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The effects of in utero environmental tobacco smoke exposure on immune responses to allergen in adult offspring

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THE EFFECTS OF *IN UTERO* ENVIRONMENTAL TOBACCO SMOKE EXPOSURE ON IMMUNE RESPONSES TO ALLERGEN IN ADULT OFFSPRING

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by
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This manuscript is dedicated to:

my wife, Stella, whose appreciation of the quest for knowledge and whose willingness to do without many things has made this physically possible;

my mother, Anna, who has been my only lifelong champion and true-believer and whose unwavering support has made this mentally possible;

my father, Roderick, who in the eyes of this child was a great educator and whose presence and absence has been the motivation that has made this possible.

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ABSTRACT

Fetal stress has been linked to adult atherosclerosis, obesity, and diabetes. Epidemiology studies have associated fetal exposure to maternal smoking and post-natal exposure to environmental tobacco smoke (ETS) with increased asthma risk. We tested the hypothesis, in a mouse model of asthma, that ETS exposure *in utero* alters airway function and respiratory immune responses in adult offspring.

Pregnant BALB/c mice were exposed daily to ETS or filtered air (AIR). Neonatal gene expression was assessed. Offspring inhaled aerosolized ovalbumin (OVA) or saline in weeks 7-8. Regardless of whether they inhaled OVA or saline, mice were sensitized by OVA injections in weeks 11 and 13 followed by OVA aerosol challenge in weeks 14-15. At weeks 6, 10, and 15, we assessed OVA-specific serum immunoglobins, bronchoalveolar lavage cells and cytokines, lung and nasal histopathology, lung gene expression, and airway hyperresponsiveness (AHR).

Neonatal mice demonstrated slight but potentially critical differences in gene expression related to their exposure to ETS *in utero*. At 6 weeks, there were no significant differences between mice exposed to ETS *in utero* and those exposed to AIR *in utero.* At 10 weeks, following OVA aerosol, mice exposed to ETS *in utero* displayed greater AHR than mice exposed to AIR *in utero* ($\alpha = 0.05$), unaccompanied by changes in histopathology, cytokine profile, or antibody levels. However, there were significant differences in gene expression between these 10 week groups. At 15 weeks, mice that had inhaled saline in weeks 7-8 developed airway inflammation: eosinophilia ($\alpha = 0.05$), IL-5 ($\alpha = 0.05$) and AHR ($\alpha = 0.05$) were greater in mice exposed to ETS *in utero* vs. mice exposed to AIR *in utero*. Mice that had inhaled OVA in weeks 7-8 demonstrated no

airway inflammation after sensitization and challenge and those exposed to ETS *in utero* had suppressed immune and inflammatory responses. Consistent with other findings at 15 weeks, there were significant differences in gene expression between mice receiving ETS exposure *in utero* and those receiving AIR exposure *in utero*.

ETS exposure *in utero* exacerbates subsequent adult responses to initial allergen exposure and causes altered gene expression, especially with additional lung perturbation.

CHAPTER 1
ENVIRONMENTAL INFLUENCE ON THE INCREASE IN INCIDENCE OF HUMAN ASTHMA

There has been a notable increase in the incidence and prevalence of asthma and allergy within the world population during the past fifty years (Mannino et al. 2002; Pearce et al. 2000; Sunyer et al. 1999). Although these increases have been worldwide, the majority of the changes are found in industrialized, or developed, nations (Rottem et al. 2005). These increases have occurred over such a brief period in time that even a dominant gene would not have had time to spread through the human population. Therefore, attention has focused on global environmental changes that might be responsible for increases in asthma and allergy.

Environmental exposures in developed versus non-developed regions of the world are of particular interest. Many theories have evolved around perturbations to the developing immune system, including the incrimination of vaccines, decreased parasite exposure, decreased viral infections, and increased environmental pollutants (Woodruff and Fahy 2001). To date, no clear causative discovery has been made. Specifically, little has been done on the ability of inhaled environmental contaminants to interact with susceptibility genes and thus alter the development of asthma and allergy.

Typically, as developed nations continue to mature, ambient environmental pollution becomes a concern and measures are taken to reduce environmental pollution. As a result, ambient environmental pollution has declined markedly in industrialized nations (Heinrich et al. 2000; Laden et al. 2006). However, the identity and character of remaining environmental contaminants are still an issue. Remaining pollutants (e.g., those arising from combustion of fossil fuels, manufacture of synthetic materials, and use of cigarettes) may be of a more synthetic or complex nature. The wide spread use of these materials has occurred only within the last one

hundred years with the major increase within the past fifty years. This coincides with the changes and progression seen in asthma and allergy.

Environmental tobacco smoke (ETS) is "second-hand" smoke. It is composed of approximately 90% sidestream smoke and 10% exhaled mainstream smoke. Sidestream smoke is smoke arising from the burning end of the cigarette. This smoke does not pass through the cigarette filter and is inhaled as a component of the ambient air. In the United States, the vast majority of cigarettes contain an attached cellulose fiber filter. Mainstream smoke is drawn through the cigarette, through this filter, and directly into the airway in a concentrated form. However, exhaled mainstream smoke has been treated by a sophisticated and efficient filter prior to inhalation and has been further filtered by the respiratory components responsible for the continuous treatment of inhaled air. By convention, sidestream smoke is usually used as a surrogate for ETS, or "second-hand" smoke. We maintain this convention in our studies.

ETS has been examined by many researchers and has been associated with exacerbation of adult asthma and allergy symptoms (Jaakkola et al. 2003; Jindal et al. 1994). A number of studies have evaluated the role of ETS in initiation of allergy or asthma in the perinatal period (DiFranza et al. 2004; Hong et al. 2003; Jaakkola et al. 2006). Previously, we conducted studies concerning the health effects of inhaled ETS, including ETS exposure effects on development of asthma and allergy in adult mice and cardiovascular disease in adult mice that were exposed to ETS *in utero*. The latter studies indicated that ETS exposure *in utero* promoted the development of atherosclerotic plaques in adults (Yang et al. 2004). In contrast, adult ETS inhalation did not alter the response of mice to subsequent allergen exposure (Bowles et al. 2005).

Findings from *in utero* studies are consistent with the ideas of David Barker (Barker 2002) and the resultant developmental "Barker Hypothesis". Although Barker initially described a relationship between prenatal, or maternal, malnutrition and subsequent adult cardiovascular

disease, the hypothesis has been extended to include other common chronic adult diseases and other developmental perturbations. The tenets of the hypothesis include; (1) there is a disturbance in the normal uterine environment during a critical period of gestation; (2) the developing fetus has plasticity, or the ability to adapt to the disturbance, in a way to maximize the chances for survival; (3) beyond a critical point, fetal programming occurs thereby limiting the ability of the fetus to return to pre-disturbance responses; (4) upon chronic exposure to a "normal" environment the adult is unable to mount "normal" responses and therefore chronic adult disorders develop (hypertension, insulin resistance, hypercholesterolemia, and hyperuricemia). We hypothesize that *in utero* environmental toxin exposures can serve to disturb the "normal" uterine environment and that the chronic disorders that can develop include allergy and asthma. To test this hypothesis, we conducted *in utero* exposures of pregnant mice to ETS and followed the responses of their offspring to allergen exposure and challenge.

This dissertation describes studies involving ETS exposure *in utero* and the subsequent effect on immune responses to allergen in the adult offspring. The dissertation is divided into five chapters. Chapter one is this introductory chapter. The second chapter addresses the rationale, material, methods, and outcome of the non-genetic findings of ETS exposure *in utero*, before and after allergen exposure in the adult offspring. Chapter three describes the rationale, material, methods, and findings of the gene expression studies of the mice at the final time point described in chapter two (15 weeks of age) and evaluates the correlation of these findings with the physical findings at that same time point. Chapter four examines the gene expression findings at the other time points examined in chapter two and relates those findings to the physical findings for those time points. The final chapter is a concluding chapter with critical and forward-looking remarks based on this study.

The majority of this information has been previously published in peer-reviewed journals as two large manuscripts, one covering the information from chapter two (Penn et al. 2007) and the other detailing the information from chapter three (Rouse et al. 2007). Chapter four contains data that have not been published as of this writing. In the final chapter, concluding remarks summarize the findings of all of these studies and the implications of these findings for present and future research. The potential of other methodologies and techniques in, and new or alternate directions for, this research is also discussed.

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CHAPTER 2

EXPOSURE TO ENVIRONMENTAL TOBACCO SMOKE *IN UTERO* POTENTIATES ADULT RESPONSES TO ALLERGEN IN BALB/c MICE[1]

INTRODUCTION

The incidence of asthma has escalated over the past twenty years. Increasingly, initial asthma episodes are observed early in life (Weiss 1998; Holt 1998). In 2004, nearly 30% of the thirty million diagnosed asthmatics in the United States were under 18 years of age. The highest asthma prevalence rate (148/1000) exists in 5-17 year-olds (American Lung Association 2006). Despite the often early onset of this disease, chronic adult asthmatics comprise the majority of asthma patients.

Stresses in the fetal environment can promote early onset of chronic adult diseases. Initial epidemiological studies related maternal nutritional deficits to low live birth weights and to premature cardiovascular disease in adult human offspring (reviewed in Barker 2002). In humans, neurological response to fetal stress has been recently reviewed (Amiel-Tison et al. 2004). In experimental studies, fetal anemia in sheep (Davis et al. 2005) and hypoxia in pregnant rats (Li et al. 2004) have resulted in chronic disease in adult offspring. In pregnant mice, viral infection (Niklasson et al. 2006), pesticide treatment (Cory-Slechta et al. 2005), and environmental tobacco smoke (ETS) exposure (Yang et al. 2004) have also resulted in chronic adult disease in offspring.

Exposure to ETS has been associated with exacerbated asthmatic responses in children (Gilliland et al. 2000; Lindfors et al 1999; Mannino et al. 2001). Altered lung function, increased risk of asthma, and persistent lung function deficits in children have been linked with *in utero* exposure to maternal smoking and/or post-natal exposure to ETS (Gilliland et al. 2000;

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2001; 2003; Li et al. 2000; Zlotkowska and Zejda, 2005). The synergistic action of tobacco smoke exposure with sensitization to non-tobacco allergens may increase the prevalence of allergy and asthma (Oryszczyn et al. 2000). Detailed experimental studies focused on pro-asthmatic responses resulting from *in utero* ETS exposure combined with post-natal exposure to non-tobacco antigens have not been reported.

In rodent models of allergic asthma, ovalbumin (OVA) is a commonly employed antigen for eliciting allergic responses. Sensitization by intraperitoneal (i.p.) injection, followed by inhalation challenge with OVA elicits expansion of the T helper-2 (Th2) lymphocyte population. Production of Th2 cytokines follows, leading to airway hyperresponsiveness (AHR) and inflammation characterized by eosinophilia and OVA-specific immunoglobin E (IgE; Zhang et al. 1997).

This sensitization/challenge protocol fails to mimic the typical human experience of aerosol-only sensitization and challenge (Bice et al. 2000; Persson et al. 1997). However, aerosol-only OVA exposure of mice results in little or no OVA-specific serum IgE, and no eosinophilic inflammatory response. This has been attributed to the induction of immunological tolerance specifically affecting IgE production (Astori et al. 2000; Holt et al. 1981; McMenamin and Holt 1993; Seymour et al. 1998). Interleukin (IL)-10 from regulatory T cells in the lung (Akbari et al. 2001) favors the production of IgG_1 antibodies and inhibits isotype switching to IgE (Jeannin et al. 1998). This, combined with the induction of T cell tolerance (Buer et al. 2005), protects most individuals from developing allergic airway diseases to commonly encountered allergens (Tsitoura et al. 2000). While genetic polymorphisms and underlying regulatory faults in IL-4 and IL-13 production can impair this safety mechanism (Heinzmann et al. 2000; Howard et al. 2001; Izuhara et al. 2000; Seah et al. 2001; Shirakawa et al. 2000;), environmental exposures might also contribute to loss of airway tolerance.

We designed experiments to simulate the respiratory consequences to offspring of pregnant, non-smoking women exposed daily to ETS. We combined daily ETS exposure of pregnant BALB/c mice with post-natal OVA inhalation to test the hypothesis that ETS exposure *in utero* can alter post-natal respiratory and immune responses to non-tobacco allergens.

We posed the following questions:

Is exposure to ETS *in utero* sufficient to:

compromise respiratory responses in young mice? in adults?

promote cytokine production in OVA-exposed mice?

influence AHR or lung histopathology following OVA provocation?

interfere with establishment of aerosol tolerance to OVA or with production of OVA-specific antibodies?

MATERIAL AND METHODS

Animal Protocols

We housed and handled BALB/c mice (Harlan, Indianapolis, IN) according to the NIH Guide for the Care and Use of Laboratory Animals. The LSU IACUC approved all animal procedures, guaranteeing humane treatment and alleviation of suffering. Mice, on a 12-hour light/dark cycle, had food and water *ad libitum* except when in the exposure chambers. We began breeding eight-week old mice (1 male/ 2 females) the evening prior to initiation of ETS exposure. After exposures ended (day 19), we housed pregnant mice in separate cages until weaning (day 21 after birth). A barbiturate solution (Beuthanasia-D, Schering-Plough, Kenilworth, NJ) was used via intraperitoneal injection (0.2 ml per mouse) for all sacrifices.

ETS Exposures

Sidestream smoke, which comprises ~90% of ETS, served as a surrogate for ETS (Penn and Snyder 1993, Bowles et al. 2005). A 30-port smoking machine (AMESA Technologies,

Geneva, Switzerland) generated smoke from 2R4F filtered research cigarettes (Univ of KY, Lexington, KY). We diluted ETS with HEPA-filtered air (AIR) to establish a suspended particle load of 10mg/m^3. A MIRAN sapphIRe infrared spectrometer (The Foxboro Co., Foxboro, MA) and a DustTrak particle monitor (STI, St. Paul, MN) continuously monitored carbon monoxide and total suspended particle levels, respectively, in exposure chambers. Gravimetric analyses were performed every 2 hrs by weighing 0.45mm membrane filters (Gelman, Ann Arbor, MI) through which measured amounts of smoke had passed. We exposed 8-week old mated mice to ETS or AIR (14 air changes/hr, 5 hr/day, 19 consecutive days) in 1.3m^3 stainless steel and plexiglass dynamic exposure chambers (T^o=71^o ± 1.5^{o}F; RH=53 ± 3%). No offspring were exposed to ETS after birth.

Aerosol Exposures

We exposed mice in a 16-compartment, 16.7-liter plexiglass inhalation exposure chamber to aerosolized sterile saline or 1% (w/v) OVA (Grade V >98% pure; Sigma-Aldrich, St. Louis, MO) in saline (20 min/day; 10 days; 7-8 weeks of age). A flow rate of 4L/min was maintained through an Airlife sidestream high-efficiency nebulizer (Cardinal Health, Dublin, OH).

OVA Sensitization/Challenge

We sensitized mice to OVA by (i.p.) injections (80 µg OVA in 2.0 mg alum), one each at 11 and 13 weeks of age. OVA challenge included three 20-minute inhalation exposures to 1% OVA in saline, every other day at 14 weeks, plus a 20-minute exposure one day before sacrifice at 15 weeks.

Bronchoalveolar Lavage (BAL) Cell and Fluid Collection

Following euthanasia, we lavaged the right lung of each mouse 4X with 0.3 ml warm PBS and re-suspended the centrifuged BAL cell pellet in PBS for BAL differential counts (200

cells). Smears were stained with a modified Wright's stain. The BAL supernatant was stored at -80°C for cytokine analyses.

Creation of Standard Sera for Isotype-specific ELISAs

We pooled and assayed sera from sensitized/challenged BALB/c mice for OVA-specific IgG_1, IgG_{2a}, and IgE by isotype-specific ELISA at varying dilutions (IgG_1 1:10000; IgG_{2a} 1:100; IgE 1:10). The pooled sera constituted a standard in subsequent isotype-specific ELISAs on test sera. The isotype-specific optical density (O.D.) value from the isotype standard was set as one ELISA Unit (EU).

OVA-specific IgG_l, IgG_{2a} and IgE ELISA

We used isotype-specific ELISAs to quantitate OVA-specific antibody levels in sera (Seymour et al. 1997) from blood obtained at sacrifice. For IgG_1 and IgG_{2a}, OVA-coated (100 µl/well of 0.1% OVA) 96-well flat bottom plates (Fisher, Pittsburgh, PA) captured OVA-specific antibodies. Horseradish peroxidase-conjugated (HRP) rat anti-mouse IgG_l or IgG_{2a} antibodies (BD-Pharmingen, San Diego, CA) marked captured antibody. For OVA-specific IgE detection, monoclonal rat anti-mouse IgE (5ug/ml in PBS; BD-Pharmingen) coated (100ul/well) 96-well Immulon4 plates (Thermo Electron Corp., Waltham, MA) captured serum IgE. Biotinylated-OVA (2.5mg/ml; Pierce EZ-Link LC; Pierce, Rockford, IL) and HRP-streptavidin (Pierce) marked captured OVA-specific antibody. For all ELISAs, TMB substrate (100ul/well) detected marked antibodies. We used a standard ELISA reagent kit (OptEIA Reagent Set B, BD-Pharmingen) and converted $O.D._{450}$ readings of the samples to EU.

Histopathological Analysis of Lungs

Following BAL collection, we perfused lungs with 0.3 ml of freshly prepared 0.02M periodate-0.1M lysine-0.25% paraformaldehyde (PLP) fixative in phosphate buffer (pH 7.4), then excised and stored the lungs in PLP for 24-48 hr. We sectioned the cranial, middle and

caudal lobes of the right lung along the plane of each lobar bronchus. Following post-fixation in 70% ethanol, we embedded samples in paraffin and dehydrated them directly in graded alcohols to eliminate further exposure to formaldehyde. We stained 3-4 μm sections with hematoxylin and eosin. We used a seven category weighted scoring system (bronchus-associated lymphoid tissue, lymphocytes, plasma cells, eosinophils, neutrophils**,** mucus metaplasia and total cellularity) for histopathological evaluation of lung sections (Bowles et al. 2005). A board-certified veterinary pathologist, blinded to the treatments, evaluated histopathological samples. A higher score (maximum score = 25) is indicative of greater tissue responsiveness.

Histochemical Analysis of Nasal Tissue

We fixed heads in buffered-neutral formalin then decalcified them for 3 days in 15% formic acid before sectioning (Bahnemann et al. 1995). A pathologist, blinded to the treatments, scored nasal sections for inflammation by cell type and epithelial changes, according to the type of epithelium (squamous, respiratory, and olfactory).

Cytokine Quantitation in BAL Fluid

We used a fluorescence-based murine Th1/Th2 cytometric bead array assay (BD-Pharmingen) to analyze aliquots of BAL supernatants on a BD FACSCaliber instrument. The cytokines included in the assay were IL-2, IL-4, IL-5, TNF-α and IFN-γ. Their limits of detection were 5.0, 5.0, 5.0, 2.5 and 6.3pg/ml, respectively. A single bead assay for IL-13 had limits of detection of 7.3pg/ml.

Pulmonary Function Testing

We assessed AHR in unrestrained mice by whole body plethysmography (Buxco, Troy, NY). Data were expressed as a dimensionless value, Penh, or 'enhanced pause' (Hamelmann et al. 1997a). Following acclimation and aerosol saline exposure, we challenged mice with graded

doses (1.56-50.0 mg/ml) of nebulized methacholine (Sigma-Aldrich, St. Louis, MO) to assess AHR. We recorded post-exposure Penh values over 5 minutes for each dose.

While some concern has been expressed regarding the limitations of Penh measurements obtained via unrestrained plethysmography (Lundblad et al. 2002), there are many reports of strong correlations between increases in Penh values and bronchoconstriction, demonstrating that Penh serves as a reliable monitor of changes in lung mechanics (Delorme and Moss 2002; Hamelmann et al. 1997b; Singh et al. 2003).

Statistical Analysis

We used the SAS statistical package (version 9.1.3; SAS Institute, Inc., Cary, NC) GLM procedure for all analyses. In addition, where appropriate, we conducted an analysis of variance (ANOVA) by sampling time with various endpoints (BAL cells, histopathology scores, cytokine levels) as the response variables, and treatment, gender, and treatment-gender interaction as effects in the model. We analyzed antibody data by a one-way ANOVA across treatment groups and plethysmograph data in an ANOVA on repeated measures. In considering limits of detection, we ranked cytokine data and carried out a Kruskal-Wallis test (one-way ANOVA) on the ranks. When the overall model indicated significance, we conducted *post hoc* pair-wise comparisons with Tukey's HSD test for main effects comparisons (Siegel 1956; Steele and Torrie 1980). Pair-wise comparisons of significant interaction effects were conducted with t-tests of least square (adjusted) means. In all cases, we considered comparisons significant at $\alpha = 0.05$.

To test the trend and relationship of one treatment group to another, we paired (blocked) the means of the various response variables and compared the two treatment groups with respect to these paired mean values. The null hypothesis was that the treatment groups were the same based on the paired comparisons. We used the Wilcoxon matched-pairs test, Pearson correlation

test, and a regression analysis on the matched groups to examine the potential trend. We considered all analyses significant at $\alpha = 0.05$ (Neter and Wasserman 1974).

Exposure and Sampling Schedules

We randomly assigned mated females to gestational ETS or AIR exposure groups. At weaning, mice were segregated by sex with no more than 4-5 mice per cage. We sacrificed groups of 6-week old mice exposed to ETS or AIR *in utero* to determine effects of ETS exposure *in utero* on lung responses of young mice. Half the remaining mice inhaled OVA (EO, AO) aerosol at 7-8 weeks of age. The other half inhaled saline aerosol (ES, AS). Each of these groups contained 35 to 39 mice representing 6 to 10 litters. We sacrificed mice in each group at 10 weeks of age to determine the effects of *in utero* ETS exposure, plus subsequent OVA inhalation, on lung responses of young adult mice. We sacrificed the remaining mice, following OVA-sensitization and -challenge, at 15 weeks of age to determine the effects of *in utero* ETS exposure on responses to OVA sensitization (ESO, ASO) and OVA tolerance (EOO, AOO). We analyzed antibody levels, BAL cytology and Th1/Th2 cytokine levels, lung and nasal histopathology, and pulmonary function at all time points.

RESULTS

Forty to fifty percent of ETS and AIR females had litters. There were no significant differences in delivery rate or litter size between ETS and AIR dams. However, *in utero* ETS offspring were of slightly lower birth weight and experienced a higher mortality rate between birth and three weeks of age than did *in utero* AIR offspring (12% vs. 2%, $\alpha = 0.05$). Thereafter, survival rates were similar and any inequity in weight or growth rate was lost. In both groups, the male to female offspring ratio was approximately 1:2. At weaning, offspring exposed to ETS *in utero* totaled 89 from 19 litters and offspring exposed to AIR *in utero* totaled 94 from 19 litters.

We detected no upper respiratory tract changes, including evidence of inflammation or congestion, in any treatment groups at any time point during this study, as determined by evaluation of nasal histopathology. We determined no gender differences in responses of mice exposed *in utero* to ETS and subsequently to OVA. There are no equivalent experimental asthma studies with which to compare gender responses directly, since the ETS/OVA exposure schedule we followed has not been reported previously in a mouse model of asthma. There are, however, reports of gender differences with regard to asthma endpoints, following post-natal ETS and OVA exposures in Balb/c mice **(**Melgert et al. 2005; Seymour et al. 2002**).** Humans display gender differences with regard to asthma endpoints, as well. More adult females than males have asthma, although this relationship is reversed in children (American Lung Association 2006**).** Mortality due to asthma is higher among human females than among males.

SIX WEEKS OF AGE

Results

Results from this time point (Figure 2.1) were analyzed to determine whether ETS exposure *in utero* altered basic lung development in either structure or function. No significant differences were noted in antigen specific antibody levels, BAL cell counts, BAL cytokines, or

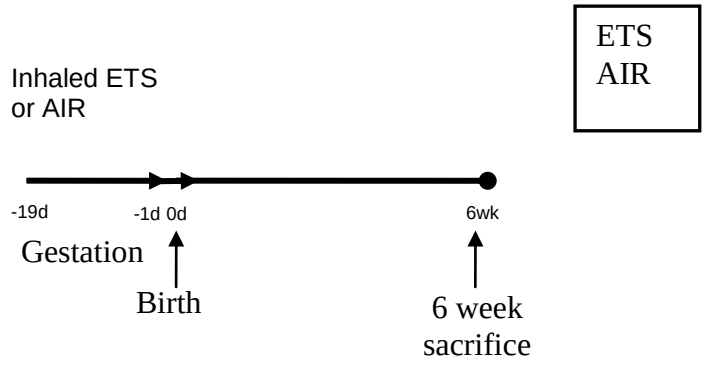


Figure 2.1: Six week timeline demonstrates the exposure of pregnant mice for nineteen consecutive days (-19d to -1d) to either ETS or HEPA-filtered air (AIR) followed by birth one to two days later (0d). Offspring received no further exposure to ETS or AIR, were weaned at 3 weeks of age, and sacrificed at 6 weeks of age.

histopathology. Exposure to ETS *in utero*, without any subsequent treatment, had no overall effect on pulmonary function (the entire curves are not significantly different), although at the two highest methacholine doses a significant AHR increase ($p \leq 0.017$) was noted in ETS mice (Figure 2.2).

Discussion

Results from six-week old mice indicate that ETS exposure *in utero*, without further lung challenge, has no observable effect on pulmonary function or histology. The only significant finding at this age is elevated AHR in ETS mice at the two highest methacholine challenge doses. These points are insufficient to produce a significant difference in the total response curve. However, they may reflect an ETS-induced AHR sensitivity that becomes more apparent with additional lung stress or challenge.

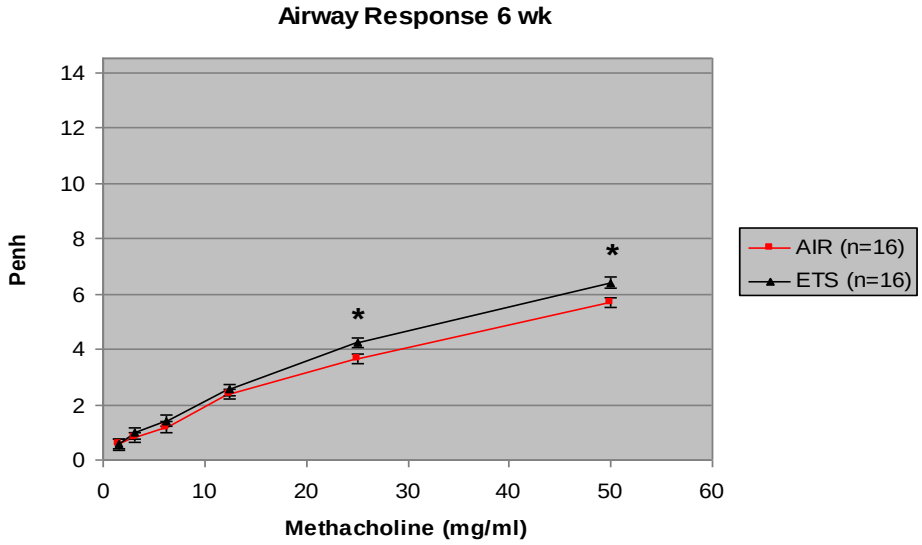


Figure 2.2: Mice received graded doses (1.56-50 mg/ml) of nebulized methacholine. Airway hyperresponsiveness (AHR) to bronchoconstriction is captured in the Penh value as determined by non-invasive, whole-body plethysmography for increasing doses of methacholine (bronchoconstrictor). Low doses demonstrate no differences, but at the two highest doses there were significantly different responses ($p=0.017$ at 25 mg/ml and $p<0.001$ at 50 mg/ml). (*) significant difference $p \leq 0.017$; these points were not sufficient to create a significant difference in overall response curves.

Aerosol OVA exposure in the absence of adjuvant results in OVA-specific aero-tolerance to future OVA aerosol exposure. Data from this time point were used to investigate whether ETS exposure *in utero* would alter immune responses to OVA and overcome the establishment of OVA tolerance. Timing of exposures is demonstrated in Figure 2.3. At 10 weeks of age, EO and AO mice had consistently differing OVA- specific antibody profiles, IgG_1 (EO > AO) and IgG_{2a} (AO > EO). These differences, however, were not significant for either isotype (data not presented). We detected no OVA-specific IgE in sera of OVA-aerosol exposed mice. As expected, we detected no OVA-specific IgG_1, IgG_{2a}, or IgE in control mice, regardless of whether their exposure *in utero* was to ETS (ES) or AIR (AS).

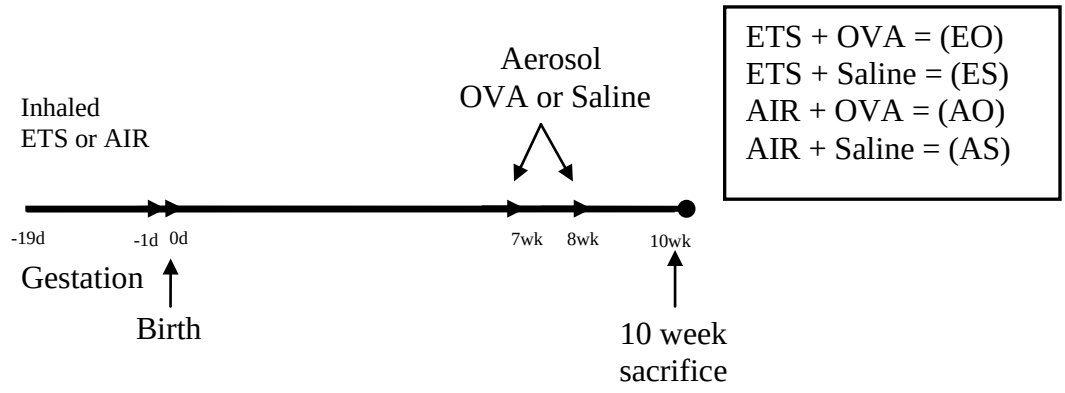


Figure 2.3: Ten week timeline duplicates the six-week timeline through birth and weaning then deviates with allergen (OVA 1%) or saline aerosol exposures (20 minutes per day for 10 days) at seven and eight weeks of age followed by sacrifice at ten weeks of age. OVA=ovalbumin.

Results

With the exception of TNFα, all cytokine levels were below limits of detection for all groups at 10 weeks. TNFα was significantly higher ($p < 0.05$; data not presented) in AO mice compared to other groups. Aerosol OVA exposure at 7 - 8 weeks of age had little overall effect on BAL cell distribution at 10 weeks. As at 6 weeks of age, BAL cells in all groups were mostly

(94 - 100%) mononuclear cells. Lung histopathology scores were low, ranging from 6.47 - 7.15. There were no significant differences between any groups in BAL cell distributions or lung histopathology.

Ten-week old AO mice demonstrated significantly increased AHR compared to AS mice (Figure 2.4) at methacholine levels ≥ 12.5 mg/ml. This response was amplified in mice exposed to ETS *in utero*. Ten-week old EO mice exhibited significantly elevated AHR compared to all other groups, at methacholine levels ≥ 6.25 mg/ml. At the highest methacholine concentration (50 mg/ml), the AHR of EO mice was 2 – 4X greater than that of other groups. Overall response curves were not significantly different between ES and AS mice. However at the highest methacholine level, ES mice had increased AHR ($p < 0.0001$) relative to AS mice.

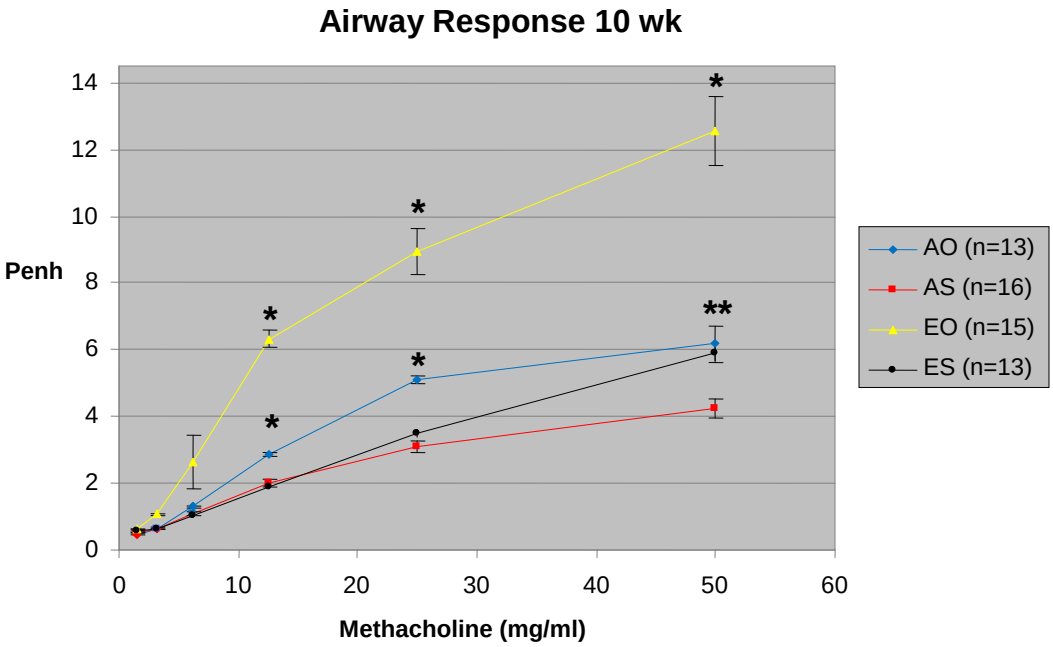


Figure 2.4: Mice received graded doses (1.56-50 mg/ml) of nebulized methacholine. AHR, expressed as Penh values, was determined by non-invasive, whole-body plethysmography. ETS exposure *in utero* increased AHR in 10-week old mice; (*) significant difference between all groups; (**) significant difference between AS and both AO and ES; p<0.0001; bars represent mean ± SE.

Discussion

At 10 weeks of age, groups of both ETS and AIR mice were exposed to aerosol OVA in the absence of any adjuvant, including LPS, to produce mice that were tolerant to airway inflammation by OVA sensitization and challenge. The antibody profiles of these animals were as expected. There were significant OVA-specific antibody responses (IgG_1 and IgG_{2a}) indicating recognition and allergen-specific activation, but no indication of significant effector response (eosinophilia, mucus hyperplasia, cytokine elevation, or OVA-specific IgE). These findings are consistent with prior studies (Holt et al. 1981; Swirski et al. 2002). As previously reported (Renz et al. 1992), AHR was significantly elevated in OVA mice relative to non-OVA mice. However, the AHR of EO mice was increased significantly relative to AO mice, demonstrating an ETS-dependent exacerbation of AHR to initial allergen exposure. This exacerbation was independent of classical inflammatory or Th2 endpoints, an occurrence that has been previously described (Barrett et al. 2002). As was the case in 6-week old ETS mice, 10 week-old ES mice demonstrated significant increase in AHR compared to AS mice at the highest methacholine dose. Again, this was not sufficient to create a significant difference in the total response curve.

FIFTEEN WEEKS OF AGE

Results

The OVA sensitization and challenge protocol used at fifteen weeks of age, and shown in Figure 2.5, elicits systemic and local allergic responses to OVA, thereby establishing an asthma phenotype. This protocol was used in OVA-tolerant mice (OVA aerosol at 7-8 weeks of age) to determine whether ETS exposure *in utero* overcomes OVA-tolerance induced by OVA aerosol exposure. This same protocol was used in mice without prior OVA exposure (saline aerosol at

7-8 weeks of age) to examine whether ETS exposure *in utero* enhanced or otherwise altered immune response to OVA sensitization and challenge.

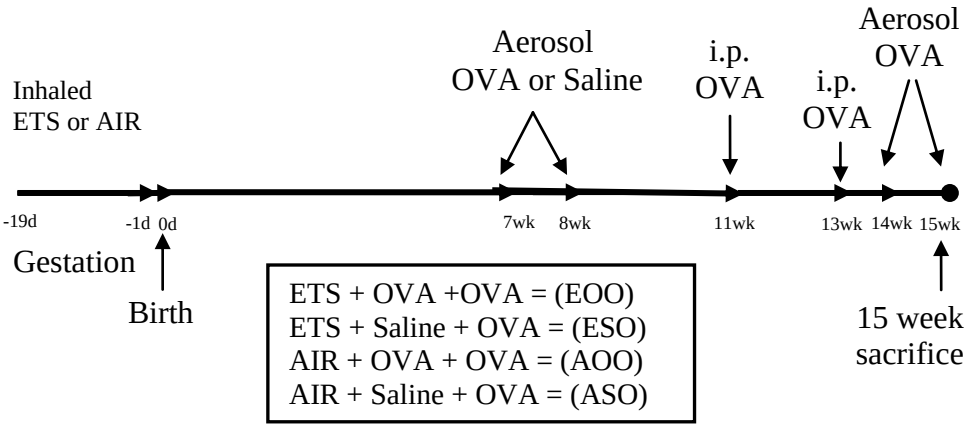


Figure 2.5: Fifteen week timeline mirrors the ten-week timeline through the seven- to eight-week of age aerosol exposures after which it deviates, with OVA sensitization and challenge exposures beginning with i.p. injections at eleven and thirteen weeks of age followed by aerosol exposures every other day at fourteen weeks of age and sacrifice at fifteen weeks of age.

We analyzed all fifteen week-old mice for the effects of *in utero* ETS exposure on responses to the OVA sensitization and challenge. There were no significant differences in OVA-specific IgG_1, IgG_{2a} and IgE levels between groups of mice exposed to OVA for the first time (ASO and ESO) or between groups previously exposed to OVA (AOO and EOO; Figure 2.6). In the former, there were significantly lower levels of OVA-specific IgG_{2a} and significantly higher OVA-specific IgE levels than in the latter.

All measured cytokines were higher in ESO vs. ASO mice, though not significantly (Table 2.1)**.** IL-4, IFNγ and TNFα were significantly lower in EOO vs. AOO mice. Levels of IL-4, IL-5, IL-13, and TNFα were significantly lower in EOO vs. ESO mice. IL-13 and IL-5

consistently higher in mice exposed to OVA for the first time (ESO and ASO) than in mice previously exposed to OVA (EOO and AOO). Levels of IL-2 did not differ between any groups.

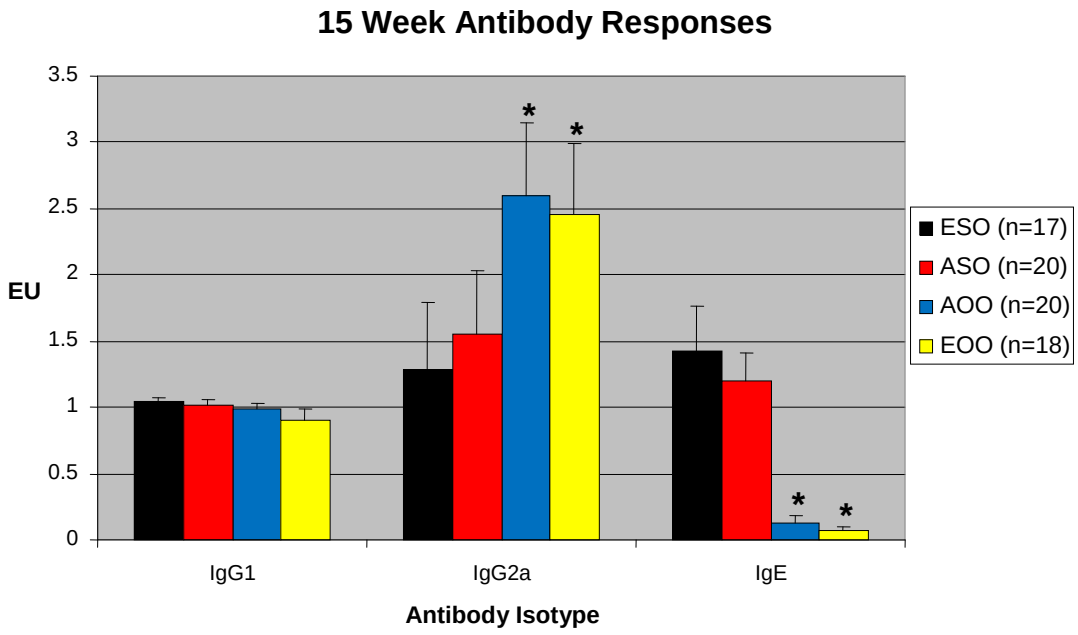


Figure 2.6: ETS exposure *in utero* has no effect on OVA-specific serum antibody (IgG_1, IgG_{2a}, IgE) levels in 15-week old mice. (*) indicates a significant difference from both ASO and ESO; $\alpha = 0.05$; bars represent ± SE.

Table 2.1: ETS Exposure *in Utero* Alters BAL Cytokine Responses of 15-Week Old Mice

	IL-13	IL-4	IL-5	TNFα	IFNγ	IL-2
AOO (n=21)	1.05*	42.76**	7.24*	31.45**	37.21**	9.07
EOO (n=18)	1.00*	20.14*	1.17*	11.56*	18.36*	2.83
ESO (n=14)	4.57**	48.82**	26.43**	31.96**	35.61	7.54
ASO (n=19)	3.18	39.79**	18.37**	21.53	35.40	4.00

Data were ranked to account for values below the limit of detection. Ranked data were analyzed using a Kruskal-Wallis test (one-way ANOVA). When significant, Tukey's HSD test was used for post-hoc comparisons of effects. For each cytokine, values marked (*) are significantly different from values marked (**); $\alpha = 0.05$. Values without an asterisk are not significantly different from any other value.

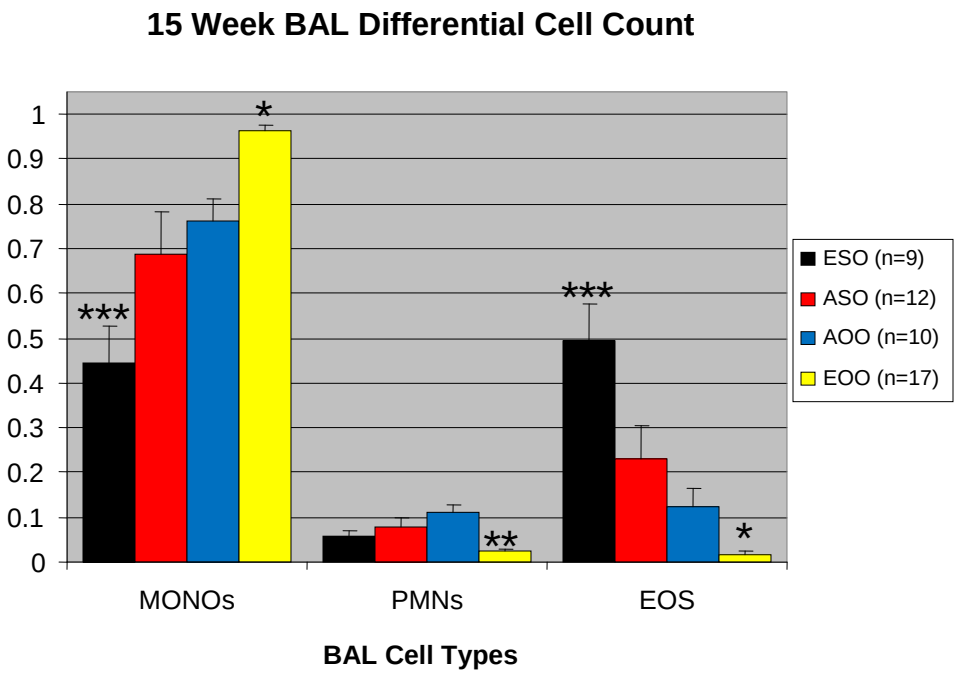
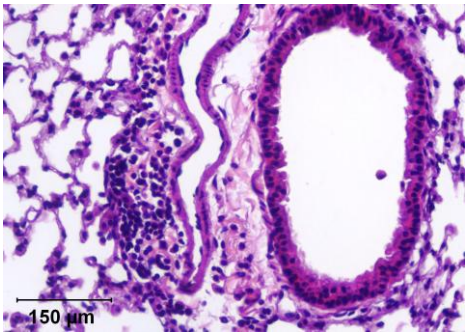


Figure 2.7: ETS exposure *in utero* affects BAL cell differentials in 15-week old mice. A 200-cell differential count was performed on BAL cells from each mouse in every group; (*) significant difference between all groups; (**) significant difference between EOO and both AOO and ASO; (***) significant difference between ESO and both AOO and EOO. Data was considered significant at $\alpha = 0.05$; bars represent mean ± SE. Values are proportion of total cell count.

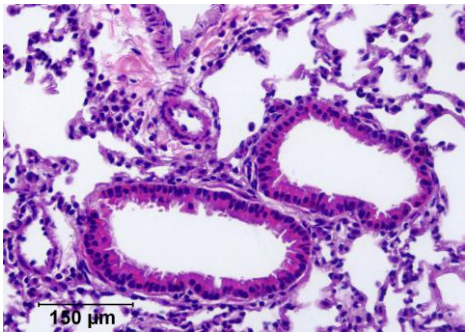
Eosinophil levels in BAL fluid were significantly higher in ESO and ASO vs. AOO and EOO mice (Figure 2.7). Eosinophil and PMN levels were significantly lower in EOO vs. AOO mice. Mononuclear cells accounted for > 96% of the BAL fluid cells in EOO mice. There were no BAL cell differences between AOO and ASO mice.

Increased lung inflammation was evident in both ESO and ASO mice (Figure 2.8). Lung inflammation in mice previously exposed to OVA (EOO and AOO) was significantly reduced compared to that in mice exposed to OVA for the first time (ESO and ASO). Again, the largest

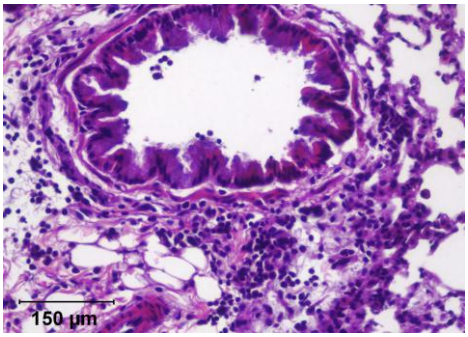
difference was between ESO and EOO mice. There were no differences between EOO and AOO or between ESO and ASO.



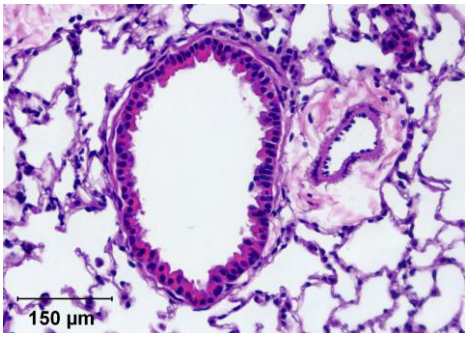
4a. ASO



4b. AOO



4c. ESO



4d. EOO

Figure 2.8: Inflammatory responses are most pronounced in ESO mice and least pronounced in EOO mice at 15 weeks. Inflammation responses are reflected by perivascular and peribronchial cellular infiltrate, increased airway mucus production, and increased interstitial density. Photomicrographs (100x) were from the median histopathology score mouse of each group.

There was increased AHR, as expected, at methacholine levels from 12.5-50 mg/ml in mice exposed to OVA for the first time (ESO and ASO; Figure 2.9) compared to mice that had previous OVA-exposure (EOO and AOO). However, AHR in ESO mice was also significantly elevated compared to ASO mice. Overall, the most striking differences in AHR were between the responsive ESO and the relatively non-responsive EOO mice. The EOO mice also exhibited significantly attenuated AHR compared to AOO mice.

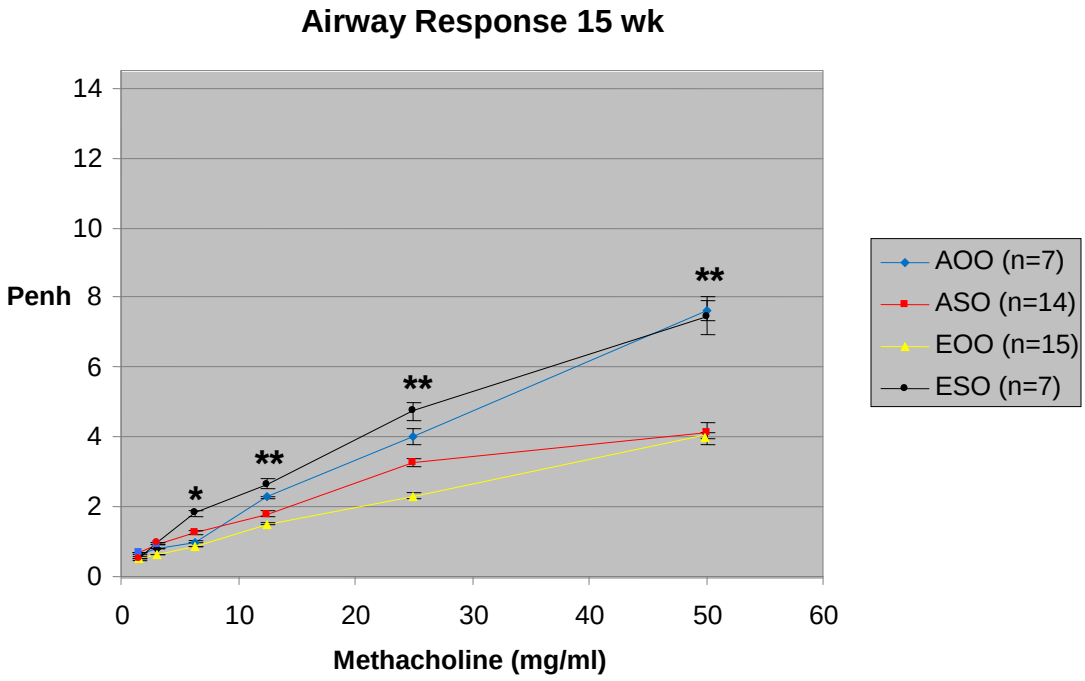


Figure 2.9: ETS exposure *in utero* alters AHR in 15-week old Balb/c mice transiently exposed to methacholine. One day post-OVA challenge in week 15, methacholine provocation and non-invasive plethysmography were used to gauge AHR; (*) significant difference between ESO and all other groups ($p<0.0001$); (**) significant difference between ESO and both ASO and EOO ($p\leq0.013$) and a significant difference between EOO and both AOO and ESO ($p\leq0.023$); bars represent mean ± SE. EOO differed significantly from ASO only at 25mg/ml methacholine. This one point did not create a significant difference in overall response curves.

As we analyzed our data, we noted what appeared to be trends in overall treatment group responses. To further investigate this observation, we identified eleven endpoints that have a direct relationship to airway inflammation and/or Th2 responses (i.e., they all increase as inflammation and/or Th2 responses increase). We used specific tests for trend to compare paired group means for these endpoint measures in mice exposed to ETS *in utero* and in those exposed to AIR *in utero* mice (Table 2.2). Analyses confirmed significant trends in the relationship between ETS and AIR mice.

Table 2.2: ETS Exposure *in Utero* Alters Initial Immune/Inflammatory Responses

	BAL Cytokines[a]				BAL	Methacholine[c]			Path[d]	Antibodies[e]	
	IL-13	IL-4	IL-5	TNFα	Eos[b]	12.5	25	50	Score	IgG1	IgE
ESO versus ASO											
ESO	4.46	48.82	26.43	31.96	50.0%	2.86	4.90	7.68	19.3	1.28	0.20
ASO	3.18	39.79	18.37	21.53	23.0%	1.74	3.20	4.04	17.7	0.46	0.18
EOO versus AOO											
EOO	1.00	20.14	1.17	11.56	1.5%	1.51	2.31	4.05	10.4	0.90	0.08
AOO	2.30	42.76	7.24	31.45	11.9%	2.28	4.00	7.62	12.8	1.00	0.13

(a) Bronchoalveolar lavage (BAL) cytokines are ranked values; (b) Eos = eosinophils;(c) Airway hyperresponsiveness (AHR) is expressed as an index value, Penh, at three methacholine concentrations (12.5, 25, and 50 mg/ml); (d) Path score is a cumulative histopathological index value; (e) Serum antibody is expressed in ELISA units (EU).

Pair-wise comparisons of group means at 15 weeks (Table 2.2) revealed significant differences for some responses of EOO vs. AOO mice (e.g., BAL differentials, cytokines, and AHR) and some responses of ESO vs. ASO (e.g., BAL differentials and AHR). However, in all cases, responses were most attenuated for EOO mice and most pronounced for ESO mice.

The Wilcoxon test on paired sign data confirmed that responses in EOO mice were uniformly lower ($p = 0.001$) than in AOO mice, indicative of a significant trend. This was further strengthened by a Pearson correlation value of 0.96 and a ranked regression yielding an adjusted R^2 of 0.91. This analysis supports the conclusion that exposure to ETS *in utero* suppressed responses to OVA sensitization/challenge in mice previously exposed to OVA. The Wilcoxon test on paired sign data also demonstrated that responses in ESO mice were consistently higher ($p = 0.001$) across all data compared to responses in ASO mice. This trend was further supported by a Pearson correlation value of 0.99 and a ranked regression yielding an

adjusted R^2 of 0.98. These results support the conclusion that ETS exposure *in utero* exacerbates subsequent adult responses to initial allergen exposure.

Discussion

At 15 weeks of age, following OVA sensitization and challenge, ESO and ASO mice were the most responsive, as reflected by increased Th2 and inflammatory cytokines, respiratory eosinophilia, AHR, and OVA-specific IgE. The EOO and AOO mice were OVA-tolerant with regard to cytokines, IgE, and airway inflammatory markers especially when compared to the ESO and ASO mice. Overall, EOO mice were significantly less responsive than AOO mice in AHR, inflammatory cytokines, and BAL eosinophils and neutrophils. ASO mice exhibited classical markers of airway inflammation but no increased AHR. AOO mice had no signs of airway inflammation but had increased AHR. These results, combined with those of 10 week-old EO mice, support the conclusion that adaptive and innate immune factors may be sufficient, but are not essential, for enhanced AHR in this murine asthma model. In addition, the results at 10 and 15 weeks demonstrate that ETS exposure *in utero* aggravates AHR following initial exposure to OVA, regardless of the route of OVA administration.

ESO mice had enhanced initial responses to OVA sensitization and challenge while EOO mice had dampened responses upon re-exposure to OVA. The reason for this response divergence is not intuitively obvious. The enhanced responses measured in ESO mice at 15 weeks of age reflect their initial response to OVA. Due to the "memory" inherent in adaptive immune responses, the suppressed responses in EOO mice at 15 weeks of age are secondary responses that reflect the initial response to aerosolized OVA at 10 weeks of age. Since that exposure was without adjuvant, establishment of tolerance to OVA was expected. In the case of EOO, the decreased responsiveness at 15 weeks of age implies increased resistance to OVA sensitization and challenge. Our data do not support a role for ETS exposure *in utero* in

determining the type (sensitization or tolerance) of immune response to allergen. However, ETS exposure *in utero* appears to significantly influence the magnitude of response, which may increase sensitivity to allergen or severity of disease.

Adult exposure to diesel exhaust particles, another major environmental combustion product, has been linked with increased incidence of allergies and asthma (Davies et al. 1998; Peterson and Saxon 1996), and rodent studies have confirmed that diesel exhaust particles promote allergic and asthmatic immune responses (Nel et al. 1998; Pandya et al. 2002;). These adult studies suggest that ETS and possibly other environmental exposures do not cause immune deviation but have an adjuvant-like effect on immune response to allergen. We demonstrate that, in addition to aggravating AHR, ETS exposure *in utero* promotes initial immune responses to allergen sensitization and challenge in adults (ESO vs. ASO).

Residual oil fly ash (ROFA) overcomes aerosol tolerance in young mice if the ROFA and OVA exposures are simultaneous (Hamada et al. 2000). Similarly, concurrent administration of inhaled OVA and Th2-adjuvant prevented establishment of OVA-specific IgE tolerance (Hurst et al. 2001). Once tolerance had been established, however, it could not be completely overcome by later simultaneous administration of OVA and the Th2 response-provoking agents. The latter finding is supported by our results, where tolerance resulting from OVA aerosol at 7-8 weeks of age persisted at least 8 weeks and was not overcome by ETS exposure *in utero*. In fact, the dampened responses of adult EOO mice suggest increased resistance to sensitization and challenge. These results are consistent with reports that smoking-associated AHR increases can occur independent of changes in levels of Th2 cytokines and IgE (Robbins et al. 2005), and that smoking-allergen interactions may be allergen-specific (Jarvis et al. 1999).

While the mechanism underlying the ETS-mediated responses to initial OVA exposure has not been identified, preliminary results point to the involvement of arginase-1 (ARG1) in this process. Up-regulation of ARG1 has been identified as an important step in the pathophysiology of asthma (Erdely et al. 2006; Zimmermann and Rothenberg 2006; Zimmerman et al. 2003). Nitric oxide (NO) production, which is vital for relaxation of airway smooth muscle cells, depends critically on the presence of adequate levels of arginine. Up-regulation of ARG-1 leads to increased catabolism of arginine, decreased NO production, and subsequently increased AHR. Consistent with this, PCR analysis revealed that ARG1 was up-regulated in lungs of ESO vs. EOO mice at 15 weeks of age (Rouse et al. 2007).

The results of the 15-week study indicate that while ETS exposure *in utero* suppresses adult responses to repeated exposures of the same antigen (EOO), it also aggravates adult responses to initial antigen exposure (EO at 10 weeks of age and ESO at 15 weeks of age). If this heightened sensitivity extends beyond OVA to unrelated antigens, as seems likely, mice exposed *in utero* to ETS and then to other antigens as adults (e.g., Aspergillus, cockroach antigen, bacterial lipopolysaccharide or respiratory viruses) would have more exaggerated responses than mice not exposed *in utero* to ETS.

Our studies were designed to address three distinct, but related issues in a mouse model of asthma: 1) whether there are any long-term, asthma-related consequences arising from exposure to ETS *in utero*, in the absence of any subsequent lung provocation; 2) what effects, if any, ETS exposure *in utero* has on adult responses to sensitization and challenge with an otherwise innocuous antigen (OVA); and 3) whether *in utero* exposure to ETS affects establishment of aerosol tolerance.

Human epidemiological studies suggest that ETS exposure *in utero* may be sufficient to increase the incidence and severity of asthma. Temporal associations between tobacco smoke

exposures of fetuses/young children and subsequent altered lung responses have been investigated. The association between smoke exposure and subsequent decreases in forced expiratory volume (a measure of lung function) was stronger when exposure was prenatal (Cunningham et al. 1994). The asthma risk for children exposed *in utero* via maternal smoking was >2X that for children receiving postnatal ETS exposure (Cunningham et al. 1996).

A 2001 review of the literature concluded "…that both *in utero* and, to some degree, passive (environmental) tobacco smoke (ETS) exposure adversely affect pulmonary function, and predispose to asthma symptoms" (Lodrup Carlsen and Carlsen 2001). A questionnaire-based survey of parents of 4000+ children revealed that *in utero* exposure to maternal smoking was a strong risk factor for wheezing and physician-diagnosed asthma in young children (Lannero et al. 2006). Neonates of non-smoking mothers exposed to ETS during pregnancy had higher serum levels of cotinine, (a major nicotine metabolite) than their mothers (Perera et al. 2004). This tendency to bioaccumulate smoke components could cause *in utero* ETS-exposed children to be particularly susceptible to respiratory problems.

Rodent studies have confirmed that responses to ETS exposure *in utero* can be critical to subsequent respiratory and immune system responses. Rats exposed to sidestream smoke for the first 100 days of life displayed no altered lung function or reactivity to methacholine (Joad et al. 1993). In contrast, female rats exposed continually to sidestream smoke *in utero* from day 3 of their gestation through week 10 after their birth, demonstrated a 24% decrease in lung dynamic compliance and a 20X increase in methacholine reactivity compared with rats exposed to smoke only during gestation or only after birth (Joad et al. 1995). The timing effect was further refined when rats were exposed to filtered air or sidestream smoke ($1mg/m^3$) *in utero* from day 3 of gestation through day 21 after their birth (Joad et al. 1999). At 8 weeks of age, they displayed

significant increases in AHR and pulmonary artery pressure and decreases in dynamic compliance compared to controls.

A recent report associated increased tumor incidence and growth with *in utero* exposures to mainstream smoke (Ng et al. 2006). Offspring of B6C3F1 mice exposed *in utero* to smoke from gestational day 4 to birth, were injected with lymphoma cells at 5 and 10 weeks of age. Males exhibited a >2X increase in tumor incidence and more rapid tumor growth relative to controls. This was accompanied by decreased cytotoxic T-lymphocyte activity without changes in natural killer cell activity, cytokine levels, lymphoid organ histology, or immune cell subpopulations.

The conclusions of our study answer the questions posed in determining the effect of ETS exposure *in utero* on constitutive lung structure and function as well as its impact on the establishment of sensitivity or tolerance to an aeroallergen. These conclusions also further implicate exposure to ETS *in utero* with altered AHR and immune responses to aeroallergen in juvenile and adult offspring, as previously reported both in human epidemiology and in rodent experimental literature.

CONCLUSION

Four main conclusions emerge from this investigation.

First, ETS exposure *in utero* alone does not alter respiratory structure or function in healthy mice.

Second, ETS exposure *in utero* exacerbates initial adult responses to allergen as demonstrated by the EO vs. AO responses at 10 weeks (AHR) and the entire range of ESO vs. ASO responses at 15 weeks. This effect is independent of whether OVA first is encountered as a tolerizing aerosol (EO at 10 weeks) or as part of a sensitization and challenge protocol (ESO at 15 weeks).

Third, in this mouse model of asthma, increased AHR upon initial allergen exposure is not necessarily coupled with changes in histopathology, cytokine profile, or antibody levels (compare responses after OVA exposure at 10 vs. 15 weeks) but is related to *in utero* ETS exposure. AHR is sometimes the only significant finding. Coincidentally, in human asthmatic episodes, AHR is the preliminary clinical response and often the only initial finding.

Fourth, OVA tolerance is not overcome by ETS exposure *in utero*.

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CHAPTER 3

ENVIRONMENTAL TOBACCO SMOKE EXPOSURE *IN UTERO* ALTERS GENE EXPRESSION IN LUNGS OF ADULT BALB/c MICE[2]

INTRODUCTION

The incidence of asthma and allergy has increased dramatically during the past thirty years, primarily in industrialized countries (Eder et al. 2006). Although improved availability and quality of medical care account for some of this increase, the magnitude of the increase surpasses the rate of improvement in healthcare delivery within these countries. Furthermore, the rate at which this increase has occurred exceeds the generational time of these countries, thereby eliminating a strictly genetic etiology. As a result, environmental exposures have become the focus of research on the rising incidence of asthma and allergy as well as many other complex diseases. The National Institute of Environmental Health Sciences spearheads programs specifically to define and measure environmental exposures critical in human disease. The new Exposure Biology Program within the Genes and Environment Initiative of the National Institutes of Health targets gene and environmental exposure interactions resulting in human disease.

Development of complex diseases or disorders, including asthma, allergy, atherosclerosis, diabetes and obesity, has been linked to multiple genes or quantitative trait loci within mammalian genomes (Casas et al. 2006; Shah et al. 2006). Multi-gene interactions are now suspected in most complex diseases (Chan et al. 2006; Motsinger et al. 2007; Yang et al. 2005). Increasingly, gene-environment interactions also are being examined for a role in the etiology of complex diseases (Colilla et al. 2003; Criswell et al. 2006; van Dellen et al. 2005;

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Williams et al. 2006). Through generalized fetal stress or specific biochemical reactions, environmental exposures *in utero* appear to mediate complex chronic diseases (cardiovascular disease, obesity, diabetes, and asthma) having recognized genetic components (Genuis 2006; Martinez 2007; Okubo and Hogan 2004). Recent findings implicate environmental exposures *in utero* in developmental disorders like autism (Ashwood et al. 2006). Although mild to moderate environmental exposures may not alter basic genetic information (DNA sequence), these exposures can determine the expression or repression of essential genes at developmentally critical points (Dolinoy et al. 2007; Li et al. 2003), thus contributing to chronic disease.

Altered lung function, increased asthma risk, and persistent lung function deficits in children (Gilliland et al. 2000; Gilliland et al. 2001; Li et al. 2000) have been associated with maternal smoking during pregnancy (*in utero* exposure). Environmental tobacco smoke (ETS) aggravates childhood asthmatic responses (Gilliland et al. 2000; Lindfors et al. 1999; Mannino et al. 2001), and promotes premature adult cardiovascular disease in mice exposed to ETS *in utero* (Yang et al. 2004). Altered lung function, exacerbation of symptoms, and acceleration of the disease processes seen with smoke exposure might arise from direct injury suffered by a developing fetus, by alteration of fetal gene expression, or through a combination of fetal injury and protective alteration of gene expression. Fetal sensitivity to ETS may be heightened or ETS components may be bio-accumulated, as demonstrated by higher cotinine levels in neonates compared to their non-smoking mothers who had received ETS exposure during pregnancy (Perera et al. 2004).

Ovalbumin (OVA) is an allergen commonly used in rodent models of allergic asthma. OVA-sensitization by i.p. injection followed by inhalation challenge with OVA aerosol elicits expansion of the T helper-2 (Th2) lymphocyte population. Production of Th2 cytokines follows, leading to airway hyperresponsiveness (AHR) and inflammation characterized by eosinophilia

and appearance of OVA-specific immunoglobulin (Ig) E (Zhang et al. 1997). This sensitization and challenge protocol does not mimic the typical human experience of aerosol-only sensitization and challenge (Bice et al. 2000). However, aerosol-only OVA exposure of mice results in little or no OVA-specific serum IgE, and no eosinophilic inflammatory response.

Within this context, we designed experiments (Figure 2.1) to simulate the respiratory consequences to offspring of daily gestational exposures to ETS or filtered air (Penn et al. 2007). We combined daily ETS exposure *in utero* with post-natal OVA inhalation to test the hypothesis that ETS exposure *in utero* alters airway function and immune responses in adults. At 10 weeks of age, *in utero* ETS and *in utero* air mice received aerosol OVA exposures. The mice exposed to ETS *in utero* displayed significantly increased AHR without significant changes in histopathology, cytokine profiles or antibody levels. At 15 weeks of age following OVA sensitization and challenge, mice exposed to ETS *in utero,* but not mice previously exposed to OVA, exhibited significantly increased AHR compared to *in utero* air controls. ETS mice previously exposed to OVA demonstrated decreased numbers of bronchoalveolar lavage (BAL) eosinophils and PMN's, diminished AHR, and lower levels of IL-4, TNFα, and IFNγ compared to air controls.

In the present study, we extracted mRNA from lungs of adult mice and examined differential gene expression profiles from four treatment groups (ESO, ASO, EOO, AOO; Figure 3.1). Lung samples for mRNA extraction were taken concomitantly with lung samples for histopathology, clinical pathology, and immunology assays (Penn et al. 2007). No gender differences were detected in immune responses, lung function or histopathology of offspring in that study. Nevertheless, RNA for individual microarray analyses in this study was examined from females only (4 females/treatment group) to eliminate any unrecognized gender effects. We used PCR to further examine selected asthma-related and inflammatory genes in these subsets of each

treatment group and to determine whether the differential gene expression levels were consistent with the pathophysiology and the immune system responses that we previously reported (Penn et al. 2007).

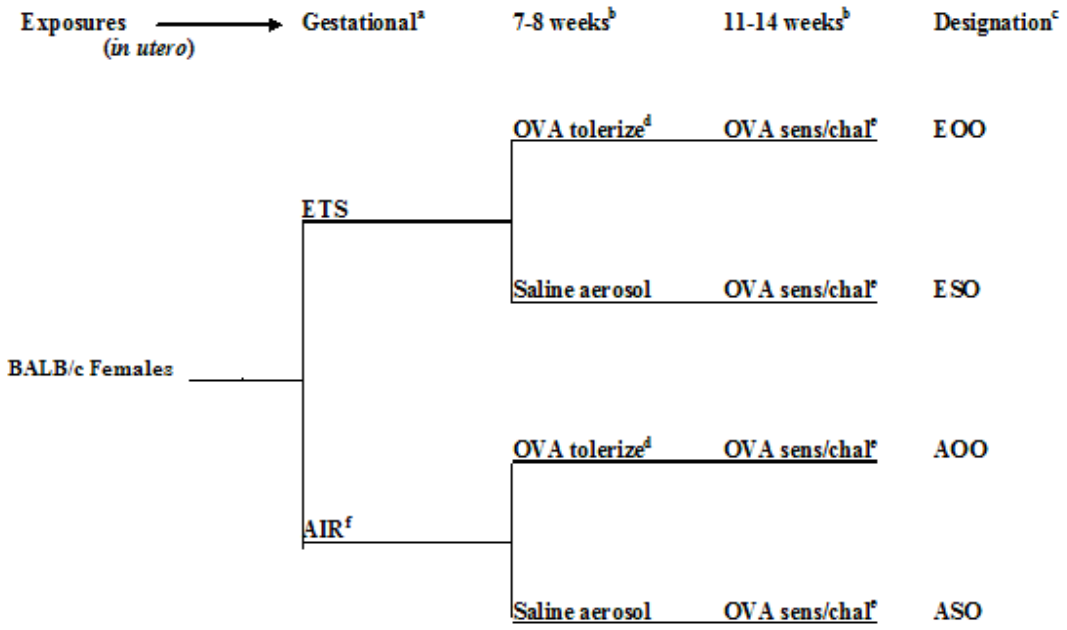

Figure 3.1: [a]days 1-19 of gestation; [b]weeks = age of offspring; [c]Treatment group designation based on gestational, 8 week, and 11-14 week exposures; [d]tolerize=OVA tolerization established through inhalation of 1% OVA aerosol (in saline), 20 minutes daily for 10 days; [e]sens/chal = OVA sensitization was established by (i.p.) injections (80 µg OVA in 2.0 mg alum), one each at 11 and 13 weeks. Three 20-minute inhalation exposures to 1% OVA in saline, every other day at 14 weeks plus an additional 20-minute exposure one day before sacrifice at 15 weeks constituted OVA challenge; [f]AIR = HEPA-filtered air.

MATERIALS AND METHODS

Animal Protocols

Eight-week old BALB/c mice (Harlan, Indianapolis, IN), were housed and handled according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory

Animal Resources. 1996. Washington D. C., National Academy Press). The Louisiana State University Institutional Animal Care and Use Committee approved all animal procedures. All animals were handled humanely and care was taken to assure alleviation of pain and suffering. A barbiturate solution (Beuthanasia-D, Schering-Plough, Kenilworth, NJ) was used via intraperitoneal injection (0.2 ml per mouse) for all sacrifices.

Exposures

Pre- and post-natal exposure protocols have been described in detail (Penn et al. 2007). Briefly, pregnant mice were exposed in dynamic exposure chambers to either ETS mixed with HEPA-filtered air (final total suspended particle concentration =10mg/m^3; carbon monoxide concentration 44.5 ppm) or to HEPA-filtered air 5 hours per day for days 1-19 of gestation. Half of the mice from each of these groups inhaled 1% OVA aerosol (20 min/day; 10 days; 7-8 weeks of age) to produce tolerance, while the other half inhaled saline aerosol. All mice were OVA-sensitized and challenged (Figure 3.1), then sacrificed one day following a final OVA challenge at 15 weeks of age to determine the effects of ETS exposure *in utero* on responses to OVA sensitization/challenge in mice not previously exposed to OVA (ESO, ASO) and on OVA tolerance in mice previously exposed to OVA (EOO, AOO). No offspring were exposed to ETS after birth.

Lung Harvest and mRNA Extraction

Left lung lobes were excised, placed in RNAlater (Ambion, Austin, TX), and stored at -80°C. Subsequently, these samples were transferred to 1mL TRIzol (Invitrogen, Carlsbad, CA) and homogenized with a Mixer Mill MM 300 (Qiagen, Valencia, CA) with a copper bead. Chloroform was added to the homogenate, mixed by inversion, and centrifuged @ 4°C to separate phases. The RNA-containing aqueous phase was transferred to an RNeasy Micro Kit (Qiagen, Valencia, CA). The manufacturer's protocol was followed, omitting Buffer RLT.

Remaining DNA was eliminated using a column RNase-Free DNase Set (Qiagen, Valencia, CA). An additional Buffer RPE wash was used to remove residual salts followed by an additional 2 minute spin to evaporate residual ethanol.

RNA samples were checked for quantity and purity with a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE). Values generated from the NanoDrop for all samples fell into the following ranges, 260/280 ratio: 2.09-2.17, 260/230 ratio: 2.20-2.27, concentration: 1200-1900 ng/uL. Further quality assays were performed on 1:5 dilutions of RNA samples with an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA) and Agilent RNA 6000 Nano Series II Kits. All samples fell into the following ranges, 28S/18S ratio: 1.3-1.8, RNA Integrity Number: 8.5-9.6.

Microarray Assay

Global gene expression in the lung was assessed in individual mice (4 female mice/treatment group) on Affymetrix Mouse Genome 430 2.0 Arrays representing more than 39,000 transcripts with over 45,000 probe sets. The arrays were processed at the Research Core Facility of Louisiana State University Health Science Center-Shreveport.

Double stranded cDNA synthesized from total RNA was used to create cRNA, which was then biotinylated, fragmented, and added to a hybridization cocktail that included probe array controls, bovine serum albumin, and herring sperm DNA. This cocktail was then hybridized (16 hours; 45°C) to oligonucleotide probes on a GeneChip Mouse 430 2.0 Array in a GeneChip Hybridization Oven 640. Immediately following hybridization, the array underwent an automated washing and staining protocol on a GeneChip Fluidics Station and was scanned with a GeneChip Scanner 3000. Data collection and processing of initial raw data were performed by a GeneChip Workstation. All gene chips and instrumentation were from Affymetrix (Santa Clara, CA).

Gene Expression Analysis

GeneChip Workstation data were sent to Expression Analysis Systems (Durham, NC). Initially, a principal component analysis was used to determine clustering of experimental units. The analysis revealed clustering by treatment group with greater variation between groups than within groups (data not presented), thus validating subsequent comparisons between treatment groups. Prior to making these comparisons, data were subjected to reduction of invariant probes (REDI) to remove previously determined mal-performing probes from the data set. Pairs of treatment group data underwent permutation analysis for differential expression (PADE), that accounts for false positives by tabulating a false discovery rate (FDR) based on a permutation-generated reference curve (technical information on REDI and PADE analyses available at www.expressionanalysis.com). Following REDI and PADE, expression data were tabulated for each remaining transcript, including individual transcript p-value, FDR, fold change, Affymetrix probe ID, gene symbol, and functional summary. For inclusion in the analysis transcripts had to be present in every sample. All transcripts included in this study had a fold change of at least 1.5 (up or down), and both an individual p-value and FDR <0.05

Pathway Analyses

Gene expression data were analyzed with the network- and pathway-building software, Ingenuity Pathways Analysis 4.0; gene networks and canonical pathways were examined using the Ingenuity Analysis Knowledge Database (Ingenuity Systems, Redwood City, CA). Identified networks and pathways were scrutinized for phenotypic relevance. Select genes were identified from the literature for confirmation by quantitative real-time PCR analysis. Custom networks were created to demonstrate the connections between the genes identified in our expression analyses.

Quantitative Real Time PCR (qRT-PCR)

*C*ytokine changes that were apparent at the protein level (IL-4, IL-5, IL-10, IL-13, TNFα; Penn et al. 2007) were not seen in the filtered microarray data. In addition, several asthma-related genes did not pass all of the filtering criteria and consequently were not identified in the filtered data as being differentially expressed, although preliminary data suggested otherwise. To clarify the status of these genes, the same fifteen-week female mouse RNA samples that had been subjected to the microarray assay underwent qRT-PCR to determine differential expression of 14 genes previously associated with asthma by other investigators (*Arg1*, *Ccl8*, *Ccl11*, *Ccl24*, *Ear11*, *Mcpt1*, *Sprr2a*, *Chi3l3*, *Chi3l4*, *Chia*, *Pde4b*, *Pde4d*, *Slc7a2*, *Tgfb1*), as well as 7 cytokine genes associated with asthma and/or pulmonary inflammation (*Ifnγ*, *Il1b*, *Il4*, *Il6*, *Il10*, *Il13*, *Tnfa*). The results of the 4 individuals in each treatment group were averaged, as they were with microarray analysis, to arrive at a fold change value for the treatment group.

RNA from each sample was converted to cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on cDNA with a Model 7300 Real-Time PCR System with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems) for the selected genes. All data collected and analyzed here adhere to the guidelines for Minimal Information About a Microarray Experiment (MIAME).

RESULTS

An ETS trend detected in pathophysiological data (Penn et al. 2007) is supported here by differential gene expression in ESO versus ASO mice. Following REDI and PADE analyses, 80 transcripts in our study met all filtering criteria (fold change ≥ 1.5, FDR < 0.05, transcript p-value < 0.05). After removal of duplicate, unmapped, and poorly characterized transcripts, 60 unique transcripts (51 down- and 9 up-regulated) remained that were differentially expressed in

the lungs of ESO versus ASO mice (Table 3.1). The actual limits of filtering criteria for these genes were: fold change 3.25 to 1.67 and -3.37 to -1.58, FDR < 0.04, transcript p-value < 0.005. None of the asthma-related genes that we targeted for qRT-PCR analysis (Table 3.2) were differentially expressed according to filtered microarray data in ESO versus ASO mice.

Table 3.1: ETS Exposure *In Utero* Changes Expression Levels of Genes in the Lungs of OVA Sensitized Mice

Affymetrix ID	Fold Change	FDR	p-value	Gene Symbol	Function	Process
1448303_at	3.25	0.040	0.0018	Gpnmb	Integrin binding	Inhibit proliferation
1441557_at	2.12	0.040	0.0008	Setd8	Histone (H4) methylation	Control cytokinesis
1424706_at	2.02	0.037	0.0003	Zfp51	DNA/protein binding	Unknown
1444396_at	2.01	0.040	0.0008	Trp53inp2	Unknown	Unknown
1424704_at	1.98	0.037	0.0002	Runx2	Transcription inhibitor	Inhibit proliferation
1421525_a_at	1.92	0.037	0.0002	Birc1e	Unknown	Macrophage activation/death
1450541_at	1.89	0.037	0.0003	Pvt1	Unknown	Unknown
1453281_at	1.88	0.040	0.0006	Pik3cd	VEGFactivation	Promote immune responses
1434500_at	1.67	0.037	<.0001	Ttyh2	Chloride channel	Chloride channel regulation
1456868_at	-3.37	<.001	0.0004	Mrcl3	Cytoskeleton actin binding	Motility, elasticity, and apoptosis
1457610_at	-2.91	<.001	0.0006	Rbck1	Zinc and protein binding	Ubiquitinization
1433774_x_at	-2.81	<.001	0.0006	Cog1	Protein binding	Golgi transport
1441840_x_at	-2.78	<.001	0.0001	Kiaa0963	Unknown	Unknown
1447530_at	-2.77	<.001	0.0012	F8a	Endosomal binding/motility	Endosomal formation/function
1440180_x_at	-2.76	<.001	<.0001	Zbtb3	Dna/protein binding	Unknown
1458354_x_at	-2.62	<.001	0.0006	Krt28	Unknown	Unknown
1443829_x_at	-2.55	<.001	0.0001	Coasy	Adenylyltransferase	Coenzyme A regulation
1455853_x_at	-2.43	<.001	0.0009	Tspan31	Transmembrane protein	Proliferation, differentiation, growth
1449804_at	-2.36	0.040	0.0036	Pnmt	Tyrosine metabolism	Growth
1458325_x_at	-2.34	0.040	0.0043	Bmf	Cytoskeleton myocin binding	Cell repair/ induce apoptosis
1436149_at	-2.23	0.037	0.0020	Cox5b	Cytochrome C subunit	Oxidative phosphorylation
1427527_a_at	-2.20	<.001	0.0001	Pthlh	Calcium binding	Proliferation, differentiation, growth
1443752_at	-2.17	<.001	0.0009	Kiaa1529	Unknown	Unknown
1438638_x_at	-2.17	0.037	0.0020	Fam116b	Unknown	Unknown
1459766_x_at	-2.14	<.001	0.0004	Sf1	Activate transcription	Proliferation, differentiation, growth
1421788_x_at	-2.13	<.001	0.0011	Klk13	Unknown	Unknown
1421621_at	-2.10	<.001	0.0003	Rasgrf2	Guanyl nucleotide exchange	Growth and signaling
1452538_at	-2.08	<.001	0.0006	Igh-V3609N[b]	Antigen binding	Proliferation, differentiation, growth
1456746_a_at	-2.08	<.001	0.0005	Cd99l2	Unknown	Unknown
1441935_at	-2.07	<.001	0.0002	Ankra2	Activate transcription	Proliferation, differentiation, growth
1419216_at	-2.04	0.040	0.0032	Azi1	Unknown	Unknown
1426325_at	-2.02	0.037	0.0015	Kif1c	Cytoskeleton binding protein	Motility and Golgi transport
1424845_a_at	-1.94	0.037	0.0011	Cep68	Unknown	Unknown
1449218_at	-1.91	0.037	0.0019	Cox8b	Cytochrome assembly	Oxidative phosphorylation
1425155_x_at	-1.88	0.037	0.0017	Csf1	Activate transcription	Proliferation, differentiation, growth
1433792_at	-1.87	0.040	0.0026	Nrip2	Inhibit transcription	Unknown

(Table 3.1 continued)

1439745_at	-1.84	0.037	0.0010	Cacng7	Calcium channel	Calcium channel regulation
1425120_x_at	-1.83	0.040	0.0019	Fam14a	Unknown	Unknown
1454634_at	-1.83	0.037	0.0014	Fuk	Glycoprotein phosphorylation	Leukocyte trafficking
1419387_s_at	-1.83	<.001	0.0005	Muc13	Transmembrane protein	Unknown
1440368_at	-1.83	0.040	0.0023	Jmjd2b	Unknown	Unknown
1459977_x_at	-1.81	0.037	0.0011	Cox10	Heme synthesis	Oxidative phosphorylation
1450704_at	-1.81	0.037	0.0010	Ihh	Transferase	Proliferation, differentiation, growth
1416518_at	-1.77	0.040	0.0019	H1foo	DNA and chromatin binding	Chromatin folding
1417305_at	-1.77	0.040	0.0019	Des	Cytoskeleton binding protein	Smooth Muscle motility/elasticity
1435015_at	-1.75	<.001	0.0005	Zfp787	DNA/protein binding	Unknown
1426274_at	-1.74	<.001	0.0001	Slc9a8	Sodium/hydrogen exchange	Unknown
1439144_at	-1.74	<.001	0.0001	Cwf19l1	Unknown	Cell cycle control
1430236_s_at	-1.74	<.001	0.0001	Gdsm2	Unknown	Unknown
1450538_s_at	-1.72	<.001	0.0003	Mcpt9	Peptide catabolism	Mast cell degranulation
1448927_at	-1.70	0.040	0.0012	Kcnn2	Potassium channel	Potassium channel regulation
1427409_at	-1.68	0.037	0.0010	9-Mar	Unknown	Unknown
1436083_at	-1.67	0.037	0.0009	Lrp3	Lipoprotein binding	Adhesion, apoptosis
1434463_at	-1.65	0.037	0.0009	Bfsp2	Cytoskeleton component	Motility, elasticity, and structure
1459073_x_at	-1.63	0.040	0.0011	Fgf14	Ion channel protein binding	Sodium channel regulation
1421975_a_at	-1.63	0.040	0.0015	Add2	Cytoskeleton actin binding	Motility, elasticity, and structure
1421329_a_at	-1.61	0.037	0.0004	Smyd1	Inhibit transcription	Chromatin remodeling
1426493_a_at	-1.61	<.001	0.0001	Kifc2	Cytoskeleton binding protein	Motility and Golgi transport
1432883_at	-1.59	0.037	0.0006	Wdr87	Unknown	Unknown
1420569_at	-1.58	<.001	<.0001	Chad	Unknown	Chrondrocyte adhesion

Genes differentially expressed by microarray in OVA-sensitized mice exposed *in utero* to ETS or AIR (ESO vs. ASO)[a]; [a] Mice were exposed *in utero* to ETS or AIR, then OVA-sensitized by i.p. injections and challenged with aerosol OVA as described in Material and Methods; [b]Human homologue IGH-1A.

However, when examined by qRT-PCR, four asthma-related genes were identified as up-regulated (*Arg1*, *Ccl24*, *Slc7a2*, *Mcpt1*). In each case, the fold change from the microarray analysis was very similar to that determined by qRT-PCR (Table 3.2). Neither microarray analysis nor qRT-PCR revealed differential expression of cytokine genes in ESO versus ASO mice (Table 3.3).

A significant ETS effect demonstrated pathophysiologically (Penn et al. 2007) is supported here by differential gene expression in EOO versus AOO mice. Following REDI and PADE analyses, 85 transcripts were initially identified. Filtering criteria for these transcripts

Table 3.2: Microarray and qRT-PCR Evaluations of Differential (fold-change) Expression of Asthma-Related Genes Yield Similar Results

	Arg1		Ccl8		Ccl11		Ccl24		Ear11		Mcpt1		Sprr2a	
	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip
AOO v ASO	-5.8	-6.3[d]	-2.9	-1.8	-3.6	-3.8[a]	-4.7	-2.6[d]	-5.0	-4.6[a]	-2.0	-1.3[d]	-17.3	-5.6[d]
ESO v ASO	2.4	2.2[d]	1.0	-1.1[d]	1.2	1.3[d]	2.1	1.8[d]	1.4	1.3[d]	1.7	1.2[d]	-1.0	1.1[d]
EOO v ESO	-26.8	-22.0	-8.2	-5.2	-7.7	-7.0	-17.0	-5.2[d]	-41.3	-23.8	-11.2	-1.5	-131.	-7.3[c]
EOO v AOO	-1.9	-1.6[d]	-2.8	-3.2	-1.7	-1.5[d]	-1.8	-1.1[d]	-5.7	-4.1[d]	-3.2	1.0[d]	-7.9	-1.4[e]

	Chi3l3		Chi3l4		Chia		Pde4b		Pde4d		Slc7a2		Tgfβ1	
	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip
AOO v ASO	-3.6	-1.5[e]	-18.5	-12.6	-3.9	-2.2[d]	1.3	1.6[a]	1.1	1.1[e]	-1.6	-1.3[e]	-1.1	-1.1[d]
ESO v ASO	1.2	1.1[e]	-1.1	-1.0[d]	1.2	1.2[d]	1.1	-1.1[e]	1.0	1.1[e]	1.7	1.7[a]	1.2	-1.1[d]
EOO v ESO	-17.6	-3.6[b]	-228.5	-45.7	-8.3	-4.3	1.0	4.8	1.1	1.1[e]	-4.0	-1.6[a]	-1.8	-1.6[d]
EOO v AOO	-4.1	-2.1[b]	-13.6	-3.7[d]	-1.8	-1.6[d]	-1.1	-1.1[e]	1.1	1.1[e]	-1.5	-1.2[e]	-1.4	-1.3[d]

a did not pass FDR

b average of two transcripts

c average of two transcripts, one did not pass FDR

d did not pass transcript p-value or FDR

e average of two transcripts, did not pass transcript p-value or FDR

were: fold-change 2.13 to 1.50 and -3.24 to -1.50, FDR $\leq$ 0.041, transcript p-value < 0.005. Removal of duplicate, unmapped, and poorly characterized transcripts left 31 down-regulated and 41 up-regulated transcripts differentially expressed in EOO mice relative to AOO controls (Table 3.4). Among the down-regulated genes, one asthma-related gene (*Ccl8*) met the filtering criteria. qRT-PCR analysis verified results for *Ccl8* and indicated a down- regulation of 10 other asthma-related genes (*Arg1*, *Ccl11*, *Ccl24*, *Ear11*, *Mcpt1*, *Sprr2a*, *Chi3l3*, *Chi3l4*, *Chia*, *Slc7a2*)

in EOO versus AOO mice (Table 3.2). Evaluation by qRT-PCR demonstrated down-regulation of 6 cytokine genes in EOO mice relative to AOO mice (*Il4*, *Il6*, *Il10*, *Il13*, *Tnfa*, *Il1b***;** Table 3.3**).** Again, differential expression of these cytokines was not detected by microarray. Gene expression comparisons of ASO versus AOO or ESO versus EOO mice, reveal that differences exist primarily as a result of airway inflammation present in mice not previously exposed to OVA (ASO and ESO) and absent in mice previously exposed to OVA (AOO and EOO). AOO mice had 673 down- and 1037 up-regulated transcripts relative to ASO mice, while EOO mice had 847 down- and 1465 up-regulated transcripts relative to ESO mice (data available upon request). In each of these cases, unidentified, duplicate, and unmapped transcripts accounted for 15-25% of the total transcripts.

Table 3.3: Differential (fold-change) Expression of Cytokine Genes: Microarray vs. qRT-PCR

	Ifnγ		Il1b		Il4		Il6		Il10		Il13		Tnfα	
	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip	PCR	Gene Chip
AOO v ASO	-1.1	-1.1[d]	-1.1	-1.1[d]	-2.1	-1.2[e]	-1.6	-1.0[d]	-1.7	-1.1[d]	-4.9	-1.4[e]	-1.2	-1.1[d]
ESO v ASO	1.1	-1.0[d]	-1.1	-1.4[d]	1.3	1.3[d]	-1.3	-1.1[d]	-1.2	-1.1[d]	-1.1	-1.3[d]	1.0	-1.1[d]
EOO v ESO	-1.6	-1.0[d]	-1.6	-1.3[d]	-4.7	-1.7[a]	-2.2	-1.0[d]	-4.8	-1.2[d]	-17.2	-1.1[d]	-2.1	-1.2[d]
EOO v AOO	-1.3	-1.0[d]	-1.6	-1.6[d]	-1.8	-1.1[d]	-1.9	-1.0[d]	-3.2	-1.0[d]	-3.8	-1.0[d]	-1.8	-1.1[d]

a did not pass FDR

b average of two transcripts

c average of two transcripts, one did not pass FDR

d did not pass transcript p-value or FDR

e average of two transcripts, did not pass transcript p-value or FDR

Table 3.4: *In Utero* ETS Exposure Changes Expression Levels of Genes in the Lungs of OVA Tolerant Mice Subsequently Challenged with OVA

Affymetrix ID	Fold change	FDR	p-value	Gene Symbol	Function	Process
1425206_a_at	2.13	<.001	0.0002	Ube3a	Ubiquitin conjugation	Protein catabolism
1415854_at	1.94	<.001	0.0006	Kitlg	Erk, Mapk, Akt activation	Proliferation signaling
1417188_s_at	1.94	<.001	0.0009	Hip2	Activate transcription	Ubiquitin regulation
1449217_at	1.87	0.041	0.0028	Casp8ap2	NFκB activation	Apoptosis/survival signaling
1450924_at	1.83	<.001	0.0005	Hdgfrp3	Bind growth factor	Regulation of growth factor
1422975_at	1.8	<.001	<.0001	Mme	Degrade bradykinin/elastin	Extracellular matrix repair
1428025_s_at	1.8	<.001	0.0004	Pitpnc1	Phosphatidylinositol binding	Phosphatidylinositol regulation
1436917_s_at	1.76	0.041	0.0031	Gpsm1	Activate GTPases	Regulate G-protein receptor activity
1421230_a_at	1.74	0.041	0.0033	Msi2h	Unknown	Unknown
1418489_a_at	1.73	<.001	0.0001	Calcrl	Mobilize calcium/cAMP	Control smooth muscle migration
1451866_a_at	1.73	<.001	0.0003	Hgf	Erk, Mapk activation	Suppress dendritic cell activation
1453139_at	1.72	<.001	0.0002	Nudt12	Hydrolase	Nucleotide regulation
1425845_a_at	1.68	0.041	0.0012	Shoc2	Co-inhibitor of Raf1	Regulate Ras pathway
1447926_at	1.67	0.041	0.002	Arl5	Unknown	Unknown
1417069_a_at	1.67	0.041	0.0015	Gmfb	NFκB activation	Apoptosis/survival signaling
1448665_at	1.67	0.041	0.0015	Dmd	Actin binding	Cytoskeletal anchoring
1451146_at	1.66	0.041	0.0016	Zfp386	Chromatin/DNA binding	Unknown
1449888_at	1.66	0.041	0.0016	Epas1	Activate Vegf	Control vascular remodeling
1418231_at	1.64	0.041	0.0011	Lims1	Pi3k, Akt activation	Integrin signaling
1420514_at	1.64	0.041	0.0016	Tmem47	Unknown	Unknown
1437784_at	1.63	<.001	0.0002	Runx1t1	Increase Myc, Jun expression	Proliferation/growth
1437668_at	1.63	0.041	0.0014	Ccrl1	Activate cytokines	Immune signaling
1426517_at	1.61	<.001	0.0002	Gnaz	GTP/Erk, Mapk activation	Differentiation signaling
1447944_at	1.61	<.001	0.0001	Zkscan1	Unknown	Unknown
1425370_a_at	1.6	0.041	0.0021	Erg	Increase Tgfb2 expression	Inhibit apoptosis
1451827_a_at	1.6	0.041	0.0011	Nox4	NADPH Oxidase	ROS metabolism/inhibit proliferation
1428345_at	1.58	0.041	0.0018	Ppapdc2	Diphosphate phosphotase	Inhibit PMN-mediated inflammation
1429776_a_at	1.57	0.041	0.0022	Dnajb6	Co-chaperone(hsp70)	Protein transport and folding
1419805_s_at	1.57	0.041	0.0021	Ggps1	Prenyltransferase	Sterol synthesis
1437302_at	1.56	0.041	0.001	Adrb2	Erk, Mapk activation	Motility/adhesion signaling
1418780_at	1.55	<.001	0.0001	Cyp39a1	Hydroxylase	Lipid metabolism
1437982_x_at	1.54	0.041	0.0015	Cox15	Cytochrome c assembly	Oxidative phosphorylation
1438530_at	1.54	<.001	0.0002	Tfpi	Inhibition of F10	Coagulation regulation
1416701_at	1.53	0.041	0.0016	Rnd3	GTP-linked protein binding	Inhibit smooth muscle contraction
1452328_s_at	1.53	0.041	0.0018	Pja2	Ubiquitin conjugation	Ubiquitin regulation
1418429_at	1.53	0.041	0.0013	Kif5b	Increase microtubule mobility	Cell mobility
1429434_at	1.52	0.041	0.0015	Pik3ca	Erk, Mapk, Akt, Rho activation	Proliferation signaling
1459973_x_at	1.51	<.001	0.0001	Dpp4	Cytokine proteolysis	Inhibit Tcell activation

(Table 3.4 continued)

1453908_at	1.51	0.041	0.0011	Ptprb	Sodium channel regulation	Proliferation signaling
1452385_at	1.51	<.001	<.0001	Usp53	Ubiquitin-specific peptidase	Ubiquitin regulation
1420019_at	1.5	0.041	0.0015	Tm4sf3b	Integrin binding	Activation/growth signaling
1448872_at	-3.24	0.041	0.003	Reg3g	Unknown	Epithelial cell regeneration
1419684_at	-3.19	0.041	0.0042	Ccl8	Activate WBC cytokines	Immune cell activation
1418930_at	-2.99	0.041	0.0037	Cxcl10	Mast cell/monocyte chemotaxis	Inflammatory response
1438148_at	-2.81	<.001	0.0009	Gm1960c	Macrophage chemotaxis	Inflammatory response
1448377_at	-2.63	<.001	0.0007	Slpi	Bind peptidase/inhibit NFκB	Inactivate leukocyte peptidase
1449401_at	-2.42	<.001	0.0009	C1qc	Complement activation	Blood cell activation
1450849_at	-2.26	0.041	0.0037	Hnrpu	Tranport snRNA to cytoplasm	RNA processing
1419128_at	-2.19	<.001	0.0008	Itgax	Adhesion/signaling	Immune response
1421326_at	-2.17	0.041	0.0016	Csf2rb1	Erk, Mapk, Akt activation	Proliferation, differentiation, survival
1440801_s_at	-2.1	0.041	0.0022	Adrbk2	G-protein receptor activation	Activate receptor signaling
1416871_at	-2.08	0.041	0.0025	Adam8	Metalloendopeptidase	Release of IgE low affinity receptor
1419699_at	-2.02	0.041	0.0012	Scgb3a1	Akt inhibition	Epithelial cell differentiation
1447071_at	-1.85	0.041	0.0025	Tcf7l2	Activate transcription	Tcell extravasation
1437864_at	-1.83	<.001	0.0001	Adipor2	Activate Ampk	Lipid metabolism
1417025_at	-1.8	0.041	0.001	H2-Eb1d	MHC II antigen binding	Antigen presentation
1456022_at	-1.78	0.041	0.0015	Hipk2	Increase Tgfβ, Jnk transcription	Cell cycle progression signaling
1425477_x_at	-1.73	0.041	0.0008	H2-Ab1e	MHC II antigen binding	Antigen presentation
1446050_at	-1.71	<.001	0.0001	Magi1	ATP-dependent Akt activation	Motility/adhesion signaling
1425294_at	-1.69	0.041	0.001	Slamf8	Unknown	Immune response
1450355_a_at	-1.63	0.041	0.0009	Capg	Caps actin filaments	Leukocyte motility and phagocytosis
1422725_at	-1.63	0.041	0.0014	Mak	cATP/nucleotide binding	Cell differentiation signaling
1415871_at	-1.61	0.041	0.0004	Tgfbi	Integrin binding	Motility/adhesion signaling
1419315_at	-1.61	0.041	0.0006	Slamf9	Unknown	Immune response
1424758_s_at	-1.59	0.041	0.0012	Serpina10	Inhibition of F10	Coagulation regulation
1433963_a_at	-1.56	0.041	0.0006	BC032204f	Unknown	Unknown
1429475_at	-1.56	0.041	0.0008	Sts1	Ubiquitin conjugation	Suppress Tcell signaling/endocytosis
1419721_at	-1.55	0.041	0.0005	Gpr109b	Inhibit cAMP	Lipid metabolism
1426324_at	-1.54	0.041	0.0006	H2-D1g	MHC I antigen binding	Antigen presentation
1425837_a_at	-1.53	0.041	0.0009	Ccrn4l	Activate RNA polymerase II	Circadian rhythm
1416985_at	-1.51	<.001	<.0001	Sirpa	Erk, Mapk, Akt inhibition	Immune signaling
1428942_at	-1.5	0.041	0.0002	Mt2a	NFκB activation	Apoptosis/survival signaling

Genes differentially expressed by microarray in OVA-tolerant mice exposed *in utero* to ETS or AIR (EOO vs. AOO)[a]; [a] Mice were exposed *in utero* to ETS or AIR, then exposed to OVA-aerosol at 7 to 8 weeks of age then OVA-sensitized by i.p. injections and challenged with aerosol OVA at 15 weeks of age as described in Material and Methods; [b]Human homologue IGH-1A.

For ESO versus ASO and EOO versus AOO comparisons, genes were organized into networks and were identified within canonical pathways based on reference literature. The analyses allowed identification of experimentally denoted genes within defined functional pathways and networks based upon previously identified gene-gene or protein-protein relationships. Within the ESO-ASO comparison, 34 genes (8 up-regulated and 26 down-regulated) had demonstrable network connections (Figure 3.2). Among the down-regulated genes were nine involved in promoting proliferation/differentiation/growth (*Tspan31*, *Pmnt*, *Pthlh*, *Sf1*, *Rasgrf2*, *Igh-V3609N*, *Ankra2*, *Csf1*, *Ihh*) and four associated with increased ubiquitination/apoptosis (*Mrcl3*, *Rbck1*, *Bmf*, *Lrp3*). Among the up-regulated genes were three associated with inhibition of proliferation (*Gpnmb*, *Setd8*, *Runx2*) and one promoting inflammatory and immune responses (*Pik3cd*), as well as four asthma-related genes (*Arg1*, *Ccl24*, *Slc7a2*, *Mcpt1*) identifiable only through qRT-PCR.

Within the EOO-AOO comparison, 71 genes (43 down-regulated and 28 up-regulated) had documented network connections (Figure 3.3) including the 14 genes identified by qRT-PCR. Of 43 down-regulated genes, 32 enhance immune and inflammatory responses or have been implicated in asthma pathogenesis, including six cytokine (*Il1b*, *Il4*, *Il6*, *Il10*, *Il13*, *Tnfa*), four chemokine (*Ccl8*, *Ccl11*, *Ccl24*, *Cxcl10*), and eight asthma-related genes (*Arg1*, *Ear11*, *Mcpt1*, *Sprr2a*, *Chi3l3*, *Chi3l4*, *Chia*, *Slc7a2*). Among the up-regulated genes were six involved in positive regulation of proliferation/differentiation/growth (*Kitlg*, *Runxlt1*, *Gnaz*, *Pik3ca*, *Ptprb*, *Tmrsf3*), seven associated with increased ubiquitination/apoptosis (*Ube3a*, *Hip2*, *Casp8ap2*, *Gmfb*, *Erg*, *Pja2*, *Usp53*), and four involved in suppression of immune responses (*Hgf*, *Ppapdc2*, *Rnd3*, *Dpp4*). The majority of genes with altered expression (up or down) play roles in the immune system. The non-cytokine immune genes with *in utero* ETS-exposure mediated changes in expression are presented in Table 3.5.

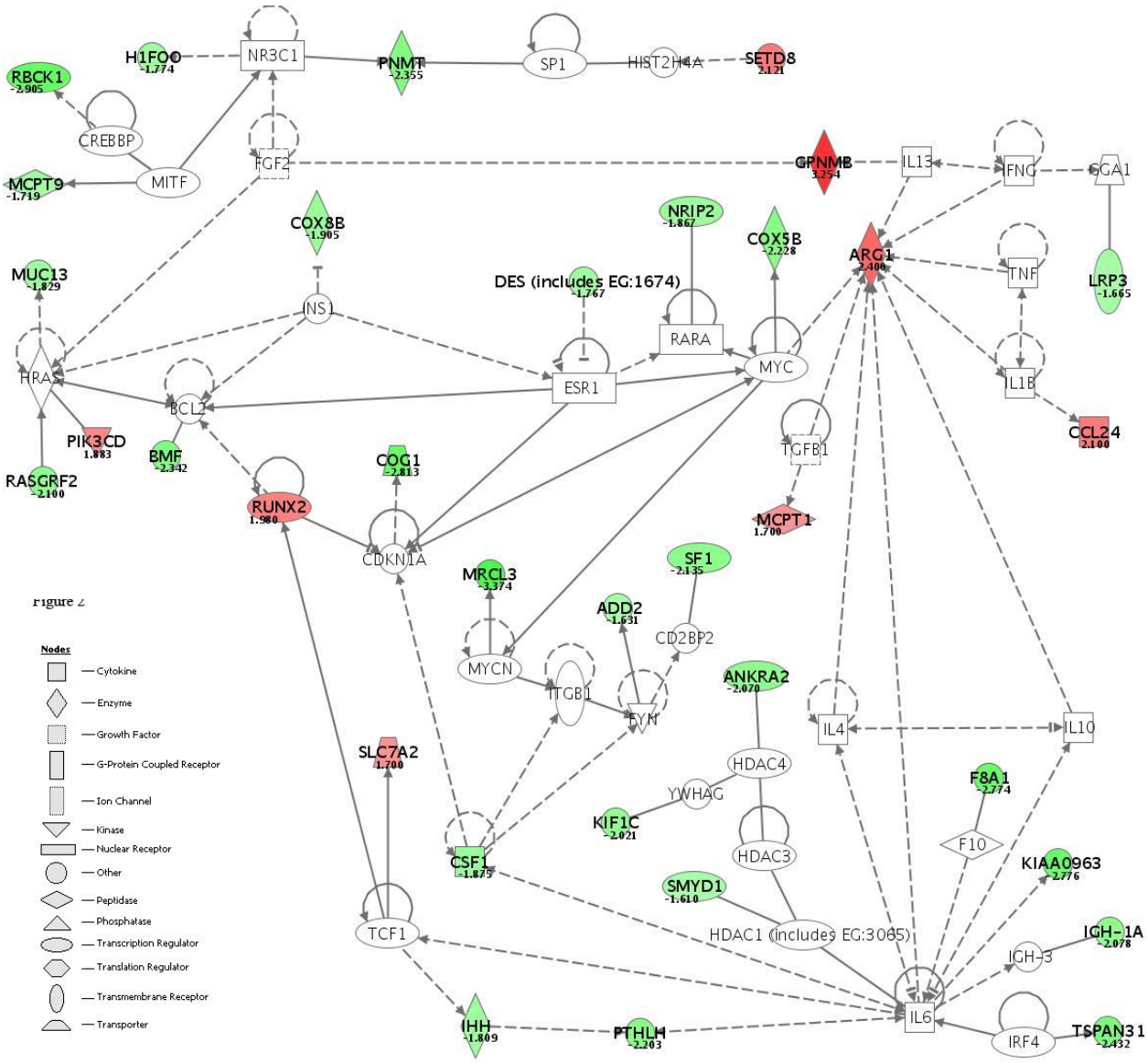

Figure 3.2: ETS Exposure *in Utero* Creates a Network of Altered Gene Expression in OVA Sensitized Mice. Green represents down-regulation and red up-regulation (fold-changes ≥ 1.5); white nodes represent fold-change < 1.5. Intensity of color increases with increasing fold-change (numbers represent fold-change values). Dotted lines indicate indirect relationships and solid lines direct interactions. All gene symbols are human homologue designations. Network constructed with Ingenuity Systems software.

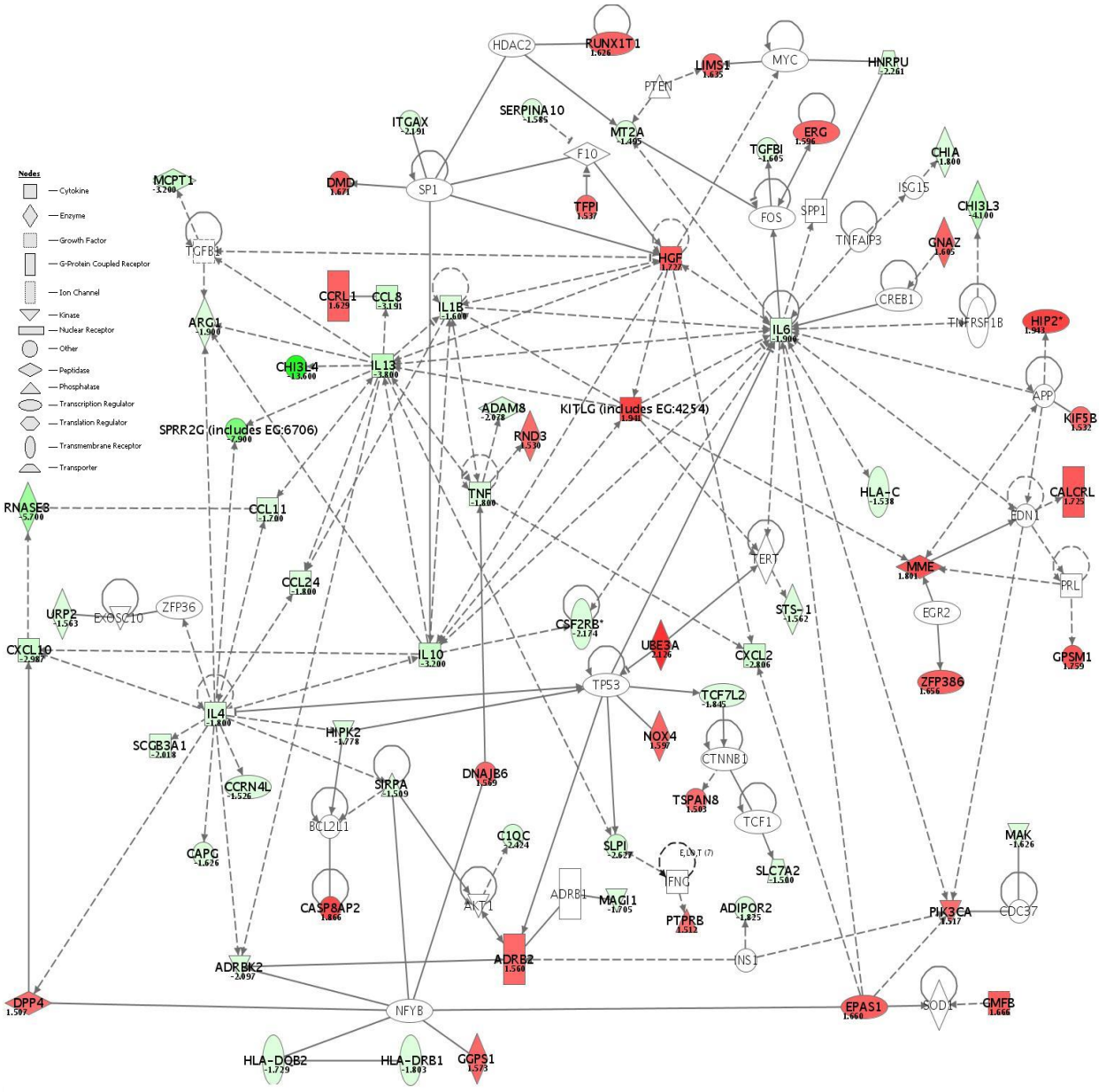

Figure 3.3: ETS Exposure *in Utero* Creates a Network of Altered Gene Expression in OVA Tolerant Mice Subsequently Challenged with OVA. Green represents down-regulation and red up-regulation (fold-changes ≥ 1.5); white nodes represent fold-change < 1.5. Intensity of color increases with increasing fold-change (numbers represent fold-change values). Dotted lines indicate indirect relationships and solid lines direct interactions. All gene symbols are human homologue designations. Network constructed with Ingenuity Systems software.

Table 3.5: Gene Expression Changes Mediated by ETS Exposure *In Utero* Diminish Immune Responses in OVA Tolerant Mice Re-exposed to OVA

Gene	Process	Influence	Change	Reference
Hgf	Dendritic cell antigen presentation	inhibits	up	(Okunishi et al. 2005)
Ppapdc2	PMN activation	inhibits	up	(Fukunaga et al. 2006)
Rnd3	Leukocyte migration	inhibits	up	(Sanchez-Madrid and del Pozo 1999)
Dpp4	Cytokine cleavage	promotes	up	(Boonacker and Van Noorden 2003)
H2-Ab1	Antigen presentation	promotes	down	(Stevenson et al. 2000; Wolk et al. 2000)
H2-Eb1	Antigen presentation	promotes	down	(Stevenson et al. 2000; Wolk et al. 2000)
H2-D1	Antigen presentation	promotes	down	(Stevenson et al. 2000; Wolk et al. 2000)
Ccl11	Pro-inflammatory signaling	promotes	down	(Mantovani et al. 2004)
Ccl24	Pro-inflammatory signaling	promotes	down	(Mantovani et al. 2004)
Ccl8	Pro-inflammatory signaling	promotes	down	(Mantovani et al. 2004)
Cxcl10	Pro-inflammatory signaling	promotes	down	(Mantovani et al. 2004)
Cxcl2	Pro-inflammatory signaling	promotes	down	(Mantovani et al. 2004)
Slamf8	Lymphocyte activation signaling	promotes	down	(Graham et al. 2006)
Slamf9	Lymphocyte activation signaling	promotes	down	(Graham et al. 2006)
Tcf7l2	T-cell maturation/development	promotes	down	(Willinger et al. 2006)
Slpi	Control of Mast cell/PMN proteases	promotes	down	(Wright et al. 1999)
Sts1	T cell signaling modulation	promotes	down	(Carpino 2004)
Adam8	Soluble IgE release	promotes	down	(Fourie et al. 2003)
Igfbi	Pro-inflammatory cytokine (IL6) production	promotes	down	(Moller et al. 1994)
Capg	Macrophage mobility/phagocytosis	promotes	down	(Parikh et al. 2003)
Sirpa	Modulates macrophage phagocytosis	promotes	down	(van den Berg et al. 2004)
Itgax	Dendritic cell maturation	promotes	down	(Hebel et al. 2006)
C1qc	Antigen recognition/uptake	promotes	down	(Sontheimer et al. 2005)

DISCUSSION

This is the first report to identify gene expression changes in the adult lung following ETS exposure *in utero*. In addition, the gene expression results presented here (microarray and RT-PCR) provide a molecular framework within which to consider the pathophysiological, lung function and inflammatory responses we recently reported (Penn et al. 2007).

The ETS exposure level (TSP=10mg/mm^3) used in this study is the same that was used in our other *in utero* (Yang et al. 2004) and earlier adult exposure studies (Bowles et al., 2005; Penn and Snyder, 1993). The associated steady-state CO levels are approximately double those detected in a typical indoor smoking area (Penn et al. 1994). This exposure level, while higher

than that found in a smoking household, is well below that used in numerous published studies. In light of the indirect (*in utero*) nature of these exposures and the lack of literature on how the dose delivered to the fetus varies with the levels of maternal ETS exposure, we selected an exposure that we felt would be sufficient to elicit a detectable response while not being beyond physiological reality.

We have carried out multiple ETS exposure *in utero* experiments with 13-30 mice per mixed-gender treatment group (Penn et al. 2007 and unpublished observations). In these studies, there were no demonstrable gender-related differences among offspring in immune responses, histopathology, lung function, or clinical pathology. These results do not predict a significant gender difference in gene expression. Nevertheless, only female offspring samples from our earlier study (Penn et al. 2007) were used in the present gene studies to rigorously control for subtle, undetected gender influences. The gene expression results we report here for those female offspring correlate well with the pathology and lung function results from that earlier study.

Another recent *in utero* study demonstrated differential DNA methylation leading to modulated gene expression as a result of altered maternal diet (Dolinoy et al. 2006). No gender differences were detected within that study, as well. Gender differences have, however, been reported for some adult responses to *in utero* stresses. In an earlier collaborative study, we demonstrated that ETS exposure *in utero* (identical to that used in this experiment) produces significant acceleration of atherosclerotic plaque development in male apoE -/- mice (Yang et al. 2004). A similar trend existed within female mice, but it did not reach a level of statistical significance. In contrast, there is a report that male, but not female, Balb/c mice that had been exposed *in utero* to mainstream smoke from 1R3F research cigarettes (not ETS from the 1R4F cigarettes used here) displayed increased tumor incidence and size associated with decreased

cytotoxic T-lymphocyte activity, following injection of EL4 lymphoma cells (Ng et al. 2006). These varied gender-effects illustrate the difficulty in predicting adult gender-specific responses associated with prior *in utero* stresses and strongly suggest that different molecular mechanisms operate depending on test agent, exposure conditions, subject strain, and experimental endpoints.

In ESO versus ASO mice, we identified differentially expressed genes that participate primarily in cell proliferation, cell motility/elasticity, cell survival, and general cell metabolism (Table 3.1). These biological functions are mediated at the molecular level by down-regulation of adhesion/apoptosis signaling molecules, transcription activating DNA-binding motifs, ATP/GTP metabolic reagents, and cytoskeletal structural components and binding elements. Up-regulated genes contribute to these biological processes through negative regulation of cell proliferation, repression of transcription, and chromatin remodeling. Again, this is accomplished through altered adhesion, transcription, metabolism, and cytoskeletal elements

A single kinase gene (*Pik3cd*) known to participate in immune and inflammatory signaling through *Nfκb, Erk*, and *Mapk* pathways was up-regulated in ESO versus ASO mice. *Nfκb, Erk*, and *Mapk* participate in inflammation processes (Roux and Blenis 2004), including those in the lung (Wuyts et al. 2003). *Pik3cd* also has been linked to the increased vascular permeability observed in inflammation (Lee et al. 2006). Four genes linked to asthma or lung inflammation (*Arg1*, *Ccl24*, *Slc7a2*, *Mcpt1*) that did not pass all the microarray filtering criteria were confirmed by qRT-PCR as being up-regulated in ESO versus ASO mice, consistent with an enhanced lung response in ETS-exposed mice. Both *Arg1* and *Slc7a2* have been implicated in arginine metabolism (Zimmermann et al. 2003). Arginine serves as a substrate for both arginase 1 and nitric oxide (NO) synthetase. Thus, increases in arginine metabolism catalyzed by arginase 1 are believed to decrease the production of NO via NO synthetase. Normal relaxation of smooth muscle is NO dependent, and decreases in NO are thought to contribute to smooth

muscle mediated broncho-constriction, such as that seen in asthma. Scl7a2 (CAT2) is a cationic amino acid transporter involved in arginine transport. Altered arginine transport affects the arginine metabolic pathway and may also alter NO production. Ccl24 (eotaxin 2) plays a role in eosinophil recruitment to the lung in allergic asthma (Panina-Bordignon and D'Ambrosio 2003). The Mpct family contains mast cell-specific proteases that participate in mast cell activation and degranulation (Small-Howard and Turner 2005). Changes in cytoskeletal elements and binding can confer relaxed cell-to-cell attachment and facilitate infiltration of inflammatory elements. The gene expression profile reported here indicates that adult mice exposed *in utero* to ETS have increased lung inflammation in response to OVA relative to *in utero* air-exposed cohorts. These results support the functional, histopathological and immune system changes we have documented in OVA-sensitized and challenged mice (Penn et al. 2007).

Figure 3.2 illustrates a literature-based gene network to which our differentially regulated gene findings (ESO versus ASO comparison) have been applied. This network brings together, through our data, groups of relationships that become associated for the first time, creating a custom network of differential gene response for our experiment. This custom network shows that the differences seen between our treatment groups are based upon differential regulation of a dispersed and varied group of genes in clustered relationships with inflammatory/asthma genes that are not differentially regulated. The majority of these differentially regulated genes does not have direct ties to the immune system or previously defined inflammatory responses. None of the well defined asthma/inflammatory genes (*Il1b, Il4, Il10, Il13, Il6, Tnfa, Infg, Tgfb1*) are differentially expressed, although they connect many of the genes that are. This is not surprising, as the comparison is between two groups (ESO, ASO) that both exhibit marked eosinophilic airway inflammation following classic OVA sensitization/challenge. However, the few asthma/inflammatory genes that are differentially

expressed (*Pik3cd*, *Arg1*, *Slc7a2*, *Ccl24*, *Mcpt1*) are all up-regulated in ETS-exposed mice. The mild enhancement of inflammatory/immune response indicated by these gene changes is reflected in the pathophysiological responses of the two groups (Penn et al. 2007) and raises concern that ETS may mediate increased inflammatory injury or alter immune response upon initial exposure to an infectious agent, such as respiratory syncytial virus (Phaybouth et al. 2006).

In EOO versus AOO mice, differentially expressed genes participate in adaptive immune responses, cell proliferation and survival, and cell cycle control (Table 3.4). These processes are controlled through down-regulated adhesion, major histocompatibility complexes, immune, and pro-inflammatory molecule genes, including cytokines and chemokines. Up-regulated genes influence these processes through alterations in G-protein receptor signaling and cell proliferation, differentiation, metabolism, and morphology. Consistent down-regulation of critical inflammatory genes in ETS- and doubly OVA-exposed mice was confirmed through qRT-PCR, revealing down-regulation of 17 asthma-related and/or inflammatory genes (*Arg1*, *Ccl8*, *Ccl11*, *Ccl24*, *Ear11*, *Mcpt1*, *Sprr2a*, *Chi3l3*, *Chi3l4*, *Chia*, *Slc7a2*, *Il4*, *Il6*, *Il10*, *Il13*, *Il1b*, *Tnfa*).

Genes directly or indirectly inhibiting immune responses were up-regulated in EOO versus AOO mice. Genes promoting immune responses were down-regulated, including genes for the pro-inflammatory/Th2 cytokines IL1b, IL4, Il6, IL10, IL13, and TNFα. Pro-inflammatory cytokine genes (*Il1b, Il6, Tnfa*) were down-regulated in EOO mice, decreasing innate inflammatory potential, as seen in endotoxin tolerance (Cook 1998). Down-regulation of Th2 cytokine genes (*Il4*, *IL10*, *Il13*), as described in our data, has been reported in alloreactive T cells in which tolerance has been induced and immune rejection suppressed (Taylor et al. 2002). The gene for IL10, which is essential for the eosinophilic inflammation seen in asthma (and in

this mouse model), is down-regulated, resulting in decreases in mucus production and eosinophilic inflammation without decreased IgE or IL4 (Yang et al. 2000).

Changes in non-cytokine immune gene expression also support ETS-associated dampening of immune responses in OVA tolerant mice subsequently challenged with OVA (Table 3.5). The product of *Hgf* suppresses pulmonary dendritic cell antigen-presenting capacity, thereby inhibiting the immune response to inhaled allergen (Okunishi et al. 2005). *Ppapdc2* produces a phosphatase that inhibits PMN activation (Fukunaga et al. 2006). *Rnd3* product is a Rho GTPase that negatively regulates the polarization and cytoskeletal rearrangements that are necessary for leukocyte migration (Sanchez-Madrid and del Pozo 1999). Dpp4 protein, CD26, cleaves a number of cytokines involved in leukocyte chemotaxis and targeting, as well as in progenitor cell proliferation and recruitment (Boonacker and Van Noorden 2003). This inactivation of cytokines serves to modulate the immune response.

The type II MHC genes, *H2-Ab1* and *H2-Eb1*, and the type I MHC gene, *H2-D1*, were down-regulated in EOO mice. Down-regulation of these molecules results in diminished antigen presenting capacity (Stevenson et al. 2000; Wolk et al. 2000). Pro-inflammatory chemokine genes *Ccl11 (MIP2), Ccl24 (Eotaxin2), Ccl8 (MCP2), Cxcl10 (IP10), and Cxcl2 (GROβ)*, produce chemoattractant ligands for the entire range of white blood cells (Mantovani et al. 2004). Their down-regulation would inhibit the attraction and accumulation of effector cells necessary for an appropriate immune response.

The signaling lymphocyte activation molecule (SLAM) family of receptor molecules provides signals modulating innate and adaptive responses and polarizing T cells toward Th2 responses (Graham et al. 2006). Hence, down-regulation of *Slamf8* and *Slamf9* would reduce Th2-mediated allergic responses. T cell factor 7 like 2 (Tcf7l2) is produced by T cells and, with other T cell factors, participates in T cell development through the Wnt pathway (Willinger et al.

2006). Down-regulation of the *Wnt* pathway could interrupt the differentiation and maturation of T cells. *Slpi* is a protease inhibitor gene whose product suppresses the inflammatory action of mast cell and PMN proteases (Wright et al. 1999). Suppressor of T cell signaling gene *(Sts1)* protein negatively regulates the T cell receptor (TCR), thereby dampening the immune response even in the face of antigen-TCR binding (Carpino 2004). ADAM8, a disintegrin metalloprotease, catalyzes cleavage of CD23 (low affinity IgE cell surface receptor). The resulting soluble CD23 causes up-regulation of IgE and inflammatory cytokine production (Fourie et al. 2003); therefore, down-regulation of *Adam8* diminishes inflammatory response. Inducible TGFβ (Tgfbi) has a direct positive regulatory effect on production of the pro-inflammatory cytokine, IL6 (Moller et al. 1994). Down-regulation of *Tgfbi* decreases IL6 and consequently inflammation. *Capg* down-regulation alters actin filament capping, disturbs the cytoskeleton, and results in reduced macrophage motility and phagocytosis (Parikh et al. 2003). *Sirpa* produces an immunoglobulin superfamily protein that inhibits phagocytosis receptors on macrophages and diminishes the innate immune response. *Itgax* codes for one chain of the heterodimeric mature dendritic cell integrin, CD11c. This molecule is critical to maturation of the dendritic cell into an antigen presenting cell, and down-regulation would decrease the recognition and uptake of antigen by dendritic cells (Hebel et al. 2006). C1QC is a subunit of the recognition member of the complement cascade that promotes antigen recognition, isolation, and up-take (Sontheimer et al. 2005). Down-regulation of the gene responsible for this molecule would help suppress innate immune response.

All alterations in expression functionally support inhibition or, more correctly, dampening of the immune response. The only apparent inconsistency among genes influencing the immune response was an up-regulation of *Ccrl1*, which encodes a receptor for dendritic and

T cell cytokines involved in chemotaxis (Gosling et al. 2000). However, the possibility of Ccrl1 involvement in specific negative regulation of dendritic and/or T cells cannot be eliminated.

Almost all differentially expressed genes participating in the immune, inflammatory, and asthma reactions are modulated toward reduced responsiveness in the mice that were exposed to ETS *in utero* (EOO versus AOO). The effect of this differential expression--decreased antigen presenting capacity and dampened immune signaling--implies decreased innate and adaptive immune responses to new challenges, as well as reduced inflammation. Previously, we demonstrated these dampened responses in OVA-tolerized mice that were subsequently sensitized and challenged (Penn et al. 2007).

Figure 3.3 illustrates a literature-based network of relationships between differentially expressed genes that we identified in the EOO versus AOO comparison. This network connects, through our data, groups of previously described relationships that have not been previously associated. The resulting custom network demonstrates that the significant, observed, immune response differences seen in this comparison (EOO versus AOO) are related to differential regulation of genes that collectively suppress immune responsiveness. In Figure 3.3, major inflammatory/immune genes (*Il1b*, *Il4*, *Il10*, *Il13*, *Il6*, *Tnfa*) that are down-regulated in EOO mice relative to AOO mice connect many up- and down-regulated genes that negatively regulate the immune response. The specificity of this suppressive modulation is of concern. Would exposure to alternate antigens within this environment of immune down-regulation elicit appropriate innate and/or adaptive responses? Agents, identified by MHC molecules or neutralized by effector cells that are down-regulated by ETS exposure *in utero*, could escape detection or elicit sub-optimal immune responses.

While our main interest was on the effect of ETS exposure *in utero* on lung responses to OVA in both sensitized and tolerized mice, it is noteworthy that the number and magnitude of

differences between ESO and EOO mice greatly exceed those between ASO and AOO mice (Tables 3.2 & 3.3). In all the asthma-related genes examined, ESO-EOO differences were consistently greater (qRT-PCR) than ASO-AOO differences. This net effect is a result of the mild to moderate increase in responsiveness of ESO mice compared to ASO mice and the much greater suppression of response in EOO mice relative to AOO mice. The relative responsiveness or reactivity of these 4 groups of mice, indicated by their general gene expression profiles as well as by their relative expression of specific asthma/inflammatory genes, is supported by histopathology, BAL cell counts and cytokine analyses, immunoglobulin levels, and AHR (Penn et al. 2007). In each comparison, additional candidate genes (white nodes in Figures 3.2 & 3.3) warrant future investigation.

In our comparison of gene expression between ESO mice and ASO mice, it was evident that genes for cell proliferation, growth, and general metabolic processes were more suppressed in the most reactive group (ESO). Inflammation is associated with proliferation of innate and adaptive immune cells. However, activation of the p38 pathway (Diehl et al. 2000) and Pik3cd (Donahue and Fruman 2004) can result in cell cycle arrest and halt cell proliferation and growth in lymphocytes. Findings in the EOO versus AOO mice indirectly support the findings in ESO versus ASO mice. The least responsive group (EOO) had a differential expression profile that was indicative of increased cell proliferation, growth, and metabolism. Since our lung samples represent total lung RNA, proliferation status of non-immune cells is also reflected in the differential expression. Fibroblasts have been implicated in maintenance of the local environment (extracellular matrix) within tissues, including down-regulation of inflammatory responses (Buckley et al. 2001). Proliferation of regulatory fibroblasts might overshadow down-regulation of proliferation in immune cells.

The fate of T cells, activation or anergy, has been tied to both cell cycle progression (Wells et al. 2000) and intracellular calcium flux (Gavin et al. 2002). Evidence for regulation of both the cell cycle and of ion channels is presented in our data (Tables 3.1 & 3.4). The ultimate fate of the immune response will depend on the character of the T cells involved (T effectors versus T regulatory) as well as on the presence of co-stimulators, MHC expression, and innate immune elements. An attenuation of innate and adaptive responses, if non-specific, might threaten the ability of the least responsive group (EOO) to respond appropriately to an infectious agent. Likewise, excessive airway inflammation might impair defenses or increase injury during infection (Beisswenger et al. 2006) in the most responsive group (ESO). Thus, either extreme of the ETS-based responsive dichotomy may be undesirable (Clark and Kupper 2005).

Our data comparing two aerosol-tolerized groups (EOO, AOO) illuminate the complexity of immune tolerance. The dynamic nature of tolerance, involving the down-regulation and inhibition of numerous biological processes that collectively potentiate the immune system, is well illustrated by our data. While anergy of specific T cell lines plays a significant role in tolerance, many other "players" (dendritic cells, PMN's, mast cells, macrophages, and eosinophils) are suppressed via multiple mechanisms, including structural and enzymatic changes yielding functional impairments. *In vivo*, tolerance is the sum of suppressed stimulation/activation (decreased antigen presentation, T cell anergy, dampened intracellular signaling) and impaired effector function (depressed chemotaxis, protease inhibition, suppressed cell mobility). Our data define multiple sites and diversities in mechanisms involved in inhibiting the immune system and achieving tolerance.

This study also demonstrates that varying degrees of responsiveness can exist within the tolerant population. While both EOO and AOO mice demonstrated no IgE or eosinophilic inflammation (and thus were considered tolerant), the AOO mice had higher levels of Th2

cytokines, BAL inflammatory cells, airway hyperresponsiveness, and airway pathology and in many of these cases the differences were significant (Penn et al. 2007). Differential gene expression data reveal more gene alterations inhibiting the immune response in EOO versus AOO mice. These differences, initiated by an environmental exposure limited to the gestational period, may not be totally antigen-specific and therefore are potentially detrimental. Certainly, the difference between responsiveness in EOO and AOO mice is a reflection of the mechanisms involved in tolerance induction.

Alteration of gene expression mediated by *in utero* environmental exposure represents a change in phenotype determined by gene-environment interaction. While the mechanism by which these gene expression changes are orchestrated is not yet defined, epigenetic involvement is likely (Drake et al. 2005; Rahnama et al. 2006; Waterland and Jirtle 2003). Given the number and variety of genes that we found to be differentially regulated along with the indirect and relatively mild nature of exposures that culminated in demonstrable response differences in immunology, histopathology, clinical pathology, and lung function, the search for single candidate genes may need to evolve to an examination of global epigenetic alterations. In most instances, gene-environment interactions may cause transient, reversible, or non-critical alterations in gene expression, or no detectable change in phenotype. However, the ability of relatively mild *in utero* environmental exposures during embryonic development to modulate adult gene expression may move chronic adult diseases (atherosclerosis, obesity, diabetes, allergy, asthma) into the realm of early developmental disorders, such as childhood diabetes, leukemia, and autism that also appear to be environmentally modulated.

Genes recognized in this study are present in other tissues and may have varied and diverse functions beyond those described here. However, the functions delineated within this study are consistent with the pathologic and physiologic differences defined by the study

exposures. Gene ontogeny has been examined in context of the complexity of an *in vivo* system. Further refinement of specific cell types involved and specific mechanisms of differential gene regulation are ongoing, particularly gene-specific and global changes in epigenetic modifications.

CONCLUSION

In utero ETS exposure alters gene expression in the lung of adult BALB/c mice in response to OVA exposure, regardless of whether that exposure was sensitizing or tolerizing. The results support a gene-environment interaction that results from ETS exposure *in utero* and that alters the phenotype of adult mice as defined by their gene expression and inflammatory responses to an allergen (OVA). The strength of these findings is re-enforced by the consistency of relative group responses across gene expression data, airway function (reactivity) changes, presence of airway inflammatory mediators (cytokines, cells), and lung histopathology. Our data implicate milder, wide-spread gene expression changes rather than (or in addition to) larger more discrete alterations in single gene expression as the mechanism through which these *in utero* exposures alter adult lung responses.

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CHAPTER 4

EXPOSURE TO ENVIRONMENTAL TOBACCO SMOKE *IN UTERO* ALTERS GENE EXPRESSION IN THE ADULT LUNG IN THE ABSENCE OF HISTOPATHOLOGY

INTRODUCTION

Human epidemiology studies and experimental animal studies have implicated various *in utero* exposures to subsequent adult disease (Baraldi et al. 2002; Barker 1995). Experimental studies have demonstrated phenotypic and gene expression changes in adult animals mediated by prenatal exposure to different environmental toxins (Holladay 1999; Yang et al. 2004). These studies have reported gene expression changes seen with the identification of an altered phenotype. Gene expression changes associated with altered phenotype help define that phenotype change. However, these expression changes hold no clue to the initiating events that lead to change in phenotype.

The inception of gene expression changes occurs at the time of exposure. For most developmental and complex diseases, the ability to recognize these early changes is undeveloped because we do not yet understand the complex interactions and multiple pathways involved in progression of these diseases. Therefore, the mechanisms through which these changes take place are difficult to define. Time series studies that look for changes at time points prior to identification of the new phenotype are a first step towards identifying initiating changes. In designing our study, several early time points were included in an attempt to determine early gene expression changes associated with altered phenotype.

We have previously reported on changes in immune responses induced in 15 week-old mice by exposure to environmental tobacco smoke (ETS) *in utero* subsequently challenged with the allergen, ovalbumin (OVA). Our studies showed ETS-related changes in bronchoalveolar lavage (BAL) fluid cytokine levels and differential cell counts, plethysmography defined airway

responsiveness, and lung histopathology (Penn et al. 2007) and gene expression (Rouse et al. 2007). Changes in lung function, physiology, and histopathology were consistent with and at least partially explained by the demonstrated gene expression changes. At earlier time points (0, 6, and 10 weeks of age), no consistent significant differences were detected in BAL fluid or histopathology. Minor differences between *in utero* ETS- and AIR-exposed mice were found in airway responsiveness (as measured by plethysmography at 6 and 10 weeks of age). However, no other significant differences were seen (Penn et al. 2007).

Although functional changes were minimal and physical changes undetected at these earlier times, the molecular alterations mediated by ETS exposure *in utero* that lead to subsequent differences in physical and functional endpoints at 15 weeks of age must be present at these times. We examined gene expression at these earlier time points in an attempt to detect the etiology of changes demonstrated at 15 weeks.

MATERIALS AND METHODS

Animal Protocols

Eight-week old BALB/c mice (Harlan, Indianapolis, IN), were housed and handled according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources. 1996. Washington D. C., National Academy Press). The Louisiana State University Institutional Animal Care and Use Committee approved all animal procedures. All animals were handled humanely and care was taken to assure alleviation of pain and suffering. A barbiturate solution (Beuthanasia-D, Schering-Plough, Kenilworth, NJ) was used via intraperitoneal injection (0.2 ml per mouse) for all sacrifices.

Exposures

Pre- and post-natal exposure protocols have been described in detail (A Penn et al. 2007). Briefly, pregnant mice were exposed in dynamic exposure chambers to either ETS mixed with

HEPA-filtered air (ETS) or to HEPA-filtered air (AIR) 5 hours per day for days 1-19 of gestation. ETS exposures had average final concentrations of total solid particles n =10mg/m^3 and CO = 44.5 ppm. Ten mice from each group were sacrificed at one day of age (neonates). At 6 weeks of age, eight males and eight females were sacrificed from each group (ETS, AIR). Half of the remaining mice from each of these groups inhaled 1% OVA aerosol (20 min/day; 10 days; 7-8 weeks of age) to produce tolerance, while the other half inhaled saline aerosol. All mice were OVA-sensitized and challenged, then sacrificed one day following final challenge at 15 weeks of age to determine the effects of ETS exposure *in utero* on responses to OVA sensitization/challenge in mice not previously exposed to OVA (ESO, ASO) and on OVA tolerance in mice previously exposed to OVA (EOO, AOO). No offspring were exposed to ETS after birth.

Lung Harvest and mRNA Extraction

Left lung lobes were excised, placed in RNAlater (Ambion, Austin, TX), and stored at -80°C. Subsequently, these samples were transferred to 1mL TRIzol (Invitrogen, Carlsbad, CA) and homogenized using a Mixer Mill MM 300 (Qiagen, Valencia, CA) with a copper bead. Chloroform was added to the homogenate, mixed by inversion, and centrifuged @ 4°C to separate phases. The RNA-containing aqueous phase was transferred to an RNeasy Micro Kit (Qiagen, Valencia, CA). The manufacturer's protocol was followed, omitting Buffer RLT. Remaining DNA was eliminated using a column RNase-Free DNase Set (Qiagen, Valencia, CA). An additional Buffer RPE wash was used to remove residual salts followed by an additional 2 minute spin to evaporate residual ethanol.

RNA samples were checked for quantity and purity with a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE). Values generated from the NanoDrop for all samples fell into the following ranges, 260/280 ratio: 2.09-2.17, 260/230 ratio: 2.20-2.27,

concentration: 1200-1900 ng/uL. Further quality assays were performed on 1:5 dilutions of RNA samples with an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA) and Agilent RNA 6000 Nano Series II Kits. All samples fell into the following ranges, 28S/18S ratio: 1.3-1.8, RNA Integrity Number: 8.5-9.6.

Microarray Assay

Individual Affymetrix Mouse MOE 430A Arrays were used to assess neonatal gene expression in each of six mice from both groups (ETS or AIR). Sex was not determined at sacrifice and was therefore random in the neonatal microarray analysis. At 6 weeks of age, gene expression in each group (ETS, AIR) was assessed on 6 microarrays. Four females of each group were hybridized to 3 microarrays, one array with 2 samples pooled and 2 arrays with individual samples. Likewise, 4 male samples from each group were hybridized to 3 microarrays, one array with 2 samples pooled and 2 arrays with individual samples. The 4 groups (ES, AS, EO, AO) analyzed at 10 weeks of age were examined using 7 to 10 microarrays per group representing 11 to 19 mice (Table 4.1).

Double stranded cDNA synthesized from total RNA was used to create cRNA, which was then biotinylated, fragmented, and added to a hybridization cocktail that included probe array controls, bovine serum albumin, and herring sperm DNA. This cocktail was then hybridized (16 hours; 45°C) to oligonucleotide probes on a GeneChip Mouse MOE 430A Array in a GeneChip Hybridization Oven 640. Immediately following hybridization, the array underwent an automated washing and staining protocol on a GeneChip Fluidics Station and was scanned with a GeneChip Scanner 3000. Data collection and processing of initial raw data were performed by a GeneChip Workstation. All gene chips and instrumentation were from Affymetrix (Santa Clara, CA). Probe hybridization and cDNA synthesis were performed at the LSU Health Sciences Center in New Orleans in the spring of 2005.

Table 4.1: Number of Microarrays and Samples Used for 10 Week Analysis

Samples	AO		EO		ES		AS	
	Male	Female	Male	Female	Male	Female	Male	Female
single	1	5	2	1	1	2	1	1
2-pooled	1	2	2	5	2	3	1	6
3-pooled							1	
arrays	2	7	4	6	2	5	3	7
mice	3	9	6	11	3	8	6	13

Gene Expression Analysis

GeneChip Workstation data were sent to Expression Analysis Systems (Durham, NC). Initially, a principle component analysis was used to determine clustering of experimental units. The analysis revealed clustering by treatment group with greater variation between groups than within groups (data not presented), thus validating subsequent comparisons between treatment groups. Prior to making these comparisons, data were subjected to reduction of invariant probes (REDI) to remove previously determined mal-performing probes from the data set. Pairs of treatment group data underwent permutation analysis for differential expression (PADE) that accounts for false positives by tabulating a false discovery rate (FDR) based on a permutation-generated reference curve (technical information on REDI and PADE analyses available at www.expressionanalysis.com). Following REDI and PADE, expression data were tabulated for each remaining transcript, including individual transcript p-value, FDR, fold change, Affymetrix probe ID, gene symbol, and functional summary. All transcripts included in this study had a fold change of at least 1.5 (up or down), and both an individual p-value and FDR <0.05.

Pathway Analyses

Gene expression data were analyzed with the network- and pathway-building software, Ingenuity Pathways Analysis 4.0; gene networks and canonical pathways were examined using

the Ingenuity Analysis Knowledge Database (Ingenuity Systems, Redwood City, CA). Identified networks and pathways were scrutinized for phenotypic relevance. Select genes were identified from the literature for confirmation by quantitative real-time PCR analysis. Custom networks were created to demonstrate the connections between the genes identified in our expression analyses.

PCR Confirmation

PCR confirmation of gene expression changes in these samples was not possible. These samples were stored in -80°C freezers at LSU Health Sciences Center in New Orleans, Louisiana at the time of Hurricane Katrina and were subsequently lost after the prolonged loss of electricity and the inaccessibility of the samples. However, with some exceptions (detailed in our earlier publication; Rouse et al, 2007), later samples demonstrated consistency between microarray and PCR findings.

NEONATE: ONE TO THREE DAYS OF AGE

Table 4.2: ETS Exposure *In Utero* Suppresses Angiogenesis and Tissue Growth Factors in Neonates

Affymetrix ID	Fold change	FDR	p-value	Gene Symbol	Function	Process
1436448_a_at	-1.50	0.000	0.0003	Ptgs1	(Cox-1); prostaglandin synthesis	angiogenesis control
1452114_s_at	-1.71	0.000	0.0009	Igfbp5	enhances growth factors secretion	growth, proliferation
1449319_at	-2.05	0.000	0.0044	Rspo1	Unknown	Wnt signaling
1423954_at	-2.16	0.000	0.0050	C3	Endopeptidase inhibitor	Complement activation

Results

When the relatively stringent criteria described in Materials and Methods was applied to neonatal gene expression data, only 4 genes (Table 4.2) were differentially expressed between neonates receiving ETS *in utero* versus those receiving AIR *in utero*.

Discussion

Although only 4 genes passed all of the screening criteria, these genes could have significant impact on lung structure and function. *Ptgs1* produces a protein that is essential for constitutive synthesis of prostaglandin E2 which plays a critical role in specific lymphocyte development (Carey et al. 2003) and angiogenesis (Jones et al. 1999). *Igfbp5* plays a role in cellular growth and proliferation through its interaction with insulin-like growth factor (Laursen et al. 2007) and has independent effects on extracellular matrix (Pilewski et al. 2005). *Rspo1* protein is believed to function in Wnt-signaling (Kamata et al. 2004) which is associated with embryonic development and adult tissue homeostasis. The *C3* protein is needed for activation of both the classical and alternative complement pathways. Complement activation is an innate immune response to infection or injury (Thurman et al. 2007; Walters et al. 2002).

SIX WEEKS OF AGE

Results

At 6 weeks of age, only 2 genes passed the filtering criteria, *Sult1a1 and Klf15*. The protein of *Sult1a1* is an enzyme that conjugates sulfur to phenol or phenol-like compounds, including drugs, xenobiotics, hormones, and neurotransmittors (Windmill et al. 1998). *Klf15* produces a transcription factor that has been implicated in glucose regulation (Gray et al. 2002) and adipocyte differentiation (Mori et al. 2005). Both of these genes had FDR's < 0.000 and *Sult1a1* p-value was less than 0.0000 while p-value of *Klf15* was equal to 0.0019. Both genes were up-regulated (*Sult1a1* 1.69 and *Klf15* 1.73).

Discussion

While the 2 genes up-regulated by *in utero* ETS exposure at 6 weeks of age could potentially have significant functional effects through metabolism of xenobiotics (*Sult1a1*) or the regulation of glucose (*Klf15*), neither gene has been previously associated with either the disease

processes or the endpoints examined within this study (allergy, asthma, pulmonary inflammation, or the immune system). Certainly, this does not preclude them participating in the responses described at later time points. However, we have no solid basis for implicating them at this time.

TEN WEEKS OF AGE

Results

Using pre-determined filtering criteria (fold change ≥ 1.5, FDR < 0.05, transcript p-value < 0.05), 12 transcripts were found to be differentially regulated (all up-regulated) following REDI and PADE analyses of raw Affymetrix data from ES versus AS mice. Following removal of duplicate transcripts and undefined or unmapped transcripts, 9 unique genes were found to be up-regulated (Table 4.3). In this gene list, fold-change in expression ranged from 3.28 to 1.59, p-value from 0.0021 to 0.0194, and FDR from 0.000 to 0.047.

When similarly filtered following REDI and PADE analyses, the raw data from EO versus AO mice revealed 16 differentially regulated transcripts (6 up-regulated and 10 down-regulated). Removal of duplicate and unmapped transcripts yielded 9 unique genes (3 up-regulated and 6 down-regulated) in mice exposed to OVA (Table 4.4). Expression fold-change in these genes ranged from 2.17 to 1.54 and from -1.50 to -3.58, p-value from < 0.0000 to 0.0024, and FDR's were all < 0.000. Confirmation via qRT-PCR was not possible with these 10 week samples. All RNA samples and cDNA derived from these samples were stored in New Orleans at the time of Hurricane Katrina and were subsequently lost.

Differential expression data revealed that small gene networks significantly affected in both ES versus AS and EO versus AO comparisons. These networks are represented in Figures 4.1 and 4.2, respectively. All of the differentially regulated genes in the OVA-exposed mice (EO vs. AO) were identified within a network. Only 6 of the genes from the ES versus AS comparison were found within a single network, one gene was identified but was outside the

network, and two genes were not related to any of the other genes. Since very few genes met all of the filtering criteria, identification of significant canonical pathway involvement was not attempted in either comparison.

Table 4.3: Differentially Expressed Genes in ES Mice versus AS Mice are Pro-Inflammatory and Metabolic Response Genes

Affymetrix ID	Fold Change	FDR	p-value	Gene Symbol	Function	Process
1448872_at	3.28	0.000	0.0021	Reg3g	Proteoglycan binding	Anti-microbial
1449434_at	2.37	0.022	0.0101	Car3	Carbonate dehydratase	Single carbon metabolism
1422651_at	2.36	0.047	0.0194	Adipoq	Hormone activity	Fatty acid metabolism
1451263_a_at	2.27	0.047	0.0153	Fabp4	Fatty acid binding	Pro-inflammatory
1417867_at	2.03	0.022	0.0111	And (Cfd)	Peptidase	Complement activation
1424737_at	1.98	0.022	0.0080	Thrsp	Transcription regulator	Lipid metabolism
1415994_at	1.93	0.022	0.0112	Cyp2e1	Monooxygenase	Xenobiotic metabolism
1422230_s_at	1.64	0.022	0.0050	Cyp2a	Monooxygenase	Xenobiotic metabolism
1449319_at	1.59	0.022	0.0056	Rspo1	Unknown	Wnt signaling

Table 4.4: Differentially Expressed Genes in EO Mice versus AO Mice Represent Stress Response Genes.

Affymetrix ID	Fold Change	FDR	p-value	Gene Symbol	Function	Process
1438211_s_at	2.17	0.000	0.0001	Dbp	Activate transcription	Control of Cyp2a4
1419248_at	1.69	0.000	0.0001	Rgs2	Signal transdution	Regulation of G-protein signaling
1422122_at	1.54	0.000	0.0000	Fcer2a	Low affinityIgE binding	B-cell activation & proliferation
1422474_at	-1.50	0.000	0.0006	Pde4b	cAMP hydrolase	Second messenger signaling
1416288_at	-1.62	0.000	0.0010	Dnaja1	Protein folding/binding	Nrf2-mediate oxidative stress response
1416755_at	-1.66	0.000	0.0004	Dnajb1	Protein folding/binding	Nrf2-mediate oxidative stress response
1416077_at	-2.00	0.000	0.0002	Adm	Hormone	Hypotension & cell proliferation
1452388_at	-3.17	0.000	0.0009	Hspa1a	Protein folding/binding	Stress response
1427126_at	-3.58	0.000	0.0024	Hspa1b	Protein folding/binding	Stress response

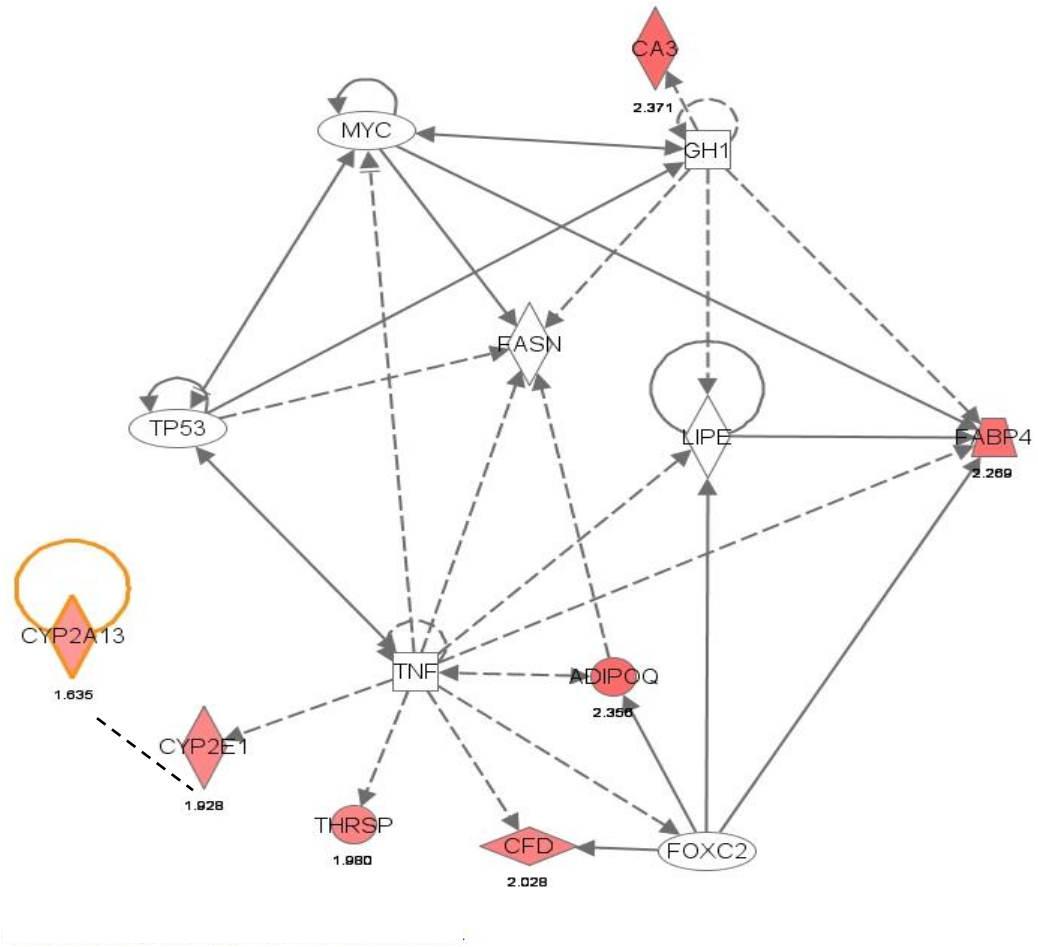

Figure 4.1: Pro-Inflammatory Network of Genes Up-Regulated in Ten-Week Old ES Mice versus AS Mice. Green represents down-regulation and red up-regulation (fold-changes ≥ 1.5); white nodes represent fold-change < 1.5. Intensity of color increases with increasing fold-change (numbers represent fold-change values). Dotted lines indicate indirect relationships and solid lines direct interactions. All gene symbols are human homologue designations. Network constructed with Ingenuity Systems software. For explanation of nodes shapes see Figure 3.2 or 3.3 in Chapter 3.

Discussion

At birth, offspring had only been exposed to ETS or AIR *in utero.* Perinatal gene expression analysis examined ETS-mediated constitutive differences in gene expression. At 6 weeks, offspring had only been exposed to ETS or AIR *in utero.* Therefore, 6-week gene expression analysis evaluated ETS-mediated constitutive differences in gene expression at an

additional time point and in a more mature developmental state. By 10 weeks of age, mice had been challenged with either OVA or saline aerosol. Examination at this point investigated changes that occur in the course of producing OVA-tolerance (AS vs. AO and ES vs. EO), as well as the effect of ETS exposure *in utero* on the development of OVA-tolerance (EO vs. AO). AIR exposure *in utero* and were then given mild respiratory perturbation (in this case, aerosolized saline) by examining ES vs. AS.

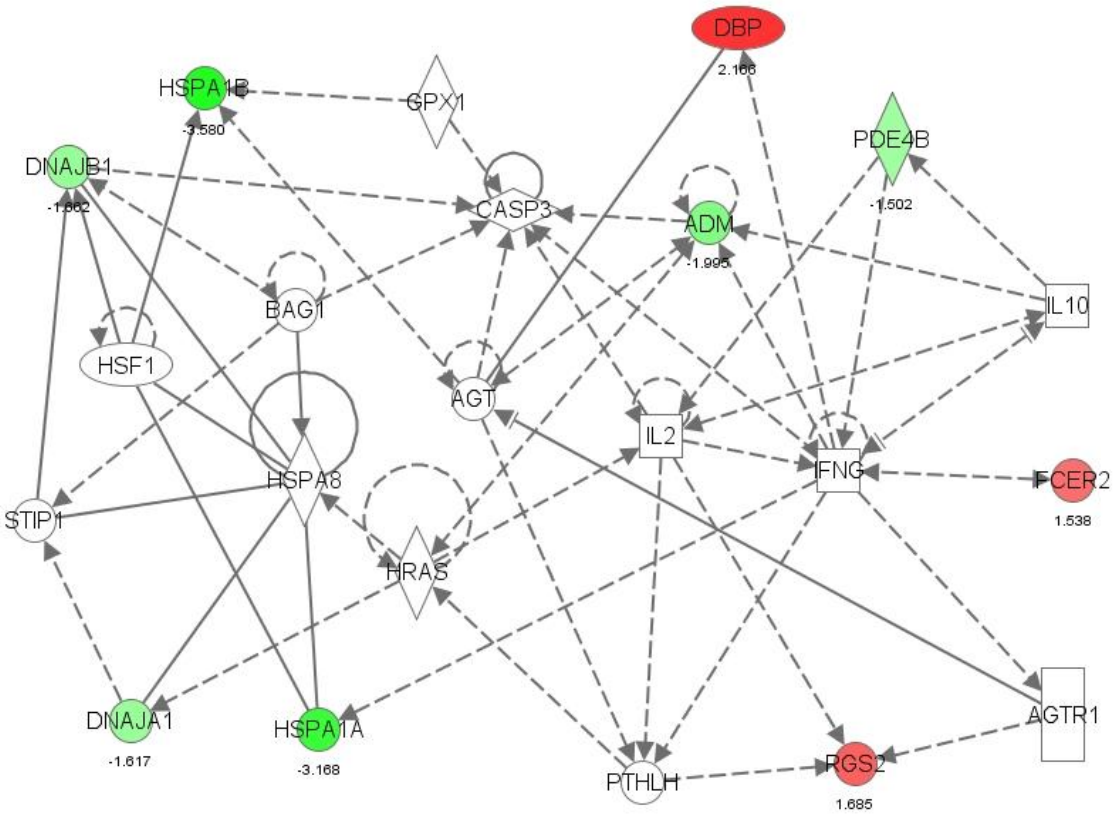

Figure 4.2: Stress Response Gene Network Primarily Down-Regulated in Ten-Week Old EO Mice versus AO Mice. Green represents down-regulation and red up-regulation (fold-changes ≥ 1.5); white nodes represent fold-change < 1.5. Intensity of color increases with increasing fold-change (numbers represent fold-change values). Dotted lines indicate indirect relationships and solid lines direct interactions. All gene symbols are human homologue designations. Network constructed with Ingenuity Systems software. For explanation of nodes shapes see Figure 3.2 or 3.3 in Chapter 3.

At 10 weeks of age, the few gene expression differences noted suggest that ETS-exposure *in utero* alters gene expression in juvenile mice. In comparing ES mice with AS mice, all of the significantly altered genes (passing the filtering criteria) are up-regulated and are directly or indirectly associated with *Tnf* (Figure 4.1). Two genes (*Reg3g, Rspo1*) that are significantly up-regulated do not appear in the gene network. *Reg3g* is a gene whose product is not well defined but has been described as an acute phase, inflammatory molecule that binds proteoglycans and through that binding has an anti-microbial effect (Cash et al. 2006). The product of *Respo1* has unknown molecular function but has been theorized to bind to iron or sulfur elements within organic molecules, resulting in Wnt signaling (Kamata et al. 2004). Of the 7 remaining genes, 4 (*Adipoq, Fabp4, Adn, Thrsp*) are related to lipid metabolism and have been associated with mediation of inflammatory conditions. The protein of *Adipoq* (adiponectin) increases insulin sensitivity and has anti-inflammatory and anti-atherogenic effects. Fatty acid binding protein 4 (product of *Fabp4*) has been implicated in macrophage cholesterol metabolism and the ability of macrophages to mount an inflammatory response (Makowski et al. 2005). The protein product of *Adn,* or adipsin, is also known as complement factor D and is essential for activation of the alternate pathway. Defects in adipsin have been associated with accumulation of immune complexes (Abrera-Abeleda et al. 2007). *Thrsp* protein, SPOT14, mediates de novo lipogenesis in specific tissues (LaFave et al. 2006). *Car3* protein is found in skeletal muscle, adipocytes, and hepatocytes where it is one of several isoforms that function in CO_2 metabolism (Kim et al. 2004). Two cytochrome p450 genes (Cyp2a5, *Cyp2e1*) were up-regulated in ETS exposed mice. Both of these genes produce enzymes previously shown to metabolize nitrosamines (Pelkonen et al. 2000; Seree et al. 1996), a major component of cigarette smoke. The genes up-regulated by *in utero* ETS exposure collectively represent slight enhancement of lipid-associated immune

responses and metabolism of organic nitrogen, especially nitrosamines which are ETS components.

In comparing OVA exposed mice (EO vs. AO), the majority of altered genes are down-regulated and are involved in cellular stress and immune responses. The network of EO mice versus AO mice (Figure 4.2) shows a prevalence of heat shock protein (HSP) genes, Il2, and Ifng in its center. There are three up-regulated genes (*Dbp, Rgs2, Fcer2a*) in the network. *Dbp* protein positively controls *Cyp2a* expression (Gachon et al. 2006). The protein product of *Rgs2* is a modulator of G-coupled protein receptor signaling associated with control of vascular smooth muscle contraction and hypertension (Tang et al. 2003). *Fcer2a* produces CD23 the low affinity binding molecule for the Fc region of IgE that could be instrumental in modulating allergic immune responses (Ford et al. 2006). Six genes (*Pde4b, Dnaja1, Dnajb1, Adm, Hspa1a, Hspa1b*) are down-regulated in the network. *Pde4b* is the predominant phosphodiesterase in immune cells and is essential to LPS-mediated release of the pro-inflammatory cytokine, TNF (Jin and Conti 2002). Down-regulation of *Pde4b* would decrease the innate inflammatory response. *Adm* produces a hypotensive peptide found in airway smooth muscle cells where disruption of the gene results in smooth muscle cell hyperplasia and hyper-responsiveness (Yamamoto et al. 2007). The remaining down-regulated genes are HSP genes from the HSP40 family (*Dnaja1, Dnajb1*) or the HSP70 family (*Hspa1a, Hspa1b*). The protein products of these genes work together chaperoning new proteins for folding and damaged proteins for re-folding (Yoneda et al. 2003). They have also been implicated in apoptosis signaling and facilitation of antigen presentation through their interaction with damaged cellular proteins (Kobayashi et al. 2000). The genes altered in EO mice versus AO mice support a composite effect of altered immune signaling and suppressed HSP function.

CONCLUSION

Results at birth indicate that without any additional local or systemic treatment or perturbation there are no detectable differences in gene expression traceable to *in utero* ETS exposures. Two points to consider in evaluating findings at this time point are; 1) the influence on microarray results of the extensive developmental RNA production seen at this time; 2) the stringent filtering criteria used to determine gene expression in our study carries an increased possibility or false negative results. Relaxation of filtering criteria (especially FDR) might yield valid and essential results presently discarded.

Results at one day of age and at 6 weeks of age, demonstrate that ETS exposure *in utero* yields no constitutive changes in gene expression. At each time point, there was no alteration in gene expression as defined by the rather stringent criteria outlined in Materials and Methods. At 10 weeks of age, mice that have had no allergen (OVA) exposure but that did receive an airway perturbation (saline aerosol) exhibit altered gene expression related to their ETS exposure *in utero*. The changes seen support an airway environment predisposed toward inflammation and metabolism of nitrosamines, such as those in cigarette smoke. Mice that have had aerosol OVA-challenge prior to 10 weeks of age also demonstrate differential gene regulation based on their ETS *in utero* experience. In this case, the gene changes suggest an airway with impaired stress responses, and altered immune responses, as well as potential up-regulation of nitrosamine metabolism.

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CHAPTER 5

CONCLUSIONS ON THE EFFECTS OF ENVIRONMENTAL TOBACCO SMOKE EXPOSURE *IN UTERO* IN A MURINE MODEL OF HUMAN ASTHMA AND THOUGHTS FOR FUTURE STUDIES

CONCLUSIONS

These experiments in a BALB/c mouse model of human asthma investigate the effects of environmental tobacco smoke (ETS) exposure *in utero* on allergen-specific immune responses in the lungs of adult offspring. Results demonstrate altered immune responses in adult mice that received ETS exposure *in utero*. ETS-associated changes in immune response are seen as an enhanced primary response to an allergen, ovalbumin (OVA). ETS exposure *in utero* does not alter establishment of immune tolerance or sensitization, nor does it alter basic lung structure or constitutive gene expression within the lung.

Mice exposed to ETS *in utero* demonstrate an increased sensitivity to i.p. injections of allergen (ESO vs. ASO) at 15 weeks of age. Mice exposed to ETS *in utero* and to allergen aerosol at 7 to 8 weeks of age show an increased resistance to i.p. sensitization at 15 weeks of age (EOO vs. AOO). Therefore, the development of immune sensitization or tolerance is not altered, but the magnitude of these responses is changed, based upon whether or not there was ETS exposure *in utero.*

Airway responsiveness, bronchoalveolar lavage cytokines and cytology, histopathology, and gene expression results all provide evidence for enhanced initial responses to allergen. ETS exposure *in utero* yields enhanced airway hyperresponsiveness (AHR) upon initial allergen exposure (EO and ES at 10 weeks of age; ESO at 15 weeks of age). BAL cytology and cytokines and histopathology data mirror the results described for AHR at 15 weeks of age. No appreciable changes are seen in the non-AHR endpoints at 10 weeks of age. Major changes in

gene expression are detected only after induced respiratory stress, or perturbation. The gene expression changes described following respiratory perturbation (ES vs. AS and EO vs. AO at 10 weeks of age; ESO vs. ASO and EOO vs. AOO at 15 weeks of age) reflect, both in process and in magnitude, the non-genetic changes seen as a result of ETS exposure *in utero.* In some cases, the changes in individual non-genetic endpoints are relatively minor or do not achieve statistical significance. However, in all cases, when viewed comprehensively, these alterations are sufficient to define a consistent, statistically significant change in response linked to ETS exposure *in utero*.

Certainly, the changes seen in all of the major endpoints are determined prior to their actual measurement especially are probably set in motion during the ETS exposure *in utero.* This is supported by the fact that adult ETS exposure does not yield the same changes. Therefore, timing of the *in utero* exposure relative to fetal development is essential to defining the molecular mechanism through which alterations are achieved. In this study, we did not attempt to narrow the exposure window, and thereby, more closely define the critical point(s) of exposure.

We did attempt, however, to detect changes prior to and during exposures to OVA. Neonatal gene expression changes indicate that some key factors in immune responses are changed at birth, prior to OVA exposure. Given the stringent criteria for acceptance (present in all samples, $p<0.05$, FDR<0.05, fold-change ≥ 1.5), it is not surprising that only a few genes are described as altered in expression. However, the majority of these genes have functions that would be consistent with alterations in immune response. For the most part, constitutive expression may not be different between treatment and control mice in this study. Differences may only become demonstrable with additional respiratory system perturbation to induce physiological or pathological responses that are accompanied by gene expression changes.

This might explain the minimal findings at 6 weeks of age when mice have developed considerably from birth, but have yet to receive allergen exposure or the corresponding control treatment (saline). At this time point, only 2 genes are recognized as having altered expression between the ETS-exposed and control groups. Neither of these genes has direct ties to the innate or adaptive immune system and their responses, although some, as of yet, undiscovered link may be possible. Respiratory perturbation at 6 weeks of age might reveal expression differences in genes induced by stress.

At 10 and 15 weeks of age, mice had received further respiratory system stress through OVA or saline aerosol and, in some cases, systemic OVA sensitization. At both time points, significant alterations in gene expression were seen as a result of ETS exposure *in utero*. The more challenged and manipulated 15-week old mice demonstrated the most pronounced changes. Even with fewer expression changes and the lack of physiological or pathological changes at 10 weeks of age, altered gene expression related to ETS exposure is important due to the function of these genes in cellular stress, antigen presentation, and immune signaling.

Gene expression changes seen at fifteen weeks of age are much more pronounced than those seen at 10 weeks of age and correlate very well with the histopathology and physiological changes seen at 15 weeks of age. Many of these genes have previously established relationships to innate or adaptive immune responses. The number of gene expression changes seen and the consistency of magnitude and direction of these changes suggest a non-random alteration of responses related to ETS exposure *in utero*. Statistical analyses further support this conclusion via correlation coefficients and regression analyses.

CRITICAL THOUGHTS FOR FUTURE STUDIES

Several steps could be taken to strengthen and further the results of this study:

1) The exposure timing should be further investigated to determine whether the effect of ETS exposure is confined to the time of fetal lung development, or is more far-reaching.

2) Given the minimal physical findings at early time points (prior to any additional respiratory exposures), the question of whether respiratory perturbation is necessary to unveil induced differences in response has arisen. We are presently conducting further studies to address whether an inducement is needed to yield measurable differences in response.

3) Many of the endpoint techniques have been improved and new equipment has been ordered that would undoubtedly provide stronger results within the same analyses. This includes improved cytokine ELISA and RNA extraction protocols, refined plethysmosgraphy, new resistance-compliance equipment, and more proficient sampling techniques. These things were all optimized as a result of this study but in some cases this optimization was only obtained after the fact. For future studies continued improvement of techniques and protocols is critical.

4) Consistency was a problem in the initial handling of gene expression data. Initial RNA samples were processed through a different laboratory and on a different Affymetrix microarray than were the fifteen week samples, which provided the greatest amount of data. The earlier samples were all lost after initial analysis and were not available for re-evaluation or confirmation testing. Much of this

was unavoidable and a consequence of Hurricane Katrina and its impact on New Orleans which was the site of initial microarray analyses.

5) Handling of raw gene expression data limited the scope and volume of conclusions concerning the impact of ETS exposure *in utero*. By using very stringent filtering criteria, we limited false positive results. However, given the nature of the exposures and the interaction of genes, it is inconceivable that we did not introduce a number of false negative results in each analysis. In each analysis, and cumulatively, this limits the conclusions that can be made about the resultant data. Many researchers now believe that in some cases fold-changes as low as 1.2 may be significant if the changes are across multiple genes within a pathway. This is especially true when evaluating mild to moderate changes in expression, such as those that would be expected from *in utero* or low-level environmental exposures. Unlike the large expression changes in knockout/knockin mice or those associated with more severe insults or treatments, changes resulting from mild environmental exposures, such as those in our study, are expected to yield less dramatic expression changes and may only result in disorder or disease over a chronic period of time (as occurs in atherosclerosis, diabetes, and obesity). In the future, enrichment programs could be used that would allow an enhanced evaluation of the up-regulation of critical processes and pathways and less emphasis on the behavior of or impact on individual genes.

6) Although we did not detect sex differences in histopathology, BAL cytology/cytokines, or AHR in our studies, others have reported sex-based differences. In non-ETS studies, we have detected an apparent increase in

AHR in males. Given the inconsistency in the literature and in our own findings, the role of sex can not be dismissed as insignificant. Since we had not detected sex-related differences in other endpoints, some of our gene expression studies involved only females. Revisiting our gene studies, we find that inclusion of both sexes can make a difference in gene expression profiles. This supports the claim that sex-related differences in response (at least at the gene expression level) can occur. We now are examining both sexes, together and independently, in gene expression analyses, as we have in analyses of our other endpoints.

7) Increased proteomic-based interpretation of the outcome of altered gene expression would strengthen the biological relevance of these findings. Alternate transcriptional splicing, translational and post-translational modifications impact the relevance of gene expression changes. This impact can be estimated by examining the actual changes in protein relative to changes in gene expression. If these changes are not relatively similar, then non-transcriptional and/or alternate transcriptional influences must be evaluated.

In concluding, it needs to be pointed out that this study was very successful, resulting in peer-reviewed publications and acceptance into the body of literature on ETS and the growing literature on adult responses to *in utero* exposures. However, there are many aspects of this study that could be improved, or new approaches attempted, as indicated above. The basic mechanisms that translate ETS exposure *in utero* into measurable, long-term changes in immune responses are still a mystery and must be pursued. The impact of this type of exposure on non-immune, but potentially very significant, basic metabolic processes has not been examined but is

almost mandatory, given some of the gene expression changes seen following ETS exposure *in utero* (data not presented).

VITA

Rodney Lamar Rouse was born in Lake Charles, Louisiana, in January of 1957 to Roderick Lamar Rouse, Head of the Accounting Department at McNeese State University, and Anna Scarborough Rouse, housewife. Rodney has one sibling, Roger, born in 1965. Roderick succumbed to malignant melanoma in 1968 and the brothers were raised by their mother.

Rodney graduated as salutatorian from Barbe High School in Lake Charles in 1975 and moved to Baton Rouge to attend Louisiana State University as a Top One Hundred Scholar. After three years in the animal science curriculum, Rodney was accepted into the Louisiana State University School of Veterinary Medicine where he served as the treasurer of the National Student Chapter of the American Veterinary Medical Association (1980-1982), performed as a summer fellow at the Northeast Regional Primate Center, Angell Memorial Hospital, and Harvard University in 1980, and graduated in the top ten percent of his class in 1982.

Rodney practiced privately from 1982 to 2002, owning and operating a multi-location veterinary service organization supplying general and emergency veterinary care in the Baton Rouge area. He returned to Louisiana State University in 1998 and received a Master of Business Administration degree in 2001. In 2003, he again returned to Louisiana State University beginning this quest for a Doctor of Philosophy degree. Over the years, Rodney produced four children and is married to Stella Manrique Rouse, who is beginning her career as an assistant professor of political science at the University of Maryland. Rodney is pursuing a research position in the Washington, D. C. - Baltimore area.