

# Combined Inhaled Diesel Exhaust Particles and Allergen Exposure Alter Methylation of T Helper Genes and IgE Production *In Vivo*

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Changes in methylation of CpG sites at the interleukin (IL)-4 and interferon (IFN)- $\gamma$  promoters are associated with T helper (Th) 2 polarization *in vitro*. No previous studies have examined whether air pollution or allergen exposure alters methylation of these two genes *in vivo*. We hypothesized that diesel exhaust particles (DEP) would induce hypermethylation of the IFN- $\gamma$  promoter and hypomethylation of IL-4 in CD4<sup>+</sup> T cells among mice sensitized to the fungus allergen *Aspergillus fumigatus*. We also hypothesized that DEP-induced methylation changes would affect immunoglobulin (Ig) E regulation. BALB/c mice were exposed to a 3-week course of inhaled DEP exposure while undergoing intranasal sensitization to *A. fumigatus*. Purified DNA from splenic CD4<sup>+</sup> cells underwent bisulfite treatment, PCR amplification, and pyrosequencing. Sera IgE levels were compared with methylation levels at several CpG sites in the IL-4 and IFN- $\gamma$  promoter. Total IgE production was increased following intranasal sensitization *A. fumigatus*. IgE production was augmented further following combined exposure to *A. fumigatus* and DEP exposure. Inhaled DEP exposure and intranasal *A. fumigatus* induced hypermethylation at CpG<sup>-45</sup>, CpG<sup>-53</sup>, CpG<sup>-205</sup> sites of the IFN- $\gamma$  promoter and hypomethylation at CpG<sup>-408</sup> of the IL-4 promoter. Altered methylation of promoters of both genes was correlated significantly with changes in IgE levels. This study is the first to demonstrate that inhaled environmental exposures influence methylation of Th genes *in vivo*, supporting a new paradigm in asthma pathogenesis.

**Key Words:** environmental exposure; respiratory sensitization; cytokines; inhalation toxicology; epigenetic modification.

The incidence of asthma is higher among low-income minority children who are more likely to reside near traffic-related air pollution and industrial sites (Brauer *et al.*, 2002; Gehring *et al.*, 2002; Venn *et al.*, 2001). Most likely, the development of asthma in urban areas is influenced by multiple factors, including genes that predispose toward the development

of an allergic phenotype and regulators of their molecular signals, and environmental exposures characteristic of the inner city, such as diesel exhaust particles (DEP), other air pollutants, and indoor allergens (Miller, 1998).

The role of T helper (Th) 2 interleukin (IL)-4, IL-5, IL-13 cytokines in promoting allergic sensitization and asthma, and of Th1 cytokine interferon (IFN)- $\gamma$  in protecting against allergic sensitization and asthma, has been an important area of research for over two decades (Mosmann *et al.*, 1986). The molecular mechanisms responsible for the early and persistent skewing of the cytokine milieu toward a more proallergic phenotype have become a much more recent area of interest. CpG methylation is an example of an epigenetic modification that can affect chromatin remodeling, Th gene locus accessibility, and therefore changes in gene expression that occur in the absence of alterations in DNA sequences. This covalent addition of a methyl group to cytosines in CpG dinucleotides is believed to begin the process by which the Th cells lose their plasticity and differentiate productively toward the Th1 versus the proallergic Th2 pattern of cytokine gene expression. CpG methylation also may confer heritable stabilization of gene expression patterns and maintain Th cell differentiation (Ansel *et al.*, 2003). In some cases, epigenetic alterations may be transmissible beyond a single generation (Anway *et al.*, 2005; Thompson *et al.*, 2001; Vickaryous and Whitelaw, 2005).

It has become evident from several *in vitro* studies that demethylation of sites at the proximal promoter and conserved intronic regulatory element (CIRe) in the first intron of the IL-4 gene, and hypermethylation of sites in the IFN- $\gamma$  promoter, result in efficient IL-4 production (Agarwal and Rao, 1998; Jones and Chen, 2006; Lee *et al.*, 2002; Tykocinski *et al.*, 2005; Yano *et al.*, 2003). Th1 differentiation is associated with methylation of a highly conserved DNaseI-hypersensitive region at the 3' end of the IL-4 gene (Lee *et al.*, 2002; Yano *et al.*, 2003). The environmental triggers of such altered methylation in Th cytokines have not been identified.

Although DEP exposure has been associated with upregulation of Th2 cytokine and chemokine production in multiple

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*in vitro* and *in vivo* studies (Bommel *et al.*, 2000; Fujieda *et al.*, 1998), only a few studies have examined whether exposure to DEP or other air pollutants can induce or inhibit gene methylation or other epigenetic alterations. *In vitro* exposure to particulate matter less than 10  $\mu\text{m}$  in aerodynamic diameter (PM)<sub>10</sub> induced chromatin remodeling by histone acetylation in human lung type II alveolar-like epithelial cells (Gilmour *et al.*, 2003). Inhaled DEP and black carbon have been shown to cause methylation of the p16<sup>INK4a</sup> gene in lung tumors in rats (Belinsky *et al.*, 2002). But no previous studies have examined the role of ambient air pollution, including DEP, on methylation patterns *in vivo* among genes associated with Th differentiation or the asthma phenotype. Finding an association between inhaled diesel exposure and Th regulation would provide a critical link in our understanding of the mechanisms involved in gene–environment interactions in asthma causation.

We hypothesized that chronically inhaled exposure to DEP would induce hypermethylation of the IFN- $\gamma$  gene promoter, and demethylation of the proximal promoter and the CIRE of IL-4 gene in CD4<sup>+</sup> T cells among mice sensitized to the allergens from the fungus *Aspergillus fumigatus*. We also hypothesized that DEP-induced methylation changes would affect immunoglobulin (Ig) E regulation, a biomarker associated with allergic sensitization and asthma. A finding that ambient air pollution in the setting of allergen exposure can modulate airway disease through epigenetic modification would suggest a new paradigm for asthma etiology. Ultimately, this work may have important implications for asthma prevention.

## MATERIALS AND METHODS

**Overview of study design and sensitization to *A. fumigatus*.** The study consists of four groups of five female BALB/c mice: (1) aerosol vehicle (saline) alone and negative control air; (2) aerosol vehicle alone and DEP; (3) *A. fumigatus* only and negative control air; and (4) *A. fumigatus* and DEP. All protocols were approved by the IACUC of the Health Sciences Division of Columbia University and New York University.

Sensitization to *A. fumigatus* was induced according to previously described protocols that reliably elicited a strong proallergic IgE response associated with airway hyperreactivity and eosinophilia (Grunig *et al.*, 1997; Padilla *et al.*, 2005; Zimmermann *et al.*, 2003). Briefly, 11-week-old BALB/c mice were exposed intranasally to 62.5  $\mu\text{g}$  of aerosolized *A. fumigatus* protein extract (Hollister Stier, Spokane, WA) (measured endotoxin dose < 0.16 EU/ml; Endotoxin Testing Service, Cambrex Bio Science Walkersville, Inc, MD) in 50  $\mu\text{l}$  PBS every 4 days for a total of six doses.

***In vivo* exposure to DEP.** DEP exposure occurred 5 h per day for 3 weeks concurrent with the *A. fumigatus* treatment. Diesel exhaust was produced by a 5500-watt single-cylinder diesel engine generator at New York University (Yanmar, Model YDG 5500E) that contains a 406 cc displacement air-cooled engine. The engine is operated using Number 2 Diesel Certification Fuel (Phillips Chemical Company) and 40 weight motor oil (Rotella T, Shell). Exhaust was diluted approximately 150:1 and routed to a 1-m<sup>3</sup> flow-through exposure chamber. The exhaust composition from the single-cylinder engine is consistent with “typical” diesel exhaust reported in the literature (McDonald *et al.*, 2004) and a larger on-road engine, a 2000 Model Cummins 5.9L ISB

engine operated on a variable duty cycle on a dynamometer using the same fuel and lube-oil. The average particle concentration was 1.28 mg/m<sup>3</sup>. This system reliably mimics outdoor exposure (McDonald *et al.*, 2004). The control animals were exposed to HEPA (high efficient particle) filtered ambient air in parallel and for the same duration of time as the diesel exposure.

**IgE production.** Sera were collected by retro-orbital bleed prior to the inhaled administration of *A. fumigatus* and 4 days after the sixth dose. Levels of IgE were measured by enzyme-linked immunosorbent assay using complementary capture and detection antibody pairs (Pharmingen, San Diego, CA). IgE levels were calculated based on a standard curve using recombinant IgE.

**Bronchoalveolar lavage and histological analysis of the lungs.** Bronchoalveolar lavage (BAL) was performed with normal saline and collected lavage fluid centrifuged for 10 min at 250  $\times$  g at 4°C. Slides were prepared, air-dried, fixed in methanol, and stained (Wright-Giemsa; Scientific Products). For differential cell counts, 10 fields of cells were enumerated for each sample and identified as macrophages, lymphocytes, neutrophils, or eosinophils. After lavage, dissected lungs from each mouse were fixed in 10% formalin, mounted in paraffin, sectioned, and stained with hematoxylin and eosin. The slides were analyzed by a blinded reader using a Nikon EclipseE800 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

**CD4<sup>+</sup> cell isolation/genomic DNA purification.** Splenic cell suspensions from harvested organs were prepared by gently pressing tissues through a nylon mesh cell strainer. CD4<sup>+</sup> cells were isolated using MACs sorting kits (CD4 (L3T4) microbeads; Miltenyi Biotec, Auburn, CA). Genomic DNA was purified from CD4<sup>+</sup> cells using Wizard SV Genomic DNA Purification System from Promega (Madison WI) as per manufacturer’s instruction.

**Pyrosequencing/determination levels of gene methylation.** Purified DNA underwent bisulfite treatment (i.e., to convert unmethylated cytosines into uracils and keep unchanged methylated cytosines) and PCR amplification as recommended by Biotage/EpigenDx (Worcester, MA, <http://www.pyrosequencing.com>). Briefly, the MethylDetector Bisulfite Modification Kit (Active motif, Carlsbad, CA) was used for bisulfite treatment according to the manufacturer’s instructions. Differential methylation was measured at all six CpG sites in the proximal promoter of the IFN- $\gamma$  gene: CpG<sup>-205</sup>, CpG<sup>-190</sup>, CpG<sup>-170</sup>, CpG<sup>-53</sup>, CpG<sup>-45</sup>, CpG<sup>-34</sup> (relative to the transcription starting site) (Jones and Chen, 2006; Kersh *et al.*, 2006). CpG<sup>-408</sup>, CpG<sup>-393</sup>, CpG<sup>-339</sup>, and CpG<sup>-314</sup> in the IL-4 proximal promoter region, and CpG<sup>+101</sup>, CpG<sup>+113</sup>, CpG<sup>+185</sup>, CpG<sup>+225</sup>, and CpG<sup>+229</sup> in the first intron (all positions relative to the first start codon of the IL-4 gene) were selected for testing based on previous reports of hypomethylation in Th2 cells and hypermethylation in Th1 and naïve T cells (Tykocinski *et al.*, 2005). PCR amplification of converted and purified DNA was conducted with the primers listed in Table 1. PCR reactions were performed using Eppendorf PCR Mastermix (Westbury, NY) with cycling parameters consisted of heating at 95°C for 5 min, and 45 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 30 s. Samples subsequently were sent to EpigenDex for commercial pyrosequencing. Briefly, the methods involves sequencing-by-synthesis in real time and provides a rapid and accurate quantification of consecutive CpG methylation sites with built-in quality controls (Dupont *et al.*, 2004; Tost and Gut, 2007). The analysis is based on an indirect bioluminescent assay of the pyrophosphate that is released from each deoxynucleotide upon DNA-chain elongation. The extent of methylation at each CpG site was automatically calculated and is sensitive enough to detect 2–5% changes in methylation levels (<http://www.pyrosequencing.com>).

**Statistical analysis.** Data analysis started with calculation of descriptive statistics and examination of the distribution of each variable and bivariate association between variables. The outcome variables include the methylation levels of IL-4 and IFN- $\gamma$  genes. One mouse failed to generate an IgE response following administration of *A. fumigatus* and was classified as an outlier and dropped from all future analyses. All tests were two-sided with significance level of 0.05. Mann–Whitney tests were used to compare means between groups. Correlations using rank order nonparametric testing were conducted to compare methylation with IgE levels.

TABLE 1  
Primers Used for PCR Amplification and Pyrosequencing Experiments

Primer name	Gene	Type of primer	Primer sequence 5'–3'	5' Modification
ADS156FP	IFN- $\gamma$ promoter	PCR forward	TGGTGTGAAGTAAAAGTGTTTTAGA	
ADS156RPB	IFN- $\gamma$ promoter	PCR reverse	TACACCTCTCTAACTTCCAATTTT	Biotin
ADS156FS1	IFN- $\gamma$ promoter	Pyrosequencing	GAATGGTATAGGTGGGTA	
ADS156FS2	IFN- $\gamma$ promoter	Pyrosequencing	AAAACAAATTTGTGAAAATA	
ADS018FPB	IL-4 promoter	PCR forward	GGAAGTAGTTAGGTTTAGGTGTGT	Biotin
ADS018RP	IL-4 promoter	PCR reverse	CCCCCTTTTTTTTAAATCTACAA	
ADS018RS	IL-4 promoter	Pyrosequencing	CAACATAAAAAATTACACCA	
ADS017FP	IL-4 CIRE	PCR forward	GGATGYGATAAAAAATTATTTGAGAG	
ADS017RPB	IL-4 CIRE	PCR reverse	TAATCCTACCTCAACCACCTA	Biotin
ADS017FS1	IL-4 CIRE	Pyrosequencing	AAAAATTATTTGAGAGAGAT	

## RESULTS

As shown in Figure 1, total IgE production was increased following sensitization to *A. fumigatus*. IgE production was augmented further following combined exposure to *A. fumigatus* and DEP. In addition, the mean eosinophil percentage on BAL was greater following sensitization to *A. fumigatus* and increased even further following combined exposure to *A. fumigatus* and DEP. Histopathological analysis revealed a greater degree of goblet cell hyperplasia and eosinophilic and mononuclear cell inflammatory infiltrate around the airways and blood vessels of mice sensitized to *A. fumigatus* alone or in combination with DEP compared with saline or DEP-treated mice.

For the CpG<sup>-53</sup> site of the IFN- $\gamma$  promoter, greater methylation was detected following concomitant *A. fumigatus* and DEP exposure compared with *A. fumigatus* alone. For CpG<sup>-45</sup> and CpG<sup>-205</sup>, the increase in methylation following concomitant *A. fumigatus* and DEP exposure reached borderline significance (Fig. 2). Also at CpG<sup>-53</sup>, greater methylation may have been detected following concomitant *A. fumigatus* and DEP exposure compared with DEP alone. DEP, in the absence of concomitant exposure to *A. fumigatus*, did not alter methylation patterns in the IFN- $\gamma$  promoter (Fig. 2). These

results suggest that at select CpG sites, combined exposure to allergen and DEP may induce methylation of the IFN- $\gamma$  promoter. In addition, at CpG<sup>-205</sup>, CpG<sup>-53</sup>, CpG<sup>-45</sup>, and CpG<sup>-34</sup>, the extent of methylation correlated significantly with IgE production. A similar trend at CpG<sup>-190</sup> was noted as well (Fig. 3). These findings suggest that the altered methylation may exert functional downstream effects associated with a heightened allergic immune response.

Furthermore, hypomethylation in the IL-4 promoter was detected following concomitant exposure to *A. fumigatus* and DEP, when compared with exposure to either saline, DEP or *A. fumigatus* alone at CpG<sup>-408</sup>. Altered methylation was not detected at any of the other sites tested. The extent of hypomethylation was associated significantly with a reduction in IgE when mice that received *A. fumigatus* (in presence or absence of DEP) were compared (Fig. 4).

## DISCUSSION

Epigenetic regulation appears to be an important modifier of disease susceptibility. Environmental exposures are believed to induce these epigenetic changes. Previous studies have shown that altered methylation affects *in vitro* Th differentiation, raising the possibility that the risk for developing allergic sensitization or asthma also may be affected. We show for the first time that concomitant exposure to inhaled DEP and allergen can induce hypermethylation at select CpG sites in the IFN- $\gamma$  promoter, and induce hypomethylation at one proximal CpG site in the IL-4 promoter *in vivo*. In addition, these patterns were associated with changes in IgE production, suggesting that altered methylation exerts molecular effects downstream.

In general, the methylation levels of CD4<sup>+</sup> splenocytes at select CpG sites in the IFN- $\gamma$  promoter averaged higher than observed in previous murine studies of CD4<sup>+</sup> thymocytes (Jones and Chen, 2006), especially following combined inhaled exposure to DEP and allergen. However, the levels appeared lower than measured from human CD4<sup>+</sup>CD45RA<sup>+</sup>

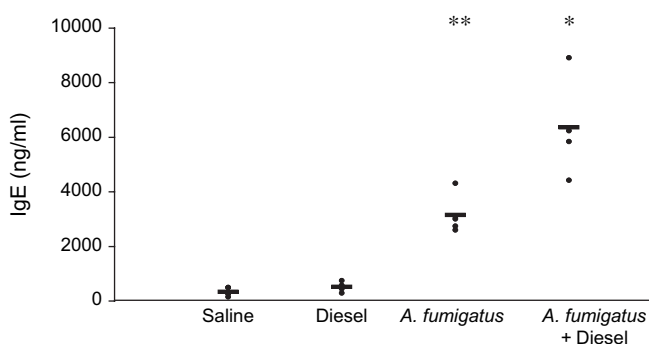
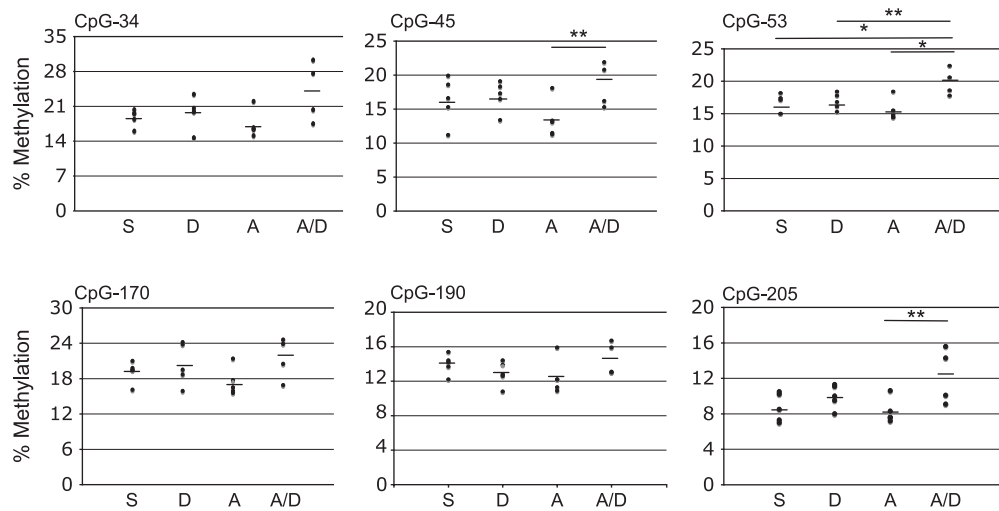


FIG. 1. IgE production following exposure to *Aspergillus fumigatus* and DEP. \* $p < 0.05$  two-tailed  $t$  test, compared with saline, DEP or *A. fumigatus* alone. \*\* $p < 0.05$  two-tailed  $t$  test, compared with saline, or diesel.

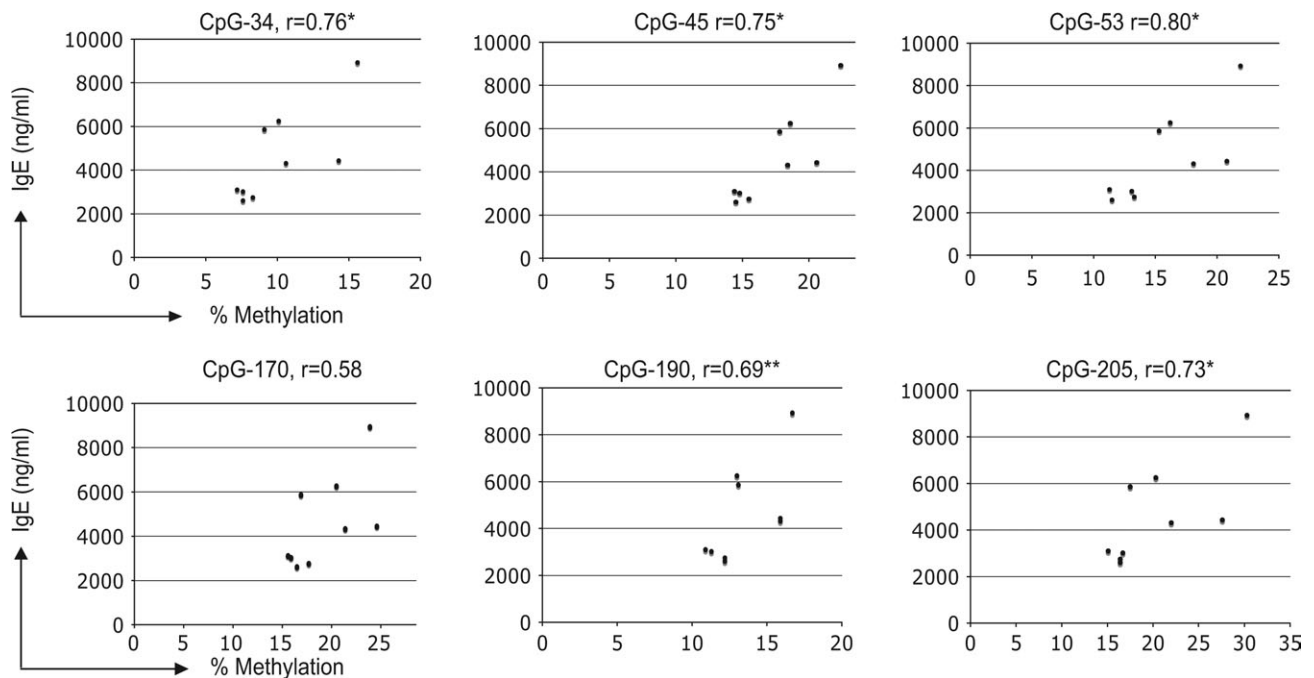


**FIG. 2.** *Aspergillus fumigatus* and DEP exposure are associated with increased methylation of IFN- $\gamma$  promoter at select CpG sites *in vivo*. S, intranasal saline; D, diesel exhaust particles; A, *A. fumigatus*; A/D, *A. fumigatus* + diesel exhaust particles. \* $p < 0.05$  two-tailed *t* test; \*\* $p < 0.05$  one-tailed *t* test.

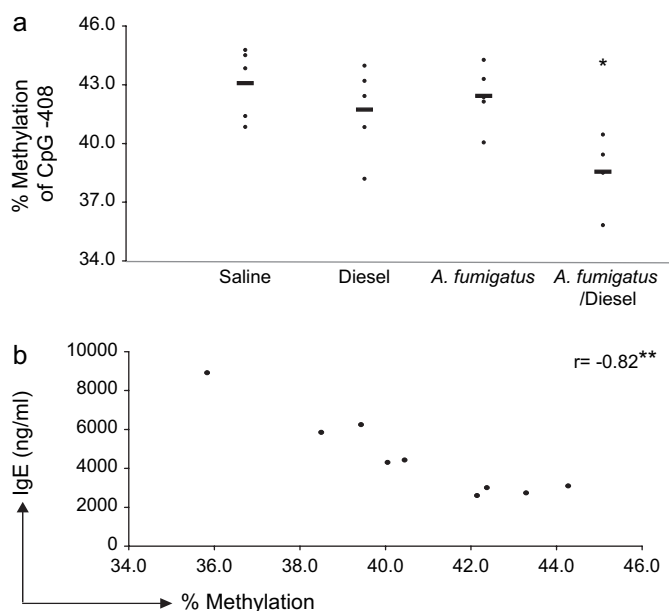
naïve T cells following *in vitro* Th2 polarizing conditions, or isolated from adult atopics (White *et al.*, 2002, 2006), suggesting fundamental differences exist across species and cell types.

A growing body of *in vitro* data has begun to reveal how these methylation patterns may modulate downstream molecular signaling pathways. For example, CpG<sup>-53</sup> and CpG<sup>-190</sup> are conserved in rat, dog, chimpanzee, and human (Jones and

Chen, 2006; White *et al.*, 2002). CpG<sup>-53</sup> is located in the proximal AP1-binding site of the IFN- $\gamma$  promoter and its methylation resulted in a change in factor binding (Young *et al.*, 1994). A subsequent study demonstrated that methylation of CpG<sup>-53</sup> in the IFN- $\gamma$  promoter significantly inhibited cAMP response element binding protein (CREB) and ATF2/c-Jun transcription factor binding to the CpG-containing AP1 site, augmenting proallergic Th2 polarization. Methylation of



**FIG. 3.** Level of IFN- $\gamma$  methylation correlates with IgE production. Methylation level at each CpG site was plotted versus IgE level among mice that underwent sensitization to *A. fumigatus* in the absence or presence of DEP exposure. \* $p < 0.05$  two tailed, Rank order correlation; \*\* $p < 0.05$  one tailed, Rank order correlation.



**FIG. 4.** *In vivo* *Aspergillus fumigatus* and DEP exposure are associated with hypomethylation of IL-4 promoter at CpG<sup>-408</sup> and decreased IgE production. (a) Decreased methylation occurs following *A. fumigatus* and DEP exposure when compared with either saline or *A. fumigatus* alone. (b) Level of methylation is associated inversely with IgE production. Methylation level at CpG<sup>-408</sup> was plotted versus IgE among mice that underwent *A. fumigatus* sensitization in the absence or presence of DEP exposure. \* $p < 0.05$  two-tailed compared with saline or *A. fumigatus*. \*\* $p < 0.05$  two tailed, Rank order correlation.

CpG<sup>-53</sup> alone was sufficient to inhibit the IFN- $\gamma$  promoter-driven reporter gene expression in a Th1 cell line (Jones and Chen, 2006). CpG<sup>-190</sup> also interacted with AP-1-CREB DNA binding complexes (White *et al.*, 2002; Ye *et al.*, 1994).

In the present study, DEP and *A. fumigatus* sensitization induced demethylation in only one of the studied CpG sites of the IL-4 promoter (i.e., CpG<sup>-408</sup>), and not in any of the sites in the conserved element in the first intron (CIRE). However, demethylation along the IL-4 gene tends to progress in sequential order during Th2 differentiation from 5' to 3' (Ansel *et al.*, 2006). Specifically, demethylation during Th2 differentiation has been shown to begin at CpG<sup>-408</sup> (Tykocinski *et al.*, 2005). Interestingly, CpG<sup>-408</sup> has been identified as a putative transcription factor AP-2 site, although not associated with IL-4 gene transcription to date (Comb and Goodman, 1990).

We recognize several limitations to our approach, including the possibility that DEP and/or *A. fumigatus* exposure also could act via more distant elements in the IFN- $\gamma$  or IL-4 genes or other genes. Additional airway studies (i.e., airway hyperreactivity) would have provided more information on the effects at local target tissues. BALB/c mice have been shown to be predisposed toward Th2 allergic responses in some experiments (Whitehead *et al.*, 2003), raising the possibility that the selection of mouse strain may have

introduced bias. Finally, it may be difficult to distinguish the independent effects of DEP on CpG methylation from those associated with a general enhancement of Th2-mediated immune responses. Nonetheless, DEP repeatedly augmented the molecular changes observed following sensitization to *A. fumigatus* alone (Figs. 2, 4), suggesting a direct effect of DEP exposure on T cells.

In sum, chronically inhaled exposure to DEP-induced hypermethylation at several CpG sites of the IFN- $\gamma$  gene promoter, and hypomethylation of CpG<sup>-408</sup> in the proximal promoter of IL-4 gene in CD4<sup>+</sup> T cells among mice sensitized to *A. fumigatus*. Hypermethylation of the IFN- $\gamma$  gene promoter was associated with greater IgE production, and hypomethylation of the CpG<sup>-408</sup> site in the proximal promoter of IL-4 gene was associated with reduced IgE production. This study is the first to demonstrate that inhaled environmental exposures implicated in asthma can alter methylation of Th genes *in vivo*. These results suggest that combined ambient allergen exposure and air pollution can modulate airway disease through epigenetic modification, supporting a new paradigm in asthma pathogenesis.

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