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Alterations in DNA methylation and airway hyperreactivity in response to *in utero* exposure to environmental tobacco smoke

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Abstract

Growing evidence indicates that prenatal exposure to maternal smoking is a risk factor for the development of asthma in children. However, the effects of prenatal environmental tobacco smoke (ETS) exposure on the genome and lung immune cells are unclear. This study aims to determine whether *in utero* ETS exposure alters DNA methylation patterns and increases airway hyperreactivity (AHR) and inflammation. Pregnant C57BL/6 mice were exposed daily to a concentration of 1.0 mg/m^3 ETS. AHR was determined in the 6-week-old offspring by measurement of airway resistance. Global and gene promoter methylation levels in lung DNA from offspring were analyzed by luminometric methylation and pyrosequencing assays, respectively. Offspring exposed to ETS showed a marked increase in the number of alveolar macrophages in the bronchoalveolar lavage fluid and level of IL-13 in the airways compared with offspring of filtered-air exposed dams (controls). ETS exposure significantly augmented AHR compared with controls. In the methylation analysis, ETS-exposed offspring had a significantly lower level of global DNA methylation than the controls. We observed a significant increase in *IFN-γ*, and significant decrease in *IL-13* methylation levels in the ETS group compared with controls. Collectively, these data suggest that *in utero* ETS exposure increases the risk of pulmonary inflammation and AHR through altered DNA methylation, but additional studies are needed to fully determine the causal link between changes in methylation and cytokines levels, as well as AHR.

Keywords

Airway hyperreactivity; DNA promoter methylation; environmental tobacco smoke; genomic methylation; inflammation

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Declaration of interest

No potential conflicts of interest were disclosed.

Introduction

Tobacco smoke contains thousands of chemical compounds, many of which are known human carcinogens with other toxic effects. Environmental tobacco smoke (ETS) consists of around 85% side-stream smoke and 15% mainstream smoke. Side-stream smoke has a higher concentration of harmful agents and is more toxic than mainstream smoke (Wolz et al., 2002). In particular, side-stream smoke contains smaller particles that make their way into the lungs and cells of the body more easily than larger particles from mainstream smoke. The chemicals found in side-stream smoke can also cross the placenta and reach the fetus. Experimental evidence indicates that fetus' and newborns are more susceptible to carcinogens than adults because their bodies are still growing (Lackmann et al., 1999; Rice & Ward, 1982). Therefore, of the most common known early life exposures, ETS continues to be one of the most harmful and remains a major public health concern.

Prenatal exposure to tobacco smoke, primarily through maternal smoking, has been found to cause lung function impairment, airway hyperreactivity (AHR) and increase the risk of allergic asthma, especially in children. Allergic asthma is the most common form of asthma and a chronic disease that is increasing in diagnoses each year, especially among children (Eder et al., 2006). Allergic asthma starts in early life and is associated with AHR and bronchial inflammation (Holt et al., 2010). A recent systematic review and meta-analysis of 13 studies examining the effects of prenatal smoking reported a 42–50% increased risk of asthma in infants and adolescents exposed to smoke prenatally (Burke et al., 2012). Several animal model studies have clearly demonstrated that i*n utero* exposure to maternal smoking is a primary cause of impaired lung function, AHR and onset of asthma (Singh et al., 2011; Yochum et al., 2014). While the impact of active maternal smoking on offspring development is well established, the impact of prenatal ETS exposure has been less studied despite the large number of women exposed to ETS during pregnancy. Few studies have investigated the consequences to offspring of pregnant, nonsmoking women exposed daily to ETS (Penn et al., 2007; Xiao et al., 2012) even though numerous studies have focused on adverse immune responses to ETS exposure in adults. In addition, studies examining the effects of *in utero* ETS exposure in the child's early life are still needed.

There is growing interest in the potential role of epigenetics in environmental-related diseases. DNA methylation represents a key epigenetic mechanism that is responsible for gene regulation. While epigenetic changes are natural occurrences, they can also be easily influenced by several factors, including age, disease state and environmental exposure (Rozek et al., 2014). Aberrant DNA methylation patterns, including global hypomethylation and gene-specific hypermethylation or hypomethylation, are one mechanism by which prenatal exposures affect disease risk later in life (Reik et al., 2001). Mapping aberrant DNA methylation patterns also provides a promising approach for understanding complex diseases like asthma (Petronis, 2001). In recent years, evidence has arisen suggesting that epigenetic modifications via DNA methylation can provide a possible mechanistic explanation for the link between *in utero* tobacco smoke exposure and allergic response. Prenatal exposure to maternal cigarette smoke contributes to an increase in DNA methylation at two loci in *FRMD4A* and *Cllorf52* of asthmatic children (Breton et al., 2014). Among children suffering from atopic dermatitis, cord blood DNA methylation in three

genes was found to be associated with *in utero* maternal smoke exposure (Wang et al., 2013). These studies indicate that prenatal-exposure to maternal smoking increases the asthmatic symptoms in childhood. Furthermore, these symptoms may be mediated through epigenetic programming and environmental exposures across life. However, it remains unclear how DNA methylation contributes to the allergic response and no study to date has assessed the effects of *in utero* ETS exposure on DNA methylation status.

The aim of the present study was to elucidate the role of epigenetics in regulating pulmonary inflammation and AHR associated with *in utero* ETS exposure. We hypothesized that *in utero* ETS exposure would cause an increase in AHR and airway inflammation via alterations in global methylation and methylation in the promoter region of genes related to allergic inflammation (*IFN-γ*, *IL-4* and *IL-13*). To this end, mice were exposed daily to a concentration of 1.0 mg/m^3 of ETS for 6 h/ day during gestation. For the control group, mice were exposed only to filtered air (FA) for the same period. For quantification of DNA methylation, DNA was extracted from whole lung tissue and global methylation and the promoter regions of selected genes were analyzed using the luminometric methylation assay (LUMA) and pyrosequencing assay, respectively.

Methods

Animals

C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). All mice were maintained in pathogen-free conditions in the animal facility at either the University of California–Davis (Davis, CA) or the University of Montana (Missoula, MT). 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).

ETS exposure

The study consisted of mating two female mice, paired with one male mouse/cage to create a timed-pregnant exposure scenario. Specifically, eight female and four male mice were used for breeding. Following confirmation of a vaginal plug, four female mice were exposed to either FA or ETS throughout gestation. ETS was generated by a smoke exposure system (University of California-Davis). For the ETS exposed group, timed-pregnant mice were exposed daily to an approximate concentration of 1.0 mg/m^3 of tobacco smoke for 6 h/day. 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY) were burned at a rate of two cigarettes every 10 min with a puff volume of 35 mL over a duration of 2 s, once per minute. Both side-stream and mainstream cigarette smoke were collected via a chimney and passed to a dilution and aging chamber to achieve the target concentration of ETS. For the control group, timed-pregnant mice were handled in the same way but exposed to FA only for 24 h 7d/week for the duration of the study. The concentration of carbon monoxide in the exposure chambers was monitored and kept to 4.8 ± 0.8 ppm. A person actively smoking can attain particulate levels as high as 2.0 mg/m^3 (Jinot et al., 1992). Therefore, the total concentration of suspended particulates was maintained at 1.0 ± 0.17 mg/m^3 for this study. Once the dams gave birth, the dams and pups

were only exposed to FA until weaning and then shipped to the University of Montana via air. Upon arriving at the University of Montana, the dams and the offspring were given time to adjust to their new environment. Figure 1 illustrates the experimental design for this study.

The number of pups in a litter for each group was counted and recorded. Litter size (6.7 versus 7.1, mean for FA- and ETS-exposed dam, respectively) and sex-ratio (12.7:13.3 versus 14.8:13.2, Male:Female for FA and ETS, respectively) were not significantly different between the groups. ETS exposure did not induce any spontaneous losses in mice. Among total offspring, 24 pups from each group were randomly selected with the same sex ratio, and used for further analyses.

Lung and bronchoalveolar lavage (BAL) fluid preparation

Offspring at 6 weeks of age were euthanized with an intra-peritoneal injection of 0.1 ml pentobarbital euthanasia solution. The lungs were then immediately lavaged, resected, and snap-frozen in liquid nitrogen then stored at −80 °C for later epigenetic analysis. BAL fluid was collected for the analysis of pro-inflammatory mediators and airway inflammation.

Cell differential counts and measurement of cytokine in the BAL fluid

Lavage cells were cytocentrifuged (Cytospin 2, Thermo Fisher Scientific, Waltham, MA) and stained using a Hema3 System (Thermo Fisher Scientific). Cell differential percentages were determined by light microscopic evaluation of Hema3-stained cytospin preparations and expressed as absolute cell numbers. IL-4 and IFN-γ were measured using MesoScale Discovery Mouse V-Plex Pro-Inflammatory Panel 1 assay (Rockville, MD). IL-13 was measured using a sensitive Quantikine Mouse IL-13 immunoassay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Samples were carried out in two separate batches for this assay.

Pulmonary function measurement

We determined lung function by measuring changes in pulmonary resistance (RL) after an aerosolized methacholine (MCh) chal lenge. Specifically, offspring was anesthetized and tracheotomized, then mechanically ventilated and exposed to increasing concentrations of aerosolized MCh. Each aerosol was delivered for a period of 3 min followed by a second 3 min period and lung function was assessed by the measurement of pulmonary R_L using the pulmonary function equipment by Buxco Research Systems (Wilmington, NC).

DNA extraction

DNA was extracted from lung tissues of offspring according to the manufacturer's protocol included with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA).

Global DNA methylation assay by LUMA

Global methylation was determined using LUMA (Karimi et al., 2006a). A 500 ng total DNA sample was cleaved by the methylation sensitive restriction enzyme (*Hpa*II) and its methylation insensitive isoschizomer (*Msp*I) in parallel reactions. Additionally, *Eco*RI was

included in all reactions to normalize the amount of DNA input as described previously (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 percent of 5-methylcytosine (5-mC) was calculated using the HpaII/MspI ratio. The samples were analyzed in technical duplicates and each plate included a positive, negative and water control.

Primer design

The pyrosequencing assay was used to measure methylation levels in the promoter regions of selected genes (*IFN-γ, IL-4* and *IL-13*). Gene-specific primers for these genes were designed using the Pyro-Mark assay design software (version 2.0, Qiagen). The program automatically generates primer sets that include both PCR and sequencing primers based on selected target sequences. Primers were designed for analyzing 2-3 CpGs within the promoter regions (Figure 2). Table 1 shows the detail of the primers and PCR conditions used in this study.

Gene-specific methylation analysis

Genomic DNA first underwent bisulfite modification to convert unmethylated cytosine residues to uracil using the EZ DNA Methylation™ Kit (Zymo Research, Orange, CA) following the protocol from the manufacturer. Bisulfited DNA was analyzed by pyrosequencing assay as described previously (Yang et al., 2004). We amplified 50 ng of bisulfitetreated DNA using the PyroMark PCR kit (Qiagen) with the following conditions: 95 °C for 5 min, 45 × (95 °C for 30 s, annealing temperature of each gene specific primer sets for 30 s, 72 °C for 30 s), 72 °C for 5 min. After annealing, pyrosequencing was conducted using a PyroMark Q96 MD instrument. Following the sequencing reaction, percent methylation within a sample was subsequently determined by averaging across all interrogated CpG sites.

Statistics

The combined data from the replicate experiments were analyzed using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). Comparisons were analyzed for statistical significance by non-parametric Mann–Whitney test, with p values <0.05 being considered significant.

Results

Airway inflammation and IL-13 production

In order to evaluate whether *in utero* ETS exposure increases airway inflammation, cell differential counts in the BAL fluid were determined. Offspring exposed to ETS showed markedly increased numbers of alveolar macrophages in the airways compared with the FA group (Figure 3A). Negligible numbers of lymphocytes or granulocytes were detected in the BAL fluid of both the ETS- and FA-exposed mice. Interestingly, a small increase in the level of IL-13 production in the ETS-exposed group was observed (Figure 3C) (but no IL-4 production, data not shown). Moreover, a decrease in IFN-γ levels was observed in the

airways of ETS-exposed mice compared with the controls, although this difference was not statistically significant (Figure 3B).

Effect of *in utero* ETS exposure on changes in lung function

We examined the effects of *in utero* ETS exposure on lung function *in vivo* by measuring changes in R_L after MCh challenge. Our data demonstrate that ETS-exposed mice exhibited elevated AHR compared to FA-exposed mice (Figure 3D). These results indicate that ETS exposure induces a significant increase in AHR compared with the controls.

DNA methylation changes in response to *in utero* ETS exposure

A significant decrease in global DNA methylation level in ETS-exposed offspring mice can be seen from Figure 4(A) (70.51% for FA versus 66.50% for ETS). Additionally, changes in the methylation of genes associated with inflammation were examined in ETS-exposed offspring mice. There were no significant differences in the level of DNA methylation at the *IL-4* promoter (64.63% for FA versus 64.24% for ETS, Figure 4C) between the ETS- and FA-exposed mice. However, there was a significant increase in the level of *IFN-γ* DNA methylation in the ETS group compared with the FA group (71.46% versus 74.07%, Figure 4B). In contrast, the level of *IL-13* methylation was significantly lower in ETS-exposed mice compared with FA-exposed mice (89.25% for FA versus 87.30% for ETS, Figure 4D). Taken together, these methylation data indicate that *in utero* ETS exposure induces changes in the methylation of genomic DNA. Epigenetic changes in the promoter region of inflammation-related genes could play a major role in activation of the immune response. Furthermore, the identified hypomethylation pattern of the *IL-13* gene corresponds to our ELISA data that show a significant release of IL-13 cytokines from the ETS exposure samples.

Discussion

ETS contains over 4000 chemicals, including carcinogens that can cross the placental barrier, such as polycyclic aromatic hydrocarbons, arylamines, *N*-aromatic amines and nitrosamines (Chaichalotornkul et al., 2015). Experimental evidence indicates that the fetus and newborn are more susceptible to carcinogens than adults (Lackmann et al., 1999). Prenatal exposure to tobacco smoke, which occurs via the placental circulation, is associated with lower pulmonary function and increased prevalence of asthma in childhood and even in adulthood, suggesting a persistent heritable effect (Li et al., 2005). However, the effects of maternal ETS exposure on offspring's lung function are understudied. Therefore, we designed experiments to examine alterations of the genome and immune system in offspring from nonsmoking female mice exposed daily to ETS during pregnancy.

Allergic asthma is a chronic disease and the most common form of asthma (Eder et al., 2006). It starts in early life and is associated with airway inflammation and hyperreactivity (Holt et al., 2010). AHR, the hallmark of asthma, has been seen in animals exposed to tobacco smoke *in utero* (Wongtrakool et al., 2012). In this study, *in utero* ETS exposure resulted in a marked increase in the number of alveolar macrophages in the BAL fluid from offspring following birth (Figure 3A). A sensitive cytokine assay revealed an increase in the

level of IL-13 in the airways of prenatally ETS-exposed mice compared with FA controls (Figure 3C). Moreover, prenatal ETS exposure of mice significantly augmented AHR compared with FA-exposed mice (Figure 3D). Taken together, our results show that *in utero* ETS exposure increases the susceptibility to AHR and pulmonary inflammation in 6-week-old offspring mice. These results are consistent with previous publications (Singh et al., 2011; Wu et al., 2009). IL-13 in the airway induces pathologies that are highly characteristic of asthma, including mucus metaplasia, AHR and airway inflammation (WillsKarp, 2004). In addition to T cells, type-2 innate lymphoid cells also produce IL-13 in the lungs, essential to the development of allergic inflammation (Spits & Cupedo, 2012). Importantly, IL-13 has been shown to skew macrophage differentiation toward the M2-type involved in fibrosis and lung injury (Mills et al., 2000). Based on human and animal studies, alveolar macrophages have been found to regulate inflammatory responses in the airways, suggesting that these cells play an important role in asthma and lung injury (Balhara & Gounni, 2012). Several studies have demonstrated 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).

In our study, the *in utero* ETS-exposed group showed significant differences in the level of methylation, including global hypomethylation and gene-specific hyper- or hypo-methylation 6 weeks after birth. Previous studies indicated that the amount of global DNA methylation decreases in response to inflammation and oxidative stress (Baccarelli et al., 2009; Thomson et al., 2009). Similar to our global methylation data (Figure 4A), Breton et al. investigated global DNA methylation from children and found prenatal maternal smoking-related effects on global methylation (Breton et al., 2009). It has been clear that epigenetic mechanisms, including DNA methylation may be involved in maintaining Th1 and Th2 lineages (Jones & Chen, 2006; White et al., 2002). In recent years, epigenetic changes have been recognized as a potential mechanism underlying the establishment and maintenance of the Th2 bias in asthmatic and allergic symptoms (Kumar et al., 2009; van Panhuys et al., 2008). In response to allergens, demethylation of Th2 cytokine genes induces a change in the chromatin structure, allowing the DNA to open and recruit transcription factors such as GATA3 for immediate expression of Th2 cytokines (van Panhuys et al., 2008). CD4 + T cells revealed a significant increase in IFN-γ promoter methylation after allergen sensitization/challenge (Brand et al., 2012). Therefore, we investigated the effects of *in utero* ETS exposure on Th1/Th2 cytokine gene promoter methylation levels. In this study, we found significant differences in the level of DNA methylation at the *IFN-γ* and *IL-13* promotors between the FA-and ETS-exposed mice in lung tissue 6 weeks after birth (Figure 4B and D). Moreover, the identified hypomethylation pattern of the *IL-13* gene corresponds to our ELISA data that show a significant release of IL-13 cytokines from the *in utero* ETS exposed samples. Likewise, in a previous murine allergic asthma model, exposure to prenatal maternal smoking exacerbated AHR, IL-13 and Th2-polarizing responses in the lung (Singh et al., 2011). Infants exposed to tobacco smoke *in utero* have significantly higher IL-13 cytokine levels than those not exposed (Noakes et al., 2003). These findings support an association between *in utero* ETS exposure and regulation of a type-2 response that would be mediated through alterations in DNA methylation.

The exposure level of ETS used in our study is within a relevant particle concentration range that pregnant women might experience in public places where smoking occurs or other enclosed spaces when people burn tobacco products (Jinot et al., 1992). Actual exposure conditions to ETS during pregnancy can be highly variable – increasing the difficulty in extrapolating the effects observed in these mice following *in utero* ETS exposure to predictions related to children's health and development. However, several animal studies observed immune responses similar to ours (Avdalovic et al., 2009; Claude et al., 2012). Claude et al. (2012) used the same concentration of perinatal exposure to ETS and showed alterations in the pattern of inflammation in mice. The same amount of ETS exposure during a postnatal period is also associated with altered lung development in infant monkeys (Avdalovic et al., 2009).

In this study, we found the *in utero* ETS exposure group had a significantly lower level of methylation than FA exposed mice. Although the absolute difference in global genomic DNA methylation (4.01%) and *IL-13* methylation (1.95%) is small, it is in agreement with other previous studies measuring LINE-1 methylation and T–relevant genes, including *IL-13*. Guerrero-Preston et al. (2010) reported that the level of LINE1 methylation was 8.1% significantly lower in newborns prenatally exposed to secondhand smoke than in unexposed ones. Lee et al. (2013) also identified a 0.65% and 1.12% lower level of LINE-1 methylation and *IL-13* in a maternal smoking group than in controls. The methylation profile in our study represents the aggregate methylation profile of a complex cellular mixture, since DNA from whole lung was collected. Thus, even small changes in percentage methylation may indicate considerable differences in underlying cell populations. The potential impact of changes found in DNA methylation can modify gene expression in a small proportion, but these changes along life can result in important effects.

In addition, this study does not address a direct causal link between the changes in methylation and the changes in cytokines levels and/or AHR. Future studies should be done to determine how DNA methylation is involved in the ETS response. Additional dose-response studies with methyl donors like folic acid or the phytoestrogen, genistein, while assessing multiple time points of methylation measurement, would provide further information of causality.

Conclusions

In this study, we provide the first evidence that differences in methylation patterns occur in early age from exposure to *in utero* ETS and that these differences can be detected in lung tissue DNA from mice 6 weeks after birth. These results support a potential role of epigenetics in fetal growth and developmental programming following *in utero* ETS exposure across the life course. Furthermore, our data suggest that *in utero* ETS-induced changes in DNA methylation patterns could be related to the expression of type-2 cytokines and thereby promote AHR development and pulmonary inflammation.

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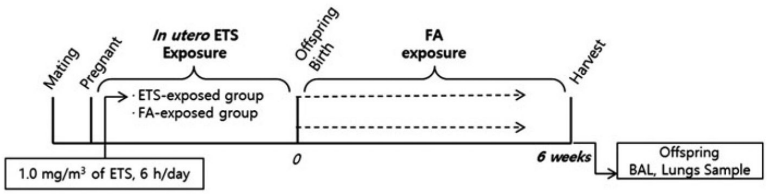

Figure 1.

Experimental model and timeline. After confirmation of vaginal plug, female mice were exposed to FA or 1.0 mg/m^3 of ETS for 6 hours/day 7 days/week. After the birth, pups were only exposed to FA until weaning. All mice were quarantined for at least 3 weeks. 6 weeks after birth, offspring were sacrificed and lungs and BAL fluid were harvested.

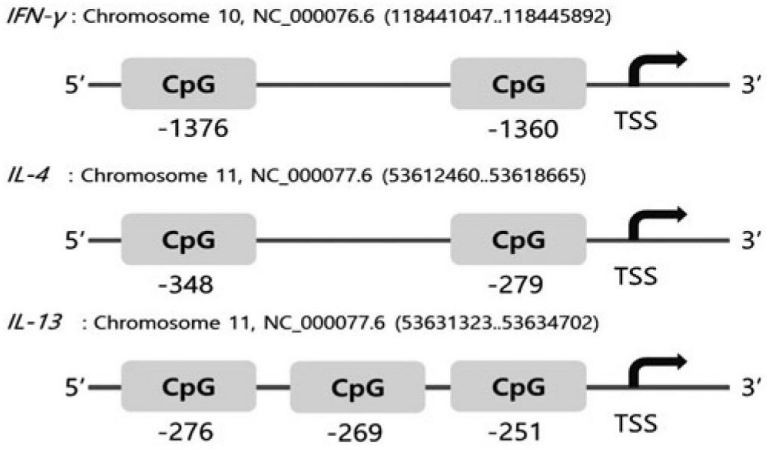

Figure 2.

Position of target genes and CpG sites in their promoter that were analyzed in the study. Pyrosequencing reactions were performed to investigate CpG sites on the *IFN-γ*, *IL-4* and *IL-13* gene promoter regions.

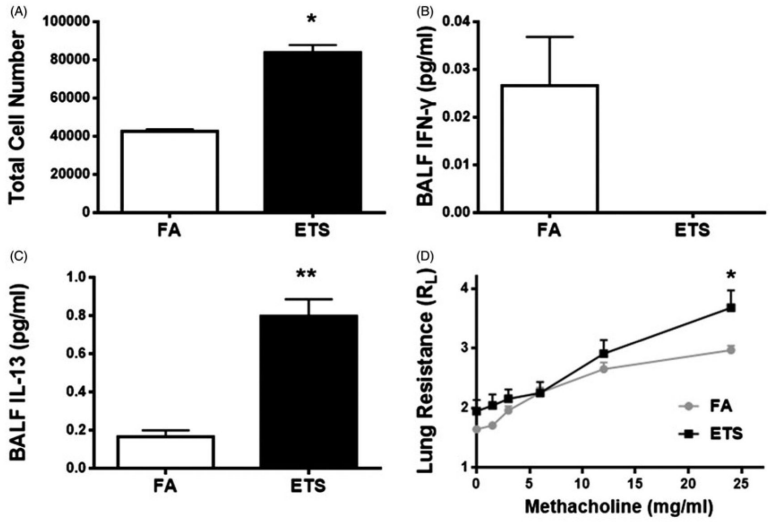

Figure 3.

In utero ETS exposure induces changes in BAL cellularity and pulmonary function in mice. (A) The total number of alveolar macrophages in the BAL. (B) The expression level of IFN-γ in the BAL. (C) The expression level of IL-13 in the BAL. (D) Changes in lung resistance (R_L) in response to different doses of MCh in FA-and ETS-exposed mice. A single asterisk indicates significance at p50.05 and two asterisks indicate p<0.01 compared with the controls. Data are mean ± SEM. n = 4–6 per condition.

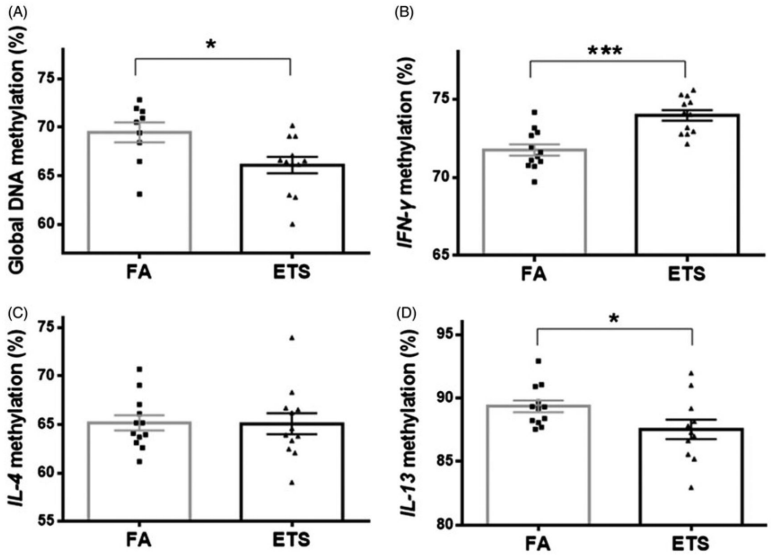

Figure 4.

Global and promoter DNA methylation status in lung tissue DNA from offspring. (A) ETS-exposed mice had a significantly lower level of global DNA methylation than the FA-exposed ones. (B) *IFN-γ* showed a significant difference in DNA methylation levels between ETS- and FA-exposed groups. (C) No changes in methylation were observed for *IL-4*. (D) *IL-13* showed a significant decrease in DNA methylation levels in ETS-exposed mice compared to the FA group. A single asterisk indicates significance at $p<0.05$ and three asterisks indicate $p<0.001$ compared to the controls. Data are mean ± SEM. $n = 9–12$ per condition.

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)
IFN-$_\gamma$ (15978)	Forward	AATGGTGTGAAGTAAAAGTGTTTTTAGA	53.4	108
	Reverse	AAAATTTCCTTTCCACTCCTTAAACTCTC[a]		
	Sequencing	ATGGTATAGGTGGGTA		
IL-4 (16189)	Forward	AGGGGTTTTTATAGTAGGAAGTAG	51.9	178
	Reverse	CCCCCCTTTTTTTTTAAATCTACAA[a]		
	Sequencing	AGATTTTTTTGATATTATTTTGTT		
IL-13 (16163)	Forward	GTTAGTATTGGGTTGGTTGTTTAGGA	51	237
	Reverse	ATTATCTAAAAACCACATCTTTACTCAT[a]		
	Sequencing	GTTGGTTGTTTAGGAG		

[a]Biotin-labeled primer.