

DNA methyltransferase 3a limits the expression of interleukin-13 in T helper 2 cells and allergic airway inflammation

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The inverse correlation between DNA methylation and lineage-specific gene expression during T helper cell development is well documented. However, the specific functions of the *de novo* methyltransferases Dnmt3a and Dnmt3b in cytokine gene regulation have not been defined. We demonstrate that the expression of Dnmt3a and Dnmt3b are induced to a greater extent in T helper 2 (Th2) cells than in T helper 1 cells during polarization. Using conditional mutant mice, we determined that Dnmt3a, but not Dnmt3b, regulated expression of T helper cell cytokine genes, with the *Il13* gene most prominently affected. *Dnmt3a* deficiency was accompanied by decreases in DNA methylation and changes in the H3K27 acetylation/methylation status at the *Il13* locus. Dnmt3a-dependent regulation of *Il13* also occurred *in vivo* because *Dnmt3a^{fl/fl}Cd4cre* mice exhibited increased lung inflammation in a murine asthma model, compared with littermate controls. Based on these observations, we conclude that Dnmt3a is required for controlling normal *Il13* gene expression and functions as a rate-limiting factor to restrict T helper 2-mediated inflammation.

de novo DNA methyltransferase | IL-13 | chromatin

Upon encountering antigen in an appropriate cytokine milieu, naïve CD4 cells differentiate into T helper 1 (Th1) or T helper 2 (Th2) cells, which secrete IFN- γ , or IL-4 and IL-13, respectively, and regulate distinct types of inflammation (1). Subset-restricted cytokine gene expression is coordinated by selective activation of transcription factors and epigenetic programming at each locus (2). Stat4 and T-bet are the primary transcription factors for Th1 polarization, whereas Stat6 and Gata3 are required for Th2 differentiation (3).

Studies on T helper cell development demonstrated that epigenetic changes are associated with cytokine gene expression (4–12). Histone modifications that are thought to regulate gene expression primarily occur on the amino termini of core histones, and combinations of modified histone residues constitutes the “histone code” (13, 14). Methylation of mammalian genomic DNA occurs on the cytosine of CpG dinucleotides and correlates with transcriptional inactivation (15, 16). Methylation-mediated gene silencing can be achieved through at least two distinct mechanisms: direct impediment to DNA binding of transcription factors or eliciting a transcriptionally repressive chromatin structure via recruitment of corepressors and chromatin modifiers (17). DNA methylation is essential for normal mammalian development and is required for biological processes such as chromosome X inactivation, gene imprinting, and silencing of transposons and retroviruses (18–21). There are three active mammalian DNA methyltransferases: Dnmt1 (22), Dnmt3a, and Dnmt3b (23). The DNA template-dependent activity of Dnmt1 replicates the parental methylation pattern onto progeny cells, leading to methylation maintenance and heritability. Dnmt3a and Dnmt3b function as *de novo* methyltransferases (24), establish new methylation by catalyzing the addition of methyl groups to previously unmethylated cytosines in response to developmental stimuli, and are indispensable for mammalian development because targeted deletion of these genes resulted in

embryonic lethality (24, 25). The development of naïve CD4 cells toward effector cells was accompanied by lineage-differential DNA methylation at multiple gene loci (7, 11, 26, 27). Promoter methylation at *Ifng*, *Il18r1*, and *Il4* gene loci is distinct between Th1 and Th2 subsets, raising the possibility that DNA methyltransferases regulate cytokine expression. Although *Dnmt1*-mediated methylation is critical for normal T-cell homeostasis and proper cytokine gene expression (28), the function of *de novo* DNA methyltransferases in the regulation of immune gene expression during T helper cell polarization is incompletely understood. In this study, we found that *Dnmt3a* deficiency led to increased cytokine gene expression and resulted in severe lung inflammation in a murine asthma model.

Results

Dnmt3a Binding at Cytokine Gene Promoters. To define the function of *de novo* DNA methyltransferases in T helper cell subsets, we first measured nuclear expression of Dnmt3a, Dnmt3b, and Dnmt1, as well as lineage-determining transcription factors in naïve CD4 T cells, nonpolarized effector cells, and Th1 and Th2 cells. Stat4 and T-bet were present in the nuclei of Th1 cells, whereas Stat6 and Gata3 were detected in the nuclei of Th2 cells, indicative of efficient polarization of these subsets (Fig. 1A). Dnmt1 was equivalently expressed in all subsets. Dnmt3a and Dnmt3b were expressed at low levels in naïve CD4 cells, but expression was increased in nonpolarized cells, in agreement with a previous report that TCR stimulation resulted in up-regulation of *de novo* methyltransferase expression (29). Notably, Th2 cells expressed more Dnmt3a and Dnmt3b mRNA and protein than nonpolarized or Th1 cells (Fig. 1A and B).

We next performed chromatin immunoprecipitation (ChIP) to determine the DNA binding of Dnmt3a and Dnmt3b at the promoters of cytokine genes. In contrast to differential binding of lineage transcription factors (Fig. S1A), Dnmt3a was detected at each gene promoter, and Dnmt3b binding was only marginally above background (Fig. 1C) in Th1 and Th2 cells. There was a greater amount of Dnmt3a binding in Th2 cells than in Th1 cells, consistent with greater Dnmt3a expression in Th2 cells. The *Il13* promoter region was associated with the most Dnmt3a binding of the promoters examined. Greater binding of Dnmt3a to *Il13* was also observed following anti-CD3/anti-CD28 activation in the absence of polarizing conditions, with only marginal effects on Dnmt3b binding, even in the absence of *Dnmt3a* (Fig. S1B–D).

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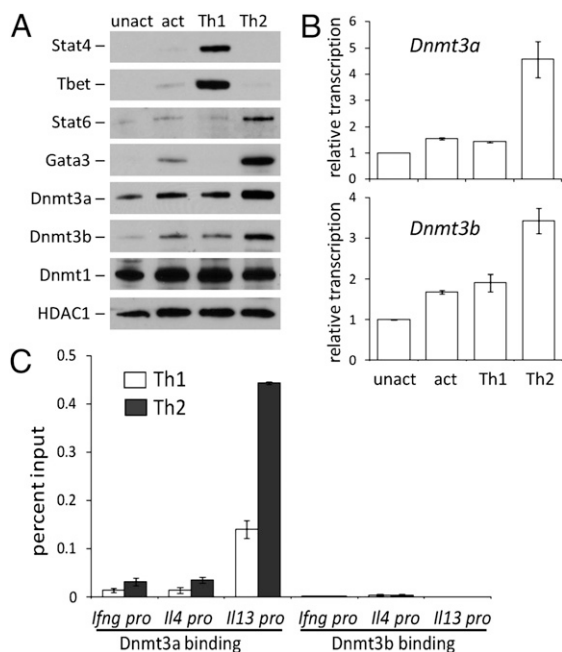


Fig. 1. Expression and DNA binding of Dnmt3a and Dnmt3b during Th1 and Th2 cell development. (A) Expression of lineage-determining transcription factors and DNA methyltransferases in nucleic extracts of naïve CD4 (unact), nonpolarized (act), Th1, and Th2 cells were analyzed by immunoblot. (B) Total cellular RNA was isolated and used for cDNA synthesis before analysis of mRNA by qPCR. *Rpl7* was used as the endogenous control. (C) Th1 and Th2 cells were subjected to ChIP by using antibodies against Dnmt3a and Dnmt3b before qPCR was used to assess precipitated DNA.

Increased Expression of Effector Cytokine Genes in the Absence of *Dnmt3a*. To determine the effects of Dnmt3a and Dnmt3b on T helper cell cytokine production, we generated mice that lacked either or both DNA methyltransferases by breeding *Dnmt3a*^{fl/fl}, *Dnmt3b*^{fl/fl}, or *Dnmt3a3b*^{fl/fl} mice with transgenic mice (*Cd4Cre*) expressing *Cre* recombinase under the control of *Cd4* promoter (collectively termed *Dnmt3*^{fl/fl}*Cd4Cre*) facilitating deletion of *Dnmt3a*, *Dnmt3b*, or both genes in T cells. The conditional deletion of *Dnmt3* was verified by PCR and immunoblot (Fig. S2A and B). Compared with their respective control mice, *Dnmt3*^{fl/fl}*Cd4Cre* mice displayed normal thymic and splenic cellularity and T-cell populations, and T cell proliferation (Figs. S2C and S3).

To define the repressive effects of Dnmt3a and Dnmt3b on cytokine gene expression, we measured expression of a panel of immune genes, comparing Th1 and Th2 cultures derived from *Dnmt3*^{fl/fl}*Cd4Cre* and littermate control mice. Gene expression and secretion of IFN γ , IL-4, and IL-13 were increased in *Dnmt3a*^{fl/fl}*Cd4Cre* and *Dnmt3a3b*^{fl/fl}*Cd4Cre*, but not *Dnmt3b*^{fl/fl}*Cd4Cre*, Th1 cells compared with littermate control cells (Fig. 2A and Fig. S4A). The transcription of *Il18r1* and *Il5* genes were unaffected by *Dnmt3a* and/or *Dnmt3b* deletion (Fig. S4A). *Dnmt3*^{fl/fl}*Cd4Cre* cultures did not express cytokine genes of the opposing lineage, suggesting that the *Dnmt3a* and *Dnmt3b* deletion was insufficient to derepress cytokine gene expression, and consistent with the expression of lineage-determining transcription factors Tbet and Gata3 being normal in the absence of *Dnmt3a* (Fig. 2B). However, when Gata3 was ectopically expressed in Th1 cells, it had a greater effect on the induction of IL-13 in the absence of *Dnmt3a* (Fig. 2C). Together, these results indicated that Dnmt3a, not Dnmt3b, played an inhibitory role on cytokine gene expression, and *Il13* was the gene most dramatically affected by the loss of *Dnmt3a*, consistent with the amount of Dnmt3a binding at the *Il13* locus. We also observed increased *Il13* expression in *Dnmt3a*-deficient Th9 cultures, (Fig. S4B).

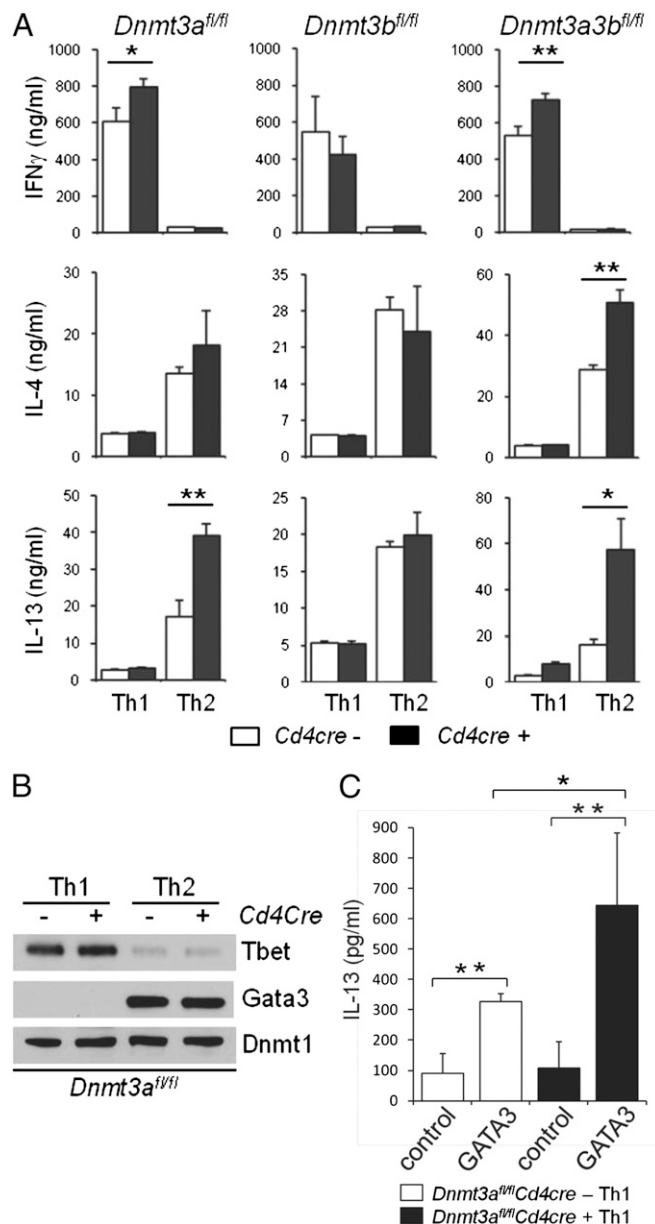


Fig. 2. Cytokine production in the absence of *Dnmt3a*. (A) CD4 T cells purified from *Dnmt3a*^{fl/fl}*Cd4Cre*, *Dnmt3b*^{fl/fl}*Cd4Cre*, *Dnmt3a3b*^{fl/fl}*Cd4Cre* and their respective littermate control mice were cultured under Th1 or Th2 conditions for 5 d and then restimulated with plate-bound anti-CD3 for 24 h before supernatants were harvested for detection of secreted effector cytokines. Data represent mean $\pm$ SD ($n = 3-4$). * $P < 0.05$; ** $P < 0.01$. Data are representative of three independent experiments. (B) Nuclear extracts of Th1 and Th2 cells derived from *Dnmt3a*^{fl/fl}*Cd4Cre* and control mice were subjected to Western blot analysis for detection of Tbet and GATA3. (C) Ectopic expression of GATA3 in *Dnmt3a*^{fl/fl}*Cd4Cre* and control Th1 cells. Th1 cells derived from *Dnmt3a*^{fl/fl}*Cd4Cre* and control mice were infected with either control or GATA3-expressing retroviruses. The retrovirus-transduced cells were sorted by flow cytometry and then activated by anti-CD3 for 24 h. The supernatants were harvested for IL-13 detection by using ELISA. Data shown represent mean $\pm$ SD ($n = 4$ per genotype and transduction condition) * $P < 0.05$; ** $P < 0.01$. Results are representative of two independent experiments.

DNA Methylation and Dnmt3a Association at the *Il13* Locus. To determine whether DNA methylation correlated with Dnmt3a-DNA binding at the *Il13* locus, we used quantitative PCR (qPCR) to measure DNA methylation-dependent digestion at 11

Table 1. Frequency of unmethylated cytosines at mouse *Il13* promoter

Cells	Cytosines unmethylated at mouse <i>Il13</i> promoter, %				
	CpG site -143	CpG site -226	CpG site -252	CpG site -270	CpG site -277
<i>Dnmt3a^{fl/fl}</i> Cd4cre- Th2	20	0	13.3	0	6.7
<i>Dnmt3a^{fl/fl}</i> Cd4cre+ Th2	33.3	33.3	13.3	13.3	6.7

Genomic DNA isolated from *Dnmt3a^{fl/fl}*Cd4cre- and *Dnmt3a^{fl/fl}*Cd4cre+ Th2 cells was bisulfite-treated and then subjected to PCR amplification, cloning, and sequencing. The percentage of unmethylated cytosines at each CpG site was calculated based on cytosine methylation status of 15 individual clones. The region interrogated is within the *Il13* promoter CNS.

encoding a catalytically inactive mutant, Dnmt3a C120S (33). Transduction of Dnmt3a into *Dnmt3a*-deficient cells reduced *Il13* expression to wild-type Th2 levels (Fig. 3F). In contrast, *Il13* mRNA was not significantly decreased by transduction of the inactive C120S mutant, and amounts were significantly higher than cells transduced with active Dnmt3a (Fig. 3F). Although not significant, there was a trend toward a partial effect of the C120S mutant, suggesting the possibility that Dnmt3a could have functions in addition to DNA methyltransferase activity.

Histone Modification Changes at the Loss of Dnmt3a. DNA methylation and histone modifications are interdependent (34, 35). To understand whether *Dnmt3a* deletion had effects on histone modifications, we performed ChIP to evaluate histone modifications at the *Il13* HpaII sites, comparing *Dnmt3a^{fl/fl}*Cd4Cre and control Th1/Th2 cells. Correlating with *Il13* expression, more histone H4 acetylation (H4ac) and H3 lysine 27 acetylation (H3K27ac) were present in Th2 than in Th1 cells, and H3 lysine 27 tri-methylation (H3K27me3) was enriched in Th1 cells (Fig. 4A). H4ac in Th2 cells was not dramatically affected by *Dnmt3a* deficiency. *Dnmt3a^{fl/fl}*Cd4Cre and *Dnmt3a3b^{fl/fl}*Cd4Cre Th2 cultures had more H3K27ac enrichment than control Th2 cells, which paralleled increased *Il13* expression in the absence of Dnmt3a. Interestingly, there was increased H3K27me3 in *Dnmt3a^{fl/fl}*Cd4Cre and *Dnmt3a3b^{fl/fl}*Cd4Cre Th1 cells, compared with control Th1 cultures, suggesting that H3K27me3 may func-

tion as an alternative mechanism to compensate for reduced repression in the absence of Dnmt3a. *Dnmt3a*-deficient Th2 cells demonstrated a greater amount of CBP and decreased HDAC2 at the *Il13* promoter, compared with controls (Fig. 4B).

***Dnmt3a^{fl/fl}*Cd4Cre Mice Displayed Excessive Lung Inflammation in an Asthma Model.** Th2-biased inflammation is implicated in the development of allergic airway disease in humans and mice (36). To explore whether *Dnmt3a* deficiency induced *Il13* expression in vivo, we sensitized and challenged *Dnmt3a^{fl/fl}*Cd4cre and control mice to induce allergic airway inflammation. *Dnmt3a^{fl/fl}*Cd4cre mice had increased airway hyperreactivity compared with littermate control mice upon increasing amounts of methacholine (Fig. 5A and Fig. S7A). The *Dnmt3a^{fl/fl}*Cd4cre mice exhibited significantly higher numbers of total bronchoalveolar lavage (BAL) cells and eosinophils and greater inflammation and mucus production than control mice (Fig. 5B–D and Fig. S7B).

To determine the effects of *Dnmt3a* deficiency on cytokine production in vivo, we purified splenocytes from sensitized and challenged *Dnmt3a^{fl/fl}*Cd4cre and control mice. Cells were either stimulated with ovalbumin (OVA) for 4 h for cytokine gene mRNA analysis or for 3 d for detection of cytokine secretion. OVA-stimulated splenocytes derived from *Dnmt3a^{fl/fl}*Cd4Cre mice had significantly higher *Il13* mRNA than control mice (Fig. 5E), with a concomitant trend toward increased *Il4* transcription. Cytokine secretion measured by ELISA also demonstrated that

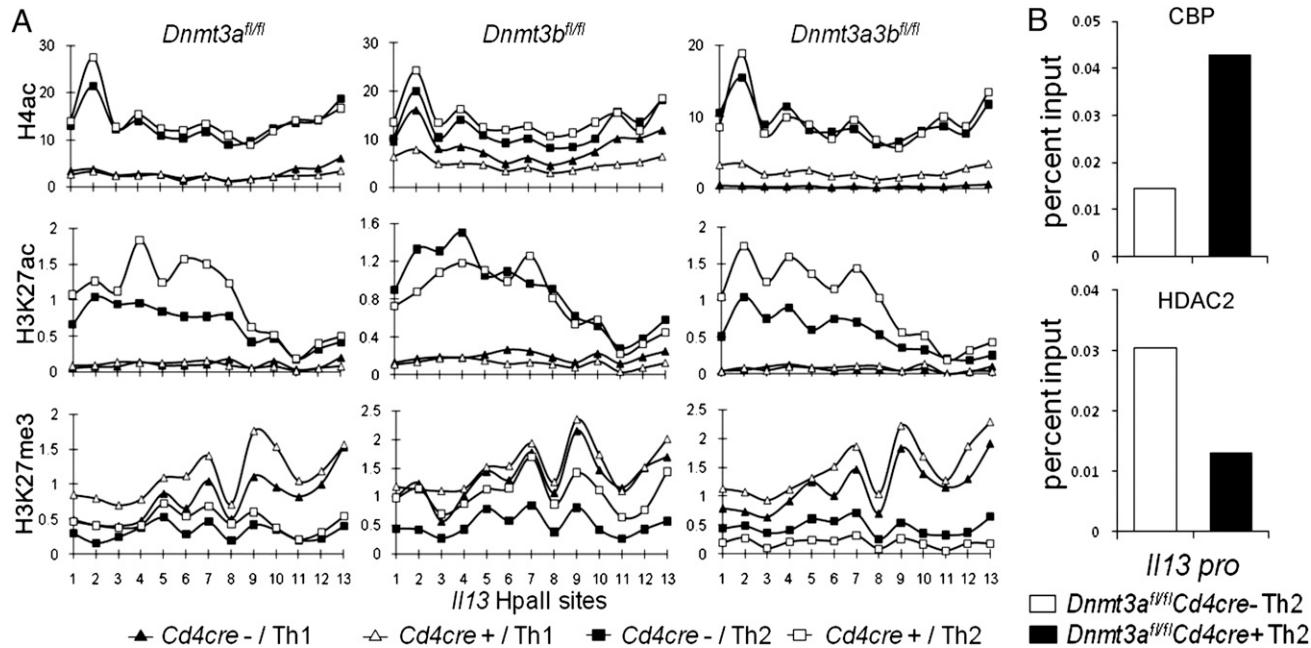


Fig. 4. *Dnmt3a* deficiency was accompanied by histone modification changes. (A) Th1 and Th2 cells derived from *Dnmt3a*-mutant mice (*Dnmt3a^{fl/fl}*Cd4cre+, *Dnmt3b^{fl/fl}*Cd4cre+, *Dnmt3a3b^{fl/fl}*Cd4cre+) and their respective littermate control mice were subjected to ChIP by using the antibodies against the indicated histone modifications at the *Il13* HpaII sites. PCR was performed with primers that flank the *Il13* HpaII sites. (B) Th2 cells derived from *Dnmt3a*-mutant and control mice were subjected to ChIP by using the antibodies against CBP or HDAC2.

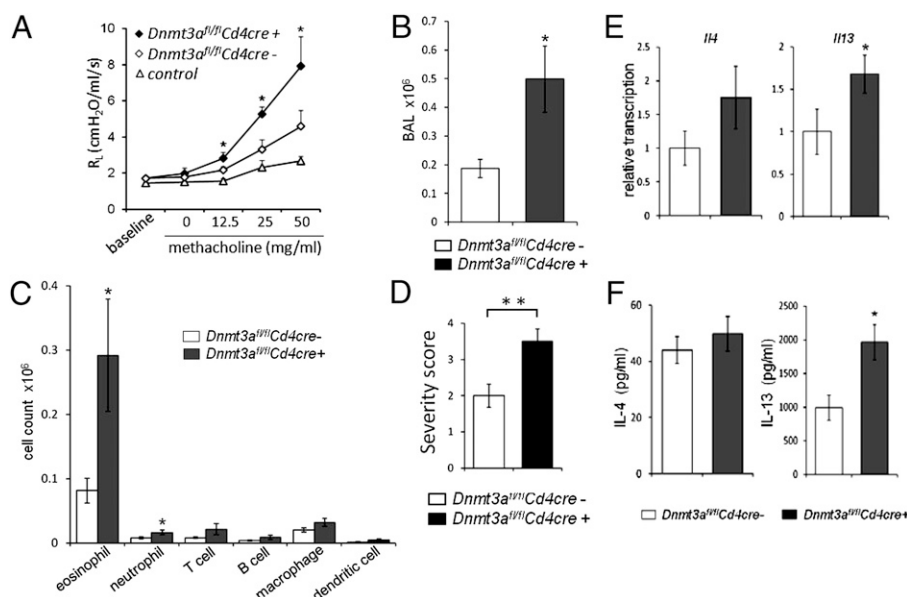


Fig. 5. OVA-induced lung inflammation in *Dnmt3a^{fl/fl}Cd4cre+* mice. (A) *Dnmt3a^{fl/fl}Cd4cre+* and littermate *Dnmt3a^{fl/fl}Cd4cre-* mice were sensitized and challenged with OVA. The airway hyperreactivity of OVA-sensitized and -challenged mice was recorded as lung resistance upon treatment with increasing amount of methacholine as indicated. (B and C) Total and differential BAL cell counts between *Dnmt3a^{fl/fl}Cd4cre+* and littermate control mice. (D) Severity of airway inflammation on H&E staining was scored by using the following scale: 0 (no inflammation), 1 (mild), 2 (moderate), 3 (moderate to severe), and 4 (severe). (E) OVA-stimulated cytokine gene expression in splenocytes from OVA-sensitized and -challenged *Dnmt3a^{fl/fl}Cd4cre+* and control mice. Splenocytes from OVA-sensitized and -challenged *Dnmt3a^{fl/fl}Cd4cre+* and control mice were stimulated with OVA (50 μ g/mL) for 4 h and then analyzed for the transcriptional levels of *Il4* and *Il13*. (F) Splenocytes from *Dnmt3a*-deficient or littermate control mice were stimulated with OVA for 3 d, and supernatants were harvested for detection of IL-4 and -13 by ELISA. Data are expressed as mean $\pm$ SEM of 3–6 mice, and results are representative of two experiments. * $P < 0.05$; ** $P < 0.01$.

IL-13 expression was significantly increased in *Dnmt3a^{fl/fl}Cd4cre* cells (Fig. 5F).

Discussion

Transcription of cytokine genes in T helper cell subsets is partly determined by DNA methylation status. In this study, we examined mice that lack de novo DNA methyltransferases in T cells to demonstrate that *Dnmt3a*, not *Dnmt3b*, was required for controlling normal expression of *Il13* in Th2 cells. *Dnmt3a* regulated DNA methylation and histone modifications at the *Il13* locus. The absence of *Dnmt3a* resulted in increased IL-13 production in vitro and in vivo and increased allergic inflammation.

Il13 expression was undetectable in Th1 cultures derived from *Dnmt3a^{fl/fl}Cd4cre* mice despite reduced DNA methylation at the *Il13* locus (Fig. S5C), which is consistent with normal expression of the lineage-defining transcription factors, even in the absence of *Dnmt3a* (Fig. 2B). Although altered DNA methylation was correlated with changes in histone modifications, the *Il13* gene in *Dnmt3a*-deficient Th1 cells did not acquire a Th2 pattern of modification, suggesting that the absence of *Dnmt3a* alone was not sufficient to generate an active chromatin configuration. Thus, although DNA hypomethylation is an important component of a coordinated regulatory network governing gene expression, effective cytokine gene activation requires the activity of lineage-specific transcription factors. Indeed, when *Gata3* was transduced into *Dnmt3a*-deficient Th1 cells, there was greater induction of IL-13 production than in wild-type cells (Fig. 2C). Our study complements another study showing that in the absence of *Dnmt3a*, there was greater switching of IL-4-secreting to IFN- γ -secreting cells when culture conditions were altered (29). A model wherein epigenetic repression of cytokine loci represents only one component of subset-specific expression is consistent with our previous work demonstrating that STAT4-dependent induction of Th1 genes only partly depends upon reducing *Dnmt3a* association with target loci (10, 11). Thus, both permissive epige-

netic modifications and appropriate transcription factors are required for lineage-restricted cytokine expression.

Dnmt3a enzymatic activity was assessed using *Il13* since expression was markedly affected by *Dnmt3a* deficiency and the *Il13* gene locus was associated with an upstream CpG island and multiple HpaII (CCGG) sites within the gene body. Our results indicated an inverse relationship between the *Il13* expression and DNA methylation during Th1 and Th2 development, compatible with previous findings on differential methylation at Th2 cytokine gene loci (7). However, the presence of *Dnmt3a* at the *Il13* locus was not the only determinant of DNA methylation, because Th2 cells had greater amounts of *Dnmt3a* bound to the locus than Th1 cells. These results suggest that other factors present within a *Dnmt3* complex impact enzymatic activity and are required for DNA methylation. However, *Dnmt3a* methyltransferase activity is required because complementation of *Dnmt3a*-deficient Th2 cells with a catalytically inactive *Dnmt3a* did not significantly repress *Il13* expression (Fig. 3F), and diminished levels of DNA methylation are present in the *Il13* gene in cells producing the highest amounts of IL-13 (Fig. S6). Thus, de novo methyltransferases are responsible, at least in part, for establishing this pattern of DNA methylation. Presumably, de novo methyltransferase-independent methylation is attributable to activity of *Dnmt1*, the maintenance methyltransferase expressed in equivalent amounts in both T helper cell subsets. Therefore, our study provides evidence to support the possibility that de novo DNA methylation acts by fine tuning gene locus-specific methylation in response to developmental instruction.

Histone modifications and DNA methylation are believed to be interdependent with respects to modulating chromatin structure and controlling gene expression. Increased *Il13* expression in the absence of *Dnmt3a* was associated with increased H3K27ac enrichment, which correlated with increased CBP and decreased HDAC2 occupancy at the gene locus. These data also suggested that *Dnmt3a*, or DNA methylation, might enhance recruitment of HDACs and block the binding of HATs to suppress gene expression

at specific loci. Together, our observations provide further evidence for a link between DNA methylation and histone modifications.

DNA methyltransferases can be recruited to target gene loci through two mechanisms: direct DNA binding via PWWP domain of DNA methyltransferase (37) or recruitment by interaction with transcriptional repressors (38). Our data also suggest that increased expression of DNA methyltransferases impacts the association with target genes. We observed that greater expression of Dnmt3a in Th2 cells, compared with Th1 cells, is associated with greater binding to the *Il13* locus. Similarly, induced expression of Dnmt3a following antigen receptor stimulation results in greater association with cytokine loci. The antigen stimulation-induced expression and DNA binding capacity of DNA methyltransferases has biological relevance in that naïve CD4 cells, upon antigen stimulation, might take advantage of methylation-mediated gene-silencing effects to limit immune responses. Similarly, Dnmt3a-expressing T helper cells may use the same strategy to prevent immune overreaction. This notion was supported by our observation that *Dnmt3a^{fl/fl}Cd4cre* mice exhibited increased lung inflammation in a murine asthma model, compared with control mice, indicating that Dnmt3a is a rate-

limiting factor restricting inflammatory reactions. Whether de novo methyltransferase-mediated negative regulation of gene expression functions as a general mechanism to manipulate immune responses needs further investigation.

Materials and Methods

Dnmt3 conditional mutant mice were established by breeding *Dnmt3a^{fl/fl}*, *Dnmt3b^{fl/fl}*, or *Dnmt3a3b^{fl/fl}* mice with Cre transgenic mice (*Cd4cre*). *Dnmt3a^{fl/fl}* mice were on a C57BL/6 genetic background. *Dnmt3b^{fl/fl}* and *Dnmt3a3b^{fl/fl}* mice were on a mixed C57BL/6 × 129 genetic background. Mice were housed in specific pathogen-free conditions in the Laboratory Animal Resource Center at Indiana University School of Medicine. All experiments were performed in accordance with procedures approved by the Indiana University Animal Care and Use Committee. Primers used for *Il13* locus analysis are indicated in Fig. S8.

Further details can be found in *SI Materials and Methods*.

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Supporting Information

Yu et al. 10.1073/pnas.1103803109

SI Materials and Methods

T-Cell Differentiation. CD4 T cells were isolated from spleens and lymph nodes of mice by using magnetic beads (Miltenyi Biotech). CD4 T cells (1×10^6 per milliliter) were cultivated in RPMI 1640 supplemented with 10% FBS, L-glutamine, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, Hepes, and 2-mercaptoethanol in the presence of plate-bound anti-CD3 (4 μ g/mL) and soluble anti-CD28 (0.5 μ g/mL). Anti-IL-4 (10 μ g/mL) and anti-IFN- γ (10 μ g/mL) were added for nonpolarizing condition. IL-12 (5 ng/mL) and anti-IL-4 (10 μ g/mL) were added for Th1 polarization, while IL-4 (10 ng/mL) and anti-IFN- γ (10 μ g/mL) were added for Th2 differentiation. Under Th9 conditions, TGF- β (3 ng/mL), IL-4 (20 ng/mL), and anti-IFN- γ (10 μ g/mL) were added. Cell cultures were expanded after 3 d and supplemented with cytokines. After a total of 5-d differentiation, cells were harvested for subsequent analyses.

Chromatin Immunoprecipitation (ChIP). ChIP was performed as described (1). The ChIP antibodies used in this study include Dnmt3a antibody (IMG-268A; IMGENEX), Dnmt3b antibody (804-233-c100; Alexis), Stat4 antibody (sc-486; Santa Cruz Biotech), T-bet antibody (sc-21003x; Santa Cruz Biotech), Stat6 antibody (sc-1698; Santa Cruz Biotech), Gata3 antibody (sc-268; Santa Cruz Biotech), H4ac antibody (06-866; Millipore), H3K27me3 antibody (cs200603; Millipore), H3K27ac antibody (07-360; Millipore), CBP antibody (sc-7300; Santa Cruz), and HDAC2 antibody (sc-7899; Santa Cruz). Please refer to Fig. S8 for sequences of primer sets for qPCR.

Proliferation Assay. Naïve CD4 cells were stained with carboxy-fluorescein succinimidyl ester (CFSE) and stimulated with anti-CD3 (4 μ g/mL) and anti-CD28 (0.5 μ g/mL) for 3 d. Cell proliferation was monitored on days 2 and 3 by flow cytometry. The proliferation index indicates average number of cell divisions.

DNA Methylation Analysis by HpaII-qPCR. This method combines methylation-sensitive HpaII restriction with qPCR. Genomic DNA of CD4 cells was purified by using the Wizard Genomic DNA Purification Kit (Promega). Genomic DNA (5 μ g) was first digested with BamHI (10 U/ μ g DNA) at 37 °C overnight to reduce genomic DNA size. One-half of BamHI-treated genomic DNA was further digested with HpaII (10 U/ μ g DNA) at 37 °C overnight, followed by heat inactivation of HpaII at 65 °C for 20 min. Then, the BamHI- and BamHI/HpaII-treated DNA was purified by phenol/chloroform extraction and ethanol precipitation. Resuspended DNA was subjected to qPCR analysis by using primers bracketing each HpaII site (for primer sequences, see Fig. S8). The arbitrary values of DNA methylation were calculated as $2^{Ct(\text{BamHI-cut DNA}) - Ct(\text{BamHI/HpaII-cut DNA})} \times 100$.

Western Blot. Nuclear extracts of CD4 cells were made by using Nuclear and Cytoplasmic Extraction Reagent (Pierce). Nuclear proteins were fractionated in SDS/PAGE and transferred to nitrocellulose membranes. Dnmts and transcription factors were probed with antibodies as indicated.

Bisulfite Sequencing. Genomic DNA was bisulfite-treated using EZ DNA Methylation Kit (Zymo Research). Bisulfite-converted DNA was amplified by nested PCR with original primer set (*il13* pro BC F and R) and nested primer set (*il13* pro BC NF and NR; see primer sequences in Fig. S8). PCR products were then

cloned into TA TOPO vector (Invitrogen), and single clones were sequenced.

Retroviral Constructs, Transfection, and Transduction. pcDNA3-Dnmt3a (a gift from Dr. En Li, Novartis Pharmaceutical Inc.) was used as a PCR template to amplify mouse *Dnmt3a* cDNA by using *Pfu* Taq polymerase (see primer sequences for *Dnmt3a* CDS F and R in Fig. S8). *Dnmt3a* gene was then cloned into a retroviral vector to generate pMIEG3-hCD4-Dnmt3a. The cysteine at the P-C domain of Dnmt3a was replaced with a serine (2) by using Quick Change II Site-Directed Mutagenesis (Stratagene) (see primer sequences for *Dnmt3a* C-S F and R in Fig. S8), to generate enzymatic inactive form of Dnmt3a, named pMIEG3-hCD4-Dnmt3a C120S. Retroviral constructs were cotransfected with Env plasmid into packaging cell line Phoenix-GP by using TransIT-LT1 Transfection Reagent (Mirus) according to the manufacturer's instructions. The recombinant retrovirus-containing supernatant was collected at 48 h posttransfection. CD4 T cells cultivated under Th1 or Th2 conditions for 2 d were infected with retroviruses by spin infection (2,000 rpm for 2 h at room temperature). The retrovirally transduced cells were stained with PE-anti-hCD4 and sorted by flow cytometry.

OVA-Induced Allergic Airway Disease. *Dnmt3a^{fl/fl}*CD4Cre and littermate control mice (8–10 wk of age) were sensitized intraperitoneally by injection of OVA (Worthington) 50 μ g, emulsified in aluminum hydroxide gel (Sigma) in a total volume of 200 μ L, on days 0 and 7. Starting from day 14, the sensitized mice were challenged intranasally with OVA 50 μ g for 5 consecutive days. Lung function tests on OVA-sensitized/challenged mice were conducted after the last OVA challenge, based on airway resistance or Penh measurements of airway hyperreactivity in response to increasing doses of aerosolized methacholine. On day 20, mice were killed for harvest of serum, spleens, bronchoalveolar lavage (BAL), and lungs. The lungs were fixed in paraformaldehyde (4%) at 25 °C overnight and were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) or Periodic acid-Schiff (PAS). Total BAL numbers were counted with a hemocytometer. Differential cell counts were calculated based on a FACS-based method as described (3).

Flow Cytometry, ELISA, and qRT-PCR. Thymic and splenic cellularity was analyzed by two-color flow cytometry. Cells isolated from thyme were stained by using anti-CD4-PE.Cy5-conjugate (BD Pharmingen) and anti-CD8-PE-conjugate (eBioscience), and splenocytes were stained with anti-CD4-FITC conjugate (BD Pharmingen) and anti-CD8-PE.Cy7-conjugate (eBioscience). IL-13 protein expression was determined by intracellular staining modified from that described (4). Briefly, differentiated Th2 cells were stimulated with anti-CD3 for 6 h (Monensin was added for the last 4 h). Cells were washed in PBS and fixed with formaldehyde. Cells were then washed with saponin-containing permeabilization buffer and incubated with PE-anti-IL-13 (eBioscience). After washes with permeabilization buffer and PBS, cells were analyzed and sorted for IL-13^{Lo} and IL-13^{Hi}-expressing cells by using a flow sorter. The sorted cells were lysed and incubated at 65 °C overnight to reverse DNA cross-linking. Purified DNA was subsequently used for DNA methylation analysis by HpaII-qPCR. Gene expression was determined by cellular RNA isolation, reverse transcription, and qPCR analysis. Total RNA of CD4 cells was purified by using TRIzol reagent (Invitrogen) and then used

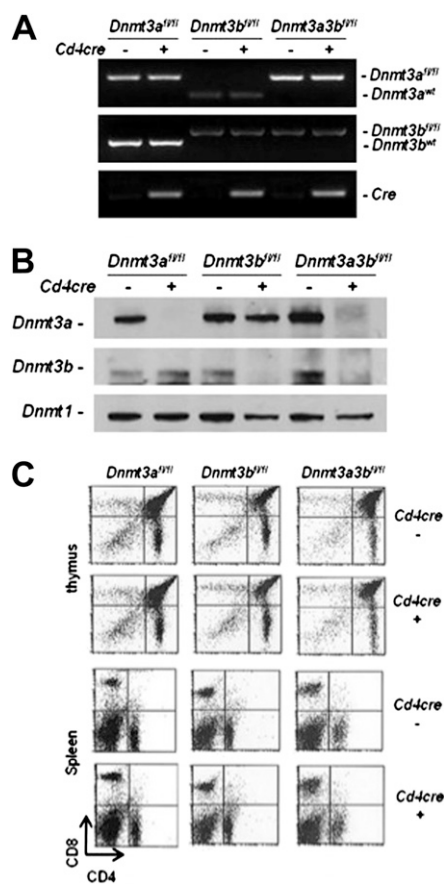


Fig. S2. Establishment of *Dnmt3* conditional mutant mice. (A) We generated *Dnmt3* knockout mice by crossing *Dnmt3a^{fl/fl}*, *Dnmt3b^{fl/fl}*, or *Dnmt3a/b^{fl/fl}* mice with *Cd4cre* mice. DNA genotyping was conducted to identify *Dnmt3a* and/or *Dnmt3b* conditional mutant mice and littermate control mice as indicated. (B) Western blot showing the absence of *Dnmt3a* or/and *Dnmt3b* proteins in corresponding conditional mutant mice. Nuclei extracts of activated CD4 cells were fractionated by PAGE and subjected to Western blot by using antibodies against *Dnmt3a*, *Dnmt3b*, and *Dnmt1*. (C) Thymic and splenic cellularity of *Dnmt3* conditional mutant mice. Cells isolated from thymi or spleens of *Dnmt3* conditional mutant mice and their respective littermate control mice were stained with anti-CD4 and anti-CD8 antibodies, and thymic and splenic cellularity were analyzed by flow cytometry.

A

Thymus	<i>Dnmt3a</i> ^{R/R}		<i>Dnmt3a</i> ^{R/R}		<i>Dnmt3b</i> ^{R/R}		<i>Dnmt3b</i> ^{R/R}		<i>Dnmt3a3b</i> ^{R/R}		<i>Dnmt3a3b</i> ^{R/R}	
	<i>Cd4cre</i> -		<i>Cd4cre</i> +		<i>Cd4cre</i> -		<i>Cd4cre</i> +		<i>Cd4cre</i> -		<i>Cd4cre</i> +	
	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
Percent												
DP	82.8	0.4	82.0	0.9	82.3	1.8	84.9	1.5	82.7	0.7	80.4	7.9
DN	5.9	0.8	6.7	0.4	7.6	0.6	5.6	0.4	6.6	0.1	9.2	4.8
CD4	8.2	0.7	8.4	0.0	8.0	1.0	7.3	1.4	7.7	0.8	7.9	2.3
CD8	3.1	0.5	2.9	0.4	2.2	0.2	2.2	0.4	3.0	0.0	2.5	0.7
CD4 MFI												
DP	772.3	22.9	760.1	29.0	811.8	30.1	845.2	26.1	766.1	24.6	776.0	27.1
CD4	967.5	6.0	927.0	19.2	977.3	8.4	948.0	8.9	1010.3	33.5	947.6	4.9
CD8 MFI												
DP	448.5	26.1	493.0	9.3	464.3	17.2	463.2	8.4	466.3	8.9	472.4	2.1
CD8	446.8	34.9	440.2	24.7	497.6	27.3	455.3	22.4	431.1	10.5	453.3	8.3

Spleen	<i>Dnmt3a</i> ^{N/N}		<i>Dnmt3a</i> ^{N/H}		<i>Dnmt3b</i> ^{N/N}		<i>Dnmt3b</i> ^{N/H}		<i>Dnmt3a3b</i> ^{N/N}		<i>Dnmt3a3b</i> ^{N/H}	
	<i>Cd4cre</i> -		<i>Cd4cre</i> +		<i>Cd4cre</i> -		<i>Cd4cre</i> +		<i>Cd4cre</i> -		<i>Cd4cre</i> +	
	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
CD4 percent	18.0	0.7	15.1	1.6	13.5	1.1	14.9	1.5	9.9	2.3	12.0	1.3
CD8 percent	10.4	0.7	11.8	1.5	5.9	1.4	7.3	2.4	5.1	1.8	8.4	1.4
CD4 MFI	101.0	5.5	96.6	6.6	102.9	0.3	96.3	3.8	98.6	5.6	100.8	3.6
CD8 MFI	1286.5	53.0	1307.4	47.9	1370.3	42.5	1353.1	19.0	1273.5	56.5	1366.7	194.5

Lympho Nodes	<i>Dnmt3a</i> ^{N/N}		<i>Dnmt3a</i> ^{N/N}		<i>Dnmt3b</i> ^{N/N}		<i>Dnmt3b</i> ^{N/N}		<i>Dnmt3a3b</i> ^{N/N}		<i>Dnmt3a3b</i> ^{N/N}	
	<i>Cd4cre</i> –		<i>Cd4cre</i> +		<i>Cd4cre</i> –		<i>Cd4cre</i> +		<i>Cd4cre</i> –		<i>Cd4cre</i> +	
	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
CD4 percent	28.4	3.0	25.9	3.8	30.8	1.7	36.1	0.5	29.3	1.2	29.6	2.0
CD8 percent	21.6	2.4	23.5	1.8	20.4	2.1	21.0	0.7	23.6	0.1	26.6	1.8
CD4 MFI	134.7	3.4	128.1	7.9	143.5	13.8	133.9	5.2	142.9	2.5	132.4	8.7
CD8 MFI	888.0	14.7	863.7	10.4	986.8	26.2	958.4	11.1	1009.8	118.7	912.9	37.8

B

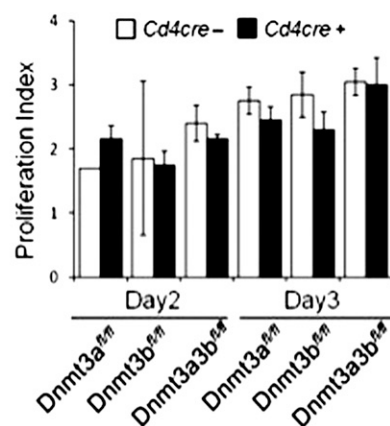
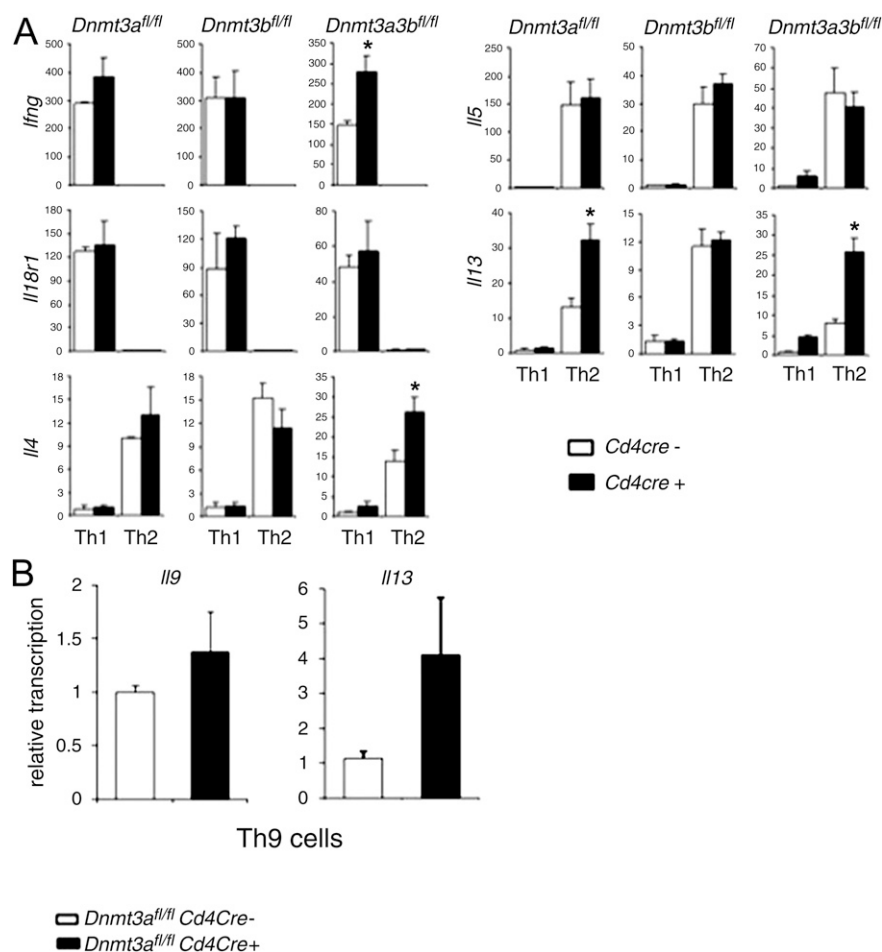


Fig. S3. (A) Percentage and mean fluorescence index (MFI) of CD4 and CD8 cells in thymuses, spleens, and lymph nodes derived from *Dnmt3^{fl/fl}* Cd4cre+ and littermate control mice. (B) Naïve CD4 cells from *Dnmt3^{fl/fl}* Cd4cre+ and littermate control mice were stained with CFSE and activated in the presence of anti-CD3 (4 µg/mL) and anti-CD28 (0.5 µg/mL). The cell proliferation was measured at days 2 and 3 by flow cytometry. All results represent mean ± SD. Data are representative of two independent experiments.



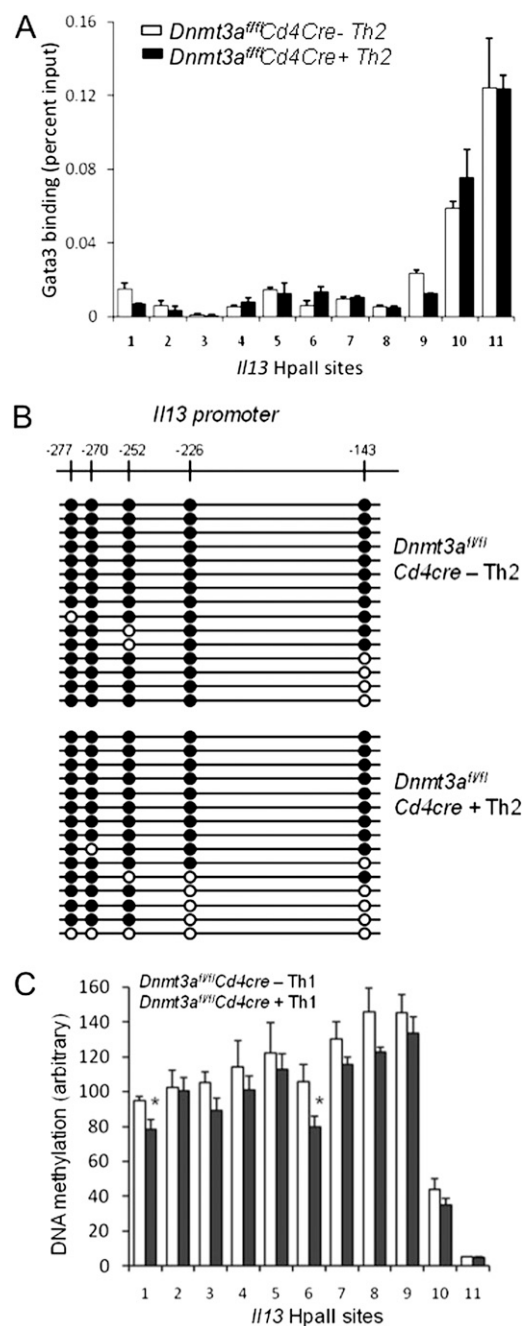


Fig. S5. GATA3 DNA binding and DNA methylation at the *Il13* promoter. (A) Th2 cells of *Dnmt3a^{fl/mf}Cd4cre+* and control mice were subjected to ChIP using the antibody against GATA3. DNA binding capacity of GATA3 was evaluated at the *Il13* HpaII sites indicated in Fig. 3A. (B) Bisulfite sequencing of mouse *Il13* promoter in *Dnmt3a^{fl/mf}Cd4cre+* and control Th2 cells. Five CpG sites are indicated as vertical lines, and their numbers are relative to transcriptional start site of *Il13* gene. Rows represent individual clones. Black and white circles denote methylated and unmethylated CpG sites, respectively. (C) Genomic DNA isolated from *Dnmt3a^{fl/mf}Cd4cre+* and control Th1 cells was subjected to HpaII-qPCR to analyze the DNA methylation levels at the *Il13* HpaII sites. Data represent mean $\pm$ SEM ($n = 4$ per genotype). Data are representative of four independent experiments. * $P < 0.05$.

Fig. S6. DNA methylation in IL-13-hi and IL-13-low cells. (A) Flow cytometry of intracellular IL-13 staining in *Dnmt3a^{fl/fl}*Cd4cre+ and control Th2 cells. (B) Flow cytometry of intracellular IL-13 staining in *Dnmt3a^{fl/fl}*Cd4cre+ Th2 cells following sorting of cells into IL-13-hi and IL-13-low populations. (C) HpaII-qPCR in IL-13-hi and IL-13-low cells using the methods described for experiments in Fig. 3. ***P* < 0.01.

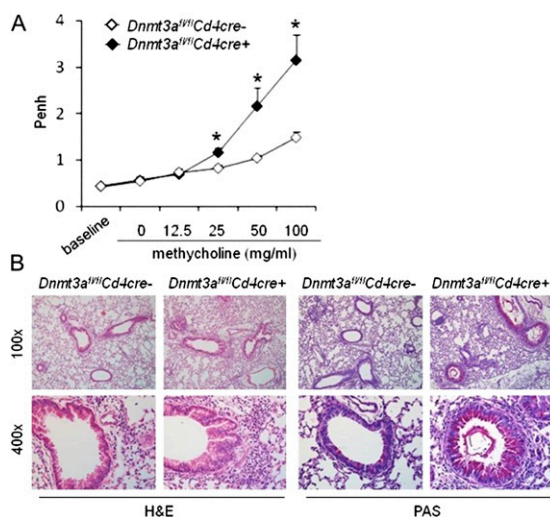


Fig. S7. Airway inflammation in the absence of Dnmt3a. (A) *Dnmt3a^{fl/fl}*Cd4cre+ (*n* = 4) and littermate control mice (*n* = 4) were sensitized and challenged with OVA. The airway hyperreactivity of OVA-sensitized and -challenged mice was recorded as Penh measurements, upon treatment with increasing amount of methacholine as indicated. **P* < 0.05. (B) Paraffin-embedded and H&E or PAS-stained lung tissues of OVA-sensitized and -challenged *Dnmt3a^{fl/fl}*Cd4cre+ and littermate control mice. Magnification is indicated to the left of the images.

Dnmt3a loxP F
Dnmt3a loxP R
Dnmt3b loxP F
Dnmt3b loxP R
Cre F
Cre R
Dnmt3a F
Dnmt3a R
Dnmt3b F
Dnmt3b R
Dnmt3a CDS F
Dnmt3a CDS R
Dnmt3a C-S F
Dnmt3a C-S R
Rpl7 F
Rpl7 R
lfnf pro F
lfnf pro R
lil4 pro F
lil4 pro R
lil13 pro F
lil13 pro R
lil13 Hpal1 1R
lil13 Hpal1 1F
lil13 Hpal1 2R
lil13 Hpal1 2F
lil13 Hpal1 3R
lil13 Hpal1 3F
lil13 Hpal1 4R
lil13 Hpal1 4F
lil13 Hpal1 5R
lil13 Hpal1 5F
lil13 Hpal1 6R
lil13 Hpal1 6F
lil13 Hpal1 7R
lil13 Hpal1 7F
lil13 Hpal1 8R
lil13 Hpal1 8F
lil13 Hpal1 9R
lil13 Hpal1 9F
lil13 Hpal1 10R
lil13 Hpal1 10F
lil13 Hpal1 11R
lil13 Hpal1 11F
lil13 pro BC F
lil13 pro BC R
lil13 pro BC NF
lil13 pro BC NR

Fig. S8. Primer sequences. Detailed sequences of primers used for HpaII-qPCR and ChIP assays as described in *SI Materials and Methods*.