ (6), IL-2 (7), and IL-3 (8), and in the regulation of other T cell lineage genes, such as perforin (9) and CD8 (3, 10), suggesting that DNA methylation may help to establish and/or maintain the different effector responses exhibited by CD4 and CD8 T cells.

In this report, we address the contribution of DNA methylation and DNA methyltransferase 1 (Dnmt1) in the regulation of T cell effector functions using T cells from CD4CreDnmt1^{2lox} mice in which the *Dnmt1* gene is efficiently deleted in double-positive thymocytes. T cells from these mice do not express Dnmt1 and have markedly reduced levels of DNA methylation, both globally and at several specific loci, including the *Ifng* and *Il4-Il13* loci (2, 3). We show that the expression of Th2 effector cytokines is broadly and substantially derepressed in T cells lacking Dnmt1. Despite this, Th1 and Th2 cytokine expression is appropriately up- and down-regulated in Dnmt1$^{-/-}$ T cells in response to polarizing culture conditions. These results support the notion that the Th2 cytokine program is coordinately silenced in a DNA methylation-dependent manner that is separable from cytokine signaling pathways, which remain intact and functional in Dnmt1$^{-/-}$ T cells.

Materials and Methods

Mice

The generation and initial characterization of the CD4Cre Dnmt^{2lox} mice has been described previously (2, 3). Age- and sex-matched CD4CreDnmt$^{2lox/+}$ littermates were used as controls. These studies were approved by the University of Washington Animal Care and Use Committee.

Analysis of gene expression in Dnmt1$^{-/-}$ and control T cells

Naive T cells were purified from spleen and lymph nodes as described elsewhere (2) and cultured at 1×10^6 cells/ml on plates coated with anti-CD3 (5 μg/ml) and anti-CD28 (10 μg/ml) in IMDM supplemented with 10% FBS, penicillin, streptomycin, 50 μM 2-ME, and 10 ng/ml IL-2 (Chiron, Emeryville, CA). Medium for type 2-polarizing culture conditions contained 10 ng/ml IL-4 (R&D Systems, Minneapolis, MN), 10 μg/ml anti-IL-12 (BioSource International, Camarillo, CA), and 10 μg/ml anti-IFN-γ (BioSource International); for type 1-polarizing conditions, medium contained 10 μg/ml anti-IL4 and 5 ng/ml IL-12 (R&D Systems); for neutral conditions, medium contained 20 μg/ml anti-IL-4, 20 μg/ml anti-IL-12, and 20 μg/ml anti-IFN-γ. Activated cells were restimulated with 50 ng/ml PMA and 0.7 μM ionomycin (Sigma-Aldrich, St. Louis, MO) for 4–6 h before harvesting for gene expression analysis. RNA was isolated and gene expression was measured by real-time quantitative PCR as described previously (2). TaqMan probes were used for all genes except for *Il10*, *Rog*, and *Eomes*, which were quantified using Brilliant SYBR Green QPCR reagents (Stratagene, La Jolla, CA). Primer and probe sequences for *Il4*, *Il5*, *IL13*, *Gata3*, and *Tbx21* (T-bet) were as described in Ref. 11; *Actb* (β-actin) and *Ifng* were as described in Ref. 2. Sequences for all other primers and probes were as follows: perforin forward, 5′-CAG GTC AGG CCA GCA TAA GAG-3′; perforin reverse, 5′-TGG TTG GTG ACC TTT GAA TCC-3′; perforin probe, 5′-6-FAM-AGC CAT GAT TCA TGC

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CAG TGT GAG TGC-TAMRA-3′; granzyme B forward, 5′-GCC TTC TTC CTC TCC TAG AGG TTA A-3′; granzyme B reverse, 5′-CGG AAG GCC GCC TAG GT-3′; granzyme B probe, 5′-6-FAM-CAG CGT CAA GAG TGT CCT TGC TCT CT-TAMRA-3′; Eomes forward, 5′-CAC GGC TTC AGA AAA TGA CA-3′; Eomes reverse, 5′-CTC TGT TGG GGT GAG AGG AG-3′; ROG forward, 5′-CTC TCT GGA GTC AGA ATC AGC TGG-3′; ROG reverse, 5′-AGC GCT GAG GAC AGA GGC TAC AGG-3′; IL-10 forward, 5′-GGT TGC CAA GCC TTA TCG GA-3′; and IL-10 reverse, 5′-ACC TGC TCC ACT GCC TTG CT-3′.

Results

Loss of DNA methylation broadly and preferentially derepresses the Th2 effector program in CD8 T cells

We previously reported that the loss of Dnmt1 derepressed IL-4 expression, resulting in high IL-4 expression by both CD8 and CD4 Dnmt1$^{-/-}$ T cells (2). IL-4 is the hallmark Th2 cytokine, suggesting that DNA methylation might play a broader role in regulating the Th2 effector program. To address this question, we compared expression of other Th2 cytokines between Dnmt1$^{-/-}$ and control CD4 and CD8 T cells. Control naive CD4 T cells expressed IL-4, IL-13, IL-5, and IL-10 after activation, but expression was below or near the limit of detection in control naive CD8 T cells (Fig. 1*A*). Expression of each of these Th2 cytokine genes was up-regulated in Dnmt1$^{-/-}$ naive CD4 T cells, particularly IL-4 and IL-10 (~20–40-fold) and, to a lesser extent (≤5-fold), IL-5 and IL-13. Most striking was the dramatic derepression of Th2 cytokine expression in activated naive Dnmt1$^{-/-}$ CD8 T cells, which increased as much as 1000-fold. As a result, expression of these cytokines by Dnmt1$^{-/-}$ CD8 and CD4 T cells was comparable and substantially greater than expression by control CD4 T cells. These findings indicate that Dnmt1 and DNA methylation are necessary to silence the Th2 effector program in naive CD8 T cells and also to limit Th2 cytokine expression in naive CD4 T cells.

Because Th2 cytokines are typically expressed by CD4 and not CD8 T cells, these results raised the possibility that the loss of Dnmt1 might more broadly disrupt normal patterns of T cell subset-specific effector gene expression. However, this was not the case. IFN-γ expression increased ~5–10-fold in both CD4 and CD8 Dnmt1$^{-/-}$ T cells, and IL-2 expression increased ~5-fold in CD8 T cells lacking Dnmt1, but the loss of Dnmt1 did not alter the normal T cell subset-specific patterns of expression. Control and Dnmt1$^{-/-}$ CD8 T cells consistently expressed higher levels of IFN-γ than CD4 T cells from the same mice, while the converse was true for CD4 T cells (Fig. 1*B*). In addition, neither perforin nor granzyme B were up-regulated in Dnmt1$^{-/-}$ CD4 T cells, demonstrating that Dnmt1$^{-/-}$ CD4 T cells have not acquired a CD8 CTL-like gene expression profile (Fig. 1*C*). Together these results indicate that DNA methylation plays an essential role in the control of T cell subset-specific effector function by broadly silencing Th2 cytokine expression in CD8 T cells.

An examination of the kinetics of cytokine gene expression demonstrated that Dnmt1$^{-/-}$ CD8 T cells consistently expressed IL-4 mRNA by 1 day after activation and expression increased over time, whereas control CD8 T cells did not express detectable IL-4 at any time point measured (Fig. 2). By contrast, differences in IL-2 and IFN-γ expression were smaller and not detectable before 2 days of activation. Neither Dnmt1$^{-/-}$ nor control T cells expressed detectable amounts of IL-4, IL-2, or IFN-γ mRNA before activation (Fig. 2, day 0). This indicates that the loss of Dnmt1 and reduction in DNA methylation did not allow basal transcription in the absence of activation signals.

Transcription factors controlling T cell effector function are not dysregulated in Dnmt$^{-/-}$ T cells

GATA-3 and T-bet are "master regulator" transcription factors that control the expression of Th2 and Th1 cytokines, respectively (12). In CD8 T cells, repressor of GATA (ROG) antagonizes the induction of IL-4 (13), and Eomes, a T box transcription factor, enhances IFN-γ expression and may repress Th2 cytokine expression

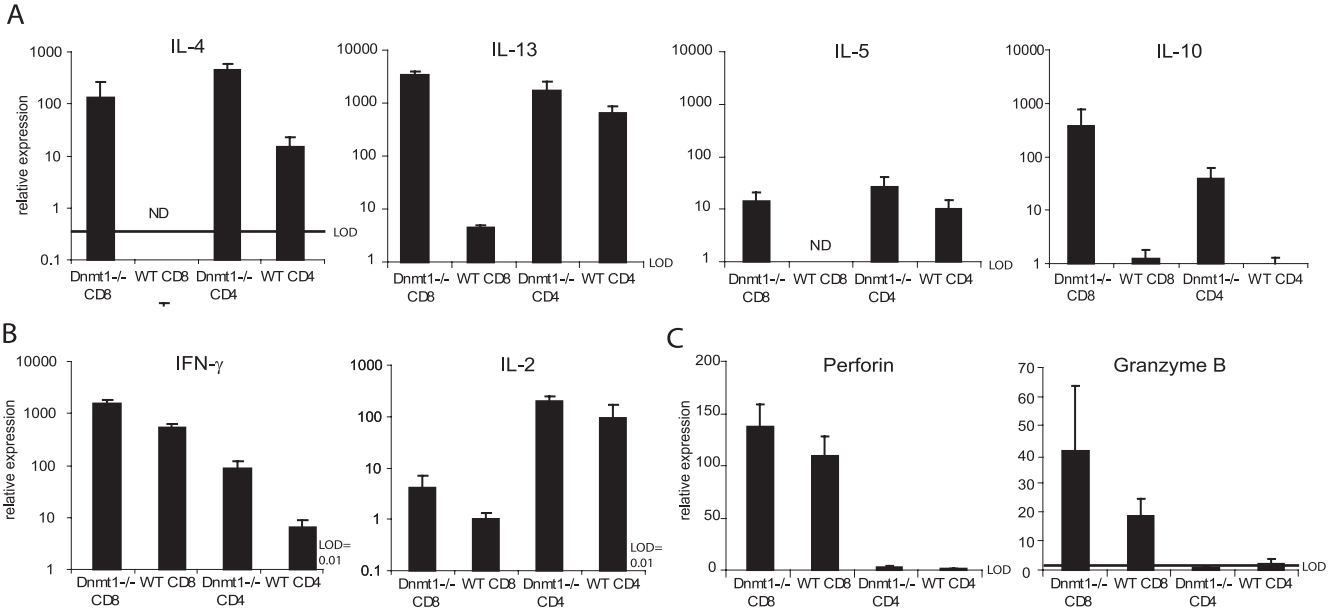

FIGURE 1. Loss of Dnmt1 and DNA methylation derepresses Th2 cytokine expression in CD8 T cells. Naive CD8 and CD4 T cells from CD4CreDnmt1^{2lox} (Dnmt1$^{-/-}$) or control (wild-type (WT)) mice were FACS purified and stimulated for 3 days on anti-CD3- and anti-CD28-coated plates. Cells were reactivated for 4–6 h with PMA and ionomycin immediately before harvesting. Relative gene expression was determined by real-time quantitative RT-PCR as described in *Materials and Methods*. For each gene, the sample with the lowest expression was set at a value of 1. The level of detection (LOD) for each assay is shown by a horizontal black line. Data represent the mean ± SD of triplicate wells from one of three to five independent experiments with similar results. ND, None detected.

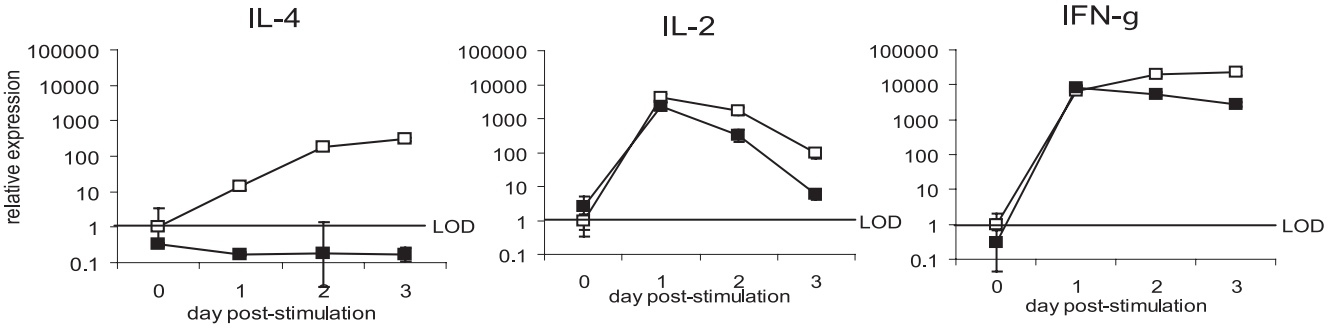

FIGURE 2. Lack of Dnmt1 allows inducible IL-4 expression early after activation in CD8 T cells. Naive ($CD44^{low/-}$) CD8 T cells from $CD4CreDnmt1^{2lox}$ (□) or control mice (■) were FACS purified and harvested immediately (day 0) or stimulated for 1–3 days on anti-CD3- and anti-CD28-coated plates. Samples from days 1–3 were reactivated for 4–6 h with PMA and ionomycin immediately before harvesting. Relative expression of IFN-γ, IL-2, and IL-4 was determined by real-time quantitative RT-PCR and calculated as described in *Materials and Methods*. The level of detection (LOD) for each gene is shown by a horizontal black line. Data represent the mean ± SD of triplicate wells from one of three independent experiments with similar results.

in CD8 T cells as T-bet does in CD4 T cells (14, 15). Thus, the up-regulation of Th2 cytokines in $Dnmt1^{-/-}$ T cells could, in principle, result from increased GATA-3 or decreased ROG, T-bet or Eomes expression. Although there was a trend toward slightly greater GATA-3 and ROG expression by $Dnmt1^{-/-}$ compared with that of control CD4 and CD8 T cells (Fig. 3), these differences were not consistently observed or statistically significant. Moreover, increased GATA-3 and ROG expression would be expected to have opposing effects on Th2 cytokine expression. These results indicate that derepression of Th2 cytokines is not mediated indirectly through changes in transcription factor expression. Furthermore, these data are consistent with our earlier observation that the up-regulation of Th2 cytokines in $Dnmt1^{-/-}$ T cells is a direct effect of reduced DNA methylation at the *Il4-Il13* locus (2).

Polarizing conditions modulate cytokine gene expression in control and $Dnmt1^{-/-}$ T cells

High-level Th2 cytokine expression by $Dnmt1^{-/-}$ T cells raised the possibility that these cells might no longer be responsive to external cytokine stimuli. To address this question, we activated naive $Dnmt1^{-/-}$ and control T cells under type 1 (IL-12, anti-IL-4), type 2 (IL-4, anti-IL-12, anti-IFN-γ), or nonpolarizing conditions (IL-2 only) for 3 days and then examined cytokine expression. Under type 2 conditions, both $Dnmt1^{-/-}$ and control CD4 T

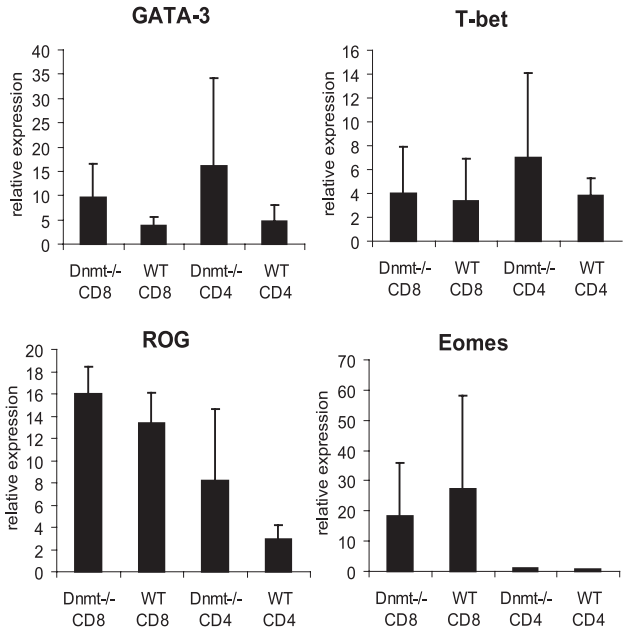

FIGURE 3. Expression of transcription factors that regulate type 1 and type 2 cytokine expression is not significantly altered in $Dnmt1^{-/-}$ T cells. Naive $Dnmt1^{-/-}$ and control T cells were purified, activated for 3 days, and relative gene expression was determined by real-time quantitative RT-PCR as in Fig. 2. Data represent the mean ± SD of relative gene expression from seven (Gata-3, T-bet) or three (ROG, Eomes) independent experiments. Values of *p* for gene expression in $Dnmt1^{-/-}$ vs control cells are: GATA-3, 0.06 (CD8), 0.12 (CD4); T-bet, 0.74 (CD8), 0.27 (CD4); ROG, 0.29 (CD8), 0.23 (CD4); and Eomes, 0.69 (CD8). Eomes expression was not reproducibly detected in $Dnmt1^{-/-}$ or control CD4 T cells.

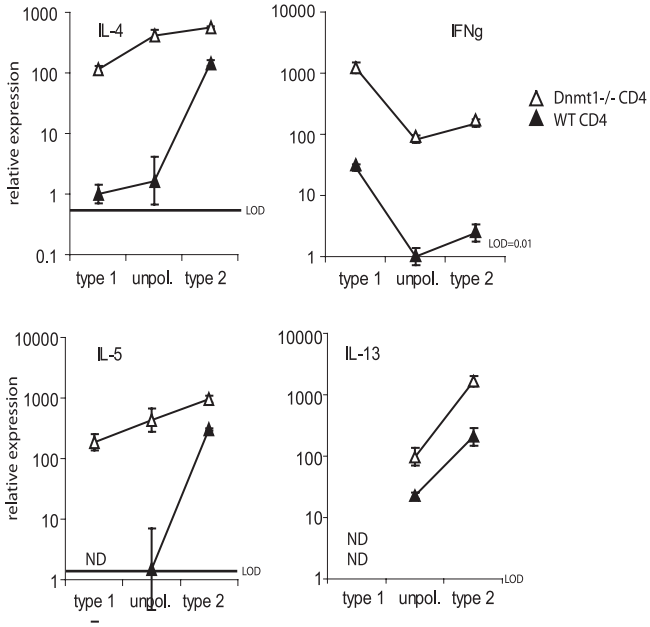

FIGURE 4. $Dnmt1^{-/-}$ CD4 T cells appropriately modulate Th2 cytokine gene expression in response to polarizing culture conditions. Relative cytokine expression by naive $Dnmt1^{-/-}$ (△) and wild-type (WT; ▲) CD4 T cells activated for 3 days under type 1, type 2, or unpolarized (unpol.) culture conditions. Samples were reactivated for 4–6 h with PMA and ionomycin immediately before harvesting. Gene expression was determined by real-time quantitative RT-PCR as described in *Materials and Methods*. For each gene, the sample with the lowest expression was set at a value of 1. The level of detection (LOD) for each assay is shown by a horizontal black line. Data represent the mean ± SD of triplicate wells from one of four to five independent experiments with similar results. ND, None detected.

cells up-regulated IL-4, IL-5, and IL-13 (Fig. 4). Under type 1 conditions, the expression of the Th2 cytokines decreased and expression of IFN-γ increased in both Dnmt1$^{-/-}$ and control CD4 T cells. Therefore, despite the fact that Dnmt1$^{-/-}$ CD4 T cells cultured in nonpolarizing conditions expressed high levels of the Th2 cytokines, these cells were able to modulate expression appropriately in response to type 1- or type 2-polarizing conditions.

Dnmt1$^{-/-}$ CD8 T cells behaved similarly. Dnmt1$^{-/-}$ CD8 T cells further up-regulated IL-4, IL-5, and IL-13 under Th2 culture conditions, and expression of these cytokines was induced in control CD8 T cells (Fig. 5*A*). Under type 1 conditions, Dnmt1$^{-/-}$ CD8 T cells decreased expression of the Th2 cytokines and increased expression of IFN-γ. These results demonstrate that Dnmt1$^{-/-}$ CD8 T cells are able to respond to polarizing signals by appropriately up- and down-regulating cytokine expression. Nonetheless, under all culture conditions, expression of IL-4, IL-5, and IL-13 was highest in Dnmt1$^{-/-}$ T cells.

IL-5 and IL-13 were not reproducibly detected in Dnmt1$^{-/-}$ CD8 T cells until 2 days after activation (data not shown), whereas IL4 was detectable at 24 h (Fig. 2). Because IL-13 and IL-5 can be up-regulated in response to IL-4 signaling (16), it was possible that the increased IL-13 and IL-5 expression observed in Dnmt1$^{-/-}$ CD8 T cells was an indirect downstream effect of increased IL-4 production. To address this question, CD8 T cells were activated in the presence of anti-IL-4, anti-IL-12, and anti-IFN-γ (neutralizing conditions) to block signaling due to these cytokines (Fig. 5*B*). There was a modest drop in IL-4 and IL-13 expression under neutralizing conditions, indicating that autocrine/paracrine IL-4 signaling did enhance expression. However, expression of IL-4, IL-5, and IL-13 was still substantially increased in Dnmt1$^{-/-}$ compared with that of control CD8 T cells. These results show that the broad derepression of Th2 cytokines in Dnmt1$^{-/-}$ CD8 T cells is not secondary to increased IL-4 expression.

Discussion

Our results show that the entire Th2 effector program is up-regulated in Dnmt1$^{-/-}$ T cells. This affect was most dramatic in CD8 T cells, which normally do not express Th2 cytokines in a cell autonomous manner. Expression of IL-4, IL-5, IL-13, and IL-10 increased up to 1000-fold in Dnmt1$^{-/-}$ compared with control CD8 T cells. As a consequence, both Dnmt1$^{-/-}$ CD4 and CD8 T cells expressed greatly increased and comparable amounts of Th2

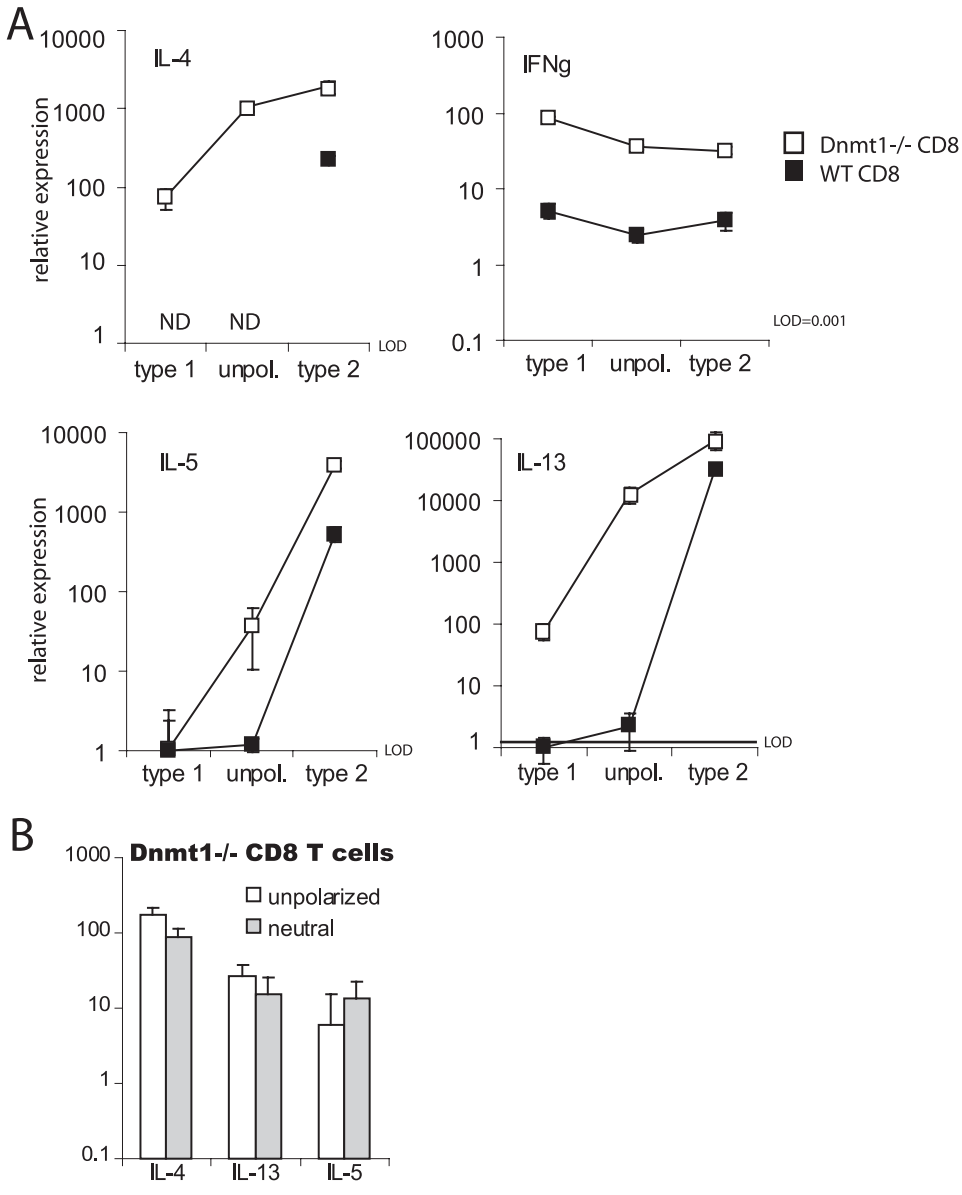

FIGURE 5. Dnmt1$^{-/-}$ CD8 T cells up-regulate Th2 cytokines in a cell-autonomous manner but still respond appropriately to polarizing culture conditions. *A*, Relative cytokine expression by naive Dnmt1$^{-/-}$ (□) and wild-type (WT; ■) CD8 T cells activated for 3 days under type 1, type 2, or unpolarized (unpol.) culture conditions. Samples were reactivated for 4–6 h with PMA and ionomycin immediately before harvesting. Gene expression was determined by real-time quantitative RT-PCR as described in *Materials and Methods*. For each gene, the sample with the lowest expression was set at a value of 1. The level of detection (LOD) for each assay is shown by a horizontal black line. Data represent the mean ± SD of triplicate wells from one of four to five independent experiments with similar results. ND, None detected. *B*, Relative cytokine expression by naive Dnmt1$^{-/-}$ CD8 T cells activated for 3 days in unpolarized (IL-2 only) or neutralizing(anti-IL-4,anti-IL-12,anti-IFN-γ) conditions. Data represent the mean relative expression ± SD of triplicate wells from one of four independent experiments with similar results.

cytokines. In contrast to these Th2 cytokines, loss of Dnmt1 had more modest effects on IL-2 and IFN-γ expression and did not alter the bias for greater expression of IL-2 by CD4 T cells and greater expression of IFN-γ by CD8 T cells. Similarly, the selective expression of perforin and granzyme B by CD8 T cells but not CD4 T cells was not altered in the absence of Dnmt1. Thus, the silencing of cell autonomous Th2 cytokine expression in the CD8 T cell lineage is dependent on Dnmt1 and DNA methylation, whereas Dnmt1 and DNA methylation may dampen expression but are not essential for maintaining proper expression of Th1 or CTL effectors.

The broad up-regulation of Th2 cytokine expression in Dnmt1$^{-/-}$ T cells did not appear to result from alterations in expression of the transcription factors GATA-3, ROG, T-bet, or Eomes, nor was it mediated indirectly by increased expression of IL-4; we have also shown previously that expression of NFATc1, NFATc2, NFATc3, STAT4, and STAT6 does not differ between control and Dnmt$^{-/-}$ T cells (2). These findings suggest that up-regulation of the Th2 cytokines resulted primarily from the loss of DNA methylation in the Th2 cytokine locus. Although loss of DNA methylation did not alter T-bet expression, it is notable that CD8 T cells from T-bet$^{-/-}$ mice, like CD8 T cells from Dnmt1$^{-/-}$ mice, express substantial amounts of IL-4, IL-5, and IL-10 (15). This has led to the suggestion that T-bet may act as a repressor of the Tc2 phenotype (15). When considered in the context of the current findings, it is possible that T-bet might initiate silencing at Th2 cytokine loci, which is then maintained through Dnmt1 and DNA methylation.

Although silencing of Th2 cytokines was lost in Dnmt1$^{-/-}$ CD8 T cells and Th2 cytokine expression was markedly up-regulated in both Dnmt1$^{-/-}$ CD4 and CD8 T cells, Th2 cytokine expression remained activation dependent and was still properly up- or down-regulated in response to polarizing signals. Similarly, expression of IFN-γ was properly up-regulated in type 1 conditions. These findings indicate that TCR and cytokine signaling pathways were intact and that cytokine signals can induce changes in gene expression even when DNA methylation is dramatically reduced. Together, these findings suggest that DNA methylation acts in collaboration with transcription factors to regulate Th2 cytokine expression.

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