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A Critical Role for Dnmt1 and DNA Methylation in T Cell Development, Function, and Survival

Peggy P. Lee,[1,7] David R. Fitzpatrick,[3,8] Caroline Beard,[4,9] Heidi K. Jessup,[1,8] Sophie Lehar,[1] Karen W. Makar,[1] Mercedes Pérez-Melgosa,[1] Marianne T. Sweetser,[2] Mark S. Schlissel,[5] Suzanne Nguyen,[4] Sara R. Cherry,[4] Jeff H. Tsai,[1] Sean M. Tucker,[1] William M. Weaver,[1] Anne Kelso,[3] Rudolf Jaenisch,[4] and Christopher B. Wilson[1,2,6]

[1]Department of Immunology
[2]Department of Pediatrics
University of Washington,
Seattle, Washington 98195
[3]The Queensland Institute of Medical Research and
The Joint Transplantation Biology Program
University of Queensland
Brisbane, QLD 4029
Australia
[4]The Whitehead Institute for Biomedical Research
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02142
[5]Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720

Summary

The role of DNA methylation and of the maintenance DNA methyltransferase Dnmt1 in the epigenetic regulation of developmental stage- and cell lineage-specific gene expression in vivo is uncertain. This is addressed here through the generation of mice in which *Dnmt1* was inactivated by Cre/loxP-mediated deletion at sequential stages of T cell development. Deletion of *Dnmt1* in early double-negative thymocytes led to impaired survival of TCR$\alpha\beta^+$ cells and the generation of atypical CD8$^+$TCR$\gamma\delta^+$ cells. Deletion of *Dnmt1* in double-positive thymocytes impaired activation-induced proliferation but differentially enhanced cytokine mRNA expression by naive peripheral T cells. We conclude that Dnmt1 and DNA methylation are required for the proper expression of certain genes that define fate and determine function in T cells.

Introduction

During their differentiation, T lymphocytes make sequential cell fate choices: T cell rather than B cell, then TCR$\alpha\beta$ or TCR$\gamma\delta$, CD4 or CD8, Th1 or Th2. These choices require the initiation of new programs of gene expression, which once initiated must be faithfully propagated from parental cells to their progeny. With the exception of the T cell receptor (TCR), these changes in gene expression occur without a change in DNA sequence.

Transcription is initiated through the ordered assembly of relevant complexes of transcription factors on regulatory regions of target genes. Thus, abundance, secondary modifications, and location of transcription factors are one means by which transcription is controlled. A second level of transcriptional control is imposed epigenetically through regulation of the accessibility of transcription factors to target genes within a highly ordered chromatin structure, thereby helping to set thresholds that transcription factors must exceed to activate gene expression (Bird and Wolffe, 1999; Reiner and Seder, 1999). In mammals, epigenetic regulation is mediated by changes in chromatin structure, which reflect in part reversible modification of histones, and by DNA methylation on cytosines in CpG dinucleotides (Bird and Wolffe, 1999; Cedar and Bergman, 1999). DNA methylation may repress gene expression directly by impeding the binding of *trans*-acting factors and indirectly through the recruitment of histone deacetylases by DNA methyltransferases and by methylated CpG binding proteins (Bird and Wolffe, 1999; Fuks et al., 2001; Robertson et al., 2000; Rountree et al., 2000); deacetylation of histones acts to compact chromatin and make it less accessible to *trans*-acting factors.

In mammals there are three known DNA methyltransferases. Dnmt1 copies the pattern of CpG methylation from the parental DNA strand to the daughter strand during S phase (Bird and Wolffe, 1999) and thereby maintains patterns of DNA methylation (Beard et al., 1995; Lei et al., 1996; Li et al., 1992). Dnmt3a and 3b are de novo methyltransferases (Okano et al., 1998). Each of these genes is essential, since mice in which either is disrupted in the germline die in utero. Maintenance of DNA methylation by Dnmt1 is also essential for proper X chromosome inactivation, parental imprinting, and silencing of parasitic retroelements (Bird and Wolffe, 1999; Jaenisch, 1997; Li et al., 1992; Walsh et al., 1998). DNA methylation correlates inversely with the expression of many tissue-specific genes, suggesting that methylation may help to confer heritable patterns of gene expression in somatic cells (Cedar and Bergman, 1999; Reiner and Seder, 1999). However, the extent to which methylation physiologically regulates or merely marks expression of tissue-specific genes is unclear and cannot be addressed in Dnmt1-deficient (Dnmt$^{N/N}$) mice, which die at gastrulation (Li et al., 1992).

In T and B lymphocytes, demethylation at the respective loci has been correlated with transcription and rearrangement of immunoglobulin and TCR genes, with cell lineage-specific expression of CD4, CD8, FcγR, and CD21, and with transcription of cytokine genes including interferon-γ (IFN-γ), IL-3, IL-4, and IL-5 (Agarwal and Rao, 1998; Asnagli and Murphy, 2001; Bird et al., 1998; Carbone et al., 1988; Cedar and Bergman, 1999; Durum et al., 1998; Fitzpatrick et al., 1998, 1999; Hamerman et al., 1997; Reiner and Seder, 1999; Sleckman et al., 1996; Young et al., 1994). To address the role of Dnmt1 and

[6]Correspondence: cbwilson@u.washington.edu
[7]Present address: Department of Biology, University of California, San Diego, La Jolla, CA 92093.
[8]Present address: Immunex Corporation, 51 University Street, Seattle, WA 98101.
[9]Present address: Department of Cancer, Immunology and AIDS, Dana Farber Cancer Institute, Boston, MA 02115.

DNA methylation in T cells directly, we generated mice with a loxP-*Dnmt1* conditional targeting allele and crossed them to mice we generated that express Cre-recombinase under the control of the lck proximal promoter or CD4 enhancer/promoter/silencer. From studies with these mice, we conclude that Dnmt1 and DNA methylation are essential for normal T cell homeostasis and play distinct developmental stage-specific roles in the regulation of T cell lineage-specific gene expression.

Results

Generation of lckCreDnmt^{2lox} and CD4CreDnmt^{2lox} Mice

To address the role of Dnmt1 in T-lineage cells, we generated mice with a loxP-*Dnmt1* targeting allele (Figure 1A). Dnmt$^{2lox/2lox}$ cells have wild-type levels of Dnmt1 protein, but following Cre-mediated deletion Dnmt1$^{1lox/1lox}$ cells lack detectable Dnmt1 protein (Jackson-Grusby et al., 2001). Dnmt$^{2lox/wt}$ ES cell clones were used to generate Dnmt^{2lox} mice. F1 progeny were intercrossed to generate Dnmt$^{2lox/2lox}$ mice or were crossed to Dnmt$^{N/wt}$ mice (Li et al., 1992) to generate Dnmt$^{2lox/N}$ mice. Unlike the Dnmt^{1lox} allele, the Dnmt^{N} allele yields small amounts (1%–2% of wild-type) of residual Dnmt1 protein (Jaenisch, 1997; Li et al., 1992). In Dnmt$^{2lox/N}$ mice, only one Dnmt^{2lox} allele must be rearranged to ablate *Dnmt1*, so the fraction of cells in a population in which *Dnmt1* has been ablated can be readily determined by comparing the ratio of 2lox to 1lox alleles. Since results with Dnmt$^{2lox/N}$ and Dnmt$^{2lox/2lox}$ mice were similar, we hereafter refer to them collectively as Dnmt^{2lox} mice.

We also generated transgenic mice in which Cre-recombinase was expressed under the control of the *lck* proximal promoter (Garvin et al., 1990) or CD4 enhancer/promoter/silencer (Sawada et al., 1994), which become active at sequential stages of T cell development. Cre mRNA expression was restricted to lymphoid tissues of these mice (data not shown). Dnmt^{2lox} and Cre-transgenic mice were then crossed and analyzed.

Efficient Developmental-Stage-Specific Deletion of *Dnmt1* and Reduced DNA Methylation in lckCreDnmt^{2lox} and CD4CreDnmt^{2lox} Mice

The major stages of T cell development in the thymus can be delineated by the expression of CD4, CD8, and the TCR, which define the TCR$^-$CD4$^-$CD8$^-$ (double-negative, DN), TCR$\alpha\beta^+$ CD4$^+$CD8$^+$ (double-positive, DP), CD4$^+$ or CD8$^+$ single-positive (SP), and CD4$^-$CD8$^-$TCR$\gamma\delta^+$ (DNTCR$\gamma\delta^+$) subsets (Kisielow and von Boehmer, 1995; Pénit et al., 1995) (Figure 1B). SP thymocytes emigrate to peripheral lymphoid organs as naive T cells. Thymocytes from lckCreDnmt^{2lox} mice showed efficient deletion of loxP-flanked *Dnmt1* as determined on Southern blots (Figure 1C) or by PCR (Figure 1D). Deletion was >50% complete in the most immature CD44$^+$ DN cells, and nondeleted alleles were not detected by PCR in CD44$^-$DN or DP plus SP thymocytes (Figure 1D, lanes 2–4). Deletion was incomplete in peripheral T cells from some (Figure 1D, lane 5) but not other (data not shown) lckCreDnmt^{2lox} mice, which appeared to reflect preferential survival of T cells derived from rare thymocytes in which deletion was incomplete (see below). As predicted, deletion began later in CD4CreDnmt^{2lox} mice, was >90% complete at the DP stage, and nondeleted alleles were not detected in CD4$^+$ and CD8$^+$ SP thymocytes or T cells (Figures 1C and 1E). Thus, targeting of *Dnmt1* was highly efficient in lckCreDnmt^{2lox} and CD4CreDnmt^{2lox} mice and occurred at sequential stages of T cell development.

Maturation of DN into DP thymocytes is accompanied by multiple rounds of cell replication, whereas proliferation of SP and TCR$\gamma\delta^+$ thymocytes and naive T cells is limited (Pénit et al., 1995; Tough and Sprent, 1998). Since Dnmt1 maintains methylation during DNA replication, we reasoned that in lckCreDnmt^{2lox} and CD4CreDnmt^{2lox} mice reduced DNA methylation should primarily occur in early thymocytes and after activation-induced proliferation of naive T cells, respectively. Global demethylation was estimated by digesting DNA with the methylation-sensitive restriction enzyme HpaII and probing Southern blots with intracisternal A particle (Walsh et al., 1998) (Figure 1F) or centromeric repeat (Beard et al., 1995) (Figure 1G) probes. Thymus but not kidney DNA from lckCreDnmt^{2lox} mice was markedly demethylated (Figure 1F), whereas splenic DNA was less demethylated, reflecting the preponderance of non-T cells in spleen; demethylation in purified subpopulations of viable thymocytes and T cells (Figure 1G) paralleled the extent of *Dnmt1* deletion (Figure 1D). In CD4CreDnmt^{2lox} mice, DNA from total thymus and DP thymocytes was only slightly demethylated, and DNA from T cells but not B cells was partially demethylated (Figures 1F and 1G); this is consistent with replication-dependent demethylation and limited replication of cells after deletion of *Dnmt1* at the DP stage.

Impaired Survival of TCR$\alpha\beta$-Lineage Cells in lckCreDnmt^{2lox} Mice

Development of TCR$\alpha\beta$-lineage cells was profoundly affected in lckCreDnmt^{2lox} mice. Although the numbers of DN thymocytes and DN thymocyte subsets were similar to littermate controls, the numbers of DP and SP thymocytes were reduced ~10-fold, and T cells in splenic, lymph node, and gut-associated lymphoid tissues were greatly reduced (Figures 2A–2C). To determine whether the reduction of thymocytes and T cells was due to a block in proliferation or to cell death, cells were pulse labeled with BrdU in vivo or stained with Annexin V in vitro. The fraction of each thymocyte subset in cycle was at least as great in lckCreDnmt^{2lox} mice as in controls (Figure 3A), although the absolute numbers of DP thymocytes in cycle was reduced >3-fold. There was a marked increase in the fraction of dead (Annexin V$^+$) thymocytes in lckCreDnmt^{2lox} mice (Figure 3A). Thymocyte death did not appear to reflect an altered balance of pro- versus antiapoptotic Bcl-2 family gene expression, which was similar to controls (Figure 3B). Nonetheless, in lckCreDnmt^{2lox} mice a lckBcl-X$_L$ transgene (Chao and Korsmeyer, 1997) substantially restored total, DP, and SP thymocyte numbers ($45.8 \pm 9.3 \times 10^6$, $28.9 \pm 5.3 \times 10^6$, and $10.5 \pm 2.9 \times 10^6$, respectively) (Figure 3C). These results suggest that impaired thymocyte development in lckCreDnmt^{2lox} mice is primarily due to reduced viability and not to a block in differentiation.

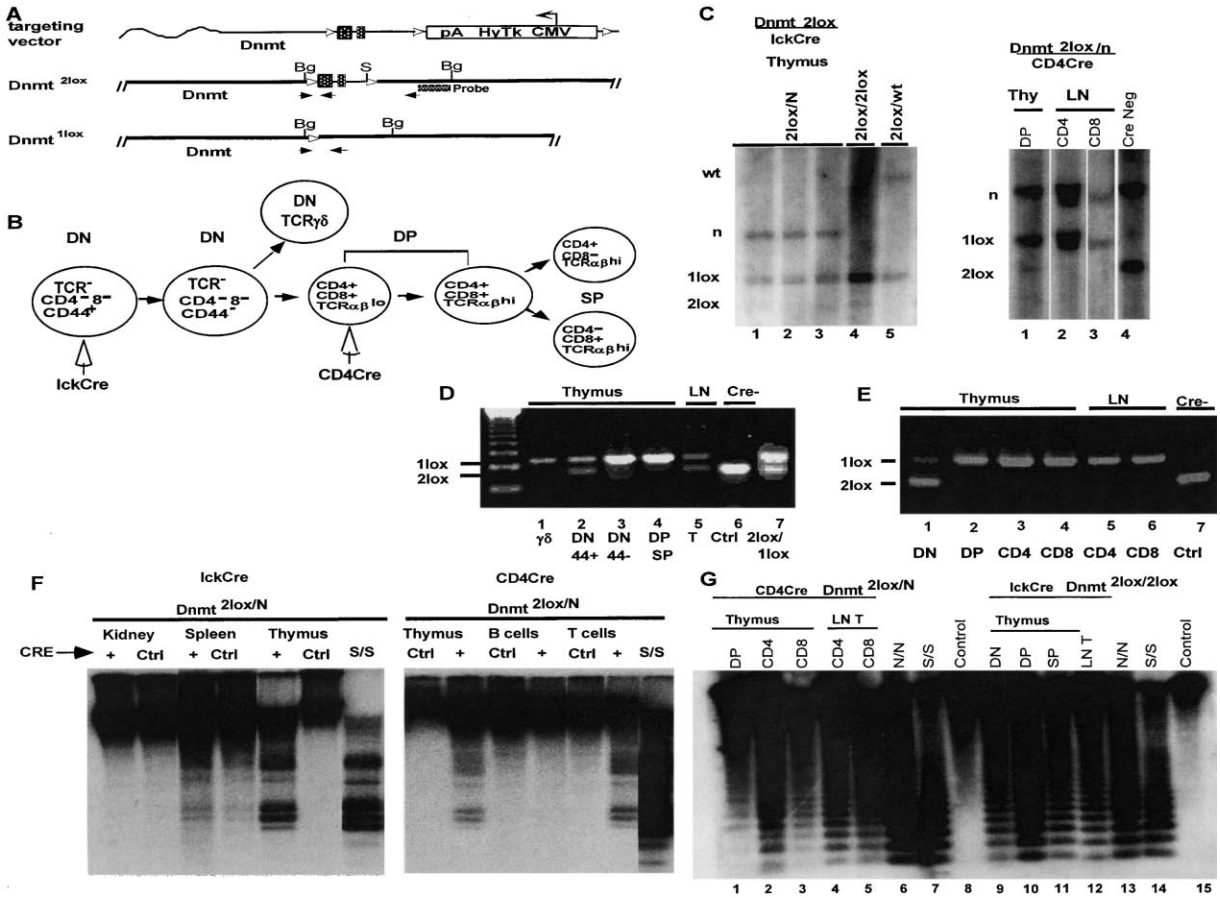

Figure 1. Targeted Deletion of *Dnmt1* in Thymocytes

(A) Targeting vector, targeted ES cells (Dnmt^{2lox}), and Cre-mediated deletion creating the Dnmt^{1lox} allele. LoxP sites are denoted by open arrowheads, and exons 4 and 5 are denoted by filled boxes.

(B) Intrathymic T cell development and stages of T cell development at which IckCre or CD4Cre transgenes are first expressed.

(C) Southern analysis of *Dnmt1* deletion in thymus and lymph node (LN) cells from IckCreDnmt^{2lox} and CD4Cre Dnmt^{2lox} mice. By densitometry, nontargeted Dnmt^{2lox} alleles in total thymocytes from the IckCreDnmt^{2lox} mice were as follows: lanes 1, 2, and 5, 0%; lane 3, 4%; and lane 4, 6%; and for CD4CreDnmt^{2lox} mice: DP thymocytes, 15%; lymph node CD4$^+$ or CD8$^+$ T cells, 0%; and thymocytes from Cre-negative controls, 100%.

(D) PCR analysis of *Dnmt1* deletion in thymocyte subsets (lanes 1–4) and LN T cells (lane 5) from IckCreDnmt$^{2lox/N}$ mice; thymocytes from Cre-negative controls (lane 6); and Dnmt$^{2lox/1lox}$ ES cells (lane 7). DNA markers are on the left. Nontargeted Dnmt^{2lox} alleles: lane 1, 9%; lane 2, 39%; lanes 3 and 4, 0%; lane 5, 41%; lane 6, 100%.

(E) PCR analysis of *Dnmt1* deletion in thymocyte subsets and LN T cells from CD4CreDnmt$^{2lox/N}$ mice and thymocytes from Cre-negative controls (lane 7). Nontargeted Dnmt^{2lox} alleles: lane 1, 90%; lane 2, 9%; lanes 3–6, 0%.

(F) Southern analysis using an intracisternal A particle probe (Walsh et al., 1998) and HpaII-digested DNA from IckCreDnmt$^{2lox/N}$ (+), CD4Cre-Dnmt$^{2lox/N}$ (+), and control (Ctrl) mice and from Dnmt-deficient (S/S) ES cells (Li et al., 1992).

(G) Southern analysis using a centromeric repeat probe (Beard et al., 1995) and HpaII-digested DNA from thymus and LN cells of CD4Cre-Dnmt$^{2lox/N}$, IckCreDnmt$^{2lox/2lox}$, and control (CD4CreDnmt$^{2lox/wt}$ and IckCreDnmt$^{2lox/wt}$) mice and from Dnmt1-deficient (N/N, S/S) ES cells (Lei et al., 1996; Li et al., 1992).

CD8αβ$^+$TCRγδ$^+$ Thymocytes and T Cells in IckCreDnmt^{2lox} Mice

In contrast to TCRαβ-lineage cells, TCRγδ$^+$ cells were more abundant in the thymus, spleen, and lymph nodes of IckCreDnmt^{2lox} mice (Figure 4A). The increase in TCRγδ$^+$ cells did not appear simply to reflect a compensatory increase in response to the decreased numbers of TCRαβ-lineage cells, since TCRγδ$^+$ cells were even further increased in IckCreDnmt^{2lox}Bcl-X$_L$ mice (Figure 4A), in which DP and SP TCRαβ thymocyte subsets were substantially restored. Many TCRγδ$^+$ cells in IckCre-Dnmt^{2lox} and IckCreDnmt^{2lox}Bcl-X$_L$ mice atypically expressed CD8 (Figure 4A), and in many of these mice the majority of CD8$^+$ thymocytes and T cells were TCRγδ$^+$ rather than TCRαβ$^+$ (Figure 4B). In some of these mice, nontargeted Dnmt 2lox alleles were found in a fraction of peripheral T cells and thymic TCRγδ$^+$ cells (an example is shown in Figure 1D), but in other mice nontargeted alleles were not detected by PCR in these cell populations; CD8$^+$TCRγδ$^+$ thymocytes and T cells were evident in mice in which nontargeted alleles were detected and in mice in which they were not. CD8$^+$TCRγδ$^+$ cells were not clonal, since they showed a diverse pattern of TCRβ and γ gene rearrangements and, as expected, lacked TCRα gene rearrangements (Figure 4C). Most CD8$^+$TCRγδ$^+$ cells were CD8α$^+$CD8β$^+$ (Figure 4D), which is character-

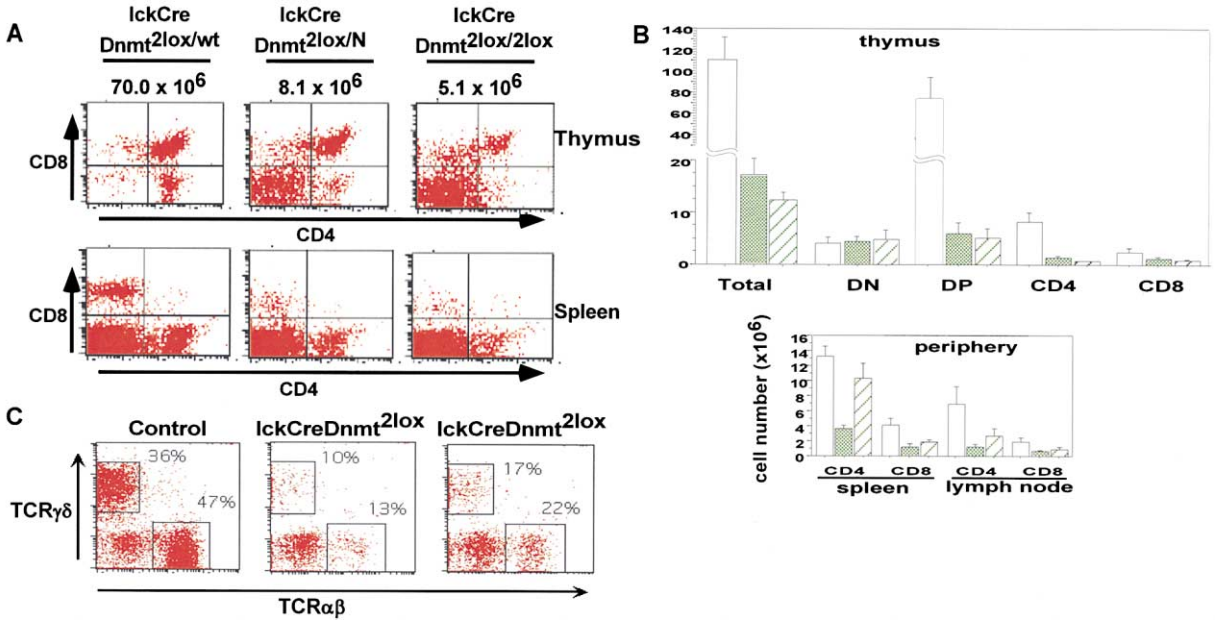

Figure 2. TCRαβ-Lineage T Cell Development in lckCreDnmt^{2lox} Mice

(A) Representative CD4 versus CD8 dot plots of thymocytes and spleen cells.

(B) Numbers of thymocytes and spleen or lymph node T cells in lckCre Dnmt$^{2lox/wt}$ littermate control (open bars, n = 10), lckCreDnmt$^{2lox/2lox}$ (filled bars, n = 7), and lckCreDnmt $^{2lox/N}$ (hatched bars, n = 5) mice.

(C) Gut-associated intraepithelial T cells (IEL). Results from one control and two lckCreDnmt^{2lox} mice are shown.

istic of thymus-derived TCRαβ-lineage T cells but not thymus- or gut-derived TCRγδ$^+$ T cells (Hamerman et al., 1997). Consistent with this, in contrast to the spleen and lymph nodes, TCRγδ$^+$ cells were markedly reduced in the gut-associated lymphoid tissues (Figure 2C). The expression of CD8 by TCRγδ$^+$ cells was selective, since expression of cell lineage-specific genes was not broadly derepressed in T-lineage cells from lckCre-Dnmt^{2lox} mice: CD8 was not aberrantly expressed on TCRαβ$^+$CD4$^+$ T cells (Figure 2A), nor did we observe the aberrant expression on T-lineage cells of proteins normally expressed on NK or B cells (NK1.1, FcγR, CD21, B220) but not on T cells (data not shown).

To explore the mechanisms for the development of CD8$^+$TCRγδ$^+$ cells in lckCreDnmt^{2lox} mice, we first evaluated methylation of the respective gene loci. Methylation of CD8 α and β was examined in regions of the genes for which, by methylation-sensitive restriction enzyme analysis, demethylation has been correlated with expression (Carbone et al., 1988; Hamerman et al., 1997). To provide a more detailed and quantitative examination of CpG methylation in these regions than is possible with restriction enzyme analysis, we sequenced multiple clones of bisulfite-modified genomic DNA (Fitzpatrick et al., 1998). The CD8α locus was markedly demethylated in CD8$^+$TCRγδ$^+$ cells and partially demethylated in DNTCRγδ$^+$ cells from lckCreDnmt^{2lox} mice compared to TCRγδ$^+$ cells from lckCreDnmt$^{2lox/wt}$ littermate controls (Figure 4E), suggesting that progressive demethylation of this locus resulted in the expression of CD8α by TCRγδ$^+$ cells when methylation fell below a critical threshold. The CD8β locus was also less methylated in the CD8$^+$TCRγδ$^+$ cells from lckCreDnmt^{2lox} mice. Overall, the degree of demethylation of the CD8α and β genes in CD8$^+$TCRγδ$^+$ cells was similar to DP thymocytes from controls. These results are consistent with the notion that CD8αβ expression is normally repressed in TCRγδ$^+$ cells by methylation and was derepressed following the loss of Dnmt1 and the reduction of DNA methylation in *cis* at the CD8α and β loci.

Demethylation of sequences in the TCRγ enhancer (Eγ) and histone hyperacetylation of the enhancers (Eδ, Eα) in the TCRδ/α locus have been correlated with expression and V(D)J recombination at these loci in T cell progenitors (Durum et al., 1998; McMurry and Krangle, 2000; Schlissel et al., 2000). This raised the possibility that the loss of Dnmt1 enhanced demethylation at these loci in T cell progenitors of lckCreDnmt^{2lox} mice and thereby favored the generation of TCRγδ$^+$ cells. Since the CD25$^+$CD4$^-$CD8$^-$TCR$^-$ thymocyte population (CD25$^+$DN) includes precursors of both TCRγδ$^+$ and TCRαβ$^+$ cells, we assessed the methylation of CpG in these enhancers by sequencing of bisulfite-modified DNA and quantified V(D)J recombination intermediates in CD25$^+$DN thymocytes and, for comparison, in DP thymocytes. These enhancers were fully demethylated in CD25$^+$DN and DP thymocytes from lckCreDnmt^{2lox}, but were also largely demethylated (>85%) in cells from control mice. Consistent with these findings, the relative abundance of TCRJγ1, 2, and 3 and TCRDβ2 RAG-mediated V(D)J recombination intermediates (Schlissel et al., 2000) was similar in cells from lckCreDnmt^{2lox} mice and controls (Figure 5), suggesting that rearrangements leading to the generation of TCRγδ$^+$ cells relative to TCRαβ$^+$ cells were not more common in these mice. There was also no evidence to suggest that the TCRγ locus remained accessible in DP thymocytes of lckCreDnmt^{2lox} mice, since unresolved recombination in-

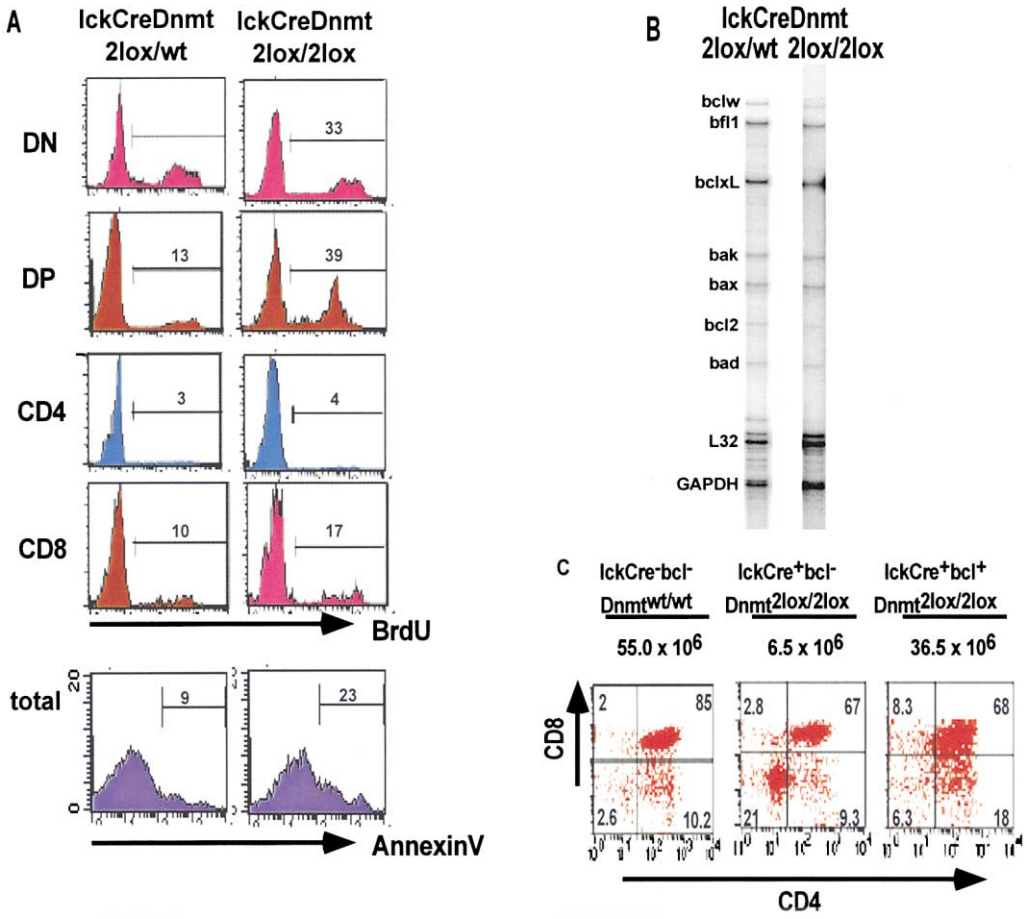

Figure 3. Impaired Survival of TCR-α/β-Lineage Cells in lckCreDnmt^{2lox} Mice

(A) In vivo thymocyte pulse labeling with BrdU. Numbers are the proportion of BrdU-positive cells. Mice received two intraperitoneal injections of BrdU (1 mg each, 2 hr apart) and were sacrificed 1 hr after the second injection. The bottom panels show Annexin V staining of total thymocytes.

(B) Expression of Bcl-2 family members by thymocytes from lckCreDnmt^{2lox} mice.

(C) An lckCreBcl-X_L transgene reduces cell death and restores thymocyte development in lckCreDnmt^{2lox} mice. Total thymocyte numbers are indicated.

termediates at the Jγ loci were not detected in these cells. Thus, the TCRγ locus closed normally as cells matured from the DN to DP stage, suggesting that rearrangement of TCRγ at the DP stage did not contribute to the generation of CD8$^+$ TCR$\gamma\delta^+$ cells in lckCreDnmt^{2lox} mice (Figure 5). Nor did we observe increased proliferation of TCR$\gamma\delta^+$ thymocytes, which was similar in controls (12% and 19% labeled) and in lckCreDnmt^{2lox} mice (8% and 12% labeled, n = 2) by BrdU labeling in vivo. Thus, the development of CD8$^+$TCR$\gamma\delta^+$ cells did not reflect or require biased generation or replication of TCR$\gamma\delta^+$ cells.

Normal T Cell Development but Altered T Cell Function in CD4CreDnmt^{2lox} Mice

T cell development in the thymus and numbers of CD4$^+$, CD8$^+$ (Figure 6A), and TCR$\gamma\delta^+$ (data not shown) T cells were similar in CD4CreDnmt^{2lox} mice and controls. This is consistent with the modest reduction in global methylation in thymocytes and resting peripheral T cells in CD4CreDnmt^{2lox} mice (Figures 1F and 1G). However, the fraction of CD44^{hi} memory T cells was reduced (Figure 6B). This suggested that replication-dependent maturation of naive into memory T cells might be impaired. Consistent with this, proliferation of naive T cells from CD4CreDnmt^{2lox} mice was reduced. The cloning frequency of naive CD4$^+$ (data not shown) and CD8$^+$ (Figure 6C) T cells from CD4CreDnmt^{2lox} mice was similar to controls, but the median number of cell divisions was 3 and none exceeded 5 ($n > 200$), whereas control clones divided >7 times after 6 days. The cloning frequency and proliferation of memory CD8$^+$ T cells from CD4CreDnmt^{2lox} mice was reduced, but $\sim$3% replicated like control cells (Figure 6C). This 3% appeared to represent the progeny of rare cells that escaped Cre-mediated deletion, since Dnmt1 mRNA was detected by RT-PCR in cultures of memory but not naive T cells from CD4CreDnmt^{2lox} mice (Figure 6D).

Cytokine expression by naive T cells is facilitated by entry into S phase and cell replication, which have been proposed to act, at least in part, by facilitating demethylation and chromatin remodeling of cytokine loci (Agar-

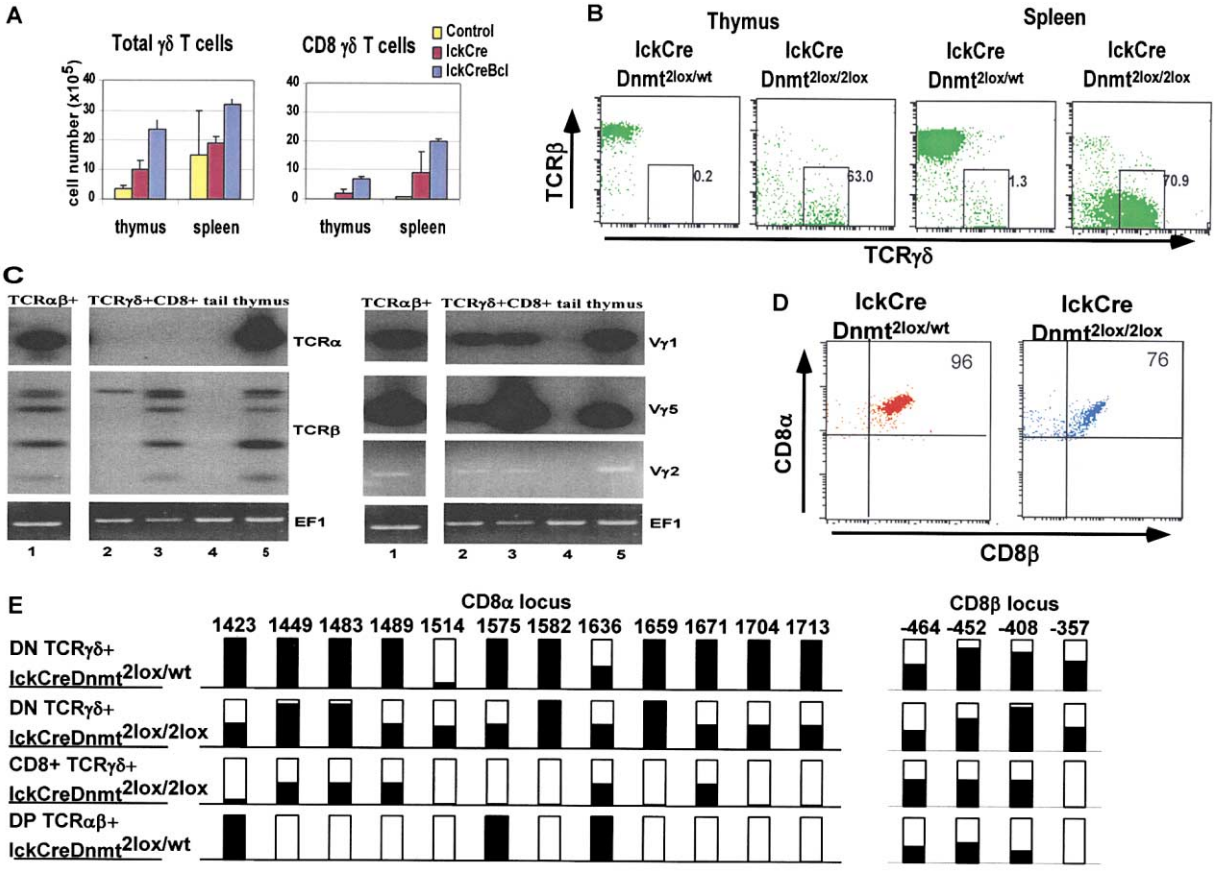

Figure 4. CD8$^+$TCR$\gamma\delta^+$ Thymocytes and T Cells in IckCreDnmt^{2lox} Mice

(A) Increased numbers of TCR$\gamma\delta^+$ cells and atypical CD8$^+$TCR$\gamma\delta^+$ cells in IckCreDnmt$^{2lox/2lox}$ and IckCreDnmt$^{2lox/2lox}$Bcl-X$_L$ mice.

(B) Representative TCR$\alpha\beta$ versus TCR$\gamma\delta$ dot plots of CD8$^+$ SP thymocytes and T cells.

(C) PCR analysis of TCRα, β, and γ gene rearrangements on DNA from CD8$^+$TCR$\gamma\delta^+$ T cells from IckCreDnmt^{2lox} mice (lanes 2 and 3 are two representative mice) compared to TCR$\alpha\beta^+$ T cells, tail, and thymocytes from controls (IckCreDnmt$^{2lox/wt}$). Shown are rearrangements of VαF3JαD558A and Vβ5-Jβ2.1-2.5; similar results were obtained with Vβ8 and Vβ11.

(D) CD8α versus CD8β expression on CD8$^+$TCR$\alpha\beta^+$ T cells from controls and CD8$^+$TCR$\gamma\delta^+$ T cells from IckCreDnmt$^{2lox/2lox}$ mice.

(E) Demethylation of CD8α and CD8β genes. The fraction of methylated (filled bar) and unmethylated (open bar) cytosines, determined by sequencing of bisulfite-modified DNA, is shown for the indicated CpG (Carbone et al., 1988; Hamerman et al., 1997). Results are from 11–12 genomic DNA clones from 2 or more cell preparations (DN and CD8$^+$ TCR$\gamma\delta^+$ cells) or 5–6 genomic DNA clones (DP thymocytes).

wal and Rao, 1998; Bird et al., 1998; Fitzpatrick et al., 1998; Gett and Hodgkin, 1998; Hu-Li et al., 2001; Lee et al., 2001; Reiner and Seder, 1999; Richter et al., 1999; Ward et al., 1998). However, in marked contrast to their reduced replication, 48 hr after activation in vitro the expression of IFN-γ, IL-2, IL-3, and IL-4 mRNAs by naive CD4$^+$ and CD8$^+$ T cells from CD4CreDnmt^{2lox} mice was consistently increased compared to controls (Figure 7A shows one experiment, and Figure 7B shows summary data), whereas IL-5 mRNA abundance was low and not detectably increased in Dnmt-1-deficient T cells (data not shown). By day 4, when replication of T cells from CD4CreDnmt^{2lox} mice ceased (Figure 6C), viability and the expression of some cytokine mRNAs declined (Figure 7A). We also used intracellular staining to determine the fraction of cells that expressed IFN-γ and tracked cell divisions using CFSE. The fraction of cells from CD4CreDnmt^{2lox} mice that contained intracellular IFN-γ was consistently increased at each cell division compared to controls (mean and range of the ratio of IFN-γ^+ cells from CD4CreDnmt^{2lox} versus control mice was 2.7, 1.3–5.2, n = 4 for CD4$^+$ T cells and 3.8, 2.0, and 7.4, n = 2 for CD8$^+$ T cells) (Figure 7C).

To determine if cytokine expression correlated with loss of methylation, we quantified cytokine mRNA (Figure 7D) and cytosine methylation (Figure 7E) over time within the IFN-γ and IL-3 genes at sites that are fully methylated in nonexpressing cell types and for which the degree of demethylation correlates with the magnitude of expression in T cells (Fitzpatrick et al., 1998, 1999; Young et al., 1994). The imprinted H19 gene was assessed as an index of global demethylation (Warnecke et al., 1998). In naive T cells from CD4CreDnmt^{2lox} mice but not from controls, methylation declined progressively with time after activation, and methylated CpG were undetectable at all loci at 4 days (Figure 7E), which coincided with the halt in cell replication (Figure 6C). The IFN-γ gene was more demethylated than the IL-3 gene in unstimulated Dnmt1-deficient T cells, and this correlated with the earlier increase in IFN-γ than

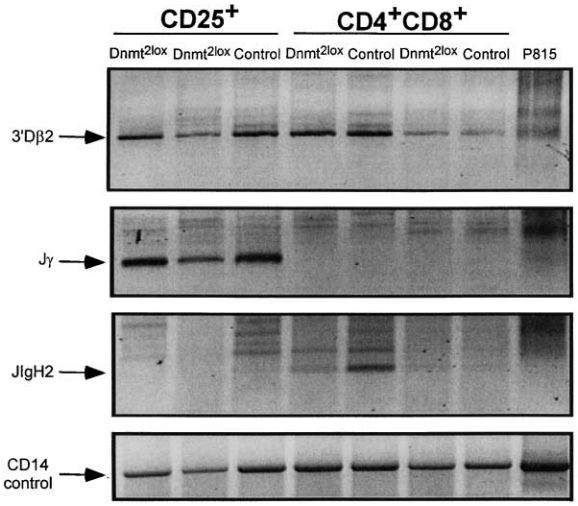

Figure 5. RAG-Mediated dsDNA Breaks in the TCRγ Locus

Recombination intermediates at TCRJγ1, 2, and 3, TCRDβ2, and immunoglobulin JH2 were determined by ligation-mediated PCR (Schlissel et al., 2000) on purified populations of $CD25^+TCR^-$DN ($CD25^+$) and DP thymocytes from lckCreDnmt^{2lox} (Dnmt^{2lox}) or littermate control mice and from the P815 mast cell line (negative control). CD14 was a control for DNA abundance.

IL-3 expression relative to controls (Figures 7D and 7E). This supports the notion that increased cytokine gene expression was due, at least in part, to demethylation in *cis*. To explore whether the increased cytokine mRNA expression by naive T cells from CD4CreDnmt^{2lox} mice might be mediated in part by additional mechanisms acting in *trans*, we evaluated the expression of nonmethylated reporter gene constructs after transient transfection. By contrast to the increased expression of the endogenous cytokine genes, transient transfection of reporter constructs into splenic T cells (Sweetser et al., 1998) from CD4CreDnmt^{2lox} mice revealed only a 2-fold increase (range: 1.4–2.8) in expression driven by the IL-2 and IFN-γ promoters and no increase in expression driven by the IL-4 promoter compared to controls. Together, these data suggest that demethylation in *cis* was the major mechanism for increased cytokine gene expression, but do not exclude *trans*-mediated influences on some cytokine genes.

Discussion

Our observations in lckCreDnmt^{2lox} and CD4CreDnmt^{2lox} mice indicate an important but selective contribution of Dnmt1 and DNA methylation to cell lineage-specific gene expression in developing and mature T cells. We also observe that DNA methylation is critical for normal T cell homeostasis.

An Essential Role for Dnmt1 and DNA Methylation in T Cell Homeostasis

Loss of Dnmt1 and of DNA methylation impaired proliferation and survival of T-lineage cells. In lckCreDnmt^{2lox} mice, deletion of *Dnmt1* preceded the period of brisk proliferation that follows the expression of TCRβ by $CD44^-$DN thymocytes, and marked demethylation and cell attrition were evident at subsequent stages of TCRαβ-lineage development. Similarly, replication of naive T cells from CD4CreDnmt^{2lox} mice ceased 4 days after activation in vitro when methylation had fallen to undetectable levels. These findings suggest that impaired homeostasis of Dnmt1-deficient T cells and T cell progenitors was due to global demethylation, although we cannot exclude the possibility that loss of Dnmt1 had deleterious effects in addition to the failure to maintain DNA methylation (Robertson et al., 2000; Rountree et al., 2000).

These results parallel those we recently reported in mouse embryo fibroblasts (Jackson-Grusby et al., 2001) and in neuronal cells (Fan et al., 2001). Similarly, Dnmt1-deficient ES cells do not survive differentiation, and Dnmt1-deficient embryos die at gastrulation (Beard et al., 1995; Lei et al., 1996; Li et al., 1992). Thus, Dnmt1 and DNA methylation appear to play a general and essential role in the homeostasis of differentiated mammalian cells. The basis for this is at present unclear. Expression of the p53-inducible CDK inhibitor $p21^{CIP/WAF}$ is increased in Dnmt1-deficient mouse embryo fibroblasts, and the replication and survival of these cells is enhanced but not fully restored by the introduction of a null p53 allele or by enforced expression of SV40 large T antigen (Jackson-Grusby et al., 2001). Expression of $p21^{CIP/WAF}$ (but not p53) mRNA was also consistently increased in naive T cells from CD4CreDnmt^{2lox} mice, but the replication of these cells was not restored by null alleles of $p21^{CIP/WAF}$ or p53 (our unpublished observations), indicating that $p21^{CIP/WAF}$- and p53-independent mechanisms are sufficient to disrupt homeostasis of Dnmt1-deficient T cells.

Selective Derepression of CD8α and CD8β on $TCR\gamma\delta^+$ Thymocytes and Peripheral T Cells in lckCreDnmt2lox Mice

In addition to the attrition of TCRαβ-lineage cells, the major phenotypic feature in lckCreDnmt^{2lox} mice was the atypical expression of $CD8\alpha\beta^+$ on many $TCR\gamma\delta^+$ cells, which correlated closely with demethylation of the CD8α and CD8β loci. The most parsimonious interpretation of these findings is that demethylation in *cis* is both necessary and sufficient for CD8 expression by TCRγδ-lineage cells, although we cannot exclude a contribution by other indirect mechanisms. This suggests that the selective demethylation of CD8α but not CD8β observed in gut-derived $TCR\gamma\delta^+$ cells (Carbone et al., 1988; Hamerman et al., 1997) is causally related to their expression of CD8αα rather than CD8αβ. Demethylation of CD8α and CD8β loci in TCRαβ-lineage cells correlates with the onset of CD8αβ expression at the DN to DP transition and with continued CD8αβ expression by mature $CD8^+$ T cells, suggesting that demethylation is also necessary for CD8 expression in the TCRαβ-lineage (Carbone et al., 1988; Hamerman et al., 1997). However, demethylation is not sufficient for continued expression of CD8αβ once commitment to the CD4 lineage occurs, since Dnmt1-deficient CD4SP thymocytes and T cells extinguished the expression of CD8 normally. Thus, in $CD4^+$ cells mechanisms other than methylation (Ellmeier et al., 1999) are sufficient to silence CD8 expression.

We also observed a modest increase in $TCR\gamma\delta^+$ thy-

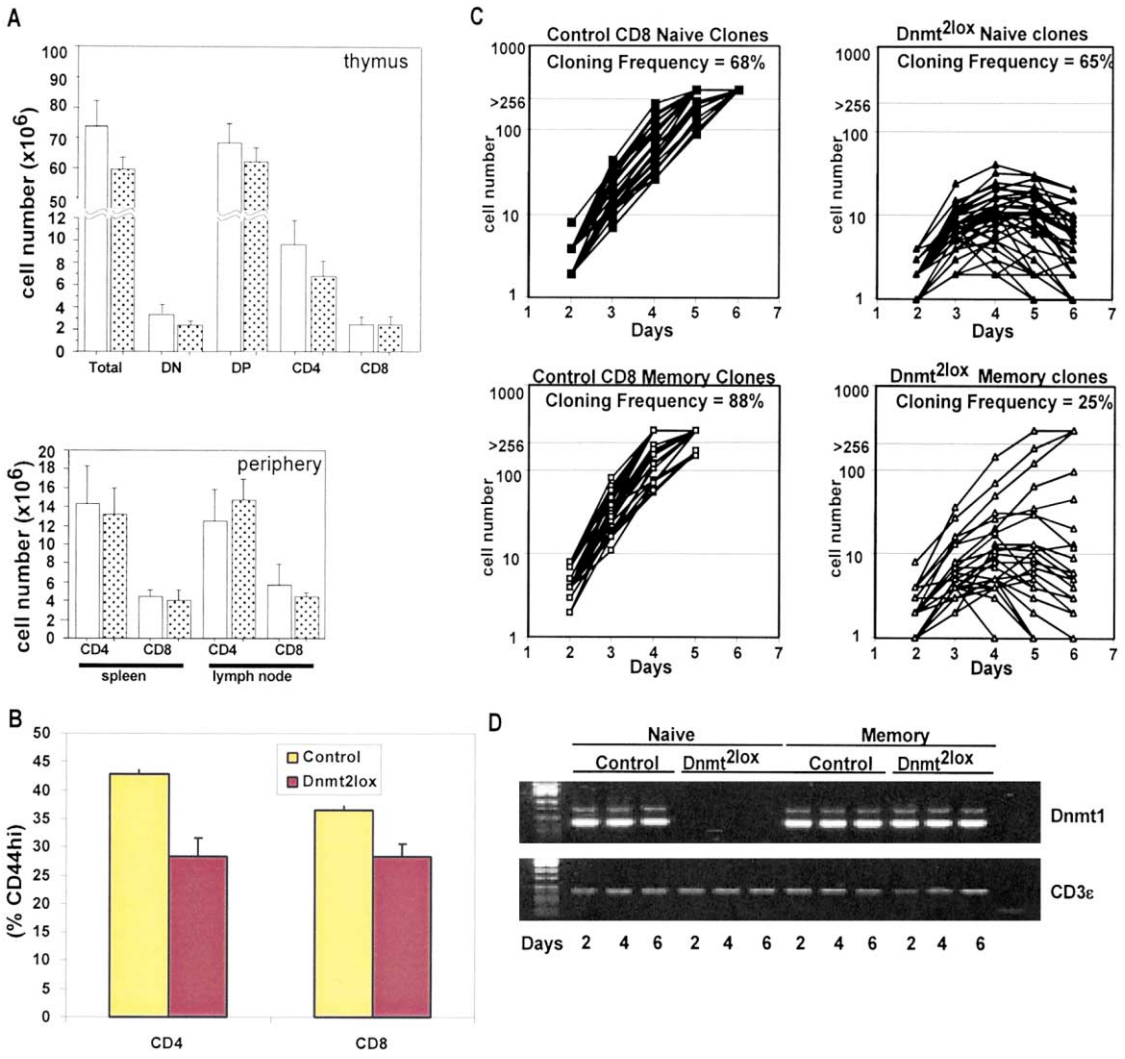

Figure 6. Normal Development but Impaired Proliferation of T Cells in CD4CreDnmt^{2lox} Mice

(A) Thymocyte, splenic, and lymph node subsets in control CD4CreDnmt$^{2lox/wt}$ (n = 10, Cre-negative Dnmt^{2lox} mice gave similar results) and CD4CreDnmt^{2lox} (shaded bars, n = 21) mice.

(B) Reduced numbers of memory (CD44^{hi}) T cells in CD4CreDnmt^{2lox} mice.

(C) Replication of single naive or memory CD8$^+$ T cells from CD4CreDnmt^{2lox} and control Cre-negative Dnmt^{2lox} mice activated as described (Fitzpatrick et al., 1998).

(D) RT-PCR analysis of Dnmt1 and CD3ε mRNA from naive and memory CD8$^+$ T cells from the same cell preparations shown in (B).

mocytes and splenic and lymph node T cells in lckCreDnmt^{2lox} mice. Their relative preservation and greater viability likely reflect in part the limited replication and already short life-span of TCRγδ-lineage cells compared to TCRαβ-lineage cells (Pénit et al., 1995; Tough and Sprent, 1998). Similar increases in absolute numbers of TCRγδ$^+$ cells have been seen in TCRβ, TCR β enhancer, pre-Tα, and lck knockout mice (Leduc et al., 2000; von Boehmer et al., 1999), which may represent a compensatory response to the reduced numbers of TCRαβ cells. Such a mechanism would not fully explain the findings in lckCreDnmt^{2lox} mice, since the numbers of TCRγδ$^+$ cells were further increased by enforced expression of Bcl-X_L, although DP and SP TCRαβ thymocyte numbers were substantially rescued.

We found no evidence to suggest that the increased numbers of TCRγδ$^+$ cells in lckCreDnmt^{2lox} mice resulted from increased replication or from preferential V(D)J recombination at the TCRγ versus TCRβ locus. Immunoglobulin and TCR loci are methylated in cells of the lymphoid lineage in which they are not recombined (Berg and Kang, 2001; Cedar and Bergman, 1999; Durum et al., 1998; Whitehurst et al., 2000), and methylation of artificial recombination substrates blocks V(D)J recombination (Sleckman et al., 1996). The failure of TCRγ rearrangement in IL-7 receptor α chain-deficient mice is associated with persistent methylation of Eγ (Durum et al., 1998), suggesting that methylation may be a barrier to recombination at this locus. We did not observe increased RAG-mediated cleavage at the TCRγ locus in thymocytes from lckCreDnmt^{2lox} mice. However, Eγ was completely demethylated in DN and DP thymocytes

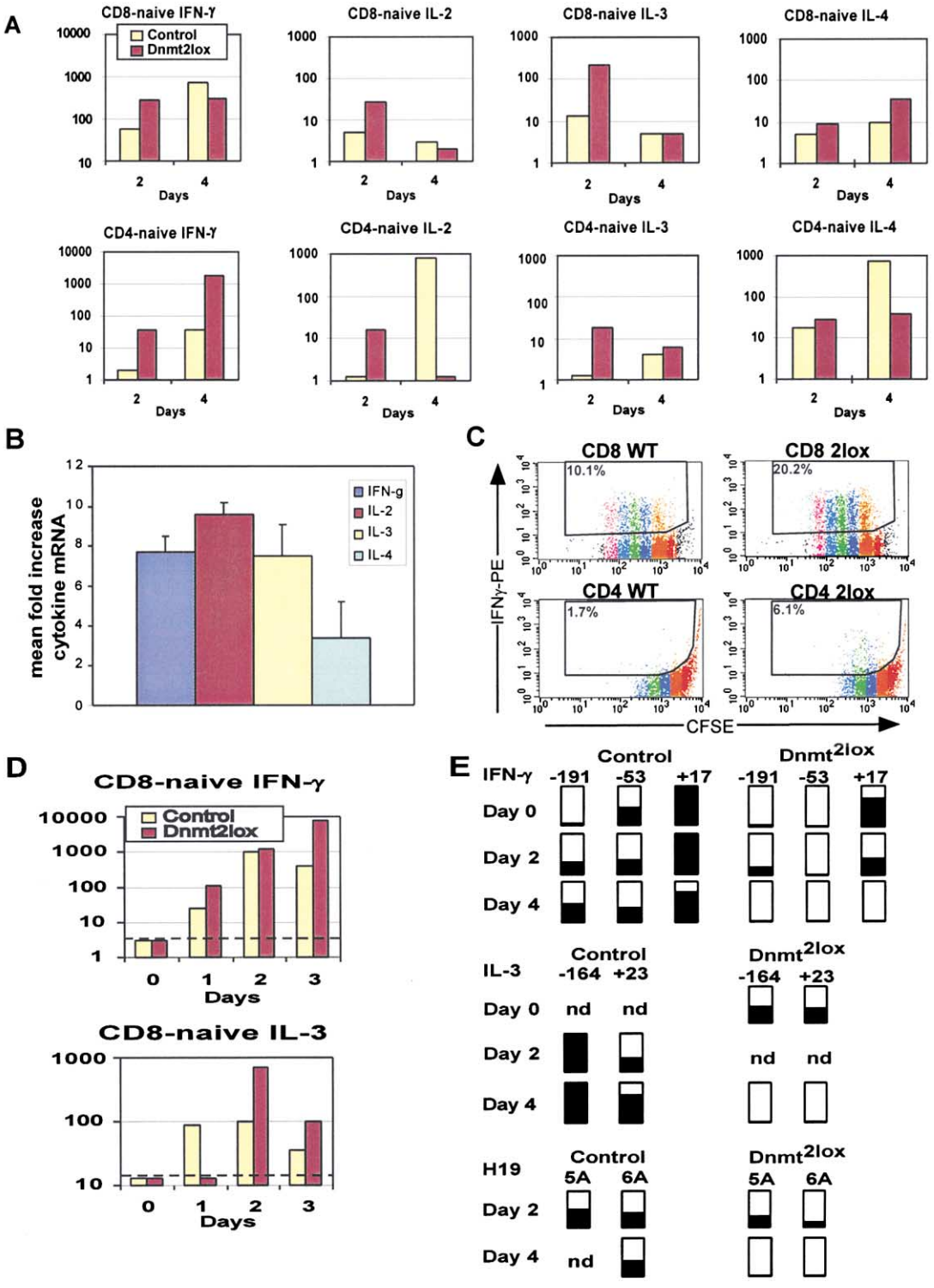

Figure 7. Enhanced Cytokine mRNA Expression by Naive T Cells from CD4CreDnmt^{2lox} Mice

(A) Representative QC-PCR analysis of cytokine mRNA expression by the naive CD8$^+$ T cells shown in Figure 6C and for naive CD4$^+$ T cells from the same mice. Results are in arbitrary units relative to CD3ϵ mRNA.

(B) Mean ± SD ratio of cytokine mRNA in naive T cells from CD4CreDnmt^{2lox} mice compared to controls 48 hr after activation in vitro (n = 3 − 5).

(C) Representative intracellular IFN-γ staining versus cell division determined by CFSE.

(D and E) IFN-γ and IL-3 mRNA expression and ([E]) fraction of methylated (filled bar) and unmethylated (open bar) cytosines at the indicated CpG in the *IFN-γ* and *IL-3* genes or in the imprinted region of the *H19* gene, which was quantified by Ms-SNuPE using bisulfite-modified DNA as template; the dashed lines in (D) indicate the limit of detectability. nd, not done due to insufficient amounts of sample.

in lckCreDnmt^{2lox} mice and >85% demethylated in controls. Thus, the absence of increased TCRγ V(D)J recombination in Dnmt1-deficient DN thymocytes does not exclude the possibility that demethylation is necessary for recombination but does indicate that methylation is not the dominant mechanism limiting accessibility in normal mice. We also found that methylation was not required for RAG-mediated cutting at this locus to be extinguished in DP thymocytes. Our findings are consistent with others suggesting that demethylation may be necessary but is not sufficient to activate V(D)J recombination at immunoglobulin loci (Cherry et al., 2000; Engler and Storb, 1999). By contrast, treatment of fetal thymic organ cultures with the histone deacetylase inhibitor trichostatin A activated TCRγ V(D)J recombination in IL-7 receptor-deficient mice (Durum et al., 1998) and allowed V(D)J recombination of the fetal-type Vγ3 locus in adult T cell progenitors (Agata et al., 2001). Although trichostatin A may have had effects other than the induction of histone hyperacetylation at the TCRγ locus (Krangel, 2001), these data suggest that histone hypoacetylation may limit TCRγ V(D)J recombination to a greater extent than does DNA methylation.

Differential Enhancement of Cytokine Gene Expression in CD4CreDnmt^{2lox} Mice

We and others have shown that the capacity of T cells to produce IFN-γ (IFN-γ) and IL-3 is correlated with hypomethylation and an open chromatin structure within the IFN-γ gene (Agarwal and Rao, 1998; Fitzpatrick et al., 1998, 1999; Melvin et al., 1995; Young et al., 1994). Similar correlations have been made for the IL-2, IL-4, and IL-5 genes (Agarwal et al., 2000; Agarwal and Rao, 1998; Bird et al., 1998; Lee et al., 2000; Ouyang et al., 2000). Although naive T cells appear to express these cytokines in the first hours after initial activation in vitro (Grogan et al., 2001), their capacity to produce effector cytokines increases as they differentiate into memory/effector T cells (Bird et al., 1998; Gett and Hodgkin, 1998; Reiner and Seder, 1999; Richter et al., 1999). This increase is paralleled by progressive demethylation and remodeling of chromatin structure at the respective cytokine loci, rendering them more accessible for engagement by activation-induced transcription factors (Agarwal and Rao, 1998; Bird et al., 1998; Hu-Li et al., 2001; Reiner and Seder, 1999). However, the relative importance of methylation, closed chromatin structure, and limited availability of key transcription factors in the repression of effector cytokine expression in naive T cells is unknown. CD4CreDnmt^{2lox} mice allowed us to explore the extent to which methylation limits cytokine gene expression by naive T cells using a direct genetic approach.

Loss of Dnmt1 and declining DNA methylation in naive T cells from CD4CreDnmt^{2lox} mice increased activation-induced expression of IFN-γ, IL-2, IL-3, and IL-4, suggesting that methylation plays an important role in limiting the expression of these cytokines by naive T cells. The increased expression of these cytokines appeared to be mediated, at least in part, by demethylation in *cis*, although we cannot exclude the possibility that demethylation at other loci upstream of these cytokines also contributed. Loss of Dnmt1 had a relatively modest effect on the expression of IL-4, suggesting that the absence of necessary *trans*-acting factors or a more repressive chromatin structure may play a greater role in limiting IL4 expression by naive T cells. Supporting this proposal is the finding that IL-4 expression by naive T cells was modestly augmented in vitro either by the DNA methylation inhibitor 5-azacytidine or by the histone deacetylase inhibitor trichostatin A; together the two inhibitors had a more robust effect and were sufficient to allow IL-4 expression by naive STAT6-deficient T cells (Bird et al., 1998). The current results are also compatible with the suggestion by Paul and coworkers that the inefficient induction of Th2 compared to Th1 cytokine production by naive T cells reflects not only limited gene accessibility but limitations in key transcription factors (Hu-Li et al., 2001).

Conclusion

To our knowledge, this is the first demonstration that Dnmt1 and DNA methylation are required for the proper expression of specific genes that help to define lineage and dictate function in T cells or in other somatic cells of a mature mammal. By contrast, the expression of other lymphoid-specific genes examined and developmental stage- and locus-specific V(D)J recombination were faithfully maintained. This suggests that even when Dnmt1 is lost and methylation declines, the fates a cell can adopt may be restricted, and that the changes observed reflect genes that are regulated physiologically and are particularly sensitive to the loss of Dnmt1 and DNA methylation at that stage of T cell development.

Experimental Procedures

Transgenic Mice

Generation of the Dnmt^{2lox} targeting vector and of Dnmt$^{2lox/wt}$ ES cells and mice have been described (Jackson-Grusby et al., 2001). The lckCre construct was created by engineering a nuclear localization signal and optimum eukaryotic translation start site at the 5′ end of Cre (tcg agc ATG gca ccc aag aag aag agg aag gtg) and inserting this downstream of the *lck* proximal promoter (Garvin et al., 1990); CD4Cre was constructed by replacing the *lck* proximal promoter with the mCD4 promoter/enhancer/silencer (Sawada et al., 1994). Southern blots of genomic DNA that had been digested with BglII and SpeI were probed to detect Cre-mediated deletion of exons 4 and 5 of *Dnmt1*. Probes and primer sequences are available as supplemental data at http://www.immunity.com/cgi/content/full/15/5/763/DC1.

Cell Preparations and Flow Cytometry

Single cell suspensions of thymus, spleen, and lymph nodes were obtained as described (Fitzpatrick et al., 1998; Kay et al., 1995). To isolate IEL, the small intestine was removed and Peyer's patches were excised. After removing fecal matter, intestines were cut longitudinally and then cut into 1 cm pieces. These were suspended in 1 mM EDTA in Hank's balanced salt solution (HBSS) at 37°C and vortexed repeatedly to separate lymphocytes from the epithelial sheaths. Aliquots were removed over time as the sheaths settled. The first two aliquots were replaced with solution, and subsequent aliquots were replaced with 5% FBS in HBSS. Aliquots were pooled, concentrated, passed over nylon wool columns, centrifuged on a 40%/75% Percoll (Sigma) gradient, and the IEL were recovered from the interface. Cells were resuspended and then stained with various combinations of monoclonal antibodies (BD-PharMingen or Caltag) as indicated. Cells were then analyzed using a FACScan or LSR flow cytometer or purified by flow cytometric cell sorting on a FACStar Plus or FACS Vantage SE (Becton Dickinson). In some experi-

ments, enrichment for the desired population was first performed by negative selection using magnetic beads (Dynal). Viability was assessed using FITC-Annexin-V. For analysis of thymocyte proliferation, mice received two intraperitoneal (i.p.) injections of BrdU (Sigma) (1 mg each, 2 hr apart) and were sacrificed 1 hr after the second injection. Cells were then fixed, permeabilized, and stained with FITC-anti-BrdU (BD-PharMingen). Intracellular cytokine staining and CFSE labeling were performed on cells that had been stimulated for 3 days with plate-bound antibodies to CD3 and CD28 and then restimulated for 6 hr with ionomycin and PMA as described (Bird et al., 1998).

T Cell Receptor V(D)J Recombination

TCRα, β, and γ gene rearrangements were determined by PCR using primers described previously (Anderson et al., 1992; Asarnow et al., 1989; Kang et al., 1998). Recombination intermediates (signal sequences that have been cleaved but not yet religated) at TCRJγ1, 2, and 3 and for comparison at TCRDβ2 and immunoglobulin JH2 were examined by ligation-mediated PCR as previously described (Schlissel et al., 2000).

Analysis of DNA Methylation

Global methylation was assessed by Southern blot as described in the Figure 1 legend. Methylation in the CD8α, CD8β, Eγ, Eδ, and Eα loci was quantified by sequencing of genomic DNA after bisulfite modification and PCR amplification (Fitzpatrick et al., 1998). Methylation in the IFN-γ and IL-3 loci and in the imprinted region of the H19 locus (Warnecke et al., 1998) was quantified by Ms-SNUPE (Gonzalgo and Jones, 1997). Genomic DNA was bisulfite modified, PCR amplified, and cytosine methylation was quantified by Ms-SnuPE using FITC-labeled dCTP or dUTP for primer extension and anti-FITC ELISA for detection. Standards were prepared from DNA that was methylated in vitro with Sss1 methylase (New England Biolabs) or was obtained from Dnmt1-deficient (S/S) ES cells (Lei et al., 1996).

Cell Proliferation and Cytokine mRNA Production

Naive ($CD44^{lo}$) $CD4^+$ and $CD8^+$ T cells were purified by flow cytometry (Fitzpatrick et al., 1998), cultured in low density bulk cultures (5×10^3 cells/ml) in DMEM (Life Technologies) supplemented with 15% FCS (Hyclone) and 600 units/ml recombinant IL-2, and activated with plate-bound MAb to CD3, LFA-1, CD28, and CD4 or CD8 for the indicated times, as previously described (Fitzpatrick et al., 1998). Cytokine mRNA abundance was determined by quantitative, competitive RT-PCR (QC-PCR) using CD3ϵ mRNA as a reference (Fitzpatrick et al., 1998). Dnmt1, CD3ϵ, GAPDH, and β-actin mRNA was evaluated on ethidium bromide-stained gels after amplification for the indicated numbers of cycles using seminested primers in a two-round PCR (Dnmt1) or one set of primers in a single-round PCR (CD3ϵ, GAPDH, and β-actin); results with GAPHD and β-actin were similar to those with CD3ϵ. Dnmt1 PCR product identity was confirmed by sequencing.

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References

Agarwal, S., and Rao, A. (1998). Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. Immunity *9*, 765–775.

Agarwal, S., Avni, O., and Rao, A. (2000). Cell-type-restricted binding of the transcription factor NFAT to a distal IL-4 enhancer in vivo. Immunity *12*, 643–652.

Agata, Y., Katakai, R., Ye, S.-K., Sugai, M., Gonda, H., Honjo, T., Ikuta, K., and Shimizu, A. (2001). Histone acetylation determines the developmentally regulated accessibility for T cell receptor γ gene recombination. J. Exp. Med. *193*, 873–879.

Anderson, S.J., Abraham, K.M., Nakayama, T., Singer, A., and Perlmutter, R.M. (1992). Inhibition of T-cell receptor beta-chain gene rearrangement by overexpression of the non-receptor protein tyrosine kinase p56lck. EMBO J. *11*, 4877–4886.

Asarnow, D.M., Goodman, T., LeFrancois, L., and Allison, J.P. (1989). Distinct antigen receptor repertoires of two classes of murine epithelium-associated T cells. Nature *341*, 60–62.

Asnagli, H., and Murphy, K. (2001). Stability and commitment in T helper cell development. Curr. Opin. Immunol. *13*, 242–247.

Beard, C., Li, E., and Jaenisch, R. (1995). Loss of methylation activates Xist in somatic but not in embryonic cells. Genes Dev. *9*, 2325–2334.

Berg, L.J., and Kang, J. (2001). Molecular determinants of TCR expression and selection. Curr. Opin. Immunol. *13*, 232–241.

Bird, A.P., and Wolffe, A.P. (1999). Methylation-induced repression-belts, braces, and chromatin. Cell *99*, 451–454.

Bird, J.J., Brown, D.R., Mullen, A.C., Moskowitz, N.H., Mahowald, M.A., Sider, J.R., Gajewski, T.F., Wang, C.-R., and Reiner, S.L. (1998). Helper T cell differentiation is controlled by the cell cycle. Immunity *9*, 229–237.

Carbone, A.M., Marrack, P., and Kappler, J.W. (1988). Demethylated CD8 gene in CD4+ T cells suggests that CD4+ T cells develop from CD8+ precursors. Science *242*, 1174–1176.

Cedar, H., and Bergman, Y. (1999). Developmental regulation of immune system gene rearrangement. Curr. Opin. Immunol. *11*, 64–69.

Chao, D.T., and Korsmeyer, S.J. (1997). BCL-XL-regulated apoptosis in T cell development. Int. Immunol. *9*, 1375–1384.

Cherry, S.R., Beard, C., Jaenisch, R., and Baltimore, D. (2000). V(D)J recombination is not activated by demethylation of the kappa locus. Proc. Natl. Acad. Sci. USA *97*, 8467–8472.

Durum, S.K., Candeias, S., Nakajima, H., Leonard, W.J., Baird, A.M., Berg, L.J., and Muegge, K. (1998). Interleukin 7 receptor control of T cell receptor γ gene rearrangement: role of receptor-associated chains and locus accessibility. J. Exp. Med. *188*, 2233–2241.

Ellmeier, W., Sawada, S., and Littman, D.R. (1999). The regulation of CD4 and CD8 coreceptor gene expression during T cell development. Annu. Rev. Immunol. *17*, 523–554.

Engler, P., and Storb, U. (1999). Hypomethylation is necessary but not sufficient for V(D)J recombination within a transgenic substrate. Mol. Immunol. *36*, 1169–1173.

Fan, G., Beard, C., Chen, R.Z., Csankovszki, G., Sun, Y., Siniaia, M., Biniszkiewicz, D., Bates, B., Lee, P.P., Kuhn, R., et al. (2001). DNA hypomethylation perturbs the function and survival of CNS neurons in postnatal animals. J. Neurosci. *21*, 788–797.

Fitzpatrick, D.R., Shirley, K.M., McDonald, L.E., Bielefeldt-Ohmann, H., Kay, G.F., and Kelso, A. (1998). Distinct methylation of the interferon-gamma (IFN-γ) and interleukin-3 (IL-3) genes in newly activated primary CD8+ T lymphocytes: Regional IFN-γ promoter demethylation and mRNA expression are heritable in $CD44^{high}$ CD8+ T cells. J. Exp. Med. *188*, 103–117.

Fitzpatrick, D.R., Shirley, K.M., and Kelso, A. (1999). Cutting edge: stable epigenetic inheritance of regional interferon-γ promoter demethylation in CD44high CD8+ T lymphocytes. J. Immunol. *162*, 5053–5057.

Fuks, F., Burgers, W.A., Godin, N., Kasai, M., and Kouzarides, T. (2001). Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. EMBO J. *20*, 2536–2544.

Garvin, A.M., Abraham, K.M., Forbush, K.A., Farr, A.G., Davision, B.L., and Perlmutter, R.M. (1990). Disruption of thymocyte development and lymphomagenesis induced by SV40 T-antigen. Int. Immunol. *2*, 173–180.

Gett, A.V., and Hodgkin, P.D. (1998). Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. Proc. Natl. Acad. Sci. USA *95*, 9488–9493.

Gonzalgo, M.L., and Jones, P.L. (1997). Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucleic Acids Res. *25*, 2529–2531.

Grogan, J.L., Mohrs, M., Harmon, B., Lacy, D.A., Sedat, J.W., and Locksley, R.M. (2001). Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. Immunity *14*, 205–215.

Hamerman, J.A., Page, S.T., and Pullen, A.M. (1997). Distinct methylation states of the CD8 beta gene in peripheral T cells and intraepithelial lymphocytes. J. Immunol. *159*, 1240–1246.

Hu-Li, J., Pannetier, C., Guo, L., Lohning, M., Gu, H., Watson, C., Assenmacher, M., Radbruch, A., and Paul, W.E. (2001). Regulation of expression of IL-4 alleles: analysis using a chimeric GFP/IL-4 gene. Immunity *14*, 1–11.

Jackson-Grusby, L., Beard, C., Possemato, R., Tudor, M., Fambrough, D., Csankovszki, G., Dausman, J., Lee, P., Wilson, C., Lander, E., and Jaenisch, R. (2001). Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nat. Genet. *27*, 31–39.

Jaenisch, R. (1997). DNA methylation and imprinting. Why bother? Trends Genet. *13*, 323–329.

Kang, J., Coles, M., Cado, D., and Raulet, D.H. (1998). The developmental fate of T cells is critically influenced by TCR$\gamma\delta$ expression. Immunity *8*, 427–438.

Kay, M.A., Holterman, A.X., Meuse, L., Gown, A., Ochs, H.D., Linsley, P.S., and Wilson, C.B. (1995). Long-term hepatic adenovirus-mediated gene expression in mice following CTLA4Ig administration. Nat. Genet. *11*, 191–197.

Kisielow, P., and von Boehmer, H. (1995). Development and selection of T cells: facts and puzzles. Adv. Immunol. *58*, 87–209.

Krangel, M.S. (2001). V(D)J recombination becomes accessible. J. Exp. Med. *193*, F27–F30.

Leduc, I., Hempel, W.M., Mathieu, N., Verthuy, C., Bouvier, G., Watrin, F., and Ferrier, P. (2000). T cell development in TCR beta enhancer-deleted mice: implications for alpha beta T cell lineage commitment and differentiation. J. Immunol. *165*, 1364–1373.

Lee, H.J., Takemoto, N., Kurata, H., Kamogawa, Y., Miyatake, S., O'Garra, A., and Arai, N. (2000). GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. J. Exp. Med. *192*, 105–115.

Lee, G.R., Fields, P.E., and Flavell, R.A. (2001). Regulation of IL-4 gene expression by distal regulatory elements and GATA-3 at the chromatin level. Immunity *14*, 447–459.

Lei, H., Oh, S.P., Okano, M., Juttermann, R., Goss, K.A., Jaenisch, R., and Li, E. (1996). De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development *122*, 3195–3205.

Li, E., Bestor, T.H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915–926.

McMurry, M.T., and Krangle, M.S. (2000). A role for histone acetylation in the developmental regulation of V(D)J recombination. Science *287*, 495–498.

Melvin, A.J., McGurn, M.E., Bort, S.J., Gibson, C., and Lewis, D.B. (1995). Hypomethylation of the interferon-gamma gene correlates with its expression by primary T-lineage cells. Eur. J. Immunol. *25*, 426–430.

Okano, M., Xie, S., and Li, E. (1998). Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat. Genet. *19*, 219–220.

Ouyang, W., Lohning, M., Gao, Z., Assenmacher, M., Ranganath, S., Radbruch, A., and Murphy, K.M. (2000). Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity *12*, 27–37.

Pénit, C., Lucas, B., and Vasseur, F. (1995). Cell expansion and growth arrest phases during the transition from precursor (CD4−8−) to immature (CD4+8+) thymocytes in normal and genetically modified mice. J. Immunol. *154*, 5103–5113.

Reiner, S.L., and Seder, R.A. (1999). Dealing from the evolutionary pawnshop: how lymphocytes make decisions. Immunity *11*, 1–10.

Richter, A., Loehning, M., and Radbruch, A. (1999). Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. J. Exp. Med. *190*, 1439–1450.

Robertson, K.D., Ait-Si-Ali, S., Yokochi, T., Wade, P.A., Jones, P.L., and Wolffe, A.P. (2000). DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nat. Genet. *25*, 338-342.

Rountree, M.R., Bachman, K.E., and Baylin, S.B. (2000). DNMT1 binds HDAC2 and a new co-repressor, DMAP-1, to form a complex at replication foci. Nat. Genet. *25*, 338–342.

Sawada, S., Scarborough, J.D., Killeen, N., and Littman, D.R. (1994). A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. Cell *77*, 917–929.

Schlissel, M.S., Durum, S.D., and Muegge, K. (2000). The interleukin 7 receptor is required for T cell receptor gamma locus accessibility to the V(D)J recombinase. J. Exp. Med. *191*, 1045–1050.

Sleckman, B.P., Gorman, J.R., and Alt, F.W. (1996). Accessibility control of antigen-receptor variable-region gene assembly: Role of cis-acting elements. Annu. Rev. Immunol. *14*, 459–481.

Sweetser, M.T., Hoey, T., Sun, Y.L., Weaver, W.M., Price, G.A., and Wilson, C.B. (1998). The roles of nuclear factor of activated T cells and ying-yang 1 in activation-induced expression of the interferon-gamma promoter in T cells. J. Biol. Chem. *273*, 34775–34783.

Tough, D.F., and Sprent, J. (1998). Lifespan of gamma/delta T cells. J. Exp. Med. *187*, 357–365.

von Boehmer, H., Aifantis, I., Feinberg, J., Lechner, O., Saint-Ruf, C., Walter, U., Buer, J., and Azogui, O. (1999). Pleitropic changes controlled by the pre-T-cell receptor. Curr. Opin. Immunol. *11*, 135–142.

Walsh, C.P., Chaillet, J.R., and Bestor, T.H. (1998). Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nat. Genet. *20*, 116–117.

Ward, S.B., Hernandez-Hoyos, G., Chen, F., Waterman, M., Reeves, R., and Rothenberg, E.V. (1998). Chromatin remodeling of the interleukin-2 gene: distinct alterations in the proximal versus distal enhancer regions. Nucleic Acids Res. *26*, 2923–2934.

Warnecke, P.M., Mann, J.R., Frommer, M., and Clark, S.J. (1998). Bisulfite sequencing in preimplantation embryos: DNA methylation profile of the upstream region of the mouse imprinted H19 gene. Genomics *51*, 182–190.

Whitehurst, C.E., Schlissel, M.S., and Chen, J. (2000). Deletion of germline promoter PD beta 1 from the TCR beta locus causes hypermethylation that impairs D beta 1 recombination by multiple mechanisms. Immunity *13*, 703–714.

Young, H.A., Ghosh, P., Ye, J., Lederer, J., Lichtman, A., Gerard, J.R., Penix, L., Wilson, C.B., Melvin, A.J., McGurn, M.E., et al. (1994). Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-gamma gene. J. Immunol. *153*, 3603–3610.