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Endotoxin Inhalation Alters Lung Development in Neonatal Mice

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Abstract

Background—Childhood asthma is a significant public health problem. Epidemiologic evidence suggests an association between childhood asthma exacerbations and early life exposure to environmental endotoxin. Although the pathogenesis of endotoxin-induced adult asthma is well studied, questions remain about the impact of environmental endotoxin on pulmonary responsiveness in early life.

Methods—We developed a murine model of neonatal/juvenile endotoxin exposures approximating those in young children and evaluated the lungs inflammatory and remodeling responses.

Results—Persistent lung inflammation induced by the inhalation of endotoxin in early life was demonstrated by the influx of inflammatory cells and pro-inflammatory mediators to the airways and resulted in abnormal alveolarization.

Conclusions—Results of this study advance the understanding of the impact early life endotoxin inhalation has on the lower airways, and demonstrates the importance of an experimental design that approximates environmental exposures as they occur in young children.

Keywords

endotoxin; lipopolysaccharide; neonatal; lung development; environmental exposures; alveolarization; childhood asthma; hygiene hypothesis; bronchopulmonary dysplasia

INTRODUCTION

Asthma in children is a major public health problem in the United States and worldwide (CDC, 2011a; WHO, 2008). It affects about 10% of U.S. children (CDC, 2010b; Asher et al., 2006), thus is one of the most common chronic childhood diseases and a leading cause of childhood hospitalizations (CDC, 2010b). Children with asthma have increased rates of missed school days, emergency room visits, and mortality (CDC, 2011a). They also

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experience reduced physical endurance due to respiratory distress, and both affected child and a caregiver often experience an overall lower quality of life (CDC 2010a). Socio-economically disadvantaged and minority children have an increased risk of asthma (CDC, 2011b). The prevalence of childhood asthma has been steadily rising over the last two decades. Ultimately, asthma accounts for the second major pulmonary health expenditure in the U.S. following upper respiratory tract infections (CDC, 2011a; U.S. DHHS, 2011).

Numerous early childhood epidemiological studies support the association between environmental endotoxin and increased rates of asthma. Specifically, elevated levels of endotoxin in house dust were associated with increased prevalence of wheeze, asthma symptoms and severity, and asthma medication use during childhood (Perzanowski et al., 2006; Park et al., 2001; Rizzo et al., 1997; Michel et al., 1996). Endotoxin containing house dust is frequently found in children's play areas, bedroom floors, and beddings (Thorne et al., 2009; Thorne et al., 2005; Braun-Fahrlander et al., 2002; Michel et al., 1996). As children spend more than half of a 24-hour day in close proximity to these surfaces, they are at increased risk of exposures to dust containing endotoxin. Humans and pets are common sources of endotoxin found in indoor dust (Thorne et al., 2009; Park et al., 2001). Other environmental sources of endotoxin include a cigarette smoke (Lawson et al., 2011; Hasday et al., 1999), Gram-negative bacteria-contaminated humidifying systems (Anderson et al., 2007; Mamolen et al., 1993), outdoor urban air (Soukup and Becker, 2001), and indoor combustion products of biomass fuels (Semple et al., 2010).

Endotoxin (lipopolysaccharide, LPS), a Gram-negative bacteria cell membrane component, is one of the most important environmental triggers of airway inflammation and asthma (Heederik et al., 2007). Identified originally as the heat-stable toxic component of cholera bacteria and later as a trigger of asthma in occupational settings, environmental endotoxin has been recognized as a potent biologically active component playing role in pathogenesis of chronic lung disease (Thorne et al., 2009; Olenchock et al., 1990; reviewed in Liebers et al., 2006). Inhaled endotoxin exerts its pathogenic properties via innate immune pathways resulting in the activation of inflammatory cells and expression of numerous pro-inflammatory mediators that in turn contribute to airway injury and airway remodeling (Liebers et al., 2006; Hadina et al., 2008; George et al., 2001).

Although the effects of inhaled endotoxin on adult human lungs have been studied extensively, little is known about pathophysiological processes underlying pulmonary responses to endotoxin in children. It has been recognized that children's lungs are particularly sensitive to environmental factors and early life exposures may seriously compromise the children's respiratory health (Finkelstein and Johnston, 2004). Animal studies of early life endotoxin exposures are scarce and often utilize endotoxin exposure modes that are not representative of childhood environmental exposures, such as intra-amniotic, intra-peritoneal, or intra-tracheal endotoxin administration (Tang et al., 2010; Chelvarajan et al., 2004; Shim et al., 2008; Blackwell et al., 2011; Martin et al., 1995). Still other studies are performed in adult, rather than juvenile mice (George et al., 2001; Thorne et al., 1999), thus not evaluating endotoxin exposure effects on pulmonary and immune homeostasis during early postnatal lung development.

In this context, it is important to examine the role of endotoxin in the pathogenesis of lung disease during the early postnatal period utilizing routes and modes of exposures approximating environmental exposures in humans. We developed a murine model of newborn/juvenile endotoxin inhalation exposures utilizing two inhalation exposure methods – an inhalation of endotoxin aerosol and intra-nasal instillation of endotoxin solution. In this model we examined inflammatory and remodeling responses in the lungs.

MATERIALS AND METHODS

Animals

C3HeB/FeJ mice (Jackson Laboratories, Bar Harbor, ME) were maintained and bred in a pathogen-free, barrier, Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved animal facility, and provided standard laboratory rodent chow and water *ad libitum* (Thorne et al., 1999). All protocols were approved by the Institutional Animal Care and Use Committee, and all animal care and housing requirements of the National Institutes of Health Committee on Care and Use of Laboratory Animals were followed. All the experiments were performed according to the established laboratory protocols and utilizing calibrated devices.

Neonatal/Juvenile Endotoxin Inhalation Exposures Utilizing Whole Body Exposure Chamber

Dams and their litters were housed and exposed to aerosolized endotoxin in custom-built exposure chamber inserts with solid bottoms and stainless-steel open-mesh sides and tops designed to achieve an effective aerosol distribution during exposures and minimize the handling of the pups. These inserts were placed in a dynamic flow whole body exposure chamber (O'Shaughnessy et al., 2003), and the endotoxin aerosol was generated from a solution of purified endotoxin (lipopolysaccharide, LPS, from *Escherichia coli* O111:B4, Sigma-Aldrich, St. Louis, MO) in sterile, pyrogen-free (PF) water (LAL Water; Lonza, Inc., Walkersville, MD) using a Collison six-jet nebulizer (CH Technologies, Inc., Westwood, NJ) (Thorne, 2000). The mass median aerodynamic diameter generated by the nebulizer system was between 1-2 μm as determined in previous experiments (Thorne, 2000). Conscious and unrestrained dams and their pups were exposed 4 hours/day, 5 days/week, from the day following birth (postnatal day 1, PND 1) until day 21 of life (postnatal day 21, PND 21) and euthanized 2-4 hours after the last endotoxin exposure, as specified in experimental timeline (Figure 1). Sham-control litters were exposed to nebulized pyrogen-free water, a vehicle in endotoxin-exposed groups. Glass fiber air-sampling filters placed in the outflow exhaust line of the chamber were collected during each endotoxin and sham exposure and used for analysis of airborne endotoxin levels utilizing a modified kinetic chromogenic *Limulus* amoebocyte lysate assay (LAL assay) (Lonza, Inc, Walkersville, MD) (Thorne et al., 2003; Thorne et al., 2010).

To evaluate the effectiveness of aerosol inhalation in inducing pulmonary endotoxin-specific effects and confirm that the huddling of the pups, crouching of the nesting dams over the pups, and the age-specific anatomy of the airways did not interfere with an effective delivery of endotoxin aerosol in to the pups' lungs, we conducted an independent, acute exposure experiment on groups of 10-day old mice. Briefly, 10-day old mice were exposed to aerosolized endotoxin as described above on two consecutive days, and evaluated on day 11 (PND 11) 2-4 hours after the last exposure.

Neonatal/Juvenile Endotoxin Intra-Nasal Aspiration Exposures

In a second set of experiments, juvenile mice were exposed to endotoxin from PND 1 to PND 21 by means of intra-nasal instillation of endotoxin solution followed by aspiration under light isoflurane anesthesia. With increasing age and lung capacity, the volume of solution increased from 5-15 microliters, containing an equal dose of endotoxin per mouse per day. The pups were spontaneously breathing, thus the endotoxin was inhaled into the lungs. Mice were euthanized 2-4 hours after the last exposure. Endotoxin concentration in the instillation solution was confirmed by the LAL assay. Age-matched sham-exposed or not-exposed (sentinel) animals served as controls.

Endotoxin, sham, and sentinel groups consisted of 6-10 animals depending on litter size. Experiments were performed twice and respective groups were pooled for data analysis, or as indicated in the figure legends.

Bronchoalveolar Lavage Fluid Collection and Differential Cell Analysis

Bronchoalveolar lung lavage (BAL) fluid was collected and processed as described previously with minor modifications (George et al., 2006). Briefly, lungs of PND 11 and PND 21 mice were washed with aliquots of 1 or 2 ml of sterile pyrogen-free saline, respectively. Recovered BAL fluid was centrifuged (2,400 rpm for 5 min at 4°C), and the supernatant was aliquoted and stored at -80°C until further analysis. Cell pellets were used to obtain total and differential cell counts using a hemocytometer and Diff-Quik Stain Set (Harleco, Gibbstown, NY).

Bronchoalveolar Lavage Fluid Cytokines/Chemokines Measurement

Cytokine and chemokine levels in the BAL fluid supernatants were determined by bead-based multiplex cytokine assay (Bio-Rad Laboratories, Inc., Hercules, CA) (Kulhankova et al., 2009) including TNF- α , IL-1 β , IL-5, IL-6, G-CSF, GM-CSF, KC and MIP-1 α . Briefly, beads were plated in 96-well filter plates pre-wetted with assay buffer and washed by vacuum filtration. Fifty μ l of cell-free BAL fluid, serially diluted cytokine standards, and blanks were added and incubated overnight at 4°C. The plate was washed; detection antibody solution was added followed by streptavidin-PE and reconstitution of beads with 125 μ l of assay buffer. Microplates were read on the Luminex Bio-Plex System reader (Bio-Rad Laboratories, Inc., Hercules, CA).

Lung Processing and Histological Evaluation

Lungs of PND 11 and PND 21 mice were perfused via the trachea with 10% buffered formalin at a pressure of 15 and 20 cm H₂O, respectively. Lungs were excised from the chest cavity, immersed in formalin, processed through a graded ethanol series, and embedded in paraffin. Five micrometer thick sections were stained with hematoxylin and eosin (H&E) and examined for evidence of inflammation and remodeling (Kulhankova et al., 2009; George et al., 2006). Evidence of sub-epithelial fibrosis was examined on Masson's Trichrome stained lung sections, and epithelial metaplasia on Periodic acid-Schiff stained lung sections.

Lung Morphometric Analysis

Seventeen randomly chosen lungs of mice dosed by endotoxin inhalation and aspiration plus sentinel mice were studied by morphometric analysis as described previously (Snyder et al., 2005; Cho et al., 2005). Three non-overlapping alveolar tissue areas per mouse of 5 μ m thick H&E stained lung sections were viewed with a 20X lens using a Nikon microscope (Melville, NY), and imaged with a SPOT digital camera (Sterling Heights, MI). The pictures were uniformly enlarged and printed on photographic paper. To determine the volume density of alveolar tissue and airspace, prints were overlaid with a 121-point grid and the number of points falling on alveolar wall compartment were counted and divided by the total number of points falling over the lung tissue. To calculate the volume of individual alveoli, photographs were overlaid with a grid of 48 evenly spaced cross points. Following identification of airspace structures, the point-sampled intercept parameter was obtained as a measurement of the distance between the airspace walls along the horizontal axis of the cross, and used to calculate the volume of the individual alveoli. To calculate alveolar wall thickness, the images were overlaid with a grid of 70 lines, each 33 mm long, and the mean cord length (Lm) was determined using the total counts of intersections of airspace walls with the lines and total length of all the lines.

Statistical Analysis

Results were expressed as mean $\pm$ standard error of the mean (SEM). Statistically significant differences ($P < 0.05$) between groups were determined by analysis of variance using Fisher's protected least significant difference test and unpaired Student's t-test (SAS 9.1, Cary, NC).

RESULTS

Evaluation of Two Newborn Inhalation Exposure Methods

Both the intra-nasal aspiration and aerosol inhalation of endotoxin were well tolerated during the first 3 weeks of life, with 100% pup survival. The whole body exposure chamber was effective in generating reproducible airborne endotoxin levels as determined from the collected outflow filters (Figure 2A). The mean average airborne endotoxin concentration in the exposure chamber was $9.4 \pm 1.6 \mu\text{g}/\text{m}^3$ (mean $\pm$ SEM) during endotoxin exposures, within the level reported for an optimal pulmonary responsiveness in mice (Thorne et al., 1999); and 0.004 ± 0.001 during sham exposures ($94,000 \text{ EU (endotoxin units)}/\text{m}^3$ and $40 \text{ EU}/\text{m}^3$, respectively). We detected a minor increase of the airborne endotoxin levels by the end of the third week of sham exposures that may have been due to normal tainting of the bedding by the maturing pups and pups' increased activity level. The low levels of airborne endotoxin during sham exposures proved to be insignificant in terms of pulmonary effects.

The body weights of 21-day old endotoxin-exposed pups at the conclusion of exposures were significantly lower compared to the age-matched sham or sentinel controls (mean $\pm$ SEM): $11.2 \pm 0.2 \text{ g}$ for inhalation-exposed mice, vs. $13.5 \text{ g} \pm 0.2 \text{ g}$ for sham mice, and $12.9 \text{ g} \pm 0.6 \text{ g}$ for sentinels, $P < 0.005$ (Figure 2B). Between the two groups of endotoxin-exposed mice, body weights of inhalation- and aspiration-exposed mice did not differ: $11.2 \text{ g} \pm 0.2 \text{ g}$ vs. $11.4 \text{ g} \pm 0.8 \text{ g}$, respectively.

Effective Pulmonary Endotoxin Dose

Based on the concentration of endotoxin in the chamber outflow ($9.4 \pm 1.6 \mu\text{g}/\text{m}^3$), duration of exposure (240 min), newborn mouse respiratory frequency ($276 \pm 29.6 \text{ breaths}/\text{min}$ in PND 8-10), newborn mouse tidal volume ($7.1 \pm 1.9 \mu\text{l}/\text{g}$ in PND 8-10), and empirically determined deposition fraction of particles with aerodynamic diameter $1\text{--}2 \mu\text{m}$ (approximately 0.05% in tracheobronchial and alveolar regions), we estimated that the daily lower respiratory tract deposited dose during 4-hour endotoxin aerosol inhalation exposure was 16 EU/pup/day (Bernier et al., 2007; Cassee et al., 1999). This was equivalent to $\text{ConcE}_{\text{Air}} \times 1\text{m}^3/10^6\text{ml} \times \text{Freq}_{\text{Resp}} \times \text{TV}_{\text{Resp}} \times \alpha \times T_{\text{Exp}}$, where $\text{ConcE}_{\text{Air}}$ is airborne endotoxin concentration in the chamber, $\text{Freq}_{\text{Resp}}$ is respiratory frequency in juvenile mice, TV_{Resp} is respiratory tidal volume in juvenile mice, α is an estimated deposition fraction for $1\text{--}2 \mu\text{m}$ aerodynamic diameter particles, and T_{Exp} is duration of exposure. Alternatively, given that the pulmonary deposition fraction after intra-nasal instillation followed by aspiration to the lungs is about 4-5% in the newborn mice (Zhou et al., 2008), we estimated that approximately 16 EU/pup/day would be delivered to LRT (lower respiratory tract) when concentration of endotoxin in intra-nasal instillation solution was 300 EU. In both scenarios, endotoxin aerosol inhalation and intra-nasal aspiration, the LRT levels fall within the range of estimated human indoor endotoxin exposures: 0.05 – 163 EU/day – range of estimated endotoxin dose deposited in the lower respiratory tract (Thorne, 2000; Park et al., 2000).

Pulmonary Effects of Acute Endotoxin Inhalation Exposures in Newborn and Juvenile Mice

In order to determine whether the huddling behaviors of the pups and small anatomical size of the airways of newborn/juvenile mice would interfere with effective endotoxin delivery to the lungs, we conducted an acute endotoxin exposure experiment and examined the degree of resulting lung inflammation. Groups of 10- and 20-day old mice were exposed to

nebulized endotoxin for four hours on two consecutive days (PND 10 and 11; and PND 20 and 21), and the bronchoalveolar lavage total and differential cellularity was determined 2-4 hours after the last exposure. Non-exposed littermates served as sentinel controls. Aerosol endotoxin exposure induced significant influx of neutrophils and macrophages to the airways, thus validating the effective endotoxin delivery to the airways. While total number of cells recruited to the lungs was similar in both 11- and 21-day old pups (Fig 3A), PND 21 pups demonstrated slightly higher overall proportion of neutrophils compared to PND 11 mice (approximately 90% vs. 70%, respectively).

Interestingly, 11-day old mice demonstrated significant airway eosinophilia, with eosinophils (Eos) accounting for nearly 50% of all BAL cells in sentinel mice (Figure 3B, 3C). Acute exposure with endotoxin further increased the number of eosinophils in the airways, however, due to disproportionately higher influx of neutrophils and macrophages, an overall proportion of eosinophils among all other cells decreased to about 14% (Figure 3B). BAL eosinophilia in sentinel mice resolved with age, and eosinophils accounted for only about 1% of total BAL cells by weaning on day 21 in both sentinel and endotoxin exposed mice (Figure 3B and C, and 4B and C).

Pulmonary Effects of Sub-Chronic Endotoxin Exposures in Newborn and Juvenile Mice

Endotoxin aerosol inhalation and intra-nasal aspiration during the first three weeks of life resulted in an influx of inflammatory cells to the airways when evaluated on postnatal day 21 (Fig 4A). Similar total cellularity in the lung lavage of 11- and 21-day old pups exposed to endotoxin by aspiration suggests that both newborn and juvenile mice were receiving similar effective dose of endotoxin and had similar responses to endotoxin. While the estimated lung-deposited dose of endotoxin in both aerosol inhalation and solution aspiration group were similar, the responses in aerosol-exposed mice were significantly greater than in aspiration-exposed animals. Mice exposed to endotoxin by aerosol inhalation had 1.4-fold more total cells and 2.5-fold more neutrophils in the airways compared to mice exposed intra-nasally (both $P < 0.05$). Compared to sentinels, there was approximately a 6-fold increase in total BAL cells in aerosol inhalation-exposed mice, and a 4-fold increase in aspiration-exposed mice (both $P < 0.05$; Figure 4A). While neutrophils represented about 76% of total BAL cells in the aerosol inhalation group, they comprised only 37% in the intra-nasal aspiration group (Figure 4B). Inhalation of nebulized pyrogen-free water in sham group resulted in an increase of total BAL cells, 92% of which were macrophages, similar to those in the sentinels.

Endotoxin inhalation induced secretion of numerous inflammatory cytokines and chemokines into the airways, irrespective of the mode of endotoxin exposure. Compared to intra-nasal aspiration, cytokine and chemokine responses were greater to endotoxin aerosol inhalation (TNF- α , KC, MIP-1, IL-1 β , and IL-6; for the latter two $P < 0.05$; Figure 5).

Interestingly, compared to the acute study with exposures on only two consecutive days, the responses to sub-chronic aerosol inhalation exposures (exposures for the first three consecutive weeks after birth) were approximately 3.5-fold lower: there was $119 \times 10^3 \pm 17$ (mean $\pm$ SEM) total BAL cells in sub-chronically-exposed mice and $423 \times 10^3 \pm 118$ total BAL cells in acutely-exposed mice ($P < 0.001$). Number and frequency of neutrophils followed this trend: sub-chronic inhalation mice had $95 \times 10^3 \pm 16$ neutrophils, whereas acute inhalation mice had $380 \times 10^3 \pm 107$ neutrophils ($P < 0.001$).

Airway Histology and Morphometry

Evaluation of the lung histology of mice from the sub-chronic study revealed peribronchiolar and paravascular mixed neutrophilic and monocytic infiltrates that

manifested as foci adjacent to medium size airways and associated vessels in the aspiration-treated mice (Figure 6, top two panels). By comparison, the mice exposed to endotoxin aerosol had more severe peribronchiolar infiltrates. Both endotoxin exposure groups demonstrated diffuse inflammatory cell infiltration in the alveolar regions of the lungs with accumulation of extracellular matrix within alveolar walls. The endotoxin-exposed mice demonstrated hypertrophic tall columnar epithelial cells in a majority of the medium and small size airways. This finding is in contrast to low cuboidal epithelial cells lining airways in control animals (Figure 6, bottom panel). The sub-epithelial layer of medium and small size airways was thickened in endotoxin-exposed mice. This was likely due to an increase of intercellular matrix and infiltration of inflammatory cells. There was no evidence of subepithelial deposits of collagenous fibrous material examined on Masson's Trichrome stained sections (Figure 6, bottom panel). Additionally, we did not find evidence of epithelial cell metaplasia examined on periodic acid-Schiff stained sections (not shown).

Morphometric analysis revealed that endotoxin-exposed mice had a significantly increased alveolar tissue volume density (TVD) and decreased airspace volume density, in comparison to sentinels (Figure 7A and 7B): TVD was $42 \pm 3.1\%$ (mean $\pm$ SEM) and $45.3 \pm 1.1\%$ in inhalation- and aspiration-exposed mice ($P < 0.05$), and $34.3 \pm 1.5\%$ in sentinels ($P < 0.001$). In addition, endotoxin exposure resulted in larger simplified alveoli with thickened alveolar walls, compared to sentinel mice (Figure 8), as documented by significantly increased mean alveolar volume: $5.7 \pm 1.0 \mu\text{m}^3$ in inhalation-exposed mice vs. $3.0 \pm 0.3 \mu\text{m}^3$ in sentinels ($P < 0.01$); and alveolar wall thickness: $17.8 \pm 0.5 \mu\text{m}$ in aspiration-exposed and $18.6 \pm 1.5 \mu\text{m}$ in inhalation-exposed mice vs. $15.9 \pm 0.4 \mu\text{m}$ in sentinels ($P < 0.01$ for both; Figure 7C and 7D).

DISCUSSION

Exposure to environmental endotoxin early in life is associated with asthma exacerbations during infancy and childhood, and airway hyper-reactivity later in life (Park et al., 2001; Rizzo et al., 1997; Michel et al., 1996). While there is ample data on the pathophysiology of endotoxin-induced lung disease in adults, little is known about the effects of inhaled endotoxin during early postnatal life. Early post-natal life is unique and particularly vulnerable time of immune system and lung maturation (Finkelstein and Johnston, 2004). In humans, a significant part of lung morphogenesis occurs postnatally as a result of lung taking on the function of gas exchange organ. The number of alveoli increases by about 85% from birth throughout the young adulthood, with most of the alveolar acquisition and enlargement taking place during the first two to three years of life (Merkus et al., 1996). The immune system of the respiratory tract also undergoes a significant maturation with macrophages and dendritic cells acquiring their full functional properties later in life (Chelvarajan et al 2004). This period of post-natal development represents a window of opportunity for normal development to be interrupted by inflammatory and immune-modulatory agents (Bäckström et al., 2011; Finkelstein and Johnston, 2004; Vernooy et al., 2002). Epidemiologic evidence based on pediatric cross-sectional and birth cohort studies suggests that microorganism-associated molecular patterns, such as endotoxin, in the environment of infants and children may be associated with a lower prevalence of childhood atopic diseases, including asthma (Ege et al., 2011). This phenomenon known as hygiene hypothesis is in part explained by an immune response shift towards a pro-inflammatory Th1 phenotype and away from a pro-allergic Th2 phenotype – with each phenotype characterized by differentially polarized cytokine profiles (Adkins, 2000). Limited evidence for early life endotoxin effects comes from the clinical case studies that link endotoxin-mediated lung injuries in infants with diseases predisposing them to respiratory tract colonization with Gram-negative bacteria (Muhlebach and Noah, 2002). Therefore, to advance our understanding of the effects the early life endotoxin inhalation has on lung

homeostasis and maturation we utilized a novel newborn/juvenile murine model of endotoxin inhalation exposures. In this model we examined the inflammatory and morphological changes in the lung.

Both methods of endotoxin exposure, inhalation of airborne or intra-nasally applied endotoxin in newborn and juvenile mice proved to be well tolerated and induced significant inflammatory changes in the lung. Compared to acute inhalation exposures (exposures on two consecutive days), lung inflammatory responses to sub-chronic exposures (exposures during the first three weeks of life) were greatly diminished. This finding is suggestive of an endotoxin tolerance developed in sub-chronically exposed mice. Endotoxin tolerance has been described in both mice and humans (Mueller-Anneling et al., 2006; Biswas and Lopez-Collazo, 2009), and is characterized by less vigorous pro-inflammatory cellular and molecular responses to repeated endotoxin challenges.

Given observed inflammatory changes in the lungs, the effectiveness of endotoxin delivery by aerosol inhalation was greater compared to intra-nasal aspiration. This result may be due to interplay of several factors. First, an aerosolized endotoxin was delivered over a 4-hour period, rather than administered as a single intra-nasal bolus, resulting in prolonged delivery of inflammatory stimulus over time. Second, the nebulization of aqueous endotoxin solution yielded to generation of airborne particles with an average mass median aerodynamic diameter of 1-2 μm , the mass fraction that is readily distributed throughout the respiratory bronchioles and alveolar compartment of the lungs (Thorne, 2000). As the response to inhaled substances is strongly linked to the aerodynamic particle size (Roy et al., 2003), inhalation of aerosolized endotoxin likely yielded a higher local end-point endotoxin concentration in the terminal regions of the lungs, allowing for greater availability of endotoxin molecules to respiratory macrophages, dendritic, and epithelial cells. In addition, particle removal in the lower airways is inefficient compared to that in the upper airways and nasopharynx, and might have contributed to a higher retained lung-dose in aerosol-inhalation group. Finally, the whole body aerosol exposure method better models the natural mechanisms of particle deposition and uptake in the airways and offers a more realistic representation of human exposures to airborne environmental substances (O'Shaughnessy et al., 2003; Thorne, 2000).

In agreement with other studies of early life endotoxin exposures, we found a number of inflammatory cytokines in the bronchoalveolar lavage fluid. These included TNF- α , IL-1 β , and IL-6, a growth factor G-CSF, and chemokines MIP-1 α and KC (Cao et al., 2009; Blackwell et al., 2011). The levels of TNF- α , IL-1 β , and IL-6 assessed in bronchoalveolar lavage fluid from the endotoxin-inhalation exposed mice were an order of magnitude lower than in adult mice exposed to comparable airborne endotoxin levels (Thorne, 1999). This may be due to the limited capacity of the immature innate immune cells in juvenile mice to produce pro-inflammatory cytokines (Chelvarajan et al., 2004; Martin et al., 1995).

We demonstrated the sub-chronic inhalation of endotoxin resulted in airways remodeling in juvenile animals. Accumulation of inflammatory cells and intercellular matrix contributed to thickening of subepithelial membranes similar to that observed in adult endotoxin-exposed mice (George et al., 2001; Vernooij et al., 2002), as well as adult individuals with asthma (Chetta et al., 1997). Furthermore, we documented hypertrophic changes in the epithelium of the medium and small size airways, but did not find evidence of subepithelial fibrosis. These data are in agreement with findings in juvenile mice that are characteristic with greater proliferation and renewal rate of bronchiolar epithelial cells and propensity to developed thickened smooth muscle layer of the conducting airways rather than subepithelial fibrosis (Ohki et al., 2002). These findings are also consistent with human

studies, where smooth muscle hypertrophy and hyperplasia were reported in the airways of patients with asthma (Ebina et al., 1993).

In the present study we demonstrated that early life endotoxin inhalation induced abnormal alveolarization characterized by enlarged simplified alveoli with thickened alveolar walls. These data are in partial agreement with studies of peri-natal endotoxin exposure on newborn lung development. Specifically, the newborn pups delivered to mothers that received intra-amniotic or intra-peritoneal endotoxin demonstrated arrested alveolarization resulting in large alveolar structures accompanied by alveolar wall thinning, contrary to our finding of alveolar thickening (Moss et al., 2002; Tang et al., 2010; Cao et al., 2009). Conversely to these studies yet in agreement with our findings, intra-tracheal administration of endotoxin directly to the lungs of newborn rats induced both alveolar arrest and alveolar wall thickening with accumulation of inflammatory cells and fibrous material in alveolar walls (Shim et al., 2008). Thus, while some parameters of lung development appear to be independent of the route of endotoxin exposure, others seem to be contingent upon the presence of endotoxin directly in the lung microenvironment.

Importantly, our findings of altered alveolarization are consistent with those of bronchopulmonary dysplasia in premature infants (Bäckström et al., 2011; Speer, 2006). As the final stage of alveolarization in mice begins at about postnatal day 5, it parallels well with the lung maturation in premature infants, who are born with their lung at various stages of alveolarization and are often exposed to various inflammatory stimuli during this critical time window of lung development (Bäckström et al., 2011; Merkus et al., 1996). The arrest of alveolarization induced by early life endotoxin may be mediated by complex mechanisms. These include activated macrophages and IL-1 β , both of which we found in the BAL in the present study (Blackwell et al., 2011; Bäckström et al., 2011). The remodeling effects of pre-natal endotoxin exposure in rats lasted through the first few weeks of life suggesting long lasting effect of these changes (Tang et al., 2010). In fact, clinical studies examining the long-term effects of early life airways remodeling documented that bronchopulmonary dysplasia in infants is associated with bronchopulmonary hyper-responsiveness and decreased pulmonary function in adolescents and young adults (Baraldi and Filippone, 2007). It would be of great importance to evaluate the long term changes in lung physiology and function caused by sub-chronic endotoxin inhalation during the neonatal/juvenile period, thus this area provides an opportunity for further research.

Examining the cellular composition of the lavage fluid obtained from the newborn and juvenile mice we observed a significant airway eosinophilia in sentinel 11-day old pups that was no longer evident in 21-day old mice. We and others have previously described this finding in murine models (George et al., 2006; Livraghi et al., 2009). The observed lung eosinophilia appears to be transient, with the absence of eosinophils in 4- and 5-day old pups (Livraghi et al., 2009), the presence and peak in 10- and 11-day old pups (48% in this study; about 35% in George et al., 2006; 37% in Livraghi et al., 2009), a reduction in 16-day old pups (about 10% in George et al., 2006), negligible levels in 21-day old pups (1% in this study; about 2% in George et al., 2006; Rose et al., 2010), and virtually non-detectable eosinophils in 5- and 8-week old mice (Livraghi et al., 2009; George et al., 2006). As newborn mice' lungs are developmentally immature and ontogenetically comparable to the 30th-32nd gestational week human fetus' lungs (Merkus et al., 1996), we wished to correlate our findings to the reports of airway cellularity in premature infants. Reports on lower airway cellularity in premature infants' lungs, though extremely scarce, confirm the presence of eosinophils as well as eosinophilic cationic protein, a highly specific eosinophil marker, in premature infants' alveolar tissues and tracheal aspirates, respectively (Yamamoto et al., 1996; Raghavender and Smith, 1997). Although the significance and mechanisms of the lung eosinophilia in preterm infants is not clear, a developmental

predisposition to eosinophilia and eosinophilic responses in the fetus and newborn has been documented. Specifically, both murine and human fetus and neonate is characteristic with a predominant Th-2 immune environment and deficient Th-1 responsiveness that plays a crucial role in the immune relationship between mother and fetus and represents a specific window of an innate immune system ontogenetic maturation (Adkins, 2000). This is reflected by a large number of eosinophils, one of the hallmarks of Th-2 immune deviation, making up to one-third of circulating granulocytes during a mid-gestation of a normal healthy human fetus (Forestier et al., 1991). In addition, mild eosinophilia is not uncommon in newborns, and up to 70% of premature infants have systemic eosinophilia that is referred to as eosinophilia of prematurity (Yen et al., 2010). Predisposition to respond to non-specific stimuli by eosinophilia was documented in a study of preterm infants who responded to long-term intubation with progressively increasing levels of eosinophil cationic protein in their tracheal aspirates (Raghavender and Smith, 1997); and a study of term infants who responded with elevated eosinophils in inflammatory exudate compared to adults (Bullock et al., 1969). Eosinophilia in newborn mice' lungs may be mediated by eotaxin, a major eosinophil chemoattractant, as it was recently discovered that eotaxin plays a crucial role driving a prenatal lung development (Haley et al., 2008).

In addition to eosinophilia in sentinel young mice' lungs, we also observed an increased influx of eosinophils to the lungs in response to endotoxin. Although the mechanisms of LPS-induced eosinophil recruitment have been studied before, they are not fully understood. The role of eotaxin has been hypothesized as eotaxin was found to be released by endotoxin-activated macrophages in a STAT6-dependent manner (Tang et al., 2005; Voehringer et al., 2007). Other molecules, such as CD11b/CD18 complex, CCR3 receptor, and a *de novo* synthesized further not characterized heat-stable protein with eosinophilotactic activity was documented to induce eosinophils influx into the pleural cavity in response to intra-thoracic LPS administration (Bozza et al., 1994; Penido et al., 2001; Larangeira et al., 2001). Taken together, while the significance and role of eosinophils in the newborn/juvenile mice lungs, both naive and endotoxin-primed is not clear, they appear to reflect a period of the pulmonary and immune system ontogenetic development and be mediated by complex mechanisms.

In summary, we have demonstrated that endotoxin inhaled in early life induces an inflammatory lung disease accompanied by airway remodeling and abnormal alveolarization. Further, we showed that inhalation of airborne endotoxin is more effective at inducing pulmonary inflammation than endotoxin delivered via an intra-nasal instillation, demonstrating the importance of developing murine models which more closely mimic environmental exposures experienced by humans. Our newborn/juvenile murine model parallels human early life exposures during important period of lung morphogenesis and we have demonstrated it is a useful tool to study the effects of early life inhalation exposures.

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Figure 1.

Experimental Timeline. For the sub-chronic endotoxin exposure study, mice were exposed either to aerosolized endotoxin in a whole body exposure chamber (inhalation, E-Inhal) or intra-nasally instilled endotoxin solution (aspiration, E-Aspir) from postnatal day 1 to 21 (PND 1-21), with day of birth (DOB) being Day 0. Sham mice were exposed to aerosolized sterile, pyrogen-free water in a whole body exposure chamber. The acute endotoxin exposure study was performed on PND 10 + 11, or PND 20 + 21. Necropsy with collection of biological samples was performed after the last exposure in both acute and chronic study (PND 11 or 21).

Figure 2.

A. Daily Airborne Endotoxin Concentrations during the Endotoxin (squares) and Sham (circles) Exposures in the Whole Body Exposure Chamber. The mean concentration of airborne endotoxin over the duration of experiment was $9.4 \pm 1.6 \mu\text{g}/\text{m}^3$ (E-Inhal; mean $\pm$ SEM) and $0.004 \pm 0.001 \mu\text{g}/\text{m}^3$ during sham exposures (Sham). **B.** Body Mass on Postnatal Day 21. The body weights of 21-day old endotoxin inhalation-exposed pups (E-Inhal, n=18) after the last exposure were significantly lower compared to the age-matched sham (Sham, n=14) or sentinel controls (Sent, n=21), *P<0.05; **P<0.01. Weight of aspiration-exposed mice (E-Aspir, n=14) did not differ from inhalation-exposed mice.

Figure 3.

Acute Endotoxin Exposure Study-Total and Differential Cells in Bronchoalveolar Lavage (BAL) Fluid. **A.** Mice exposed to endotoxin by inhalation of aerosol (E-Inhal) on postnatal days 10 and 11 (PND 11) and on days 20 and 21 (PND 21) had approximately 10-fold higher total BAL cell counts than age-matched sentinel mice (Sent) (***) $P < 0.001$, compared to respective Sent). **B.** Endotoxin-exposed mice had a significantly greater proportion of neutrophils in the BAL fluid (* $P < 0.05$, compared to Sent). Mac = macrophages, PMN = neutrophils, Ly = lymphocytes, Eos = eosinophils. **C.** Differential cell analysis in BAL fluid demonstrated increased eosinophil and neutrophil counts in PND 11 mice and increased neutrophil counts with little change in eosinophils in PND 21 mice, * $P < 0.05$, compared to Sent. Symbol '<' denotes that cell count was below 0.3×10^3 BAL cells/mouse.

Figure 4.

Sub-Chronic Endotoxin Exposures Study -Total and Differential Cells in Bronchoalveolar Lavage (BAL) Fluid. Mice were exposed from postnatal day 1 to day 11 (PND 11) or 21 (PND 21). **A.** Total BAL cells: Endotoxin-exposed mice had significantly more total cells in their BAL compared to sham and sentinel mice (Sent). Mice inhaling endotoxin aerosol (E-Inhal) had significantly more total BAL cells than mice exposed by nasal aspiration (E-Aspir), * $P < 0.05$, compared to Sent; # $P < 0.05$, compared to E-Aspir. Compared to sub-chronically-exposed mice, acutely-exposed mice had significantly more inflammatory cells in the BAL (Fig. 3A). Differential cell percentages, in **B**, and differential cell numbers, in **C**, document that sub-chronic endotoxin exposure produced a neutrophilic response compared to Sham-exposed mice. Both endotoxin exposure and Sham exposure increased BAL macrophages compared to Sentinel mice. Mac = macrophages, PMN = neutrophils, Ly = lymphocytes, Eos = eosinophils, * $P < 0.05$, *** $P < 0.005$, compared to Sent; # $P < 0.05$, compared to E-Aspir; Symbol '<' denotes that cell count was below 0.5×10^3 cells/mouse.

Figure 5. Sub-Chronic Exposure Study – Cytokines and Chemokines in BAL Fluid of Postnatal Day 21 Mice. Exposure by inhalation (E-Inhal) or aspiration (E-Aspir) increased bronchoalveolar lavage fluid (BAL) concentrations of all six cytokines/chemokines compared to Shams and Sentinels (Sent). IL-1 β and IL-6 were significantly higher in E-Inhal than E-Aspir mice. Units are pg/mouse. *P<0.05, compared to age-matched Sent; #P<0.05, E-Inhal compared to E-Aspir.

Figure 6.

Representative Microscopic Images of Lung Sections of Sub-Chronically Endotoxin-Exposed Mice. Lung sections were stained with hematoxylin and eosin (H&E, top two panels), or Masson's Trichrome (MT), and photographed at 20x and 40x magnification. Compared to exposure to endotoxin by intra-nasal aspiration (E-Aspir), inhalation of endotoxin aerosol (E-Inhal) induced greater accumulation of inflammatory cells, mononuclear cells and neutrophils in subepithelial regions of the airways (A) and adjacent blood vessels (V). Both aspirated and inhaled endotoxin induced hypertrophic changes (*) of respiratory epithelium in small size and medium airways in the absence of subepithelial fibrosis. Accumulation of inflammatory cells and extracellular matrix contributing to thickening of the alveolar walls is denoted by arrowheads.

Figure 7.

Morphometric Analysis of the Alveolar Tissues in the Juvenile Lungs. Sub-chronic inhalation of endotoxin resulted in significantly increased alveolar tissue volume density (Alv Tissue VD) (A), and decreased airspace volume density (Airspace VD) (B). Alveolar wall thickness (Alv Wall Thickness) (C), and mean alveolar volume (Mean Alv Volume) (D) were increased by sub-chronic endotoxin inhalation, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 8.

Representative Photomicrographs of the Alveolar Tissues of Mice Sub-Chronically Exposed to Endotoxin. Mice were exposed to endotoxin from postnatal day 1 to 21 by either intra-nasal instillation followed by aspiration to the lungs (E-Aspir), or aerosol inhalation in an exposure chamber (E-Inhal), or were not exposed (Sent). Alveolar regions of the hematoxylin & eosin-stained lung sections were photographed at 20x and 40x magnification. Images illustrate morphological changes induced by endotoxin exposure analyzed in Figure 7, such as enlarged simplified alveoli and thickened alveolar walls.