

# DNA methylation of T<sub>H</sub>1/T<sub>H</sub>2 cytokine genes affects sensitization and progress of experimental asthma

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**Background:** Epigenetic changes in DNA methylation have recently been demonstrated to be involved in effector T-cell polarization, resulting in differential secretion of T<sub>H</sub>1 and T<sub>H</sub>2 cytokines. However, the contribution to the development of a chronic inflammatory phenotype remains still unclear.

**Objective:** We sought to investigate changes in DNA methylation in marker genes of T-cell subsets during allergen sensitization/challenge and their influence on the development of an allergic airway inflammatory response.

**Methods:** The relationship between changes in DNA methylation and phenotype development were examined in a well-established model of experimental asthma. DNA methylation was investigated at genomic loci associated with T<sub>H</sub>1 (*IFNG* promoter) or T<sub>H</sub>2 (conserved noncoding sequence 1 [*CNS1*]) cytokine production by using bisulfite pyrosequencing.

**Results:** Analysis of CD4<sup>+</sup> T cells revealed a significant increase in DNA methylation at the *IFNG* promoter after allergen sensitization/challenge, which correlated with decreased IFN- $\gamma$  cytokine expression, whereas only minor changes were observed at the *CNS1* locus. Furthermore, the increase in DNA methylation at the *IFNG* promoter could be reversed with a DNA methyltransferase (DNMT) inhibitor *in vitro* and *in vivo* with beneficial effects on sensitization status and allergic phenotype. The specific importance of the DNA methylation status in CD4<sup>+</sup> T cells could be confirmed by using adoptive transfer experiments.

**Conclusion:** We here report the novel finding that epigenetic regulation in T cells contributes to the development of experimental asthma and can be targeted pharmacologically. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

**Key words:** Epigenetics, T cells, experimental asthma

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Supported by the Deutsche Forschungsgemeinschaft (SFB/TR22), the LOEWE Excellence Centre UGMLC (Universities of Gießen & Marburg Lung Centre), and Stiftung P.E. Kempkes, Marburg (10/07).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication April 14, 2011; revised October 29, 2011; accepted for publication December 12, 2011.

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0091-6749/\$36.00

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doi:10.1016/j.jaci.2011.12.963

## Abbreviations used

|        |                                          |
|--------|------------------------------------------|
| 5Aza:  | 5-Aza-2'-deoxycytidine                   |
| BAL:   | Bronchoalveolar lavage                   |
| CFSE:  | Carboxyfluorescein succinimidyl ester    |
| CNS-1: | Conserved noncoding sequence 1           |
| DNMT:  | DNA methyltransferase                    |
| Foxp3: | Forkhead box protein 3                   |
| GAPDH: | Glyceraldehyde-3-phosphate dehydrogenase |
| LUMA:  | Luminometric Methylation Assay           |
| MNC:   | Mononuclear cell                         |
| NK:    | Natural killer                           |
| OVA:   | Ovalbumin                                |

Imbalance in effector T-cell populations plays an important role in the development of chronic inflammatory conditions, including autoimmunity and allergy. Allergic bronchial asthma is a prototypic disease of dominant T<sub>H</sub>2 activation, which, apart from genetic predisposition, is influenced by environmental stimuli present or absent during fetal life.<sup>1-4</sup> Many aspects of airway inflammation are related to the effects of the T<sub>H</sub>2 cytokines IL-4, IL-5, and IL-13,<sup>5</sup> which are not adequately counterregulated by T<sub>H</sub>1 or regulatory T-cell activities. Adequate regulation of T-cell lineage decisions and cytokine gene activation and silencing are crucial for effective immune function and prevention of allergy or autoimmunity.<sup>6</sup> The genes encoding these cytokines are reciprocally regulated during differentiation and being remodeled at the chromatin level by means of epigenetic mechanisms for appropriate transcription.<sup>7</sup> Such epigenetic mechanisms comprise methylation of DNA CpG motifs and histone modifications, both influencing the accessibility of the DNA for the transcription machinery.<sup>8,9</sup>

*In vitro* polarization experiments with naive T cells pointed out that the underlying processes of differentiation into effector T-cell populations are orchestrated by changes in DNA methylation at promoter regions, resulting in differential secretion of T<sub>H</sub>1/T<sub>H</sub>2 signature cytokines.<sup>10,11</sup> Although DNA methylation signatures in CD4<sup>+</sup> T cells differ significantly between atopic and nonatopic patients,<sup>12</sup> the contribution of this level of regulation to the development of the disease phenotype has not been investigated yet. It was therefore the aim of this study to demonstrate that modifications in epigenetic signatures during the process of allergic sensitization are sufficient to drive T<sub>H</sub>2 cell polarization and subsequent phenotype development.

## METHODS

### Animals

Six- to 8-week-old female BALB/c mice and BALB/c<sup>scid</sup> mice were obtained from Harlan Winkelmann (Borchen, Germany). DO11.10 mice on

the BALB/c background were bred at the animal facility of Marburg University.

## Sensitization and aerosol challenge

Animals were sensitized to ovalbumin (OVA) by means of 2 intraperitoneal injections of 10  $\mu$ g of OVA (grade VI; Sigma-Aldrich, Munich, Germany) emulsified in 1.5 mg Al(OH)<sub>3</sub> (Pierce Biotechnology, Rockford, Ill) in a total volume of 200  $\mu$ L on days 15 and 29 of the experimental protocol. Sham groups received PBS with Al(OH)<sub>3</sub>. At days 34, 35, and 36, all animals were exposed to aerosolized OVA (1% wt/vol diluted in PBS) for 20 minutes.

## 5-Aza-2'-deoxycytidine treatment

Mice were treated intraperitoneally with 5-Aza-2'-deoxycytidine (5Aza; Sigma-Aldrich; 0.2 mg/kg body weight dissolved in 100  $\mu$ L PBS) or PBS (control) every other day starting 2 weeks before sensitization until OVA challenge.

## Culture of splenic mononuclear cells and purification of CD4<sup>+</sup> T cells for adoptive transfer

Seven days after OVA sensitization, splenocytes of 5Aza- or sham-treated donor mice (DO11.10) were disaggregated. Erythrocytes were hemolyzed, and the washed splenocytes ( $4 \times 10^6$ /mL) were cultured for 3 days in the presence of 100  $\mu$ g/mL OVA (Sigma-Aldrich). Thereafter, CD4<sup>+</sup> T cells were isolated with the Dynal Mouse CD4 Negative Isolation Kit (Invitrogen, Carlsbad, Calif), according to the manufacturer's protocol. FACS analysis revealed a purity of greater than 95%. Isolated CD4<sup>+</sup> T cells were resuspended in sterile PBS, and 100  $\mu$ L ( $1 \times 10^7$  cells) of this suspension were injected intravenously into recipient mice (wild-type BALB/c mice). Twenty-four hours before and 2, 48, and 96 hours after transfer, the recipient mice were challenged with OVA aerosol, as described above.

## Assessment of airway reactivity

Lung function analysis was performed 24 hours after the last aerosol challenge by using noninvasive head-out body plethysmography.<sup>13</sup> Additional details on the methods are provided in the [Methods](#) section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## Measurement of antibody titers

Blood samples were taken from the axillary vessels. Levels of anti-OVA IgE, anti-OVA IgG<sub>1</sub>, and anti-OVA IgG<sub>2a</sub> antibody titers were measured by means of ELISA, as previously described.<sup>1</sup>

## Bronchoalveolar lavage and differential cell counts

Bronchoalveolar lavage (BAL) was performed as described by Neuhaus-Steinmetz et al,<sup>13</sup> with the exception that lavage was performed only once with 1 mL. Additional details on the methods are provided in the [Methods](#) section in this article's Online Repository.

## Lung histology

For histologic analyses, lungs were fixed with 6% paraformaldehyde through the trachea, removed, and stored in 6% paraformaldehyde. Lung tissues were embedded in paraffin, and 3- $\mu$ m sections were stained with periodic acid-Schiff, as previously described.<sup>13</sup>

## Differential cell counts of blood slides

Blood slides were stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany). Standard morphologic criteria were used to identify monocytes, lymphocytes, eosinophils, and neutrophils. One hundred cells were counted per slide by a person blinded to the experimental protocol. Additional

details on the methods are provided in the [Methods](#) section in this article's Online Repository.

## Cell preparation

For *in vitro* experiments, the T-cell clones LNC-2 (T<sub>H</sub>1) and L1/1 (T<sub>H</sub>2) cells were used. The *in vitro* propagation of these cells has been described previously.<sup>14</sup> Mononuclear cells (MNCs) were prepared from spleens or lymph nodes, which were meshed through a nylon sieve followed by density gradient centrifugation (Pancoll; Pan Biotech, Aidenbach, Germany).

## Measurements of cytokines

MNCs ( $1 \times 10^6$  cells/well) were stimulated for 24 hours with anti-CD3 (0.5  $\mu$ g/mL) and anti-CD28 (2  $\mu$ g/mL) mAbs (BD, Heidelberg, Germany; splenocytes) or for 72 hours with OVA (50  $\mu$ g/mL; lymph node cells) to determine *in vitro* cytokine production. Cell-free supernatants were harvested after centrifugation, and cytokine levels were assessed by using ELISA (IL-4, IL-5, IL-10, IFN- $\gamma$  [Opteia Kit; BD PharMingen, Heidelberg, Germany], and IL-13 [CytoSet, Invitrogen] production), as recommended by the manufacturers.

## Measurement of proliferation

Proliferation measurement of MNCs and CD4<sup>+</sup> T cells was performed by using carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Invitrogen), according to the manufacturer's instructions. Additional details on the methods are provided in the [Methods](#) section in this article's Online Repository.

## Quantitative PCR

Total RNA was extracted (TriFast; Peqlab, Erlangen, Germany) and reverse transcribed (Omniscript RT Kit; Qiagen, Hilden, Germany) by using oligo (dT) primers. A RotorGene (Corbett, Hilden, Germany) and the QuantiTect SYBR Green PCR Kit (Qiagen) were used for real-time quantitative PCR (qPCR) of *IL4*, *IL5*, *IL10*, *IL13*, *IFNG*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; housekeeping control). Quantitative PCR (qPCR) data were analyzed according to the previously described  $2^{-\Delta\Delta C_T}$  method<sup>15</sup> and normalized to *GAPDH* gene transcript levels. Primer sequences are detailed in [Table E1](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## Preparation and bisulfite conversion of genomic DNA, PCR, and pyrosequencing

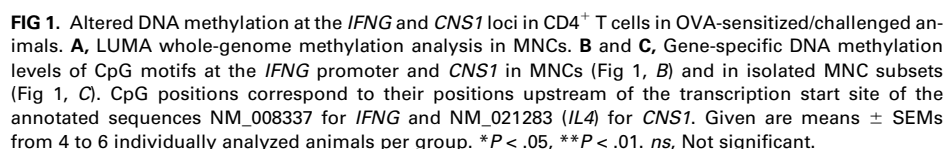
CD4<sup>+</sup> and CD8<sup>+</sup> T cells and natural killer (NK) cells were positively isolated from splenic MNCs by using magnetic cell sorting (CD4, CD8, and CD49b Microbeads), as recommended by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Bisulfite conversion of genomic DNA was performed as described elsewhere.<sup>16</sup> Template preparation was performed as described previously by Tost and Gut.<sup>17</sup> Pyrosequencing was performed with the PyroMark ID System (Biotage AB, Qiagen) and sequencing primers. Primer sequences are shown in [Table E2](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). Additional details on the methods are provided in the [Methods](#) section in this article's Online Repository.

## Assessment of whole-genome methylation status

The whole-genome methylation status was analyzed by using the Luminometric Methylation Assay (LUMA), as described by Karimi et al.<sup>18</sup> Additional details on the methods are provided in the [Methods](#) section in this article's Online Repository.

## Statistical analysis

The number of independently performed experiments is mentioned in the legends of each figure. All numeric data are expressed as means  $\pm$  SEMs and



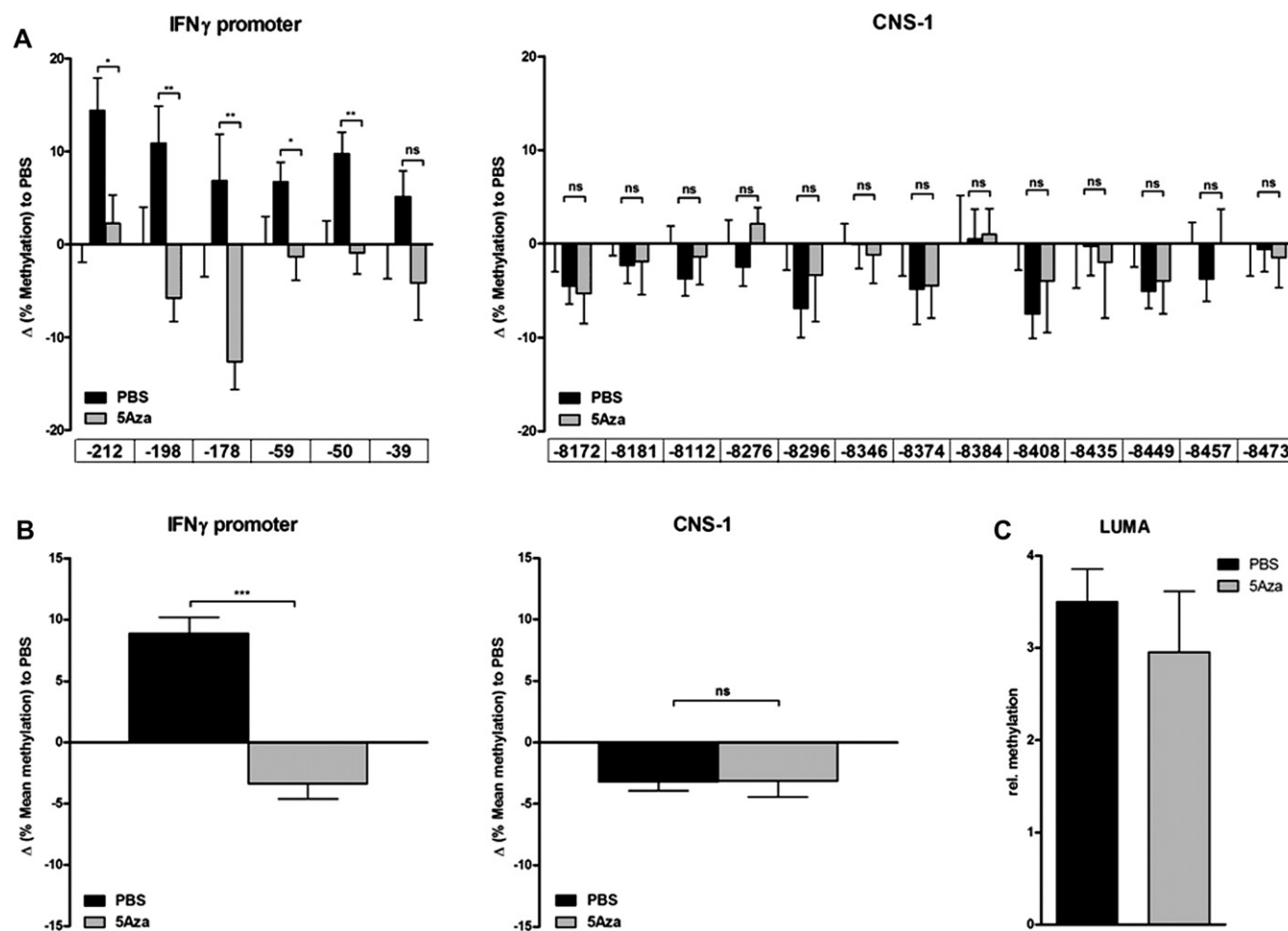


**FIG 2.** Differential gene demethylation after 5Aza treatment. **A**, DNA methylation within the *IFNG* promoter and *CNS1* of splenic CD4<sup>+</sup> T cells after OVA sensitization/challenge. CpG positions correspond to their positions upstream of the transcription start site of the annotated sequences NM\_008337 for *IFNG* and NM\_021283 (*IL4*) for *CNS1*. **B**, Mean methylation levels for the *IFNG* and *CNS1* loci after OVA sensitization/challenge with and without 5Aza treatment. **C**, LUMA whole-genome methylation analysis in MNCs. Given are means  $\pm$  SEMs from n 4 to 6 individually analyzed animals per group. \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ . ns, Not significant.

analyzed for significance by using 1-way ANOVA or the Student unpaired  $t$  test with significances of less than .05, less than .01, and less than .001. Calculations were performed by using GraphPad Prism software, version 4.01 (GraphPad Software, Inc, La Jolla, Calif).

## RESULTS

### OVA sensitization/challenge alters DNA methylation signatures at the *IFNG* promoter in CD4<sup>+</sup> T cells

The analysis of established CD4<sup>+</sup> T<sub>H</sub>1 (LNC2) and T<sub>H</sub>2 (L1/1) T-cell clones<sup>14</sup> revealed marked differences in DNA methylation levels at regulatory gene regions of signature cytokine genes, which confirmed previous results.<sup>10,19</sup> In essence, T<sub>H</sub>1 cells are characterized by a low DNA methylation level at the *IFNG* promoter and high DNA methylation levels at the T<sub>H</sub>2 locus (*IL4* and *IL5* promoter and conserved noncoding sequence 1 [*CNS1*]), whereas a reciprocal pattern was observed in T<sub>H</sub>2 cell clones (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). The possible effect of DNA methylation

on the development of experimental asthma was examined in a murine model of intraperitoneal sensitization to OVA followed by OVA aerosol challenges.<sup>20</sup> After establishment of the asthmatic phenotype based on a T<sub>H</sub>2-dominated inflammatory response in the airways, global assessment of DNA methylation in MNCs by using LUMA revealed no differences between allergic and control animals (Fig 1, A). Because this finding did not exclude gene-specific differences in DNA methylation, T<sub>H</sub>1-specific (*IFNG*) and T<sub>H</sub>2-specific (*CNS1*, *IL4*, and *IL5*) genes were selected for further analysis. The comprehensive assessment by mean of bisulfite pyrosequencing included 6 CpG motifs at the proximal *IFNG* promoter,<sup>10</sup> 13 CpG motifs at the T<sub>H</sub>2 regulatory *CNS1* region, 4 CpG motifs at the *IL4* promoter, and 6 CpG motifs at the *IL5* promoter, which are known to be involved in regulation of gene expression of the respective cytokines.<sup>21</sup> Significantly increased DNA methylation levels were detected for all analyzed CpG motifs at the *IFNG* promoter from sensitized animals compared with those seen in cells from nonsensitized mice. Only minor changes were observed at 3 of 13 analyzed CpG motifs at the *CNS1* region (Fig 1, B). No differences between the study groups

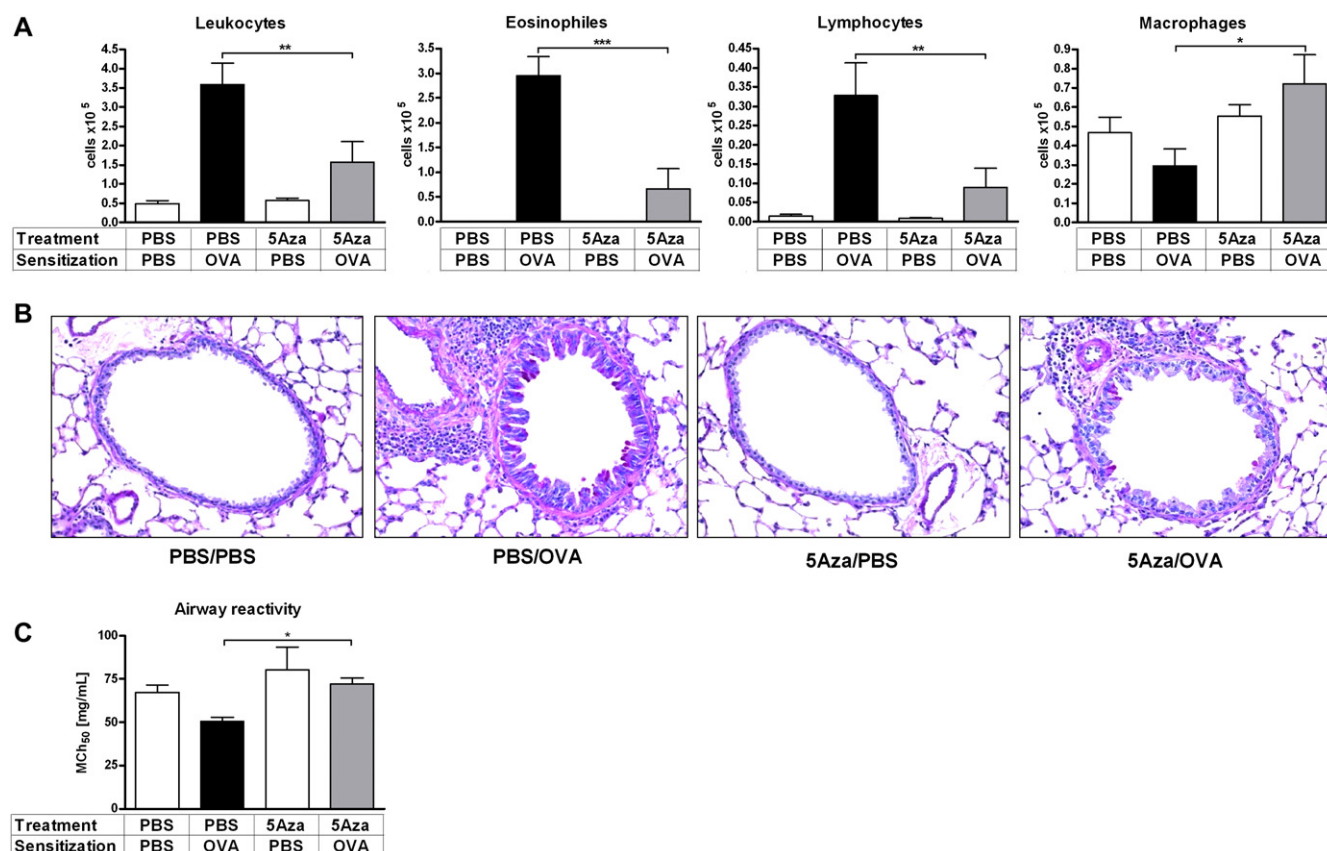


**FIG 3.** Inhibition of DNA methylation at the *IFNG* locus by treatment with 5Aza reduces features of experimental asthma. **A**, Differential leukocyte numbers in BAL fluid. **B**, Inflammation and mucus-producing goblet cells, as indicated by periodic acid-Schiff staining in the airways. **C**, Airway responsiveness to aerosolized methacholine (*MCh*) in offspring, as measured by using head-out body plethysmography. Given are means  $\pm$  SEM from 812 individually analyzed animals per group. \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ . ns, Not significant.

could be observed at the *IL4* and *IL5* promoters (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

DNA methylation was analyzed in purified CD4<sup>+</sup> and CD8<sup>+</sup> T cells and NK cells to identify the T-cell subsets in which epigenetic processes are active in asthmatic mice. Compared with MNCs, a similar effect of allergen sensitization/challenge on DNA methylation at the *IFNG* promoter was only found in the CD4<sup>+</sup> T-cell subset, whereas no significant differences could be detected in CD8<sup>+</sup> T cells and NK cells between study groups (Fig 1, C). In CD4<sup>+</sup> T cells of OVA-sensitized/challenged mice, the *IFNG* promoter revealed higher DNA methylation levels compared with those seen in PBS-exposed animals. In none of these cell populations was a significant difference in DNA methylation detected for *CNS1*.

### Increased DNA methylation within the *IFNG* promoter in CD4<sup>+</sup> T cells is inhibited by the DNMT inhibitor 5Aza

To test the influence of DNMT inhibition on DNA methylation within selective gene loci and the subsequent development of the asthmatic phenotype, animals received intraperitoneal injections of the DNMT inhibitor 5Aza (0.2 mg/kg body weight) 3 times per week over 5 weeks, which represents a dose

comparable with those used in murine models used for cancer intervention.<sup>22,23</sup>

Initial experiments were performed to exclude possible non-specific or cytotoxic effects of 5Aza treatment on T cells. *Ex vivo* proliferation of anti-CD3/anti-CD28-stimulated, CFSE-labeled CD4<sup>+</sup> T cells isolated from 5Aza-treated mice remained completely intact. In addition, no effects were observed on *in vivo* homeostatic proliferation<sup>24</sup> of CFSE-labeled CD4<sup>+</sup> T cells transferred into SCID mice, which were subsequently treated with 5Aza (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Furthermore, intraperitoneal injection of 5Aza was well tolerated by the mice because no changes in behavior, body weight, or coat appearance were observed.

LUMA analysis of whole-genome methylation revealed a nonsignificant trend toward reduced DNA methylation in 5Aza-treated mice compared with that seen in control animals (Fig 2, C). The *IFNG* promoter and the *CNS1* region of CD4<sup>+</sup> T cells were compared between OVA-sensitized and OVA-challenged animals treated with 5Aza or PBS as a control to analyze gene-specific methylation level. At the *IFNG* promoter, 5Aza treatment resulted in marked demethylation at (nearly) all positions compared with that seen in control animals. Additionally, we investigated downstream genomic loci that can contribute to regulation of *IFNG* transcription (intron 3, CNS+18), where only 1 position

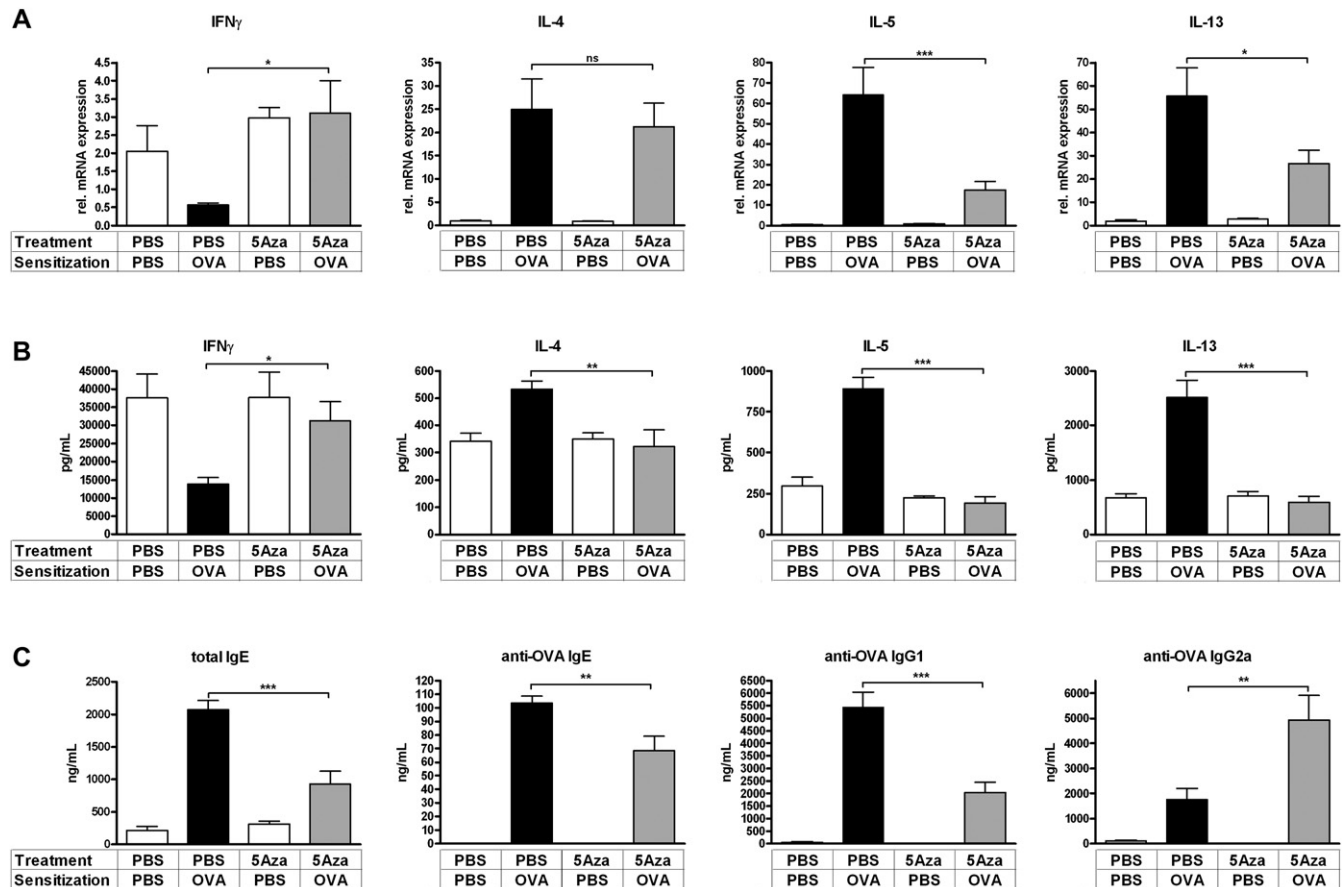


**FIG 4.** 5Aza treatment influences T-cell sensitization and is accompanied by a rebalanced  $T_H1/T_H2$  response.

**A** and **B**, IFN- $\gamma$ , IL-4, IL-5, and IL-13 cytokine mRNA expression (Fig 4, **A**) and cytokine release (Fig 4, **B**) by splenic MNCs stimulated for 24 hours with anti-CD3/anti-CD28. **C**, Levels of total IgE and OVA-specific IgE, IgG $_1$ , and IgG $_2a$  in serum. Given are means  $\pm$  SEMs from 4 to 6 individually analyzed animals per group.

\* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ . ns, Not significant.

in intron 3 was found to be significantly demethylated (see Table E2). 5Aza treatment in the *CNS1* region caused minor changes in methylation levels, which surprisingly showed a significant increase at 5 of the 13 analyzed positions (Fig 2, **A**). For each genomic region analyzed, the mean difference in DNA methylation level was calculated between 5Aza- and PBS-treated mice. The overall degree in methylation changes was significantly higher at the *IFNG* promoter compared with the average change in methylation observed in the *CNS1* region (Fig 2, **B**). These data indicate that 5Aza treatment prevents the increase in DNA methylation observed during sensitization/challenge at the *IFNG* promoter.

### Inhibition of DNA methylation at the *IFNG* locus by 5Aza prevents development of experimental asthma

The inflammatory phenotype of these mice was analyzed to investigate the effect of 5Aza-mediated inhibition of DNA methylation on asthma development. OVA sensitization and aerosol challenges in PBS-treated control animals resulted in marked inflammation of the airways mainly caused by an influx of eosinophils and lymphocytes (Fig 3, **A**). Histologic analysis of airways and lung tissue revealed a significant infiltrate of eosinophils

and MNCs together with increased mucus production by goblet cells (Fig 3, **B**). Furthermore, these mice had airway hyperresponsiveness, as indicated by a significant decrease in lung function after methacholine challenge (Fig 3, **C**). In contrast, when these animals were treated with 5Aza, the development of the asthmatic phenotype was significantly prevented. This is reflected by significantly lower cell numbers in the BAL fluid compared with those seen in the sham-treated OVA control group. Further differentiation revealed that this reduction was mainly due to a decreased number of eosinophils and lymphocytes (Fig 3, **A**). Histologic analysis of lung tissues revealed an almost complete absence of peribronchial and perivascular airway inflammation (Fig 3, **B**). Measurements of airway reactivity showed a normal level of airway responsiveness to methacholine exposure (Fig 3, **C**).

### 5Aza treatment prevents $T_H2$ polarization

IFN- $\gamma$  ( $T_H1$ ), IL-4, IL-5, and IL-13 ( $T_H2$ ) expression was analyzed after *in vitro* anti-CD3/anti-CD28 stimulation of mononuclear splenocytes harvested from the above-described 5Aza-treated mice to investigate whether the observed site-specific (de)methylation changes correlate with changes in cytokine transcription and translation levels. Compared with levels seen in nonsensitized control mice, OVA sensitization/challenge resulted

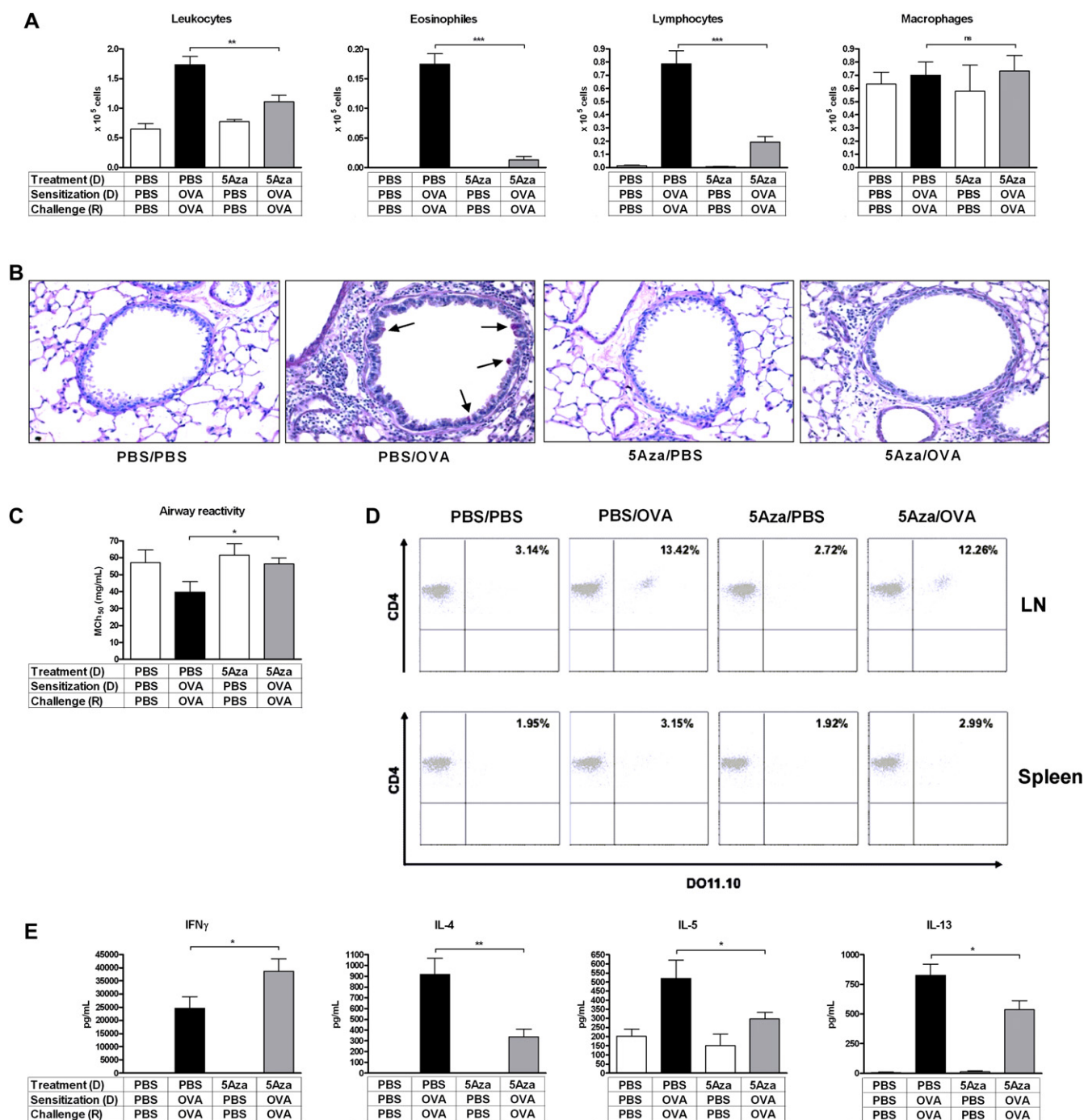


**FIG 5.** Treatment with 5Aza blocks development of an asthmatic phenotype by adoptively transferred CD4<sup>+</sup> T cells from DO11.10 donor mice. **A–C**, Differential leukocyte numbers in BAL fluid (Fig 5, A), inflammation and mucus-producing goblet cells (arrows) in the airways (Fig 5, B), and airway responsiveness to methacholine in recipients (Fig 5, C). **D**, Percentage of DO11.10<sup>+</sup> cells within CD4<sup>+</sup> T cells in lymph nodes and spleens of recipient mice. **E**, ELISA of supernatants from MNCs stimulated for 72 hours with OVA. Given are means  $\pm$  SEMs from 4 to 6 individually analyzed animals per group. \* $P$  < .05, \*\* $P$  < .01, and \*\*\* $P$  < .001. ns, Not significant.

in suppression of *IFNG* mRNA levels together with a significant shift toward a T<sub>H</sub>2 cytokine mRNA profile (Fig 4, A). This finding could also be affirmed at the protein level (Fig 4, B). We also analyzed the frequency of forkhead box protein 3 (Foxp3)-positive cells within the CD4<sup>+</sup> cell population. A slight increase in

Foxp3-expressing cell numbers was observed in 5Aza-treated animals (data not shown).

The changes in T<sub>H</sub>1/T<sub>H</sub>2 cytokine production were accompanied by an altered pattern of antigen-specific immunoglobulin production. Sensitization to OVA resulted in the production of

high anti-OVA-specific IgE and IgG<sub>1</sub> titers in contrast to low levels of IgG<sub>2a</sub> titers. After 5Aza treatment, this ratio was reversed to high IgG<sub>2a</sub> and low IgG<sub>1</sub> titers (Fig 4, C).

### CD4<sup>+</sup> T cells from 5Aza-treated animals protect against asthma development

T-cell transfer experiments were carried out to further explore that the asthma-preventive effects were indeed dependent on methylation changes in CD4<sup>+</sup> T cells. DO11.10 donor mice, which are transgenic for the OVA-recognizing T-cell receptor, were intraperitoneally treated with 5Aza in parallel to OVA sensitization. MNCs were isolated from the spleen and *in vitro* restimulated with OVA. Thereafter, CD4<sup>+</sup> cells were isolated and transferred to unsensitized but OVA aerosol-challenged wild-type BALB/c recipient mice, which were further OVA aerosol challenged. In concordance with previously published data,<sup>25,26</sup> transfer of allergen-primed CD4<sup>+</sup> T cells established the asthmatic phenotype in OVA-challenged recipients, which developed the complete phenotype of experimental asthma, as defined by significantly increased numbers of eosinophils and lymphocytes in BAL fluid (Fig 5, A), inflamed airway tissue (Fig 5, B), and development of airway hyperreactivity (Fig 5, C). FACS analysis revealed a similar percentage of treated and untreated DO11.10<sup>+</sup> T cells in local draining bronchial lymph nodes of the recipients, indicating a normal migration pattern (Fig 5, D). OVA-restimulated MNCs isolated from the draining lymph nodes of sham-treated animals showed a significant shift in cytokine production toward a T<sub>H</sub>2 profile. However, the capacity of MNCs from 5Aza-treated animals to produce IL-4, IL-5, and IL-13 was significantly reduced, whereas IFN- $\gamma$  production was augmented (Fig 5, E). In addition, transfer of CD4<sup>+</sup> T cells from 5Aza-treated DO11.10 donor animals in OVA-challenged recipients significantly prevented the inflammatory response in airways and lung tissue, as well as development of airway hyperreactivity.

### DISCUSSION

Asthma is a chronic inflammatory disease of the airways caused by both heritable and environmental factors. Although genetic studies show an effect of genetic alterations (eg, single nucleotide polymorphisms), on asthma prevalence,<sup>3,27</sup> these studies do not adequately explain the susceptibility to asthma. Therefore the analysis of epigenetics, noncoding changes in DNA modifications, such as DNA methylation, might help to provide an explanation in this regard. Such epigenetic modifications have already been shown to be influenced by environmental factors, including diet, air pollution, and cigarette smoke,<sup>4,28</sup> which are known asthma risk factors. In our study we would like to demonstrate that environmental trigger-induced epigenetic changes might be involved in skewing the immune system toward a T<sub>H</sub>2 or T<sub>H</sub>1 phenotype, thereby influencing the risk of asthma.

Epigenetic regulation is known to be an important mechanistic principle in effector T-cell development and, subsequently, in the pathogenesis of chronic inflammatory conditions, such as allergic asthma. Changes in the DNA methylation pattern of CD4<sup>+</sup> T cells have also been shown to contribute to the development of autoimmunity, such as systemic lupus erythematosus.<sup>29,30</sup> Therefore in a first step to show the contribution of epigenetic modulation to the development of allergic asthma, we analyzed DNA methylation within regulatory gene regions of the T<sub>H</sub>1 and T<sub>H</sub>2 loci (*IFNG*

promoter, *CNS1*, *IL4*, and *IL5* promoter) in different T-cell subsets (CD4<sup>+</sup>, CD8<sup>+</sup>, and NK cells) isolated from animals with experimental asthma. Here we could demonstrate that OVA sensitization/challenge led to an increase in DNA methylation within the *IFNG* promoter of the CD4<sup>+</sup> T-cell subset. This might indicate that the OVA effect during sensitization/challenge was mainly associated with the silencing of the *IFNG* gene, supporting the hypothesis that epigenetic modulation plays a role in asthma development. To further examine the functional relevance of this difference on the development of allergic asthma, we took advantage of the potent DNMT inhibitor 5Aza. 5Aza is a nucleoside analog of cytidine, which can be incorporated into DNA during replication. On incorporation, DNMT1 is inhibited by covalent binding of 5Aza, resulting in passive demethylation.<sup>31</sup> By treating animals during sensitization with this reagent, the increased DNA methylation within the *IFNG* locus in CD4<sup>+</sup> T cells was inhibited, which was associated with a prevention of T<sub>H</sub>2 polarization in favor of an intensified T<sub>H</sub>1 response, leading to a normalized T<sub>H</sub>1/T<sub>H</sub>2 pattern. These results are in line with *in vitro* data indicating that T<sub>H</sub>1/T<sub>H</sub>2 polarization is related to changes in *IFNG* promoter methylation<sup>10,32</sup> and provide evidence for *in vivo* relevance of this mechanism. In addition, we also could demonstrate that such modification of DNA methylation at the promoter regions in genes of the T-cell cytokine network is associated with differences in immunoglobulin isotype production and the development of a local inflammatory response in the lung with consequences for airway hyperresponsiveness. T-cell transfer experiments were performed to validate the significance of DNA methylation within the CD4<sup>+</sup> T-cell subset. Thereby the importance of modulation of DNA methylation in these cells for the observed asthma protective effect could be confirmed. In line with previous findings,<sup>33</sup> we observed a slight increase in Foxp3-expressing cells within the CD4<sup>+</sup> cell population. Because it is known that IFN- $\gamma$  augmentation can induce Foxp3 expression in the CD4<sup>+</sup> cell population,<sup>34</sup> we cannot exclude that 5Aza directly affects Foxp3 expression.<sup>35</sup> Thus both mechanisms might contribute to the observed phenotype.

Our findings point out the crucial role of DNA methylation in CD4<sup>+</sup> T cells in tuning the course of activation and silencing *IFNG* gene expression during experimental asthma, thus (eventually) contributing to the plasticity of CD4<sup>+</sup> T-cell populations.

Such studies of epigenetic regulation might therefore play an important role in our understanding and possible treatment of asthma and other allergic diseases, and pharmacologic interference with epigenetic mechanisms might represent a novel approach to target the development of asthma.

We thank T. Ruppertsberg, N. Disser A. Spies-Naumann, and N. Baumgartl for excellent technical assistance and M. Huber for support with the FoxP3 measurement.

**Clinical implications: Epigenetic modulation induced by pharmacologic interference with DNA methylation might represent a novel approach to target the development of asthma.**

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## METHODS

### Assessment of airway reactivity

Lung function analysis was performed 24 hours after the last aerosol challenge by using noninvasive head-out body-plethysmography,<sup>E1</sup> a method that was shown to be directly related to lung resistance measured on the basis of invasive lung function.<sup>E2</sup> Bronchial responsiveness was determined as change in midexpiratory airflow in response to consecutive exposures to increasing concentrations (3, 6, 12.5, 25, 50, 75, 100, and 125 mg/mL) of aerosolized  $\beta$ -methacholine (Sigma-Aldrich). The methacholine concentration that induced a 50% reduction of baseline midexpiratory airflow was defined as the MCh<sub>50</sub>.

### BAL and differential cell counts

BAL was performed as described by Neuhaus-Steinmetz et al,<sup>E1</sup> with the exception that lavage was performed only once with 1 mL. An automated Casy TT cell counter (Schäfer Systems, Reutlingen, Germany) was used to determine total leukocyte cell counts. Cells were centrifuged, and cell-free supernatants were stored at  $-20^{\circ}\text{C}$  for cytokine measurement. For differential cell count analysis, cytospin preparations were prepared, fixed, and then stained with Diff-Quick (Medion Diagnostics, Düringen, Switzerland). Standard morphologic criteria were used to identify macrophages, lymphocytes, eosinophils, and neutrophils. One hundred cells were counted per slide by a blinded investigator unaware of the experimental protocol.

### Differential cell counts of blood slides

Blood slides were air-dried and stained with May-Grünwald staining solution (Merck) for 3 minutes, rinsed with Aqua dest for 3 minutes, and subsequently stained with Giemsa staining solution (Roth, Karlsruhe, Germany) for 15 minutes. After staining, slides were rinsed with Aqua dest. Air-dried and differential cell counts were determined by using light microscopy. Standard morphologic criteria were used to identify monocytes, lymphocytes, eosinophils, and neutrophils. One hundred cells were counted per slide by a person blinded to the experimental protocol.

### Measurement of proliferation

MNCs from 5Aza-treated animals were labeled with  $1\ \mu\text{mol/L}$  CFSE (Molecular Probes, Invitrogen) for 15 minutes at  $37^{\circ}\text{C}$  (according to the manufacturer's instructions) before stimulation with anti-CD3 and anti-CD28 to assess the *ex vivo* proliferative capacity of cells. After 72 hours, cells were harvested and stained with anti-CD4-allophycocyanin (Becton Dickinson), and proliferation was determined by using flow cytometry.

CD4<sup>+</sup> T cells were isolated by using the Dynal CD4 Negative Isolation Kit (Invitrogen) from spleens of BALB/c mice, as recommended by the manufacturer, and labeled with  $1\ \mu\text{mol/L}$  CFSE to assess their *in vivo* proliferative capacity. Cells ( $1 \times 10^7$ ) were transferred by means of intravenous injection into BALB/c<sup>scid</sup> mice. Recipients were treated every other day with 0.2 mg/kg 5Aza intraperitoneally. At day 8, proliferation of CFSE-labeled CD4<sup>+</sup> T cells from the spleen was determined by using flow cytometry.

### Preparation and bisulfite conversion of genomic DNA, PCR, and pyrosequencing

CD4<sup>+</sup> and CD8<sup>+</sup> T cells and NK cells were positively isolated from splenic MNCs by using magnetic cell sorting (CD4, CD8, and CD49b Microbeads), as

recommended by the manufacturer (Miltenyi Biotec); resuspended in ATL buffer (Qiagen); and incubated with proteinase K (Sigma-Aldrich) at  $37^{\circ}\text{C}$  for 1 hour. Proteinase K was deactivated by means of incubation at  $95^{\circ}\text{C}$  for 15 minutes, and genomic DNA was stored at  $-80^{\circ}\text{C}$ .

Bisulfite conversion of genomic DNA was performed as described elsewhere.<sup>E3</sup> PCR products were generated in a  $50\text{-}\mu\text{L}$  reaction volume with 10 pmol of forward and reverse primer and HotstarTaq PCR Master Mix (Qiagen). Template preparation was performed as described previously by Tost and Gut.<sup>E4</sup> Pyrosequencing was performed with the PyroMark ID System (Qiagen) and sequencing primers (see below). Positions of CpG sites within the genomic sequences were determined in comparison with the transcription start site of the published full-length mRNA sequences NM\_008337 (*IFNG*), NM\_010558 (*IL5*), and NM\_021283 (*IL4*). The *IL4* transcription start site was used for numbering of CpGs within *CNS1*. Primer sequences are shown in Table E1.

### Assessment of whole-genome methylation status

The whole-genome methylation status was analyzed by using LUMA, as described by Karimi et al.<sup>E5</sup> In brief, purified genomic DNA of MNCs was digested by the methylation-sensitive restriction endonuclease isoschizomers *MspI* (insensitive to methylation) or *HpaII* (methylation sensitive). Subsequently, a single nucleotide expansion was performed with the Pyromark ID system (Qiagen) for quantifying the extent of DNA cleavage. The DNA methylation status was defined as the *MspI/HpaII* ratio, whereby unmethylated DNA would result in a ratio of 1.0. Increased DNA methylation is represented by ratios of greater than 1.0.

### FACS analysis

CD4<sup>+</sup> cells were positively isolated, as described above. Cells were stained by using fluorescein isothiocyanate-coupled CD4 antibodies (BD PharMingen) and allophycocyanin-coupled Foxp3 antibodies (eBioscience, Aachen, Germany). The Foxp3 staining buffer set from eBioscience was used for the intracellular staining of Foxp3. The following isotype controls were performed: fluorescein isothiocyanate Rat IgG<sub>2a</sub>,  $\kappa$  isotype (BD PharMingen), and allophycocyanin Rat IgG<sub>2a</sub>,  $\kappa$  isotype (eBioscience). FACS analyses were performed with the FACS-Canto (Becton Dickinson).

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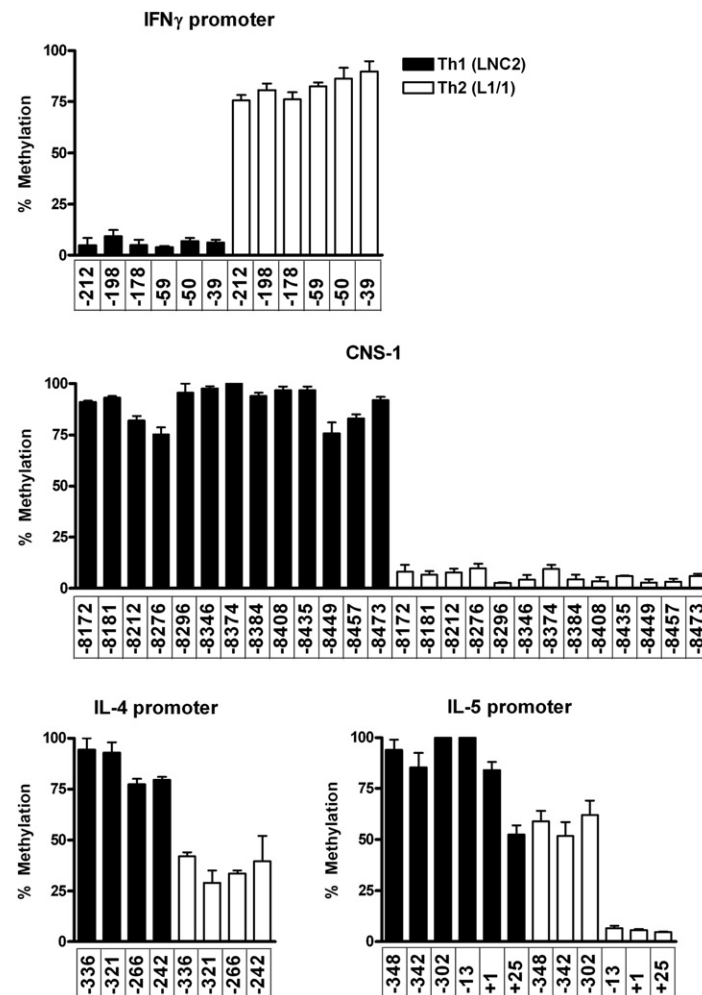


**FIG E1.** Gene-specific differences in DNA methylation of  $T_H1$  and  $T_H2$  cell clones. Gene-specific DNA methylation levels of CpG motifs at the *IFNG* promoter and the  $T_H2$  locus (*IL4* promoter, *IL5* promoter, and *CNS1*) were determined by means of bisulfite pyrosequencing in  $T_H1$  (LNC2) and  $T_H2$  (L1/1) T-cell clones. Positions of CpG sites correspond to their positions upstream of the transcription start site of the annotated sequences NM\_008337 for *IFNG*, NM\_021283 for *IL4* and *CNS1*, and NM\_010558 for *IL5*. Shown are means  $\pm$  SEMs.

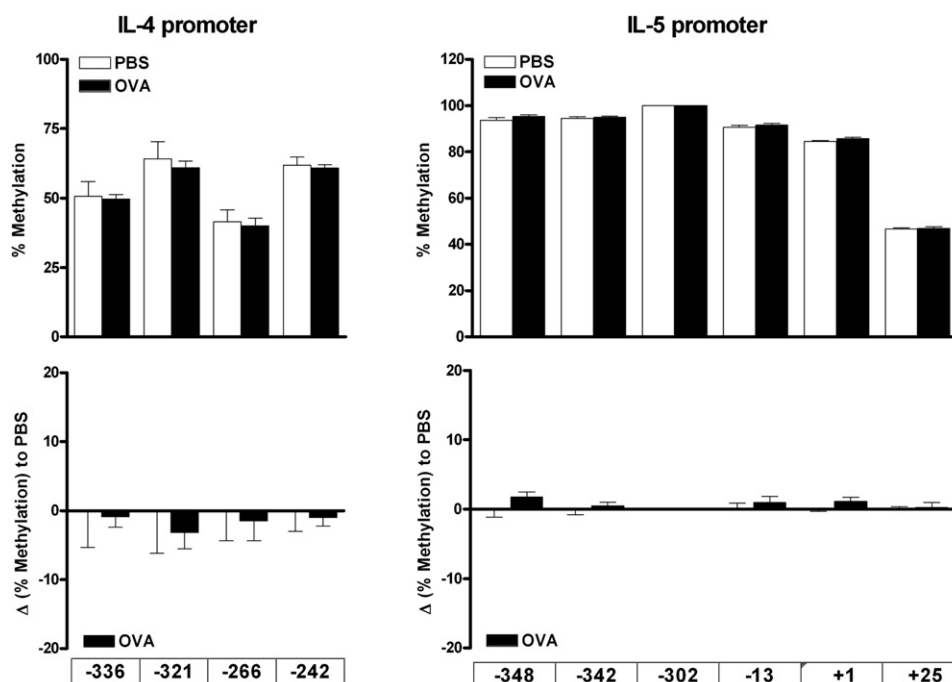


**FIG E2.** 5Aza treatment and  $T_H2$  cytokine promoter methylation. Animals were treated with intraperitoneal injections of 5Aza (body weight) 3 times per week starting 2 weeks before OVA sensitization until OVA challenge. DNA methylation within the *IL4* and *IL5* promoter of splenic  $CD4^+$  T cells was determined after OVA sensitization/challenge by means of bisulfite pyrosequencing. Shown are changes in relative methylation levels of cells from OVA-sensitized and OVA-challenged animals compared with cells from the sham-sensitized group. Positions of CpG sites correspond to their positions upstream of the transcription start site of the annotated sequences NM\_021283 for *IL4* and NM\_010558 for *IL5*. Shown are means  $\pm$  SEMs representing results of 2 independent experiments, each with 4 to 6 individually analyzed animals per group. ns, Not significant.

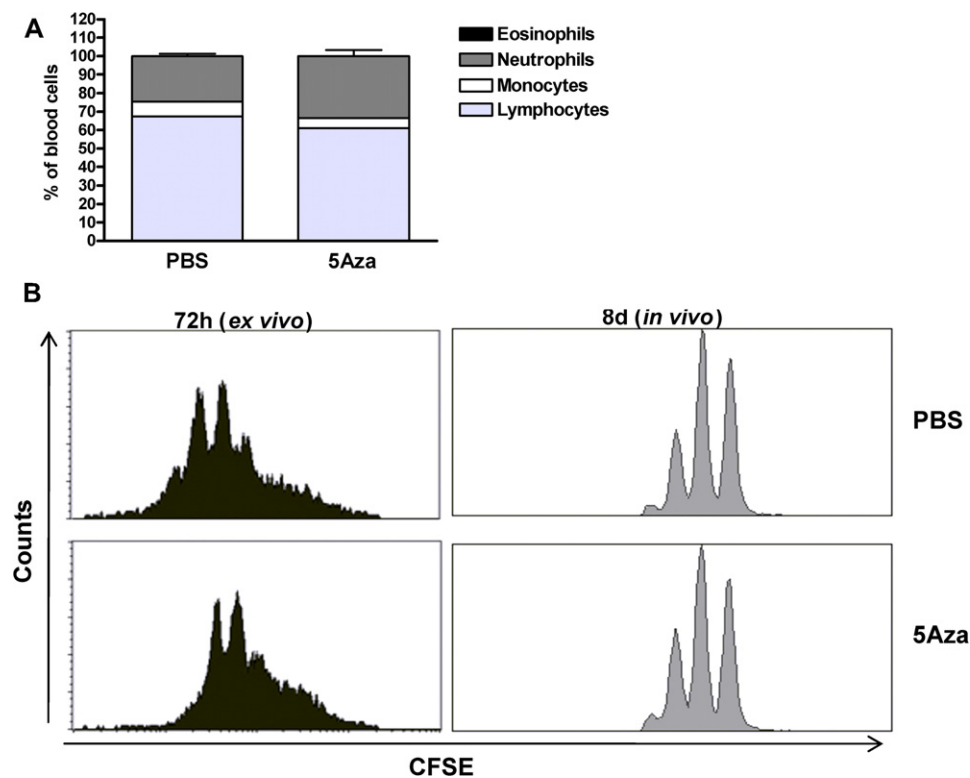


**FIG E3.** T-cell activation and proliferation is not affected by 5Aza treatment. Animals were treated with intraperitoneal injections of 5Aza (body weight) 3 times per week to exclude possible side effects of 5Aza. **A**, Three weeks after 5Aza treatment, blood smears were prepared and analyzed for major cell population frequencies after May-Grünwald-Giemsa staining. Shown are mean values  $\pm$  SEMs representing results of 2 independent experiments, each with 2 to 4 individually analyzed animals per group. **B**, MNCs were isolated from spleens of 5Aza- or PBS-treated animals, and *ex vivo* proliferation of CD4<sup>+</sup> T cells was determined after *in vitro* stimulation for 72 hours with anti-CD3/anti-CD28 by mean of anti-CD4 and CFSE labeling and subsequent FACS analysis (*left panel*). For analysis of *in vivo* proliferation, CFSE-labeled CD4<sup>+</sup> T cells were transferred intravenously into BALB/c<sup>scid</sup> mice, which were treated every other day with 5Aza or PBS. Proliferation of splenic CD4<sup>+</sup> T cells was determined at day 8 by using FACS analysis. Data are from 1 representative mouse per group and are representative of 2 experiments, each with 2 to 4 individually analyzed animals per group.

**TABLE E1.** Oligonucleotide sequences

| <b>Bisulfite PCR</b>  |  | <b>5'-3'</b>                                                    |
|-----------------------|--|-----------------------------------------------------------------|
| <i>Py-IFNG</i>        |  |                                                                 |
| s                     |  | GGT TTG AGA AGT TTA AGA GTT TTT TTA                             |
| as                    |  | <i>Biotin</i> - CCT ACA CCT CTC TAA CTT CCA ATT TTA TAC CTA ATC |
| <i>Py-CNS1</i>        |  |                                                                 |
| s                     |  | TAT AAG GGT TGT AGG AAG AGT AAT GTA GTT TTT ATA T               |
| as                    |  | <i>Biotin</i> - CAC TTT AAA CTA ATC AAA CCT AAA ACT ATA TCC ATA |
| <i>Py-IL-4</i>        |  |                                                                 |
| s                     |  | GTT AAA GTT TTA TTT TAT GGT TTT GTT TGT TTT AT                  |
| as                    |  | <i>Biotin</i> - ACA AAT AAA TTT TCC TAT AAA ATC AAA CCA ATT AA  |
| <i>Py-IL5</i>         |  |                                                                 |
| s                     |  | GGG GAT AAA AAA GAA GGT GTT TAA GAA AAG T                       |
| as                    |  | <i>Biotin</i> - AAA CAC TCA AAT ACA AAA ACA TCC TTC TCA TAA CT  |
| <i>Py-CNS+18</i>      |  |                                                                 |
| s                     |  | TAA TGT GAG TTG GAA TAT TAA GAA TTT                             |
| as                    |  | <i>Biotin</i> - TCT AAA TAA ACA AAT CAC CAA ATC TCA             |
| <i>Py-Intron 3</i>    |  |                                                                 |
| s                     |  | TGG GTA GGA GGG AGT TGA GAA GAT TTG TAT T                       |
| as                    |  | <i>Biotin</i> - ACA AAC TAA ACT ACA TAA CAC TAA AAA CTA         |
| <b>Pyrosequencing</b> |  | <b>5'-3'</b>                                                    |
| <i>IFNG</i> seq1      |  |                                                                 |
|                       |  | ATT TTA TAA GAA TGG TAT AGG TG                                  |
| <i>IFNG</i> seq2      |  |                                                                 |
|                       |  | AAA ATT AAA AAA AAA TTT GTG                                     |
| <i>CNS1</i> seq1      |  |                                                                 |
|                       |  | AAT GTA GTT TTT ATA TAT TGG TTT AT                              |
| <i>CNS1</i> seq2.1    |  |                                                                 |
|                       |  | TTG AGA GGG GAT TAT TTT TTA T                                   |
| <i>CNS1</i> seq3.1    |  |                                                                 |
|                       |  | TAT GGG GTT TTT TTT GGT TG                                      |
| <i>IL4</i> seq2       |  |                                                                 |
|                       |  | TTT TTT TGA TAT TAT TTT GTT                                     |
| <i>IL5</i> seq1       |  |                                                                 |
|                       |  | AAG AAG GTG TTT AAG AAA AG                                      |
| <i>IL5</i> seq2       |  |                                                                 |
|                       |  | TTT TTT AGA GAG AGA ATA AAT TG                                  |
| as                    |  | TCC CCA AGC AAT TTA TTC TCT C                                   |
| <i>Intron 3</i> seq1  |  |                                                                 |
|                       |  | TTT TAG TTA GGT TATAAG GAG GA                                   |
| <i>Intron 3</i> seq2  |  |                                                                 |
|                       |  | ATG AAA TTT TTA TAG TGT TAA GGG TA                              |
| <i>CNS+18</i> seq     |  |                                                                 |
|                       |  | GTT ATT ATG TTG TTG AAA GAA ATG                                 |
| <b>qPCR</b>           |  | <b>5'-3'</b>                                                    |
| <i>IFNG</i>           |  |                                                                 |
| s                     |  | GCG TCA TTG AAT CAC ACC TG                                      |
| as                    |  | TGA GCT CAT TGA ATG CTT GG                                      |
| <i>IL4</i>            |  |                                                                 |
| s                     |  | TCA ACC CCC AGC TAG TTG TC                                      |
| as                    |  | TGT TCT TCG TTG CTG TGA GG                                      |
| <i>IL5</i>            |  |                                                                 |
| s                     |  | AGC ACA GTG GTG AAA GAG ACC T                                   |
| as                    |  | TCC AAT GCA TAG CTG GTG ATT T                                   |
| <i>IL13</i>           |  |                                                                 |
| s                     |  | CCA TCC CAT CCC TAC AGA AA                                      |
| as                    |  | ATA GGC AGC AAA CCA TGT CC                                      |
| <i>GAPDH</i>          |  |                                                                 |
| s                     |  | CGT CTT CAC CAC CAT GGA GA                                      |
| as                    |  | CGG CCA TCA CGC CAC AGT TT                                      |

as, Antisense; *Py*, pyrosequencing primer; s, sense.

**TABLE E2.** Mean methylation (in percentages) of downstream *IFNG* loci

| Locus              | PBS/PBS (A) | PBS/OVA (B)   | 5Aza/PBS (C)  | 5Aza/OVA (D)        |
|--------------------|-------------|---------------|---------------|---------------------|
| Intron 3<br>+ 2169 | 63.04       | 60.37         | 58.03<br>†(A) | 8.43<br>*(A)        |
| Intron 3<br>+ 2191 | 60.12       | 58.98         | 56.49<br>*(A) | 57.0                |
| Intron 3<br>+ 2216 | 75.99       | 73.65         | 73.43         | 72.85               |
| Intron 3<br>+ 2288 | 73.67       | 71.48         | 69.05<br>‡(A) | 69.33<br>†(A)       |
| Intron 3<br>+ 2335 | 72.53       | 71.60<br>‡(A) | 69.23<br>†(B) | 68.93<br>‡(A), †(B) |
| Intron 3<br>+ 2347 | 87.42       | 84.76         | 85.77         | 86.91               |
| CNS +18<br>+ 17776 | 68.81       | 58.52         | 58.33<br>*(A) | 61.74               |
| CNS +18<br>+ 17801 | 31.18       | 33.75         | 28.78         | 36.63               |

*P* values are in relation to the group shown in parentheses (eg, A-D).

\**P* < .05.

†*P* < .01.

‡*P* < .001.