Epigenetic Changes after Prenatal Environmental Tobacco Smoke Exposure and Asthma

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EPIGENETIC CHANGES AFTER PRENATAL ENVIRONMENTAL TOBACCO SMOKE EXPOSURE AND ASTHMA

By

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B.S, B.S., Briar Cliff University, Sioux City, Iowa, 2010

Thesis

presented in partial fulfillment of the requirements for the degree of

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In Toxicology

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ABSTRACT

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Toxicology

Epigenetic changes after prenatal environmental tobacco smoke exposure and asthma

Chairperson: Yoon Hee Cho, MPH, PhD

Allergic asthma affects over 300 million people worldwide. Multiple factors have been shown to alter an individuals’ susceptibility to this allergic airway disease such as exposure to polycyclic aromatic hydrocarbons (PAHs), endotoxin, and environmental tobacco smoke (ETS). Prenatal exposure to these contaminants, especially ETS, has been shown to increase risk for an individuals’ asthma risk, however, the mechanism by which this happens is still unknown. Epigenetic alterations, particularly DNA methylation patterns, have been introduced as a proposed mechanism. The goals of this project were to use a house dust mite (HDM) murine model of asthma to determine the methylation alterations of asthma-associated genes and the immunological effects after prenatal exposure to a low dose of ETS. The asthma-associated genes used in this experiment are genes encoding for: Th2 cytokines, II-4 and II-13, Th1 cytokine, Ifn-γ, and regulatory molecule, Foxp3. Male C57BL/6 mice were exposed prenatally to 1.0 mg/m³ of ETS and later challenged with HDM at 6 weeks old. Promoter regions of the asthma-associated genes were analyzed via bisulfite sequencing, using a Qiagen® PyroMark Q96MD pyrosequencer. Cytokines in bronchoalveolar lavage fluid (BALF) were analyzed using Quantikine ELISA (R&D) for IL-13 and Mesoscale Discovery® Pro-inflammatory Panel 1 kit for IFN-γ, IL-4 and IL-5. Serum IgE levels were determined using a BioLegend IgE ELISA kit. We concluded that prenatal exposure to low doses of ETS causes a severe increase in the immune response when challenged with HDM. We also found Ifn-γ to be hypermethylated in the HDM group and further hypermethylated after prenatal ETS exposure and II-4 to be hypomethylated in the HDM group and further hypomethylated after ETS exposure. This suggests that prenatal ETS exposure increases the susceptibility to an allergic and asthmatic response when exposed to allergen, with promoter methylation as a proposed mechanism.
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LIST OF ABBREVIATIONS AND SYMBOLS

5-mC: 5-methylcytosine

AAAAI: American Academy of Allergy, Asthma, and Immunology

AHR: airway hyperresponsiveness

ANOVA: Analysis of variance

APC: antigen presenting cell

BALF: bronchoalveolar lavage fluid

bp: base-pair

CD: cluster of differentiation

CpG: C—phosphate—G

DC: Dendritic cell

DerP: *Dermatophagoides pteronyssinus* (Genus species of house dust mite)

DNA: deoxyribose nucleic acid

ELISA: enzyme-linked immunosorbent assay

EPO: eosinophil peroxidase

ETS: Environmental tobacco smoke

FA: Filtered air
Foxp3: forkhead box P3

H&E: Hematoxylin and eosin stain

HDM: House dust mite

Ifn-γ: Interferon – gamma

Ig: immunoglobulin

IL: interleukin

PAH: poly-aromatic hydrocarbons

PAS: Periodic acid-Schiff stain

SEM: standard error of the mean

SHS: Second hand smoke

Th: T helper

THS: Third hand smoke

Treg: T regulatory
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CHAPTER 1

INTRODUCTION

The prevalence of asthma, the most common chronic disease in children, has increased dramatically during the last two to three decades (Bloemen et al. 2007). According to the American Academy of Allergy, Asthma & Immunology, more than 300 million individuals are affected globally, and that statistic is predicted to increase by over 100 million before the year 2025 (AAAAI). People diagnosed with allergic asthma experience intense, recurring episodes of wheezing, shortness of breath, chest tightness and cough. In 2008, approximately 250,000 people died from preventable asthma-related complications (AAAAI). Asthma is not only a detrimental public health burden, but also an economic burden as it accounted for more than $10 billion in direct health care costs in the United States annually (Eder et al. 2006). Even with increased research, asthma continues to have a complex and poorly understood etiology, involving both genetic and environmental factors.

It is thought that early life exposures severely increase one’s risk for asthma development later in life. The early origins of adult disease hypothesis proposes that adverse influences or exposures early in development cause organs to undergo in-utero developmental programming which predetermines subsequent physiologic and metabolic adaptations during adult life which increases disease risk. This early origins hypothesis, or Barker Hypothesis (De Boo & Harding 2006), has been introduced as a possible underlying cause for development of allergic asthma. Early life or in-utero exposures to harmful environmental toxins may alter the epigenetic programming of genes related to allergic
asthma. Epigenetic alterations are heritable changes that can regulate gene activity without altering the primary DNA sequences. With respect to allergic or asthma phenotypes elicited by environmental exposures, Th2 cytokines (IL-4, IL-5, IL-13), transcriptional factors (e.g., GATA-3), and T-regulatory (Treg) factor (FOXP3) associated with allergic disease (Ho 2010; I. J. Wang et al. 2013; Bégin & Nadeau 2014) have also been shown to be modulated by epigenetic alterations.

Of early life exposures, environmental tobacco smoke (ETS) continues to be one of the most harmful environmental toxicants. ETS exposure can lead to poor respiratory health among susceptible children, and still remains a major public health concern. Especially prenatal exposure to ETS that have effects that may be transmissible from mother to fetus has been associated with later development of asthma-related outcomes in children and adults. This provides compelling evidence that in utero exposure to ETS influences fetal immune development and in programming for the susceptibility to allergic diseases (Singh et al. 2011). This early exposure results in epigenetic modification in the DNA, which may be a mechanism of early origins of adult disease.

Multiple types of environmental and occupational exposures have been shown to modify risk for asthma development; however, these factors have been unable to independently explain the cause of asthma. Discovery of preventable factors and the underlying mechanisms that exacerbate asthma is important for mitigating the health burden of this childhood disease.

1.1 Environmental Tobacco Smoke (ETS)

Despite significant declines during the past 30 years, cigarette smoking among
adults in the United States remains widespread, and significant year-to-year decreases have been observed only sporadically in recent years. Smoking has been banned in many public places, which has resulted in a general decreased population exposure to cigarette smoke. It is estimated, based on data from the 2012 National Health Interview Survey (NHIS), that the proportion of U.S. adults who smoke cigarettes is 18.1% or 42.1 million American adults (age 18+). This is not dramatically less than in 2005 when NHIS predicted 20.9% of U.S. adults identified as smokers (Agaku et al. 2014). Among smoking adults, 78.4% (33.0 million) smoked every day averaging 14.6 cigarettes per day(Centers for Disease Control and Prevention (CDC) 2011).

Second hand smoke (SHS) is defined as a mixture of the sidestream smoke, which is the smoke emitted from the burning cigarette, pipe, or cigar and the mainstream smoke which exhaled from the lungs of the smoker. SHS contains more than 4,000 chemicals, hundreds of which are known or suspected carcinogens or other contributors to adverse health effects. These chemicals include, but are certainly not limited to, ammonia, acrolein, carbon monoxide, formaldehyde, hydrogen cyanide, nicotine, nitrogen oxides, polycyclic aromatic hydrocarbons (PAHs), and sulfur dioxide (Matt et al. 2011). These chemicals are eye and respiratory irritants, mutagens, carcinogens, and cardiovascular and reproductive toxicants(Office on Smoking and Health (US) 2006). Third hand smoke (THS), sometimes referred to as the legacy of tobacco smoke, consists of residual tobacco smoke chemicals that remain on surfaces and in dust after tobacco has been smoked. These chemicals are re-emitted into the gas phase, or react with other compounds in the environment to yield secondary pollutants. The constituents of THS identified to date include nicotine, phenol, cresols, naphthalene, formaldehyde, and tobacco- specific nitrosamines, and many others
(Matt et al. 2011). These chemicals and their secondary pollutants from tobacco smoke can persist in environments long after the tobacco smoking has ceased. Some of the pollutants present in SHS predominantly remain in the gas phase and can be removed by ventilation, but a significant fraction of SHS and THS adheres to indoor surfaces which allows the toxic chemicals to persist for a longer time (Matt et al. 2011).

Exposure to environmental tobacco smoke (ETS), defined as a combination of SHS and THS, remains a major public health concern, as the decrease in adult smokers has been slight and intermittent. Pregnant women and children remain most susceptible to the highest levels of ETS exposure because, despite the increased regulations, smoking in one's home or automobile remains legal (Office on Smoking and Health (US) 2006). Parental smoking, particularly maternal smoking, is strongly linked to allergic asthma, infection in children, and other disease susceptibility. Similarly, mice exposed to SHS during early postnatal life are more susceptible to developing respiratory infections (Claude et al. 2012). There is an increase in convincing evidence that in utero exposure to ETS influences fetal immune development and programming influencing susceptibility to allergic diseases.

1.2 Allergic Asthma

Allergic asthma, the most common form of asthma, is a chronic inflammatory airway disease characterized by pulmonary eosinophilia, increased mucus production by goblet cells, elevated immunoglobulin E (IgE) levels and structural remodeling of the airway wall, leading to airway obstruction, bronchial inflammation and airway hyperreactivity (AHR) to nonspecific stimuli. The allergic process is believed to result from allergens inducing the
infiltration of CD4+ Th2 cells, eosinophils, mast cells, basophils, and other allergic mediators into the small airways. Allergen-specific CD4+ Th2 cells, producing cytokines such as IL-4, IL-5, IL-9 and IL-13 are prevalent in driving the mucosal inflammation in asthma.

Figure 1 illustrates a schematic representation of the inflammatory cascade in allergic asthma (Ho 2010). Allergen sensitization is a prerequisite to development and initiation of the inflammatory cascade. Airway epithelium is exposed to various environmental toxicants, which are then sampled by local dendritic cells (DCs). The allergen-activated DCs prime naive CD4+ T cells to differentiate into proinflammatory Th2 cells rather than infection-fighting Th1 cells, whose main effector cells are macrophages. The gradual increase in the recruitment of CD4+ T cells toward a Th2 phenotype is primarily driven by the Th2 cytokines: IL-4, IL-5, IL-9, and IL-13, which result because of heightened expression of transcription factor GATA-3. Briefly, IL-4 generally promotes IgE production and is involved in growth and activation of mast cells, IL-5 promotes eosinophil development, activation, and tissue recruitment, and IL-13 stimulates mucus production and secretion (Bloemen et al. 2007; Verstraelen et al. 2008). As for the counterpart of T cell differentiation, the Th2 cells shut off or inhibit the expression of IFN-γ and other Th1 cytokines, such as IL-2. TGF-β also plays a role in causing naive CD4+ T cells to differentiate into FOXP3+ Treg cells that play a role in immune tolerance and regulate immune processes in order to reduce allergic or inflammatory responses. Alveolar macrophages play a dual role in toxicant/allergen elimination as well as suppression of the responses for airway repair and remodeling. Once activated, Th2 cells have the capacity to recruit secondary
effector cells such as macrophages, basophils and eosinophils to the site of inflammation where these cells become primed and activated for mediator secretion (Bloemen et al. 2007).

**Figure 1.** Environmental factor–induced immune cell regulation of allergic airway responses (Ho 2010).

1.3 Epigenetics

The early origins of adult disease hypothesis proposes that adverse influences or exposures early in development cause organs to undergo in-utero developmental programming which predetermines subsequent physiologic and metabolic adaptations during adult life which increases disease risk. This hypothesis originated from a study on
English populations dating back to the early 20th century, led by Dr. DJ Barker, that determined a relationship between low birth weight and the development of coronary heart disease (Barker 2004). This study inspired other studies to correlate birth weight to disease risk in adulthood, including type II diabetes, hyperlipidemia, and even allergic asthma (De Boo & Harding 2006). The theory behind this relationship is that early life exposures to environmental and lifestyle factors affect the programming of the offspring. Programming is the process whereby a stimulus or insult during a sensitive period of time, or window, has long-term irreversible developmental effects (De Boo & Harding 2006). One of the proposed mechanisms of programming is epigenetics.

Epigenetic modifications are heritable changes that can regulate gene activity or expression without altering the primary DNA sequences. Environmental exposures and maternal diet early in development have shown to alter epigenetic patterns of offspring, especially DNA methylation (Jirtle & Skinner 2007).

1.3.1 Epigenetic Mechanisms

There are three epigenetic mechanisms studied today: histone modification, non-coding (micro) RNA, and DNA methylation. Histones are a family of alkaline proteins that associate with DNA in the nucleus to aid in the condensation of nucleosomes, the structural unit of chromatin. Histones can be modified by methylation, acetylation, phosphorylation, addition of various amino acids, ubiquitylation as well as others (Yang & Schwartz 2012). These modifications affect the condensation of DNA and may block or open transcription sites. Another epigenetic mechanism is microRNA, which are small non-coding RNA
segments that block translation of messenger RNA into proteins (Rodriguez et al. 2004). MicroRNAs can inhibit translation in two ways; the first is by binding to single stranded mRNA and not allowing ribosome to bind. Alternatively, they can bind to the ribosome itself so that the ribosome cannot bind to other mRNAs to make proteins. Non-coding RNA is the least studied epigenetic mechanism, but holds great promise for disease pathology and drug development (Yang & Schwartz 2012). Lastly, the most studied epigenetic mechanism is DNA methylation, where a methyl group is added to the 5-position of the nucleotide cytosine, particularly at cytosine-guanine dinucleotide residues (CpG). There are approximately 30 million CpG sites within the human genome and they tend to cluster within gene promoter regions (100-1000 bp upstream of the start codon), these clusters are referred to as CpG islands. Hypermethylation of these islands in gene promoter regions leads to decreased gene expression and hypomethylation elicits an increase in active gene transcription (Yang & Schwartz 2012). Figure 2 illustrates how DNA methylation, histone modification and non-coding RNA inhibit the transcription of mRNA and gene expression. White circles symbolize unmethylated CpGs and black circles denote methylated CpGs.
1.3.2 Epigenetics and Asthma

With respect to allergic or asthma phenotypes elicited by environmental exposures, epigenetics, especially DNA methylation, is thought to play a huge role in antigen presenting cell (APC), T-cell differentiation and allergic cytokine production (Yang & Schwartz 2012). Differentiation of APCs is a critical first step to the differentiation of naïve T-cells into effector T-cells (Th1, Th2, or Th17) or regulatory T-cells (Treg) (Miller & Ho 2008). In allergic asthma, there is a trend to suppress the Th1 profile and favor a Th2
response with epigenetic alterations as a proposed mechanism (Calderon et al. 2009). Th2 cytokines IL-4, IL-5, IL-13 and transcription factor GATA-3 have been shown to have promoter regions which are generally demethylated under allergic conditions (Miller & Ho 2008). In parallel, expression of IFN-γ in Th2 cells is silenced by increased promoter methylation (Jones & Chen 2006). Studies also suggest that expression of Treg factor, Foxp3, is also epigenetically regulated by DNA methylation. FOXP3 expressing T-cells play a crucial role in maintaining immune homeostasis and preventing autoimmune reactivity and hypersensitivity (Huehn et al. 2009). Excess DNA methylation on histones and within CpG islands of the promoter region block transcription factors and prevent stable FOXP3 expression in effector T cells, while cells stably expressing FOXP3 are demethylated in these regions (Lal et al. 2009). In addition, a study population of monozygotic twins discordant for asthma concluded that FOXP3 and IFN-Y were shown to be hypermethylated in white blood cells from the twins with asthma. This hypermethylation was also increased if the twins were also discordant for second hand smoke exposure (Runyon et al. 2012).

DNA methylation patterns are critical for normal T cell differentiation, but early life exposures to various agents, including tobacco smoke, polycyclic aromatic hydrocarbons (PAHs), particulate matter, and maternal diet can alter these patterns and contribute to asthma pathogenesis (Ho 2010). Few studies have been reported analyzing the effect of prenatal tobacco smoke exposure and how it may potentially contribute to asthma etiology by epigenetic mechanisms, particularly DNA methylation.

1.4 Specific Aims
This study was designed to research the effect of prenatal ETS exposure on epigenetic programming and hypothesized increased susceptibility to development of allergen-induced airway inflammation. In order to complete this goal, I used a HDM allergen-induced mouse model of asthma to examine the effect of in utero ETS exposure on susceptibility to an asthmatic phenotype via the proposed mechanism of epigenetic programming by alterations in DNA methylation of genes associated with asthma. For purposes of consistency and to minimize confounding factors, I considered only male mice for this experiment. My central hypothesis states that prenatal exposure to ETS in utero will lead to alterations in DNA methylation that will correlate with increased severity and/or risk for indicators of asthma development. I created the following aims to test this hypothesis:

**Specific Aim 1: Epigenetic Alterations**

Determine the promoter region DNA methylation of genes associated with allergic phenotype of male C57BL/6 mice exposed to 1.0 mm/m³ environmental tobacco smoke (ETS) prenatally and inoculated and challenged with HDM at 8 weeks old and relate to gene expression. My hypothesis states that promoters of regulatory molecules (Ifn-γ, Foxp3) will be hypermethylated and promoter regions of pro-allergic cytokine (Il-4, Il-13) will be hypomethylated. The effects on the following epigenetic alterations were evaluated:

(i) Promoter methylation of Th2 genes: Il-4 and Il-13

(ii) Promoter methylation of Th1 gene: Ifn-γ

(iii) Promoter methylation of Treg gene: Foxp3
Specific Aim 2: Immunologic Alterations

Determine extent of immunological susceptibility to HDM challenge after prenatal exposure to ETS. I hypothesized that mice exposed prenatally to ETS will have a more severe immune response (increased serum IgE, mucus production, presence of eosinophils, and IL-4, IL-5 and IL-13 production in BAL fluid) to HDM challenge, therefore increasing their susceptibility and severity of disease. The effect on the following responses were evaluated:

(i) Cytokines present in BAL fluid (IL-4, IL-5, IL-13, and IFN-γ)
(ii) Immune cell types present in BAL fluid
(iii) IgE levels present in serum
CHAPTER 2

METHODS

2.1 Animals

C57BL6 mice were purchased from Harlan Laboratories, Indianapolis Indiana. All mice were maintained in pathogen-free conditions in the animal facility at either University of California – Davis (Davis, CA) or the University of Montana (Missoula, MT). All experiments were performed to the guidelines of the National Institutes of Health and approved by the University of Montana Institutional Animal Care and Use Committee (IACUC).

2.2 ETS Exposure

Collaborators at UC Davis’ CHE animal facilities carried out the ETS exposure. The mice used in my experiment were a small part of the larger exposure project. The larger project consisted of mating of 2 female mice, paired with 1 male mouse/cage to create a timed-pregnant exposure scenario. Specifically, 36 female and 18 male C57BL6 mice were used for breeding. Following confirmation of a vaginal plug, 18 female mice were exposed to either filtered air (FA) or ETS through gestation. For the control group, timed-pregnant mice were exposed only to filtered air only for 24 h 7d/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/m³ of tobacco smoke for 6 h/day. Research cigarettes (3R4F, University of Kentucky) were burned at a rate of two cigarettes every 10 min with a puff
volume of 35 mL over 2 s, 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 (1.0 ± 0.17 mg/m³). The carbon monoxide level was 4.8 ± 0.8 ppm, and the average temperature was 73 °F.

Once the dams gave birth, the dams and pups were only exposed to only 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. Of the offspring, 48 males were used for my project and separated to receive HDM or PBS inoculations.

2.3 Murine Model of Asthma

![Figure 3: Experimental Model and Timeline.](image-url)
A house dust mite murine model of airway disease was used to investigate the effect of prenatal ETS exposure on increased risk or exacerbation of Th2-mediated pulmonary inflammation and AHR. There are several species of HDM, however only two are known to elicit an allergic response, one of which is *Dermatophagoides pteronyssinus* (DerP). The exoskeletons of DerP were mashed and suspended in PBS at various doses to be administered intranasally. Male offspring (6 weeks old) were sensitized and challenged to HDM allergen over a period of 2 weeks by intranasal administration of HDM (or PBS control) on day 0 (100 μg) and days 7 and 14 (50 μg), as previously described (Hammad et al., 2009). On day 16, 48 hours after last exposure, mice were harvested and blood, BAL fluid and lung tissue were collected for analysis of allergen-induced pulmonary inflammation and epigenetic studies.

### 2.4 Gene Specific Methylation Analysis

In order to measure methylation levels, the pyrosequencing assay was used for selected gene-specific methylation. Genomic DNA will be extracted from murine lung samples according to the manufacturer’s protocol included with the DNeasy Blood & Tissue Kit (Qiagen).

#### 2.4.1 Primer Design
Gene specific murine primers were designed for *Ifn-γ*, *Foxp3*, *Il-4* and *Il-13* using PyroMark Assay Design 2.0 software (Qiagen). Primers were designed for analyzing 2 CpGs within the promoter regions.

Figure 4 illustrates the CpG content of the 1000 bp promoter region, with the red bar representing the region analyzed by pyrosequencing. The Y locations represent each CpG location. In order to design the primer, CpG islands located in the promoter region (500-1000 bp upstream of start codon) are highlighted and the location on the gene is denoted. Gene regions were located using NCBI and GenBank. Next, the sequence was input into Qiagen’s Assay Design Software 2.0, which converted the original promoter sequence to represent bisulfite converted DNA. Then, a region of interest (100-150 bp in length) was selected highlighting a minimum of 2 CpG locations. After a region of interest was selected the software designs the forward, reverse, and sequencing primers, noting which primer is biotinylated, and assigned each primer set a score. For this project, no primer score below 75 was accepted. Table 1 lists the promoter regions of the genes used in the project. Table 2 lists the primers designed for this project.

*Figure 4: CpG content and region of interest within gene promoter region*
2.4.2 Bisulfite Treatment, PCR, & Pyrosequencing

For the pyrosequencing assay, 50 ng of purified DNA was processed by bisulfite treatment using the EZ DNA Methylation™ Kit (Zymo Research). PCR for the new primer sets were optimized and amplification was verified using gel electrophoresis.
PCR amplification of the 50ng of bisulfite converted DNA was conducted and PCR reactions were performed using Pyromark PCR Kit (Qiagen, Valencia) 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. Each set of amplifications will include CpGenome universal 100% methylated mouse DNA.

Following amplification, the biotinylated PCR products was purified and incubated with the sequencing primer designed to bind adjacent to the CpG sites of interest. Pyrosequencing was conducted using a PyroMark Q96 MD instrument (Qiagen), with subsequent quantification of methylation levels determined by the PyroMark-CpG software. Relative peak height differences were used to calculate the percentage of methylated cytosines at each given site. Percent methylation within a sample was subsequently determined by averaging across all interrogated CpG sites in the analysis. Non-CpG cytosine residues were used as internal controls to verify efficient sodium bisulfite DNA conversion.

2.4.3 Pyrosequencing Data Analysis

Prenatal ETS exposures and DNA methylation outcomes among offspring were also assessed. Quantitative percent changes in methylation were evaluated as a continuous dependent variable. T-test was used to examine the difference of methylation levels between ETS exposed and sham exposed groups. The difference of methylation levels by exposure status to ETS as well as asthma induction by HDM exposure was examined using
a two-way analysis of variance (ANOVA) followed by appropriate post hoc test to compensate for increased type I error. ETS exposure was considered separately and evaluated for associations using linear regression for each gene (i.e., CpG promoter regions for IL-4). Two-tailed statistical significance was defined as a probability of type I error occurring at less than 5% ($P < 0.05$). Statistical power was greater than 80% (0.8). Statistical analysis was performed using PRISM 5.0.

2.5 Immunological Assays

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

2.5.1. Measurement of Cytokines

*MSD Cytokine Analysis*

MSD, or Meso Scale Discovery, detects cytokines similarly to ELISA but is based on MULTI-ARRAY® technology, a proprietary combination of electrochemiluminescence detection and patterned arrays. Electrochemiluminescence detection uses labels that emit light when electrochemically stimulated. Background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light).

The level of 10 different cytokines in mouse BAL fluid was measured. Training on the use of the QuickPlex SQ 120 instrument and the MSD Workbench software was also
completed. The MSD Mouse V-Plex Pro-Inflammatory Panel 1 (MSD) was used and cytokines IFN-γ, IL-1β, IL-10, IL-12 p70, IL-2, IL-4, IL-5, IL-6, KC/GRO, TNF-α were measured. The dynamic range of all of the assays was 3.5-4+ logs, with sensitivities in the 10s to 100s of fg/mL for most assays.

**IL-13 ELISA**

IL-13 was measured in the BAL fluid using the Quantikine ELISA kit (R&D).

**2.5.2 Cell Differential Count**

Also from the bronchoalveolar lavage, cytospin preparations were performed on 5x10⁴ BAL fluid cells after staining the cells using a Wright-Giemsa-protocol (Hema 3 Staining kit, Fisher Scientific, Houston, TX). Cell differential percentages were determined by light microscopic evaluation of stained cells and expressed as absolute cell numbers.

**2.5.3 Serum IgE Analysis**

Blood was collected via cardiac bleed and samples were allowed to sit on ice for 20 minutes after collection. Samples were centrifuged at 14000 RPM for 10 minutes. Serum fraction was collected, making an effort not to disturb the pellet. Samples were stored in -20°C until ready to do ELISA. Serum samples were analyzed at a 1:4 dilution using BioLegend IgE ELISA kit.
2.5.4 Data Analysis

The significance of the differences between the ETS and/or HDM exposure groups was determined by t-test, one-way, or two-way ANOVA, in conjunction with post hoc test for variance, where appropriate. Graphics and analyses were performed on SPSS or Prism software, version 5 (GraphPad, La Jolla, CA), depending on the complexity of the design. The criterion for significance was set at $P < 0.05$.

2.6 Support

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CHAPTER 3

RESULTS

3.1 Specific Aim 1: Epigenetic Alterations

In allergic airway hyperactivity, a Th2 response is favored and inhibits both Th1 and Treg responses. In order to illustrate a Th2 bias the methylation level of multiple murine genes were assessed. In particular, we assessed the promoter regions of murine genes *Il-4* and *Il-13*, which when expressed allow for production of cytokines heavily involved in the Th2 response. In order to illustrate a lack of a Th1 response, the methylation of murine gene *Ifn-γ*, which translates into a Th1 cytokine, was also assessed. Lastly, the promoter methylation of a representative gene that translates into regulatory molecules, or Treg cells, *Foxp3* was assessed. In order to perform the methylation analysis, DNA was isolated from murine lungs and after bisulfite treatment was amplified with gene-specific primers and the methylation was analyzed via pyrosequencing.

3.1.1 Genes representing Th2 bias

*Il-4*

*Il-4* is a murine gene that, when expressed, produces pro-allergic cytokine IL-4. IL-4 is a multi-functional cytokine involved in Th2 cell differentiation and also in eosinophil recruitment (Bloemen et al. 2007). The promoter methylation of the gene *Il-4* was analyzed in order to determine if prenatal ETS exposure altered methylation patterns of this pro-allergic gene. Figure 5 shows significant hypomethylation of the HDM group when compared to the PBS exposed group (65.33% +/- 0.95 vs. 68.57% +/- 0.68, respectively). Significant hypomethylation was also observed when adjusting for prenatal ETS exposure
(FA: 66.09% +/- 0.90 vs. ETS: 64.57% +/- 1.00).

**Figure 5: IL-4 hypomethylation after prenatal ETS exposure.** IL-4 is significantly hypomethylated in the HDM exposed groups and is also hypomethylated further if exposed prenatally to ETS. Two-tailed ANOVA with Bonferroni Post-Hoc tests. Data are means +/- SEM, n=6 **p<0.01 ***p<0.001
**Il-13**

Murine gene *Il-13* translates to the asthma-related cytokine IL-13. IL-13 is crucial for eosinophil recruitment and is secreted primarily by Th2 cells. Figure 6 shows a trend of hypomethylation, but no significant difference of the PBS group when compared to the HDM exposed group (68.12% +/- 0.79 vs. 67.33% +/- 0.37, respectively). No significant hypomethylation was also observed when adjusting for prenatal ETS exposure (FA: 66.96% +/- 0.48 vs. ETS: 67.78% +/- 0.26).

**Figure 6: Il-13 methylation results.** Il-13 is not significantly hypomethylated in the HDM exposed group, but shows a hypomethylation trend. No significance is found when adjusting for prenatal ETS exposure. Two-tailed ANOVA with Bonferroni Post-Hoc tests. Data are means +/- SEM, n=6.
3.1.2 Gene representing Th1 response

Ifn-γ

Methylation results are displayed in Figure 7. The methylation levels show significant hypermethylation of the HDM group when compared to the PBS exposed group (70.18% +/- 0.69 vs. 72.92% +/- 0.90, respectively). Significant hypermethylation was also observed when adjusting for prenatal ETS exposure (FA: 71.78% +/- 0.91 vs. ETS: 74.06% +/- 0.89).

Figure 7: Hypermethylation of Ifn-γ after HDM and prenatal ETS exposure. Ifn-γ was significantly hypermethylated in the HDM exposed groups and is hypermethylated further if exposed prenatally to ETS. Two-tailed ANOVA with Bonferonni Post-Hoc tests. Data are means +/- SEM, n=6 *** p<0.001
3.1.3 Gene representing Regulatory Response

**Foxp3**

Figure 8 shows no significant difference in methylation levels in any exposure group. There is a no trend of hypermethylation when comparing PBS groups to HDM group (79.25% +/- 0.85 vs. 79.58% +/- 1.02). Nor is there a difference when adjusting for prenatal ETS exposure (FA: 80.17% +/- 1.05 vs. ETS: 78.98 +/- 1.27).

![Figure 8: Foxp3 methylation results.](image)

*Figure 8: Foxp3 methylation results. The methylation levels of the promoter region of Foxp3 are not significantly different between the groups. Two-tailed ANOVA with Bonferroni Post-Hoc tests. Data are means +/- SEM, n=6.*
3.2 Specific Aim 2: Immunological Responses

In order to determine if prenatal ETS exposure increased susceptibility to allergic airway disease pro-allergic cytokines, inflammatory cell numbers, and serum IgE were analyzed. The cytokines IL-4, IL-5, and IL-13 were analyzed in BAL fluid in order to determine if Th2 polarization was taking place, as these cytokines play a huge role in T-cell differentiation and the Th2 response. IFN-γ was also measured in the BAL fluid to determine the Th1 response, or lack thereof. In addition to cytokine measurement, inflammatory cell types were determined in order to distinguish an allergic response or inflammatory response. To further support the data, IgE levels in the serum were evaluated to determine the extent of allergic response present.

3.2.1 Cytokine Analysis

**IL-4**

IL-4 is a very important cytokine, it not only is produced and released by Th2 cells, but also plays a role in differentiating naïve T-cells (Bloemen et al. 2007). IL-4 plays a role in eosinophil recruitment and infiltration and also is secreted by eosinophils in order to create a positive feedback loop (Kay et al. 1997). To determine the susceptibility to allergic airway hyperreactivity after prenatal ETS exposure, IL-4 was measured in the BAL fluid by QuickPlex SQ 120 instrument and the MSD Workbench software using the MSD Mouse V-Plex Pro-Inflammatory Panel 1. Our results show that IL-4 was not produced in the PBS control groups, and was significantly increased by prenatal ETS exposure in the HDM challenged groups.
Figure 9: Increased production of IL-4 after prenatal ETS exposure and HDM challenge.

IL-4 was significantly increased in BAL fluid of mice exposed prenatally to ETS and challenged with HDM at 6 weeks old. Data are means ± SEM n=6. * indicates P values < 0.05.
**IL-5**

IL-5 is a critical cytokine in allergic airway disease and asthma. It is secreted primarily by Th2 cells but also by mast cells. IL-5 stimulates immunoglobulin secretion by B cells and is also a key mediator in eosinophil activation, along with IL-13 (Bloemen et al. 2007; Kay et al. 1997). To determine the susceptibility to allergic airway hyperreactivity after prenatal ETS exposure, IL-5 was measured in the BAL fluid by QuickPlex SQ 120 instrument and the MSD Workbench software using the MSD Mouse V-Plex Pro-Inflammatory Panel 1. Our results show that IL-5 was produced in small quantities in the PBS control groups, and was significantly increased by prenatal ETS exposure in the HDM challenged groups.
**Figure 10.** IL-5 production increased after prenatal ETS exposure and HDM challenge.

IL-5 was significantly increased in BAL fluid of mice exposed prenatally to ETS and challenged with HDM at 6 weeks old. Data are means ± SEM n=6. * indicates P values < 0.05.
**IL-13**

IL-13, as well as other Th2 cytokines, plays an important role in the remodeling process and suspected to be central mediator of physiologic changes induced by allergic inflammation in airway epithelium. In order to determine if HDM and prenatal ETS exposure influenced the allergic response, BAL fluid was analyzed for the cytokine IL-13 via ELISA. Without HDM exposure, no IL-13 was produced, and with HDM exposure, the animals with prenatal ETS exposure had significantly increased IL-13 production (FA: 6.56 +/- 3.14 vs. Pre: 53.20 +/- 15.62).
**Figure 11: Increased IL-13 production in prenatally ETS exposed group with HDM challenge.** IL-13 was significantly increased in BAL fluid of mice exposed prenatally to ETS and challenged with HDM at 6 weeks old. Data are means ± SEM n=6. * indicates P values < 0.05.
IFN-γ

IFN-γ is a cytokine that is critical for both innate and adaptive immunity to protect the system against bacterial and viral infections. IFNγ is secreted by Th1 cells and is an important activator of macrophages (Bloemen et al. 2007). When IFN-γ is present Th1 cells inhibit many Th2-induced effector functions that allergic airway disease. It has also been found that the action of IFN-γ through the airway epithelium will limit airway obstruction and inflammation (Mitchell et al. 2011). To determine the susceptibility to allergic airway hyperreactivity after prenatal ETS exposure and determine if Th2 polarization was taking place, IFN-γ was measured in the BAL fluid by QuickPlex SQ 120 instrument and the MSD Workbench software using the MSD Mouse V-Plex Pro-Inflammatory Panel 1. Our results indicated that there was little to no production of IFN-γ in any of the exposure groups. IFN-γ levels all remained at baseline.
Figure 12: IFN-γ production in BAL fluid. There was little production of IFN-γ for all of the exposure groups. Data are means ± SEM, n=6.
3.2.2 Absolute inflammatory cell number

During the inflammatory process, infiltration of inflammatory cells occurs in airways and eosinophil infiltration in airways is a hallmark of airway inflammation. Eosinophil infiltration is initiated by the secretion of Th2 cytokines IL-4, IL-5, and IL-13 (Bloemen et al. 2007). In order to evaluate whether our prenatal ETS exposure increases airway inflammation, absolute cell numbers in BAL fluid were determined. Figure 13 demonstrates that the HDM exposure induced eosinophil production (59546 +/- 2153 for FA + HDM vs. 0 for FA + PBS) and this effect was increased by prenatal ETS exposure (94278 +/- 6277 for Pre ETS + HDM vs. 59546 +/- 2153 for FA + HDM). No significant increase in cell numbers for lymphocytes, macrophages, or neutrophils was observed.

Figure 13. HDM and Prenatal ETS exposure increase eosinophils in BAL fluid. This figure represents total inflammatory cell count. Data are means ± SEM n=3. *** indicates P values < 0.001 compared to FA + HDM group.
3.2.3 Serum IgE

Atopy, or increased levels of IgE in the blood, is a strong risk factor for developing allergic asthma. To determine whether prenatal ETS exposure affects atopy, IgE levels in the mouse serum were measured by ELISA. Our results indicate that, with exception of two outliers, the levels of IgE were consistently low in all of the groups not exceeding 5 pg/mL.

Figure 14. Serum levels of IgE. IgE levels were not significantly increased among the different exposure groups, with the exception of two outliers. Data represented are individuals in each group, n=6.
CHAPTER 4
DISCUSSION

Allergic asthma is a chronic disease, which is increasing in diagnoses each year, especially among children (Eder et al. 2006). The mechanism for this disease has yet to be elucidated, although epigenetic changes have been introduced as a possible mechanism for this debilitating disease. Allergic asthma is usually exacerbated after multiple exposures to allergens or environmental pollutants, such as tobacco smoke, PAHs, pollen and house dust mite (HDM) (Bégin & Nadeau 2014). In urban areas, HDM is one of the most common allergens. It has been suggested that chronic exposure to HDM skeletal extracts induces airway remodeling and AHR via allergic immune responses. It has been proposed that HDM extracts can destroy airway epithelial and ASM cells by its proteolytic activity (Grunstein et al. 2005). In the experimental murine model of asthma, previous studies found an increase in Th2 cytokine levels and the numbers of inflammatory cells in response to HDM (Jaffar et al. 1999), illustrating that the HDM methodology represents an accurate allergen induced murine model of airway hyperreactivity.

There is an agreement that epigenetic regulation can be altered with environmental exposure and its dysregulation leads to asthma pathogenesis and susceptibility (S.-C. Wang et al. 2008; Miller & Ho 2008; Ho 2010). Increased DNA methylation (hypermethylation) within the CpG islands in promoter regions of genes alters gene transcription machinery and eventually leads to decreases in gene expression. Conversely, decreased methylation (hypomethylation) can lead to an increase in gene expression. Gene dysregulation further affects cellular functions. Global DNA methylation levels assayed by methylation sensitive pyrosequencing or PCR of the repetitive elements LINE-1 and Alu have been found to be
decreased in response to prenatal tobacco smoke (Breton et al. 2009). In this study, reduced global DNA methylation was associated with an increased risk of childhood asthma. Genomic DNA methylation, however, are not indicative of transcription level mechanisms that are occurring which may be altering asthma susceptibility. In order to illustrate the relationship between allergic asthma and DNA methylation we analyzed the gene-specific methylation, via pyrosequencing, of the promoter regions of asthma-associated genes. According to multiple studies, asthma related genes such as, IL-4, IL-13, IFN-γ, and Foxp3 have all been shown to be epigenetically regulated (Lee et al. 2002; Lu et al. 2009; White et al. 2006; Huehn et al. 2009). In order to determine if these genes were epigenetically regulated in our murine model of asthma, gene specific primers for these four genes were designed, as no murine primers for pyrosequencing analysis of the promoter regions of these genes have been published. However, there are publications illustrating the relationship of various epigenetic mechanisms in allergic disease, and also studies demonstrating how global methylation is affected by HDM and tobacco smoke exposure (Cheng et al. 2014). Currently, there are no published studies showing the relationship between altered gene-specific methylation levels after HDM or ETS exposure with correlation to immunological responses. The novelty of this project lies in having a known ETS exposure dose, an efficient model of murine allergic airway hyperreactivity, and pyrosequencing technology to determine gene specific methylation of asthma-associated genes.

IL-4 plays an important role in naïve T-cell differentiation and Th2 polarization during an allergic response. 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. Then, after Th2 differentiation they found these regions to be demethylated, suggesting an increase in expression of these genes (Lee et al. 2002). Alterations in these methylation patterns could affect how the naïve CD4+ T cells differentiate, and certain exposures may trigger these methylation patterns. Whether these methylation patterns could be altered after prenatal exposure to ETS is yet to be determined. Our study shows significant hypomethylation of Il-4 in lung DNA of prenatally ETS exposed mice after being challenged with HDM, which suggests that the gene would be up-regulated, and transcription of Il-4 increased.

Il-13 is also very important for Th2 polarization and the Th2 response, however we did not see a significant difference in the methylation patterns of the Il-13 gene. Publications have shown Il-13 to be epigenetically regulated, but by a different epigenetic mechanism known as microRNA (Lu et al. 2009). Therefore, it could be possible that Il-13 may not be epigenetically regulated by DNA methylation mechanisms. Also, many different cell types, such as eosinophils, macrophages, dendritic cells, and B-cells secrete IL-4, while only fully differentiated Th2 cells secrete IL-13. A reason why no methylation change was observed could be because the DNA used in our experiment came from the whole lung, consisting of a various cell types. It may be possible to see hypomethylation in Il-13 if sorting for the Th2 cells within the lung.

IFN-γ is thought to be highly regulated early in life and epigenetic mechanisms are theorized to play a role in its expression, especially in allergic disease (White et al. 2006). Our study illustrated significant hypermethylation of Ifn-γ after HDM challenge and was further hypermethylated if the animal experienced prenatal ETS exposure. This suggests that the gene Ifn-γ is less likely to be transcribed and its expression would be down
regulated. Down-regulated expression would lead to less cytokine production. In our experiment we did not see production of IFN-γ in any of the exposure groups.

The methylation of Foxp3, which is a transcription factor that is expressed in important regulatory T-cells, did not change among the different exposure groups. As it has been shown that FOXP3 expression is regulated by DNA methylation (Lal et al. 2009), we expected to see methylation differences. The lack of methylation change could be due to the fact that the cells expressing this transcription factor would be a small subset of the cells from which we extracted the DNA. There would be a potential for methylation differences if T-regulatory cells were sorted, which would warrant further studies.

Taken together, our epigenetic results suggest that HDM and especially prenatal exposure to ETS might trigger gene-specific DNA methylation machinery either through the use of DNA methylation transferases, hydroxymethylation, or the passive/active demethylation via DNA repair, resulting in altered gene transcription. The mechanism by which these epigenetic machineries contribute to gene transcription and its functions in airway disease remains unanswered and requires further study. As the field of epigenetics and its technology advances, increased knowledge and new validation assays will likely improve our ability to reveal markers for the prevention, diagnosis, and prognosis of asthma.

Th2 bias, eosinophilia, and AHR are the hallmarks of allergic asthma and, in animal models, prenatal plus perinatal exposure to mainstream as well as secondhand cigarette smoke increases AHR and Th2 lung inflammation (Singh et al. 2003) In this project, we showed that prenatal exposure to ETS primes the lung to dramatic increases in allergic
airway response (increased eosinophils) as well as Th2 inflammation (increased IL-4, IL-5 and IL-13) levels.

Production of IL-4 is significantly increased in BAL fluid after prenatal exposure to ETS and challenge with HDM. This correlates with our methylation data, which shows significant hypomethylation in the ETS exposed group corresponds to a significant increase in production of IL-4. This illustrates that prenatal exposure to ETS could illicit epigenetic programming, making the ETS-exposed mice more susceptible to Th2 polarization and an allergic phenotype when challenged with allergen. Also, the promoter region of Ifn-γ was hypermethylated and no significant levels of IFN-γ were observed in the BAL fluid.

Production of Th2 cytokines IL-5 and IL-13 were also significantly increased in the BAL fluid of prenatally ETS exposed mice, illustrating their increased susceptibility to asthma. The increased cytokine levels of IL-4, IL-5, and IL-13 were consistent with the cell differential data showing increased production of eosinophils. The cytokines just listed are crucial for the recruitment of eosinophils and eosinophilia is a hallmark trait of asthmatic individuals (Bloemen et al. 2007). The number of eosinophils significantly increased in the mice prenatally exposed to ETS and challenged with HDM. Unfortunately, the epigenetic modification of IL-5 was not analyzed and based on the immunological results, is an experiment worth pursuing. The epigenetic results of IL-13, however, were inconclusive and did not support the hypothesis. The cytokine IL-13 may not be epigenetically regulated as its transcription factors are activated by IL-4 (Bloemen et al. 2007), however further studies on these mechanisms need to be completed.

Serum IgE level of the mice was also evaluated. The mice in this experiment, with the exception of two outliers, did not demonstrate to have increased levels of IgE, or atopy,
after exposure to ETS or to HDM. The normal HDM protocol carried out by Jaffar et. al. is a 3 week sensitization and challenge protocol, after which all of the HDM exposed mice have severe airway hyperreactivity and a highly allergic response (Jaffar et al. 1999). In this study, in an effort not to mask the effect of ETS, the mice were only exposed to HDM using a 2-week protocol. This lessened HDM exposure allowed us to see the effects of the ETS, however, it did prevent eliciting a severe allergic response which would correspond to high serum IgE levels.

Our study also had various limitations. Lungs acquired from mice are very small and it is tedious and difficult to acquire the necessary amount of DNA from sorted cell populations, such as Th2 and Treg cells. Epigenetic patterns are altered in each specific cell type, so by extracted whole lung DNA we are diluting the actual methylation of the promoter region of interest. Also, in order to conclude a true correlation between Ifn-γ methylation and IFN-γ production, we would have liked to observe a decrease in the Th1 cytokine after prenatal ETS exposure and HDM challenge. However, since all results were baseline, this conclusion cannot be made. The methylation level across all groups, however, may be the reason that no production was observed. Stress is another factor shown to alter epigenetic patterns (Pembrey et al. 2014). Therefore, another limitation is that the mice were shipped via plane from UC Davis to the University of Montana, and this could have stressed the animals, which may have altered some of the methylation results, although these changes may not have effected the asthma-associated genes of interest.

Overall, this project strongly suggested that a developing fetus is exceptionally sensitive to even very low doses of cigarette smoke, which may promote the development
and increase the susceptibility of childhood allergic asthma. Every effort should be made to
dissuade women from being exposed to any amount of cigarette smoke during pregnancy.
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

This study was designed to research the effect of prenatal ETS exposure on epigenetic programming and hypothesized increased susceptibility to development of allergen-induced airway inflammation. In order to complete this goal, I used a HDM allergen-induced mouse model of asthma to examine the effect of in utero ETS exposure on susceptibility to an asthmatic phenotype via the proposed mechanism of epigenetic programming by alterations in DNA methylation of genes associated with asthma.

To summarize, prenatal ETS exposure altered the methylation of the promoter regions of asthma-associated genes Il-4 and Ifn-γ. Il-4 was hypomethylated, which corresponded to the increased production of IL-4 that was found in the BAL fluid. Ifn-γ promoter region was hypermethylated, which correlated in there being little to no production of IFN-γ present in the BAL fluid. There was also an increased amount of IL-13 and IL-5 found in the BAL fluid after prenatal ETS exposure, however Il-13 methylation levels did not differ among groups and IL-5 methylation levels were not assessed.

Further investigation is needed in order to determine if there is perhaps another region within the promoter of Il-13 where the methylation may take place or perhaps looking specifically at the Th2 cells’ expression of Il-13. Primers of the promoter region of Il-5 should be designed and the methylation should be analyzed to look at any correlation between its methylation and production in the BAL fluid.

Also, as GATA-3 and T-bet are important transcription factors aiding in T-cell differentiation, their epigenetic alterations may play a crucial role in Th2 polarization and the production of pro-allergic or regulatory cytokines. It is not known to what extent DNA
methylation plays in their expression and prenatal ETS exposure may alter their methylation patterns and therefore increases asthma susceptibility. Studies analyzing the promoter region methylation of GATA-3, T-bet and other allergy or inflammation related genes would allow for a much better understanding of how epigenetic mechanisms play a role in allergic asthma.

It is important to determine the mechanism by which allergic airway inflammation and asthma develop. Epigenetic mechanisms like DNA methylation, are reversible, and could potentially be used as a therapeutic for this disease. Once the genes and CpG locations that are altered in this disease are identified, further mechanistic studies should be done to determine what machinery is responsible for the methylation/demethylation, which would bring us one step closer to developing therapies for this debilitating condition.
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