



HHS Public Access

Author manuscript

Environ Mol Mutagen. Author manuscript; available in PMC 2018 July 01.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Published in final edited form as:

Environ Mol Mutagen. 2017 July ; 58(6): 423–433. doi:10.1002/em.22097.

Prenatal environmental tobacco smoke exposure increases allergic asthma risk with methylation changes in mice

Sonja Christensen¹, Zeina Jaffar¹, Elizabeth Cole¹, Virginia Porter¹, Maria Ferrini¹, Britten Postma¹, Kent E. Pinkerton², Mihi Yang³, Yang Jee Kim⁴, Luke Montrose¹, Kevan Roberts¹, Andrij Holian¹, and Yoon Hee Cho^{1,*}

¹Center for Environmental Health Sciences, Department of Biomedical and Pharmaceutical Sciences, University of Montana, Missoula, MT USA

²Center for Health and the Environment, Department of Anatomy, Physiology and Cell Biology, University of California, Davis, CA, USA

³Research Center for Cell Fate Control, Department of Toxicology, Sookmyung Women's University, Seoul, Korea

⁴Da Vinci College of General Education, Chung-Ang University, Seoul, Korea

Abstract

Allergic asthma remains an inadequately understood disease. *In utero* exposure to environmental tobacco smoke (ETS) has been identified as an environmental exposure that can increase an individual's asthma risk. To improve our understanding of asthma onset and development, we examined the effect of *in utero* ETS exposure on allergic disease susceptibility in an asthmatic phenotype using a house dust mite (HDM) allergen-induced murine model. Pregnant C57BL/6 mice were exposed to either filtered air or ETS during gestation, and their offspring were further exposed to HDM at 6–7 weeks old to induce allergic inflammation. To quantify DNA methylation, methylation in the promoter regions of allergic inflammation-related genes and genomic DNA was analyzed. Exposure to HDM resulted in the onset of allergic lung inflammation, with an increased presence of inflammatory cells, Th2 cytokines (IL-4, IL-5, and IL-13), and airway remodeling. These asthmatic phenotypes were significantly enhanced when the mice had been exposed to *in utero* ETS. Furthermore, prenatal ETS exposure and subsequent HDM (ETS/HDM)-induced asthmatic phenotypes are in agreement with methylation changes in the selected asthma-related genes, including *Il-4*, *Il-5*, *Il-13*, *Ifn-γ*, and *Foxp3*. Global DNA methylation was significantly lower in ETS/HDM exposed mice than that of controls, which coincides with the results observed in lung, spleen, and blood DNAs. Prenatal ETS exposure resulted in a severe increase in allergic inflammatory responses after an HDM challenge, with corresponding methylation changes.

Corresponding author: Yoon Hee Cho, Ph.D., Department of Biomedical and Pharmaceutical Sciences, College of Health Professions and Biomedical Sciences, The University of Montana. 32 Campus Drive, Skaggs 283, Missoula, MT 59812. Phone: 1-406-243-4529; Fax: 1-406-243-2807; yoonhee.cho@umontana.edu.

Author contributions: YHC, KR and AH designed the study and applied for Research Ethics Board approval. SC, ZJ, VP, MF, BP, and KEP performed the laboratory experiments, including ETS exposures, and collected the data. EC, and YJK contributed to analyzing the data and preparing draft figures and tables. SC, MY, LM, and YHC prepared and revised the final manuscript. All authors approved the final manuscript.

Competing interests: The authors declare that they have no competing interests

Prenatal ETS exposure may influence developmental plasticity and result in altered epigenetic programming, leading to an increased susceptibility to asthma.

Keywords

Allergic asthma; *in utero*; environmental tobacco smoke; HDM murine model; methylation

Introduction

Asthma is the most common chronic disease in children. According to the American Academy of Allergy, Asthma & Immunology, more than 300 million individuals are affected globally, and that number is predicted to increase by over 100 million before the year 2025 [Bloemen et al. 2007]. Allergic asthma starts in early life and is thought to arise as a result of aberrant T helper (Th) cell responses to noninfectious environmental antigens. In particular, the symptoms of asthma are associated with the presence of activated Th2 cytokine-producing cells (IL-4, IL-5 and IL-13) and eosinophils in the airways, leading to airway hyperreactivity (AHR), mucus hypersecretion, and smooth tissue remodeling [Robinson et al. 1992; Kay et al. 1997; Wills-Karp et al. 1998; Wills-Karp 2004; Mitchell et al. 2011]. Exposure to a variety of environmental triggers has been shown to modify risk for asthma development; however, these factors do not independently explain the cause of asthma [Karmaus et al. 2013]. Furthermore, asthma continues to have a complex and poorly understood etiology, involving both genetic and environmental factors. Therefore, discovery of preventable factors and the underlying mechanisms that exacerbate asthma are important for mitigating the health burden of this childhood disease.

Extensive evidence has shown that environmental tobacco smoke (ETS) exposure is a major public health concern that can lead to poor respiratory health in children. In particular, early life ETS exposure has been associated with development of asthma-related outcomes in children and adults [Singh et al. 2003; Singh et al. 2011]. Several epidemiologic and experimental studies have found associations between prenatal tobacco smoke exposures and greater frequency or severity of adverse health effects such as asthma, mental health disorders, and cardiovascular diseases [Hales and Barker 2001; McKeever et al. 2002; Mone et al. 2004; Selgrade et al. 2013; Leung et al. 2015]. These findings of an association between specific prenatal exposures and increased risk of poor health outcomes led to the “fetal and early origins of adult disease hypothesis” that proposes prenatal or early postnatal environmental exposures influence developmental plasticity and result in altered programming, thus leading to increased susceptibility to a variety of complex diseases [Dolinoy et al. 2007].

Epigenetic regulation offers a plausible mechanistic explanation, in addition to gene-environment interactions, for some of the molecular events linking early exposures with later disease. Evidence suggests that maternal smoking is transmissible from mother to fetus and causes *in-utero* exposures that may alter the epigenetic programming of genes related to allergic asthma, thereby increasing susceptibility to allergic diseases [Patil et al. 2013; Breton et al. 2014; Maccani and Maccani 2015]. However, there are a limited number of

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

studies examining adverse immune responses to *in utero* ETS exposure; supporting animal data confirming the role of epigenetics as an underlying mechanism are also limited. We previously demonstrated that *in utero* ETS exposure leads to an increased risk of pulmonary inflammation and AHR through epigenetic alterations in offspring. It has also been shown that *in utero* ETS exposure can induce allergen-associated hypersensitivity reactions [Lee et al. 2015]. However, additional studies examining the impact of *in utero* ETS exposure and subsequent environmental exposure to asthma triggers later in life are still needed to fully elucidate the etiology of asthma.

In this study we aimed to examine epigenetic changes induced by *in utero* ETS exposure and subsequent environmental exposure to asthma triggers and further determine their relationship to an increased susceptibility for developing allergic inflammation and asthma. To this end, we utilized a house dust mite (HDM) allergen-driven murine model to examine the capacity of *in utero* ETS exposure to induce epigenetic alterations in the promoter regions of asthma-related genes as well as in genomic DNA methylation patterns, consequently predisposing the offspring to a greater risk of developing asthma.

Methods

Animals

C57BL6 mice (Harlan Laboratories, Indianapolis, Indiana) were used in this study, and all mice were maintained in pathogen-free conditions in the animal facility at either the University of California-Davis (UC-Davis) or the University of Montana (UM). All experiments were performed according to the guidelines of the National Institutes of Health and approved by the University of Montana Institutional Animal Care and Use Committee (IACUC).

Breeding and ETS Exposure

ETS exposure was carried out in the Center for Health and the Environment's animal facilities at UC-Davis. The project consisted of mating 2 female mice paired with 1 male mouse/cage to create a timed-pregnant exposure scenario. Twelve female and 6 male mice were used for breeding. Following confirmation of a vaginal plug, 6 female mice were exposed to either filtered air (FA) or ETS throughout gestation as previously described [Lee et al. 2015]. Figure 1 indicates a timeline of this study's design. In brief, control group timed-pregnant mice were exposed to FA only for 24 hours 7 days/week for the duration of the study. For the ETS-exposed group, timed-pregnant mice were exposed daily to a concentration of approximately 1 mg/m^3 of tobacco smoke for 6 h/day. Research cigarettes (3R4F, University of Kentucky) were burned at a rate of two cigarettes every 10 minutes with a puff volume of 35 mL over 2 seconds, once per minute. Both sidestream and mainstream cigarette smoke were collected via a chimney and passed to a dilution and aging chamber to achieve the target concentration of ETS ($1.02 \pm 0.03 \text{ mg/m}^3$). The carbon monoxide and nicotine levels were $6.08 \pm 0.23 \text{ ppm}$ and $240 \pm 60 \text{ } \mu\text{g/m}^3$, respectively, and the average temperature was 70 °F.

Author Manuscript

Once the dams gave birth, the dams and pups were exposed to FA only until weaning and then shipped to UM via air. Upon arriving at UM, the dams and offspring were given time to adjust to their new environment. The litter size (6.5 vs 6.7, mean for FA- and ETS-exposed dam, respectively) and sex-ratio (19:20 vs 19:21, Male:Female for FA and ETS, respectively) were not significantly different between the groups, and ETS exposure did not induce any spontaneous losses in mice. The FA and ETS groups were separated from the 32 offspring to receive HDM or PBS inoculations.

HDM exposure

An HDM murine model of acute airway disease was used to investigate the effect of prenatal ETS exposure on increased risk or exacerbation of Th2-mediated pulmonary and airway inflammation. An HDM allergen extract in sterile PBS (30 μ l) was used for installations. *Dermatophagoides pteronyssinus* (DerP, Greer, NC), which is well-known to elicit an allergic response, was administered intranasally over a period of two weeks. Young male offspring were lightly anesthetized with isofluorane 6–7 weeks after birth and then sensitized with HDM allergen extract (100 μ g) or PBS alone (control) on day 0, followed by challenge with the allergen (25 μ g) or PBS on days 7 and 14. On day 16, mice were harvested for bronchoalveolar lavage (BAL) fluid and tissues collection.

Lung, spleen, blood, and BAL fluid preparation

Two days after the last HDM exposure, mice were euthanized with an intraperitoneal injection of 0.1 mL pentobarbital euthanasia solution and tissues harvested. BAL fluid, whole lung, spleen, and blood were collected for analysis of allergen-induced pulmonary inflammation and epigenetic studies.

Cell differential counts and measurement of Th2 cytokines

BAL was performed (3 \times 0.5 ml PBS) to collect BAL fluid for analysis. BAL fluid was centrifuged; a cell differential count was conducted, and the supernatants were used for cytokine analysis.

Cytospin preparations were performed on 5 \times 10⁴ BAL fluid cells after staining the cells using a Hema 3 Staining system (Fisher Scientific, Houston, TX), which is comparable to the Wright-Giemsa-protocol. Cell differential percentages were determined by light microscopic evaluation of stained cells and expressed as absolute cell numbers.

Meso Scale Discovery (MSD) was used to detect cytokines, which is similar to ELISA but is based on MULTI-ARRAY® technology, a proprietary combination of electrochemiluminescence detection and patterned arrays. The MSD Mouse V-Plex Pro-Inflammatory Panel 1 (MSD) was used and cytokines IFN- γ , IL-4, and IL-5 were measured. IL-13 was measured in the BAL fluid using the Quantikine ELISA kit (R&D, Minneapolis, MN) according to the manufacturer's protocol.

Lung histology

Lungs from mice were fixed, sectioned, and stained as previously described [Ferrini et al. 2013]. In brief, lung tissue was fixed in 4% paraformaldehyde and embedded in paraffin

using a Shandon Citadel tissue processor (Thermo Fisher Scientific, Pittsburgh, PA). Microtome sections were cut at 5-mm thickness and stained with H&E using a Shandon Varistain 24-4 (Thermo Fisher Scientific).

DNA preparation

DNA was extracted from lung, spleen, and blood from offspring mice according to the manufacturer's protocol included with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Purified DNA was processed by bisulfite treatment using the EZ DNA MethylationTM Kit (Zymo Research, Irvine, CA) for Pyrosequencing assay.

Gene-specific methylation

Pyrosequencing assay was used for selected gene-specific methylation analysis. Gene specific murine primers were designed for *Ifn-γ*, *Foxp3*, *Il-4*, *Il-5*, and *Il-13* using PyroMark Assay Design 2.0 software (Qiagen) as previously described [Lee et al. 2015]. Gene regions were located using NCBI and GenBank. Table 1 shows the information of each gene and its location of amplification.

PCR amplification of the bisulfite converted DNA was conducted and PCR reactions were performed using Pyromark PCR Kit (Qiagen) with cycling parameters consisting of denaturation at 95°C for 5 min, and 45 cycles of 95°C for 30 s, 51-53°C for 30 s, 72°C for 30 s and a final extension of 72°C for 5 min, with the annealing temperature varying slightly for each gene-specific primer set. Pyrosequencing was conducted using a PyroMark Q96 MD instrument (Qiagen), with subsequent quantification of methylation levels determined by the PyroMark-CpG software (Qiagen).

Global DNA methylation by luminometric methylation assay (LUMA)

Global DNA methylation was determined using LUMA [Karimi et al. 2006a]. A 400 ng total DNA sample from lung and spleen was cleaved by a methylation sensitive restriction enzyme (HpaII) and its methylation insensitive isoschizomer (MspI) in parallel reactions. Additionally, *Eco*RI was included in all reactions to normalize the amount of DNA input as previously described [Karimi et al. 2006b]. After the digestion step, the extent of cleavage was quantified by bioluminometric polymerase extension via pyrosequencing (Pyromark Q96 MD, Qiagen). The percentage of 5-methylcytosine (5-mC) was calculated using the HpaII/MspI ratio. Samples were analyzed in technical duplicates and each plate included a positive, negative, and water control.

Global DNA methylation by 5-mC quantification

We also determined global DNA methylation levels in lung and blood using ELISA-based MethylflashTM Methylated DNA Quantification Kit (Colorimetric) (Epigentek, Farmingdale, NY), which measures the 5-mC content as a percentage of total cytosine in DNA samples. The assay was performed in duplicate according to manufacturer's instructions with 100 ng total DNA. The 5-mC in DNA was detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance at 450 nm in a microplate spectrophotometer. The percentage of 5-mC in genomic DNA was calculated using the following formula: 5-mC % = [(OD_{sample} - OD_{negative control}) / (2* × slope × input DNA

amount)] $\times 100$. A methylated polynucleotide containing 50% 5-mC was used as a positive control, and a factor of 2 was used to normalize 5-mC in the positive control to 100%.

Statistical analysis

Statistical analyses involved comparison of means using an unpaired t-test for parametric scale level endpoints and a two-tailed Mann-Whitney U test for subjective ordinal level endpoints. A 2-way ANOVA was used in cases where there was more than 1 variable using the Tukey test. Bonferroni post-hoc analysis was done comparing multiple variables using a 2-sided test; statistical power was greater than 0.8. Statistical significance was defined as a probability of type I error occurring at less than 5% ($P < 0.05$). Graphics and analyses were performed on PRISM 5.0.

Results

In utero ETS increases the susceptibility of airway and pulmonary inflammation

We determined the effects of *in utero* ETS exposure and subsequent inhalation of HDM allergen or PBS on the inflammatory response and Th2 and Th1 cytokine production in the airways of young offspring mice. Eosinophil influx into the airways was greater in prenatal ETS-exposed mice that were subsequently exposed to HDM allergen (ETS/HDM) than in FA-exposed mice challenged with HDM alone (FA/HDM) (Figure 2). There was a significant increase in the number of eosinophils after allergen inhalation (94278 \pm 6277 for ETS/HDM vs. 59546 \pm 2153 for FA/HDM, $p < 0.001$); however, no significant differences in the number of lymphocytes, macrophages, or neutrophils were observed between ETS/HDM-exposed offspring and FA/HDM-exposed offspring. ETS exposure alone (ETS/PBS) did not induce any increase in the number of eosinophils in the BAL fluid.

Levels of cytokines IL-4, IL-5, IL-13, and IFN- γ in the BAL fluid were measured to determine susceptibility to allergic inflammation after prenatal ETS and/or subsequent HDM allergen exposures. As shown in Figure 3, ETS/PBS did not affect Th2 cytokine levels; however, production of the Th2 cytokines IL-4, IL-5, and IL-13 ($p < 0.05$) was significantly higher in the airways of ETS/HDM exposed mice than that of FA/HDM exposed mice. In contrast, levels of the Th1 cytokine, IFN- γ , in the BAL fluid were not significantly affected by ETS/HDM exposure. These results suggest that prenatal ETS increases the susceptibility of Th2 inflammation elicited by allergen inhalation, but not Th1 inflammation, in the young offspring mice.

Lung histological analysis using H&E staining revealed that HDM allergen-exposed mice have more pronounced pulmonary inflammation (Figure 4C) than controls (FA/PBS) (4A) or ETS/PBS-exposed mice (4B). Mice exposed to ETS/HDM showed higher levels of pulmonary inflammation than that of other groups (4D).

Prenatal ETS enhances gene-specific and global DNA methylation changes

To confirm the Th2 bias observed in the immunological experiments, methylation levels in the promoter regions of asthma-related genes and genomic DNA were assessed. In this study, *Il-4*, *Il-5*, *Il-13*, *Ifn- γ* , and *Foxp3* genes were selected based on their key role in

asthma; in particular, genes associated with Th2 polarization and differentiation (*IL-4*, *IL-5* and *IL-13*) [Kumar et al. 2009; Ho 2010] or its regulation (*Foxp3* and *IFN- γ*) [Zhu et al. 2004; Lloyd and Hessel 2010; Nadeau et al. 2010; Breton et al. 2012] were examined.

There were no significant effects from ETS/PBS exposure on *Il-4* methylation levels compared to the FA/PBS group ($71.11\% \pm 3.28$ vs. $71.52\% \pm 1.89$, respectively, $p=0.79$). However, there was a borderline significant difference between *Il-4* methylation in the FA/HDM group and the FA/PBS-exposed group ($68.98\% \pm 2.63$ vs. $71.52\% \pm 1.89$, respectively, $p=0.08$). When the offspring mice were exposed to ETS/HDM, *Il-4* was hypomethylated to a greater extent than that observed in the FA/HDM group ($65.35\% \pm 2.88$ vs. $68.98\% \pm 2.63$, respectively, $p=0.04$) (Figure 5A).

As shown in Figure 5B, *Il-5* displayed a trend in hypomethylation after prenatal ETS and/or HDM exposures, but no significant differences were observed in methylation levels between FA/PBS and ETS/PBS or FA/PBS and FA/HDM. However, there were borderline significant differences in *Il-5* methylation between ETS/PBS and ETS/HDM ($84.77\% \pm 0.81$ vs. $84.03\% \pm 0.42$, respectively, $p=0.07$), as well as between FA/HDM and ETS/HDM ($84.50\% \pm 0.46$ vs. $84.03\% \pm 0.42$, respectively, $p=0.09$).

Hypomethylation in *Il-13* was significantly greater in the ETS/PBS exposed group than that of the FA/PBS group ($93.90\% \pm 0.64$ vs. $95.01\% \pm 1.01$, respectively, $p=0.04$), while FA/HDM exposure did not result in a significant change in *Il-13* hypomethylation. *Il-13* methylation levels were significantly less in offspring mice exposed to ETS/HMD than those of mice exposed to ETS/PBS or FA/HDM only (ETS/HDM: $92.06\% \pm 1.29$ vs. ETS/PBS: $93.90\% \pm 0.64$ or FA/HDM: $93.83\% \pm 1.23$, $p=0.01$ or $p=0.03$, respectively) (Figure 5C).

Hypermethylation of *Ifn- γ* , a Th1 gene, was significantly greater in the FA/HDM group than that of the FA/PBS control ($72.90\% \pm 0.53$ vs. $71.03\% \pm 1.43$, respectively, $p=0.01$). Further, hypermethylation of *Ifn- γ* was significantly higher in offspring mice exposed to ETS/HDM than that of offspring mice exposed to FA/HDM ($74.98\% \pm 2.12$ vs. $72.90\% \pm 0.53$, respectively, $p=0.04$) (Figure 5D).

Foxp3 showed similar patterns in methylation changes to those of *Ifn- γ* . As shown in Figure 5E, ETS/PBS exposure induced a borderline significant increase in hypermethylation of *Foxp3* over that of the FA/PBS group ($78.71\% \pm 2.18$ vs. $80.45\% \pm 2.30$, respectively, $p=0.07$). *Foxp3* hypermethylation was significantly higher in offspring mice exposed to ETS/HDM than that of offspring mice exposed to FA/PBS ($82.27\% \pm 2.89$ vs. $78.71\% \pm 2.18$, respectively, $p=0.03$) (Figure 5F).

Figure 6 shows global methylation changes in lung, spleen, and blood DNAs after prenatal ETS and/or subsequent HDM allergen exposures. Global hypomethylation in offspring mice exposed to prenatal ETS and/or HDM allergen was significantly higher than that of FA/PBS offspring mice in lung tissue DNAs (Figure 6A). Global DNA hypomethylation in spleen DNAs shows similar patterns to that in lung tissue DNAs (Figure 6B). Furthermore, global methylation levels were determined in blood to ascertain whether global methylation could be used as a biomarker of exposure in a more easily accessible tissue; therefore, we further determined global DNA methylation in lung and blood DNAs by quantifying 5-Mc. As

shown in Figure 6C and D, methylation levels in mice exposed to ETS/HDM were significantly lower than in those exposed to FA/PBS.

Discussion

Allergic asthma is a chronic disease that is increasing in diagnoses each year, especially among children [Eder et al. 2006]. Epigenetic changes have been suggested as a possible mechanism for this debilitating disease that is exacerbated after multiple exposures to allergens or environmental pollutants, especially ETS and HDM [Begin and Nadeau 2014]. In particular, HDM allergen exposure results in the onset of allergic lung inflammation, with increases in Th2 cytokines and inflammatory cells, airway remodeling, and AHR [Jaffar et al. 1999; Grunstein et al. 2005]. The HDM experimental murine model of asthma has been shown to closely approximate the disease process in human asthmatics [Hammad et al. 2002; Plantinga et al. 2013]. Therefore, we utilized an HDM allergen-driven murine model of asthma to determine epigenetic changes and examine their relationship to an increased susceptibility for developing allergic inflammation and asthma in response to *in utero* ETS exposure for a better understanding of allergic asthma etiology.

In general, the two types of inflammatory responses, Th1 and Th2, are in balance; however, environmental exposures may shift the response toward either Th1 or Th2 dominance [Berger 2000]. It has been demonstrated that DNA methylation is a potential mechanism underlying the establishment and maintenance of Th1 and Th2 lineages [White et al. 2002; Jones and Chen 2006]. For allergen-induced asthmatic and allergic symptoms, Th2-polarizing responses are exacerbated via demethylation of Th2 cytokine genes and hypermethylation of Th1 cytokines [van Panhuys et al. 2008; Kumar et al. 2009; Brand et al. 2012]. Therefore, we also investigated methylation levels of the Th1 cytokine, IFN- γ , as well as the transcription factor of regulatory T (Treg) cells, Foxp3.

Our results show that exposure to HDM allergens induces allergic asthmatic phenotypes, including Th2 bias, eosinophilia, and pulmonary inflammation in mice. These asthmatic phenotypes were significantly enhanced when the mice had been exposed to *in utero* ETS. In particular, the number of eosinophils and production of the Th2 cytokines, IL-4, IL-5, and IL-13, elicited by HDM allergen were significantly increased in the BAL fluid of prenatally ETS-exposed mice, illustrating their increased susceptibility to asthma. The increased IL-4, IL-5, and IL-13 cytokine levels were consistent with the cell differential data, showing significantly increased production of eosinophils and pulmonary inflammation (Figures 2-4). These ETS/HDM-induced asthmatic phenotypes were also in agreement with methylation changes observed in selected asthma-related genes, including *Il-4*, *Il-5*, *Il-13*, *Ifn- γ* , and *Foxp3*, which were all epigenetically regulated [Miller and Ho 2008; Wang and Pinkerton 2008; Ho 2010]. Furthermore, several studies support our observations that epigenetic regulation is altered with environmental exposure and affects various cellular functions that, in turn, may lead to asthma pathogenesis and susceptibility.

IL-4 plays an important role in naïve T-cell differentiation and Th2 polarization during an allergic response. Some human studies have concluded that naïve CD4 $^{+}$ T cells are hypermethylated at the 5' end of the *IL-4* gene as well as in the intergenic *IL-4/IL-13*

region. It was found that after Th2 differentiation, these regions were demethylated, suggesting an increase in gene expression [Lee et al. 2002]. These altered methylation patterns can affect how the naïve CD4⁺ T cells differentiate. In addition, *IL-4* hypomethylation in the present study (Figure 5A) suggests that transcription of *IL-4* is increased in response to ETS/HDM exposure that, in turn, increases expression of IL-4 and corresponds with our immunological data showing significant increases in IL-4 cytokine production. These data illustrate that ETS/HDM exposure illicits irregular epigenetic programming with greater susceptibility to Th2 polarization and an allergic phenotype.

Methylation patterns of *IL-13* were similar to those of *IL-4* following exposures. IL-13 cytokine levels were higher in the ETS/HDM group than those of the other groups (Figure 5C), and this increment was in agreement with the significant hypomethylation observed for *IL-13*. IL-13, which is secreted by type-2 innate lymphoid cells, is essential to the development of allergic inflammation [Spits and Cupedo 2012], including mucus metaplasia and AHR [Wills-Karp 2004]. Furthermore, IL-13 is involved in the development of fibrosis and lung injury by skewing macrophage differentiation towards the M2-type [Mills et al. 2000]. Several studies have reported a substantial link between lung macrophages and airway eosinophilic inflammation and remodeling in asthma [Mautino et al. 1999; Moon et al. 2007], as well as development of severe asthma [Yang et al. 2012].

IL-5 is a key cytokine for Th2 polarization and induces eosinophil activation, which acts as a central regulator of allergic inflammation in a manner similar to IL-4 and IL-13. Differences in the methylation levels among treatment groups were borderline significant, which may be explained by our experimental conditions. Th2 cells, as well as master cells, are known to produce IL-5 [Roediger et al. 2015]; since we measured methylation levels of *IL-5* from the whole lung tissue DNAs, our results may have been attenuated.

We observed significant hypermethylation of *Ifn-γ* in mice challenged with HDM, an effect that was further increased in offspring mice exposed to ETS/HDM. *Ifn-γ* hypermethylation exacerbates the Th2-polarizing responses, causing down-regulation of IFN-γ. IFN-γ is thought to be highly regulated early in life and methylation alterations of *IFN-γ* are known to play a role in its expression, especially in allergic disease [Jones and Chen 2006; White et al. 2006]. Unsurprisingly, we did not see measurable production of IFN-γ in the BAL fluid from any of the exposure groups.

Hypermethylation of *Foxp3* was also observed after ETS/HDM exposure (Figure 5E). *Foxp3* is a transcription factor for the development and function of Treg cells [Fontenot et al. 2003; Nadeau et al. 2010], which are a subset of CD4⁺ T lymphocytes and play a key role in controlling the immune response [Sakaguchi et al. 2001; Xystrakis et al. 2006]. Recent studies have shown epigenetic regulation of the *Foxp3* gene [Fontenot et al. 2003; Lal et al. 2009]. Stable expression of Foxp3 is essential to normal Treg function [Huehn et al. 2009]. Hypermethylation of *Foxp3* and consequent decreases in the expression of Foxp3 may impair Treg function, and this may lead to immunosuppression of Th2 cells and increased levels of the Th2 cytokines IL-4 and IL-13 [Nadeau et al. 2010]. Kohli et al. also reported similar results, showing that exposure to second hand smoke is associated with significant

hypermethylation and decreased expression of *IFN- γ* and *Foxp3* in children [Kohli et al. 2012].

In the present study, we observed significant global hypomethylation after prenatal ETS and/or HDM exposures, an effect that was exacerbated in mice exposed to ETS/HDM (Figure 6). Breton et al. also showed prenatal maternal smoking-related effects on global methylation among children [Breton et al. 2009]. Several studies reported loss of global DNA methylation in response to inflammation and oxidative stress [Baccarelli et al. 2009]. In addition, global DNA hypomethylation is associated with development of several chronic diseases [Jones and Baylin 2002; Komatsu et al. 2012]. Therefore, the altered global methylation as well as lung histology data in the present study suggest that ETS/HDM exposure may increase the likelihood of a severe asthma phenotype and further affect the lung environment over the long-term. Furthermore, the global DNA methylation data within the lung and/or spleen allow for a better understanding of disease development. Accessibility to lung and spleen tissues in population studies is limited; therefore, methylation levels in blood DNA were examined as a viable biomarker for exposure and disease development. Our data show that global DNA methylation changes occur in the lungs after ETS/HDM exposure, and those changes correlate with DNA methylation changes in blood.

Epigenetic patterns are altered according to the specific cell type; therefore, a limitation of our study is the use of DNA extracted from whole lung tissue, which may not be representative of the actual methylation levels of specific promoter regions. Furthermore, we confirmed our methylation data by determining the levels of protein production (cytokines) rather than gene expression levels; therefore, our results should be interpreted with caution. Finally, we only used male mice aged approximately 9–10 weeks in our experiments for the sake of simplicity, which is another limitation of this study. Therefore, larger studies using mice of various ages and both sexes will be needed to confirm our study results. Despite these limitations, our study presents the first evidence that prenatal exposure to ETS might alter methylation patterns of selected genes, and this programming contributes to and increases asthma susceptibility when offspring are challenged with allergens later in life. In addition, epigenetic mechanisms such as DNA methylation are reversible, and modifications could potentially be used as a therapeutic approach for treating asthma. Therefore, our study may provide useful information to prevent or potentially treat asthma among children.

Conclusions

Prenatal ETS exposure resulted in a severe increase in the allergic inflammatory response in offspring challenged with HDM, corresponding with methylation changes. These data suggest that *in utero* ETS exposure may influence developmental plasticity and result in altered epigenetic programming, leading to an increase in susceptibility to asthma.

Acknowledgments

The authors thank Mary Buford, Melisa Schelvan, and Joong Won Lee (Center for Environmental Health Sciences at University of Montana), Dale Uyeminami and Shanie McCarty (Center for Health and the Environment at University of California, Davis) for their expert technical assistance with various aspects of this manuscript.

Research reported in this publication was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P30GM103338. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the NIH, NIGMS, or NIEHS.

References

Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua AA, Suh HH, Zanobetti A, Sparrow D, Vokonas PS, Schwartz J. Rapid DNA methylation changes after exposure to traffic particles. *Am J Respir Crit Care Med.* 2009; 179(7):572–578. [PubMed: 19136372]

Begin P, Nadeau KC. Epigenetic regulation of asthma and allergic disease. *Allergy Asthma Clin Immunol.* 2014; 10(1):27. [PubMed: 24932182]

Berger A. Th1 and Th2 responses: what are they? *BMJ.* 2000; 321(7258):424. [PubMed: 10938051]

Bloemen K, Verstraelen S, Van Den Heuvel R, Witters H, Nelissen I, Schoeters G. The allergic cascade: review of the most important molecules in the asthmatic lung. *Immunol Lett.* 2007; 113(1): 6–18. [PubMed: 17765979]

Brand S, Kesper DA, Teich R, Kilic-Niebergall E, Pinkenburg O, Bothur E, Lohoff M, Garn H, Pfefferle PI, Renz H. DNA methylation of TH1/TH2 cytokine genes affects sensitization and progress of experimental asthma. *J Allergy Clin Immunol.* 2012; 129(6):1602–1610 e1606. [PubMed: 22277202]

Breton CV, Byun HM, Wenten M, Pan F, Yang A, Gilliland FD. Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. *Am J Respir Crit Care Med.* 2009; 180(5):462–467. [PubMed: 19498054]

Breton CV, Salam MT, Wang X, Byun HM, Siegmund KD, Gilliland FD. Particulate matter, DNA methylation in nitric oxide synthase, and childhood respiratory disease. *Environ Health Perspect.* 2012; 120(9):1320–1326. [PubMed: 22591701]

Breton CV, Siegmund KD, Joubert BR, Wang X, Qui W, Carey V, Nystad W, Haberg SE, Ober C, Nicolae D, Barnes KC, Martinez F, Liu A, Lemanske R, Strunk R, Weiss S, London S, Gilliland F, Raby B, Asthma Bc. Prenatal tobacco smoke exposure is associated with childhood DNA CpG methylation. *PLoS One.* 2014; 9(6):e99716. [PubMed: 24964093]

Dolinoy DC, Weidman JR, Jirtle RL. Epigenetic gene regulation: linking early developmental environment to adult disease. *Reprod Toxicol.* 2007; 23(3):297–307. [PubMed: 17046196]

Eder W, Ege MJ, von Mutius E. The asthma epidemic. *N Engl J Med.* 2006; 355(21):2226–2235. [PubMed: 17124020]

Ferrini ME, Simons BJ, Bassett DJ, Bradley MO, Roberts K, Jaffar Z. S-nitrosoglutathione reductase inhibition regulates allergen-induced lung inflammation and airway hyperreactivity. *PLoS One.* 2013; 8(7):e70351. [PubMed: 23936192]

Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol.* 2003; 4(4):330–336. [PubMed: 12612578]

Grunstein MM, Veler H, Shan X, Larson J, Grunstein JS, Chuang S. Proasthmatic effects and mechanisms of action of the dust mite allergen, Der p 1, in airway smooth muscle. *J Allergy Clin Immunol.* 2005; 116(1):94–101. [PubMed: 15990780]

Hales CN, Barker DJ. The thrifty phenotype hypothesis. *Br Med Bull.* 2001; 60:5–20. [PubMed: 11809615]

Hammad H, Lambrecht BN, Pochard P, Gosset P, Marquillies P, Tonnel AB, Pestel J. Monocyte-derived dendritic cells induce a house dust mite-specific Th2 allergic inflammation in the lung of humanized SCID mice: involvement of CCR7. *J Immunol.* 2002; 169(3):1524–1534. [PubMed: 12133980]

Ho SM. Environmental epigenetics of asthma: an update. *J Allergy Clin Immunol.* 2010; 126(3):453–465. [PubMed: 20816181]

Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol.* 2009; 9(2):83–89. [PubMed: 19114986]

Jaffar Z, Roberts K, Pandit A, Linsley P, Djukanovic R, Holgate S. B7 costimulation is required for IL-5 and IL-13 secretion by bronchial biopsy tissue of atopic asthmatic subjects in response to allergen stimulation. *Am J Respir Cell Mol Biol.* 1999; 20(1):153–162. [PubMed: 9870929]

Jones B, Chen J. Inhibition of IFN-gamma transcription by site-specific methylation during T helper cell development. *EMBO J.* 2006; 25(11):2443–2452. [PubMed: 16724115]

Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet.* 2002; 3(6): 415–428. [PubMed: 12042769]

Karimi M, Johansson S, Ekstrom TJ. Using LUMA: a Luminometric-based assay for global DNA-methylation. *Epigenetics.* 2006a; 1(1):45–48. [PubMed: 17998810]

Karimi M, Johansson S, Stach D, Corcoran M, Grander D, Schalling M, Bakalkin G, Lyko F, Larsson C, Ekstrom TJ. LUMA (LUMinometric Methylation Assay)--a high throughput method to the analysis of genomic DNA methylation. *Exp Cell Res.* 2006b; 312(11):1989–1995. [PubMed: 16624287]

Karmaus W, Ziyab AH, Everson T, Holloway JW. Epigenetic mechanisms and models in the origins of asthma. *Curr Opin Allergy Clin Immunol.* 2013; 13(1):63–69. [PubMed: 23242116]

Kay AB, Barata L, Meng Q, Durham SR, Ying S. Eosinophils and eosinophil-associated cytokines in allergic inflammation. *Int Arch Allergy Immunol.* 1997; 113(1-3):196–199. [PubMed: 9130521]

Kohli A, Garcia MA, Miller RL, Maher C, Humblet O, Hammond SK, Nadeau K. Secondhand smoke in combination with ambient air pollution exposure is associated with increased CpG methylation and decreased expression of IFN-gamma in T effector cells and Foxp3 in T regulatory cells in children. *Clin Epigenetics.* 2012; 4(1):17. [PubMed: 23009259]

Komatsu Y, Waku T, Iwasaki N, Ono W, Yamaguchi C, Yanagisawa J. Global analysis of DNA methylation in early-stage liver fibrosis. *BMC Med Genomics.* 2012; 5:5. [PubMed: 22281153]

Kumar RK, Hitchins MP, Foster PS. Epigenetic changes in childhood asthma. *Dis Model Mech.* 2009; 2(11-12):549–553. [PubMed: 19892885]

Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, Reid SP, Levy DE, Bromberg JS. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J Immunol.* 2009; 182(1):259–273. [PubMed: 19109157]

Lee DU, Agarwal S, Rao A. Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. *Immunity.* 2002; 16(5):649–660. [PubMed: 12049717]

Lee JW, Jaffar Z, Pinkerton KE, Porter V, Postma B, Ferrini M, Holian A, Roberts K, Cho YH. Alterations in DNA methylation and airway hyperreactivity in response to in utero exposure to environmental tobacco smoke. *Inhal Toxicol.* 2015; 27(13):724–730. [PubMed: 26525176]

Leung CY, Leung GM, Schooling CM. Early second-hand smoke exposure and child and adolescent mental health: evidence from Hong Kong's 'Children of 1997' birth cohort. *Addiction.* 2015; 110(11):1811–1824. [PubMed: 26119482]

Lloyd CM, Hessel EM. Functions of T cells in asthma: more than just T(H)2 cells. *Nat Rev Immunol.* 2010; 10(12):838–848. [PubMed: 21060320]

Maccani JZ, Maccani MA. Altered placental DNA methylation patterns associated with maternal smoking: current perspectives. *Adv Genomics Genet.* 2015; 2015(5):205–214. [PubMed: 26203295]

Mautino G, Henriet C, Gouyat C, Le Cam A, Dayer JM, Bousquet J, Capony F. Increased expression of tissue inhibitor of metalloproteinase-1 and loss of correlation with matrix metalloproteinase-9 by macrophages in asthma. *Lab Invest.* 1999; 79(1):39–47. [PubMed: 9952109]

McKeever TM, Lewis SA, Smith C, Hubbard R. The importance of prenatal exposures on the development of allergic disease: a birth cohort study using the West Midlands General Practice Database. *Am J Respir Crit Care Med.* 2002; 166(6):827–832. [PubMed: 12231492]

Miller RL, Ho SM. Environmental epigenetics and asthma: current concepts and call for studies. *Am J Respir Crit Care Med.* 2008; 177(6):567–573. [PubMed: 18187692]

Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol.* 2000; 164(12):6166–6173. [PubMed: 10843666]

Mitchell C, Provost K, Niu N, Homer R, Cohn L. IFN-gamma acts on the airway epithelium to inhibit local and systemic pathology in allergic airway disease. *J Immunol.* 2011; 187(7):3815–3820. [PubMed: 21873527]

Mone SM, Gillman MW, Miller TL, Herman EH, Lipshultz SE. Effects of environmental exposures on the cardiovascular system: prenatal period through adolescence. *Pediatrics.* 2004; 113(4 Suppl): 1058–1069. [PubMed: 15060200]

Moon KA, Kim SY, Kim TB, Yun ES, Park CS, Cho YS, Moon HB, Lee KY. Allergen-induced CD11b⁺ CD11c(int) CCR3⁺ macrophages in the lung promote eosinophilic airway inflammation in a mouse asthma model. *Int Immunol.* 2007; 19(12):1371–1381. [PubMed: 17977814]

Nadeau K, McDonald-Hyman C, Noth EM, Pratt B, Hammond SK, Balmes J, Tager I. Ambient air pollution impairs regulatory T-cell function in asthma. *J Allergy Clin Immunol.* 2010; 126(4):845–852 e810. [PubMed: 20920773]

Patil VK, Holloway JW, Zhang H, Soto-Ramirez N, Ewart S, Arshad SH, Karmaus W. Interaction of prenatal maternal smoking, interleukin 13 genetic variants and DNA methylation influencing airflow and airway reactivity. *Clin Epigenetics.* 2013; 5(1):22. [PubMed: 24314122]

Plantinga M, Guilliams M, Vanheeswijnghels M, Deswarde K, Branco-Madeira F, Toussaint W, Vanhoutte L, Neyt K, Killeen N, Malissen B, Hammad H, Lambrecht BN. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity.* 2013; 38(2):322–335. [PubMed: 23352232]

Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay AB. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med.* 1992; 326(5):298–304. [PubMed: 1530827]

Roediger B, Kyle R, Tay SS, Mitchell AJ, Bolton HA, Guy TV, Tan SY, Forbes-Blom E, Tong PL, Koller Y, Shklovskaya E, Iwashima M, McCoy KD, Le Gros G, Fazekas de St Groth B, Weninger W. IL-2 is a critical regulator of group 2 innate lymphoid cell function during pulmonary inflammation. *J Allergy Clin Immunol.* 2015; 136(6):1653–1663. e1651–1657. [PubMed: 26025126]

Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M, Takahashi T. Immunologic tolerance maintained by CD25⁺ CD4⁺ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev.* 2001; 182:18–32. [PubMed: 11722621]

Selgrade MK, Blain RB, Fedak KM, Cawley MA. Potential risk of asthma associated with in utero exposure to xenobiotics. *Birth Defects Res C Embryo Today.* 2013; 99(1):1–13. [PubMed: 23723168]

Singh SP, Barrett EG, Kalra R, Razani-Boroujerdi S, Langley RJ, Kurup V, Tesfaigzi Y, Sopori ML. Prenatal cigarette smoke decreases lung cAMP and increases airway hyperresponsiveness. *Am J Respir Crit Care Med.* 2003; 168(3):342–347. [PubMed: 12791581]

Singh SP, Gundavarapu S, Pena-Philippides JC, Rir-Sima-ah J, Mishra NC, Wilder JA, Langley RJ, Smith KR, Sopori ML. Prenatal secondhand cigarette smoke promotes Th2 polarization and impairs goblet cell differentiation and airway mucus formation. *J Immunol.* 2011; 187(9):4542–4552. [PubMed: 21930963]

Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu Rev Immunol.* 2012; 30:647–675. [PubMed: 22224763]

van Panhuys N, Le Gros G, McConnell MJ. Epigenetic regulation of Th2 cytokine expression in atopic diseases. *Tissue Antigens.* 2008; 72(2):91–97. [PubMed: 18554247]

Wang L, Pinkerton KE. Detrimental effects of tobacco smoke exposure during development on postnatal lung function and asthma. *Birth Defects Res C Embryo Today.* 2008; 84(1):54–60. [PubMed: 18383132]

White GP, Hollams EM, Yerkovich ST, Bosco A, Holt BJ, Bassami MR, Kusel M, Sly PD, Holt PG. CpG methylation patterns in the IFNgamma promoter in naive T cells: variations during Th1 and Th2 differentiation and between atopics and non-atopics. *Pediatr Allergy Immunol.* 2006; 17(8): 557–564. [PubMed: 17121582]

White GP, Watt PM, Holt BJ, Holt PG. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between

human neonatal and adult CD45RO- T cells. *J Immunol.* 2002; 168(6):2820–2827. [PubMed: 11884451]

Wills-Karp M. Interleukin-13 in asthma pathogenesis. *Curr Allergy Asthma Rep.* 2004; 4(2):123–131. [PubMed: 14769261]

Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science.* 1998; 282(5397):2258–2261. [PubMed: 9856949]

Xystrakis E, Boswell SE, Hawrylowicz CM. T regulatory cells and the control of allergic disease. *Expert Opin Biol Ther.* 2006; 6(2):121–133. [PubMed: 16436038]

Yang M, Kumar RK, Hansbro PM, Foster PS. Emerging roles of pulmonary macrophages in driving the development of severe asthma. *J Leukoc Biol.* 2012; 91(4):557–569. [PubMed: 22293472]

Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, Killeen N, Urban JF Jr, Guo L, Paul WE. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat Immunol.* 2004; 5(11):1157–1165. [PubMed: 15475959]

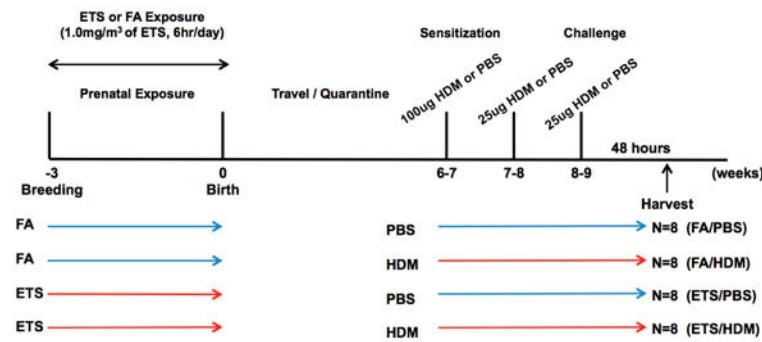


Figure 1.
Experimental model and timeline.

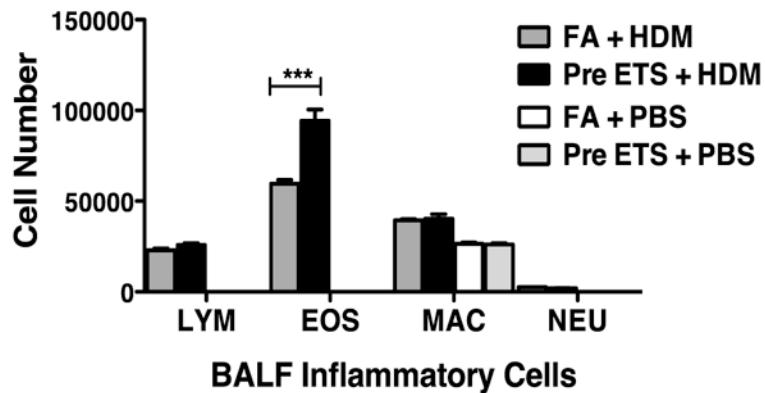
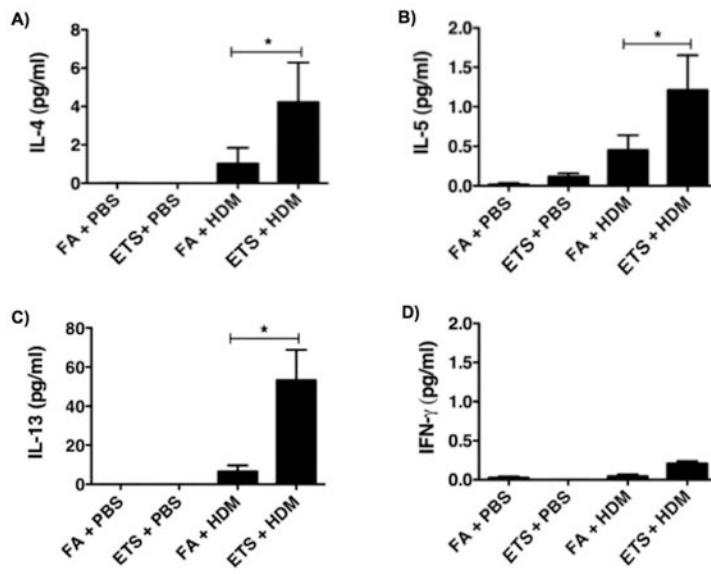


Figure 2.

Cell differential counts in BAL fluid after prenatal ETS and/or subsequent HDM exposure expressed as total cell number of lymphocytes (LYM), eosinophils (EOS), macrophages (MAC), and neutrophils (NEU). The number of eosinophils significantly increased after ETS/HDM exposure, illustrating an asthmatic response. Data are means \pm SEM n=3. *** p < 0.001.

**Figure 3.**

Measurement of cytokine production in the BAL fluid after prenatal ETS and/or subsequent HDM exposure. FA/PBS group is the double control group. IL-4, IL-5, and IL-13 levels are significantly higher in the ETS/HDM-exposed group than that of the FA/HDM group. IFN- γ was at baseline levels for all groups. Data are means \pm SEM n=6. * p < 0.05.

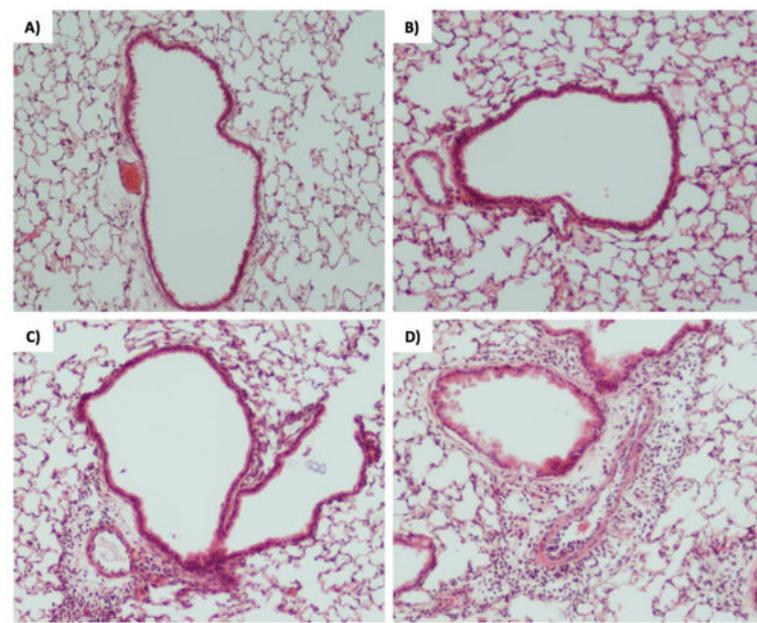
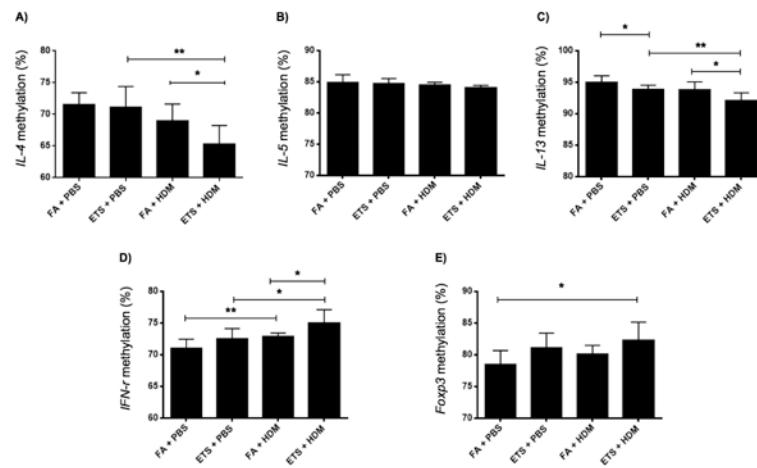
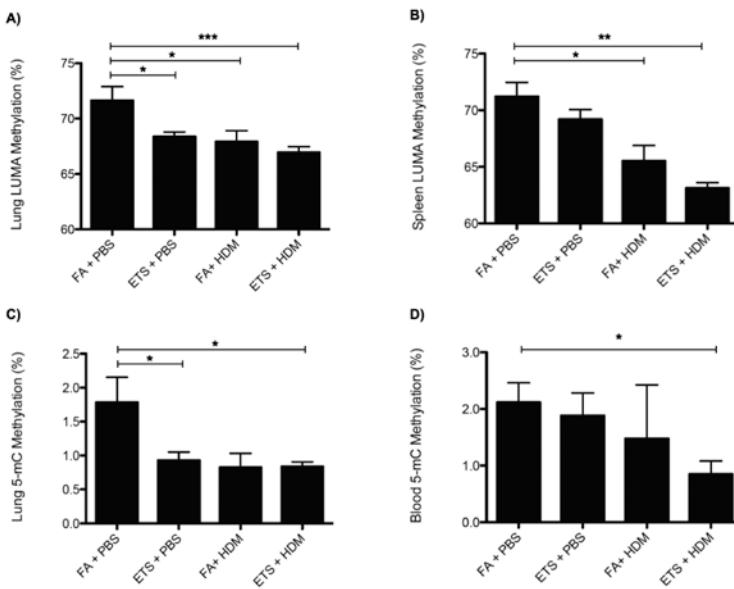


Figure 4.

Lung tissue histology from prenatal ETS-exposed and FA-exposed mice after HDM allergen or PBS (control) challenge. There is a moderate increase in pulmonary inflammation in young FA/HDM- and ETS/HDM-exposed mice. Representative pictures for each group. A: FA/PBS, B: ETS/PBS, C: FA/HDM, D: ETS/HDM

**Figure 5.**

Epigenetic alterations in the promoter regions of selected genes after prenatal ETS and/or subsequent HDM exposure. Data are means \pm SEM n=6. *p < 0.05, **p < 0.01.

**Figure 6.**

Global DNA methylation in lung, spleen, and blood DNAs after prenatal ETS exposure and subsequent HDM challenge using LUMA and 5-mC quantification. Data are means \pm SEM n=3-6. * p < 0.05, ** p < 0.01, *** p < 0.001

Table 1

Primer sequences and PCR conditions used for Gene-specific Methylation analysis

Target (Gene ID)	Primer	Sequence (5'-3')	Annealing Temp (°C)	PCR product (bp)
IL-4 (16189)	Forward	AGGGGTTTTATAGTAGGAAGTAG	51.9	178
	Reverse	CCCCCTTTTTAAATCTACAA ^a		
	Sequencing	AGATTTTTGATATTATTTGTT		
IL-5 (16191)	Forward	GAGGGGGGATAAAAAGAAG ^a	50.6	144
	Reverse	ATCCACATTACACTCCATTCTAATT		
	Sequencing	AAATTCTCTTTAAAATTATACA		
IL-13 (16163)	Forward	GTTAGTATTGGGTTGGTTAGGA	51	237
	Reverse	ATTATCTAAAACCACATTTACTCAT ^a		
	Sequencing	GTTGGTTGTTAGGAG		
IFN- γ (15978)	Forward	AATGGTGTGAAGTAAAAGTGTAGA	53.4	108
	Reverse	AAAATTCCTTCCACTCCTAAACTCTC ^a		
	Sequencing	ATGGTATAGGTGGTA		
Foxp3 (20371)	Forward	TTGTTTATTTGGTATTAATTGTGT	52	231
	Reverse	TTTACCCCTAACTACACTTAACCTTT ^a		
	Sequencing	ATTTGGGTATTAATTGTGT		

^aBiotin-labeled primer