In utero ultrafine particulate matter exposure causes offspring pulmonary immunosuppression

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Early life exposure to fine particulate matter (PM) in air is associated with infant respiratory disease and childhood asthma, but limited epidemiological data exist concerning the impacts of ultrafine particles (UFPs) on the etiology of childhood respiratory disease. Specifically, the role of UFPs in amping Th2- and/or Th17-driven inflammation (asthma promotion) or suppressing effector T cells (increased susceptibility to respiratory infection) remains unclear. Using a mouse model of in utero UFP exposure, we determined early immunological responses to house dust mite (HDM) allergen in offspring challenged from 0 to 4 wk of age. Two mice strains were exposed throughout gestation: C57BL/6 (sensitive to oxidative stress) and BALB/C (sensitive to allergen exposure). Offspring exposed to UFPs in utero exhibited reduced inflammatory response to HDM. Compared with filtered air (FA)-exposed/HDM-challenged mice, UFP-exposed offspring had lower white blood cell counts in bronchoalveolar lavage fluid and less pronounced peribronchiolar inflammation in both strains, albeit more apparent in C57BL/6 mice. In the C57BL/6 strain, offspring exposed in utero to FA and challenged with HDM exhibited a robust response in inflammatory cytokines IL-13 and IL-17. In contrast, this response was lost in offspring exposed in utero to UFPs. Circulating IL-10 was significantly up-regulated in C57BL/6 offspring exposed to UFPs, suggesting increased regulatory T cell expression and suppressed Th2/Th17 response. Our results reveal that in utero UFP exposure at a level close to the WHO recommended PM guideline suppresses an early immune response to HDM allergen, likely predisposing neonates to respiratory infection and altering long-term pulmonary health.

Significance

Particulate matter exposure causes infant respiratory morbidity and mortality, but the role of ultrafine particles (UFPs) with an aerodynamic diameter of less than 0.1 μm in asthma and respiratory tract infections is unclear. Limited mechanistic information is available concerning UFP influence on the etiology of childhood asthma or susceptibility to respiratory infections. Here we exposed two strains of mice (sensitive to oxidative stress or allergen exposure) to UFPs throughout gestation at concentrations relevant to human exposures. Our results reveal a window of pulmonary immunosuppression in offspring following in utero UFP exposure. A dampened host immune response during early development underlies increased childhood susceptibility to respiratory infections, highlighting the necessity to develop strategies to protect the fetus during this vulnerable period.


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although exposure has been associated with asthma exacerbation in children (16). UFPs represent an important component of ambient PM and traffic-related air pollution (17). However, the current National Ambient Air Quality Standard does not specifically classify UFPs as a criteria pollutant (18). A few mouse models have investigated the offspring allergic inflammatory response to inhaled allergens following in utero exposure to diesel exhaust particulate matter (DEPM), a primary component of traffic-related pollutants. Findings from the previous studies vary considerably, demonstrating either increased airway inflammation and airway hyperreactivity indicative of an asthmatic phenotype (19–21), no effect (22), or even protection from airway inflammation in response to allergen challenge (23). The source and size of particles as well as timing of both PM and allergen exposure may be critical due to the complexity of fetal and infant lung and immune system development during this period. In neonatal mice, which represent an immunologically immature population, coexposure to DEPM (with a mean diameter between 0.1 and 0.3 μm) and HDM from 0 to 3 wk of age markedly enhanced airway inflammation through a mixed Th2 and Th17 response compared with HDM treatment alone (24). Conversely, a model where neonatal mice were exposed to combustion-derived PM (CDPM) with a mean diameter of 0.2 μm before (0–1 wk) and throughout HDM dosing from 1 to 5 wk of age did not show a dampened immune response immediately after exposure in mice coexposed to CDPM and HDM, yet a predisposition to develop asthma upon rechallenge later in life (25). Collectively, these disparate outcomes may be attributable to differences in maternal/neonatal exposure conditions, type and timing of offspring PM or allergen exposure regimens, as well as variance in genetic background of the mouse model. Strain differences in allergic airway inflammation are well established, and reported BALB/c mice are more sensitive to allergens than C57BL/6 mice (26). Airway inflammatory response to DEPM also varies significantly among mouse strains. Findings from adult exposure models of prolonged low-dose DEPM exposure with 90% of particles less than 2.5 μm and ~60% of 0.33-μm particles indicate that genetic differences in host defense response to oxidative stress may underlie variance, suggesting that C57BL/6 mice are more sensitive to DEPM than BALB/c mice (27, 28).

In this study, we investigated the effects of in utero UFP exposure on offspring pulmonary immune response in a mouse model representing a period of immune maturation (Materials and Methods and SI Appendix). We also evaluated the impact of genetic background (i.e., strain difference) on offspring airway inflammatory response. An in utero exposure model was developed to challenge neonates by a subchronic HDM challenge over the juvenile period (0–4 wk). Our exposure system replicated urban UFPs, including atmospherically relevant compositions and concentrations. Since genetic background is an important determinant of airway inflammation, we exposed two common strains employed in murine asthma models, C57BL/6 and BALB/c mice, to a 24-h daily dose of 25 μg/m³ throughout gestation and assessed the pulmonary immune response in offspring.

Particle Generation Replicates Urban UFPs

Ambient fine PM is primarily composed of organics, sulfates, nitrates, ammonium, black carbon, and chloride (4–6, 29). The nitrate constituent is attributable to vehicle emissions and industrial source sites, the sulfate is due to the burning of sulfur-containing fuels or coal burning from power plants, and black carbon is produced mainly by diesel vehicles or coal burning from power plants, and black carbon is produced mainly by diesel vehicles or coal burning from power plants. In our animal model, a multicomponent aerosol mixture representative of PM chemical composition under typically polluted environment was produced and introduced into the exposure chamber in a controlled manner (SI Appendix, Figs. S1 and S2). We generated UFPs using an atomizer (SI Appendix, Fig. S1) and a diluted solution consisting of ammonium nitrate, ammonium sulfate, diesel exhaust PM (NIST, SRM 2975), and potassium chloride, with the mass fractions of 44, 39, 10, and 7%, respectively (Fig. 1A). The particle size ranged from 20 to 220 nm, with a peak diameter of about 50 nm (Fig. 1B) and a total surface area of (3.2 ± 0.3) × 10⁶ μm²/cm³. The particle number concentration was measured by a conventional particle counter. In our model, we administered UFPs to time-mated mice 6 h per day from gestation days 0–18 (Fig. 1C). Daily exposure values over the gestational period had an average mass concentration of 101.94 μg/m³ ± 0.0784 (SE), corresponding to a 24-h daily mean dose of 25 μg/m³. While there is no current regulatory standard for UFPs (18), this level is under the US Environmental Protection Agency national ambient air quality standard of 35 μg/m³ for PM₂.₅ and similar to the WHO recommended guideline of 25 μg/m³ for 24-h average exposure. In utero exposure to UFPs had no effect on litter size. Furthermore, in utero UFP exposure did not affect offspring growth, including weight and length, which were measured beginning on postnatal day 3 until 4 wk of age.

In Utero Exposure to UFPs Dampens Offspring Pulmonary Inflammatory Response to HDM

Following birth, offspring exposed to filtered air (FA) or UFPs in utero were placed onto a subchronic allergen challenge using HDM from 0 to 4 wk of age and killed on postnatal day 31 (72 h after the final dose) (Fig. 1C). In both strains of mice, total white blood cell (WBC) counts recovered from bronchoalveolar lavage (BAL) fluid were increased in FA-HDM mice compared with FA-PBS (control) offspring, as expected following HDM exposure. However, in PM-HDM mice the inflammatory response was lower than in FA-HDM mice (Fig. 2A). In C57BL/6 mice, FA-HDM offspring (170 WBC/μL) had significantly higher median BAL WBC counts in comparison with FA-PBS levels (50 WBC/μL) (P = 0.005). Conversely, levels in PM-HDM (79.5 WBC/μL) offspring did not vary significantly from the FA-PBS group (50 WBC/μL) and were significantly lower than the FA-HDM group (P = 0.032), indicating a dampened response.

Fig. 1. In utero UFP exposure model. (A) Particle chemical compositions represent mass compositions measured in typical polluted urban air. The nitrate and sulfate mass fractions, generated from ammonium nitrate and ammonium sulfate, were 44 and 39%, respectively. The chloride mass fraction, from potassium chloride, was 7%. The mass fraction of diesel soot, generated from diesel exhaust PM (NIST, SRM 2975) was 10%. (B) The particle size distribution ranged from 20 to 220 nm with a peak diameter of about 50 nm. (C) Experimental protocol. FA, filtered air; GD, gestational day; HDM, house dust mite; PBS, PBS control; UFPs, ultrafine particles; PND, postnatal day.
BAL cell differentials showed that macrophages predominated in the PBS-treated groups in comparison with HDM-treated offspring (P < 0.001). Levels of lymphocytes and eosinophils were significantly higher in HDM-treated offspring than in PBS controls in both FA and PM groups (P < 0.001). BAL WBC counts in the BALB/c strain (Fig. 2B) did not vary significantly between groups. Significant differences in the number of macrophages and lymphocytes present in the BAL were observed between the PM-PBS and PM-HDM groups (P < 0.0001). Sex differences were not apparent in offspring from either strain, likely due to unequal distribution of male and female offspring across the groups and low numbers in some groups. Six weeks after the final allergen challenge, a subset of remaining C57BL/6 mice from the same litters were killed to assess inflammatory response maintenance. BAL WBC counts decreased in all groups, and differential cell counts indicated minimal levels of lymphocytes, eosinophils, and neutrophils (SI Appendix, Fig. S3).

**In Utero UFP Exposure Reduces Overall Pulmonary Inflammation in C57BL/6 Mice and Does Not Affect Mucus Production in Either Strain**

Histological examination of the lungs confirmed the results from the BAL cell counts. Hematoxylin and eosin (H&E) stained lungs isolated from FA-HDM and PM-HDM mice showed similar types of inflammatory responses, with eosinophils as the predominant inflammatory infiltrate and macrophages as the secondary inflammatory cell type. The majority of the inflammation was centered around the bronchioles and vasculature, with alveolar eosinophils becoming apparent only in the most severe cases (Fig. 3). Notably, in C57BL/6 offspring, FA-HDM mice overall demonstrated a more severe inflammatory response, with eight of nine mice (88.9%) classified with marked inflammation and the remaining mouse with moderate inflammation. The average total score for the FA-HDM group was 6.0 (SI Appendix, Table S1). In comparison, the PM-HDM mice had 6/14 mice (42.9%) classified with marked, 5/14 (35.7%) with moderate, and 3/14 (21.4%) with mild inflammation. Their average total score was 4.5, significantly lower than that of FA-HDM mice (P = 0.0483). Average total scores in PM-PBS (0.2) and FA-PBS (0.1) mice did not vary significantly from each other, yet were higher than absolute control mice (0), reflecting a slight response to inhaled saline. Lymphoid hyperplasia was mild to moderate in PM-HDM and FA-HDM lungs, and minimal to non observable in the PBS-dosed mice and absolute controls. To determine the presence of activated T lymphocytes, we used immunohistochemistry targeting FoxP3, a putative marker for Treg cells. Mirroring the BAL WBC differential data showing higher lymphocytes, there were significantly more FoxP3-positive cells in the FA-HDM (39.7 ± 4.2) and PM-HDM (32.5 ± 4.7) groups than in their respective PBS controls, in which no FoxP3+ cells were identified (P < 0.0001). Levels between the PM- and FA-HDM groups did not vary significantly.

In BALB/c offspring, similar to the BAL data, histological examination of the lungs confirmed more variation within the inflammatory response in the FA-HDM group. In this group, two of six mice (33%) were classified as having marked inflammation, 33% mild, and the remaining 33% showed no apparent inflammation. The average total score for the FA-HDM group was 3.0 (SI Appendix, Table S1). To the HDM groups. The FA-HDM offspring did not significantly differ from the FA-PBS group across any categories. In comparison, the PM-HDM mice had 7/10 mice (70%) classified as marked, while the remaining 33% showed no apparent inflammation. Their average total score ±SD, 4.3 ± 5, was significantly higher than the PM-PBS mean total score (P < 0.001), but was not significantly different from the FA-HDM group.

Mucus production via goblet-cell hyperplasia is common in allergic airway diseases. C57BL/6 offspring from the FA-HDM and PM-HDM groups exhibited marked goblet-cell hyperplasia and mucus production compared with PBS-dosed controls (Fig. 3). The FA-HDM and PM-HDM mice scored 1.8 ± 0.2 and 1.6 ± 0.4, respectively, which was insignificantly different from each other, while the FA-PBS, PM-PBS, and absolute control mice scored significantly lower: 0.4 ± 0.2, 0.3 ± 0.2, and 0.2 ± 0, respectively (P < 0.001). Similarly, in BALB/c mice, the FA-HDM and PM-HDM groups scored an average of 1.8 ± 0.6 and 2.0 ± 0.3, respectively, which was insignificantly different from each other, while the FA-PBS and PM-PBS groups scored significantly lower: 0.6 ± 0.5 (P = 0.013) and 0.2 ± 0.1 (P < 0.001), respectively.

We determined mucus-associated gene expression with quantitative PCR (qPCR) on whole lung tissue (Fig. 4), showing a negligible difference in Muc5a expression among the groups in C57BL/6 mice. In BALB/c mice, Muc5a expression was significantly increased in the FA-HDM group, but not in the PM-HDM group (P < 0.05).

**In Utero UFP Exposure Diminishes Pulmonary IL-13 and IL-17 Expression in C57BL/6 Mice**

We further measured the expression of cytokines associated with a Th1 (IFNγ), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17), or Treg (IL-10, TGF-β) response using qPCR. In C57Bl/6 mice, expression of IL-13 and IL-17 was significantly increased in lung tissue from FA-HDM offspring versus FA-PBS controls (P < 0.05) (Fig. 4A). Notably, IL-13 and IL-17 expression in the PM-HDM group was reduced in comparison with the FA-HDM group and did not significantly differ from PM-PBS controls. In the BALB/c strain, IL-13 and IL-17 expression did not vary significantly between...
In this study, we investigated the impact of in utero UFP exposure on offspring pulmonary immune response in a mouse model representing a period of immune maturation. We generated a representative polluted environment by atomizing a mixture of UFPs representative of urban air pollution. The advantages of our model employing a peak particle concentration of 20,000 cm\(^{-3}\) represents realistic ambient levels of UFPs (4–8).

Our major conclusion is that in utero UFP exposure results in a reduced pulmonary inflammatory response to allergen challenge during a period of immune maturation. This immune-suppressive phenotype was more pronounced in the C57BL/6 strain, as reflected by significantly decreased white blood cell infiltration in BAL and histopathology; airway eosinophilia elicited by HDM was largely diminished in offspring exposed in utero to UFPs. The observed Th2/Th17-driven inflammatory response (i.e., increased pulmonary expression of IL-13 and IL-17) in the FA-HDM group was lost in the PM-HDM group and was indistinguishable from PBS-treated controls. Conversely, airway mucus production in response to HDM

groups. Levels of other cytokines measured in both strains, including IL-4, IL-5, and TGF-β, did not exhibit significant differences between groups. Levels of IL-10 were not detectable.

In Utero UFP Exposure Alters Systemic Inflammatory Response

Systemic inflammatory response was measured by quantifying circulating cytokines in offspring serum. In C57BL/6 mice, reduced systemic IL-9, MCP-1 (i.e., CCL2), and MIP-1α (i.e., CCL3) was observed in PM-HDM offspring compared with PM-PBS controls (Fig. 3A). In comparison with FA-HDM offspring, PM-HDM mice had significantly higher levels of IL-10, an important cytokine involved in Treg cell signaling. In BALB/c mice, the PM-HDM group had significantly higher levels of circulating IL-1β, IL-4, and IL-5 and lower levels of IL-3 than the PM-PBS group (Fig. 3B).

Discussion

In this study, we investigated the impact of in utero UFP exposure on offspring pulmonary immune response in a mouse model representing a period of immune maturation. We generated a representative polluted environment by atomizing a mixture of UFPs representative of urban air pollution. The advantages of our model included the real-time measurement of concentrations at relevant levels and route of exposure. We selected a 24-h dose of 25 μg/m\(^3\) (the current WHO recommended guideline for PM\(_{2.5}\)). Previously, exposure to PM\(_{2.5}\) in utero at levels of around 25 μg/m\(^3\) was attributed to adverse respiratory health outcomes in infants and children (3, 30). PM\(_{2.5}\) exposure during midgestation (during the second trimester) was also identified as a window of susceptibility for childhood respiratory disease (3, 31). Our model captured the relevant window of susceptibility. Notably, there is no current regulatory standard for UFPs. The UFP component of ambient air pollution typically contributes to a negligible portion (less than a few percentage points) of the total PM mass (5). Atmospheric measurements of particle size distributions demonstrated a high-number concentration of UFPs in urban locations because of direct emissions (4, 5) or new particle formation (6–9). For example, under relatively low PM loading, the PM\(_{0.1}\) number concentrations exceeded 200,000 cm\(^{-3}\) because of new particle formation and decreased slightly and remained at about 50,000 cm\(^{-3}\), as the particle size grew and haze events developed (9).
treatment was not attenuated by in utero UFP exposure. While our results differ from the findings of previously described models, i.e., a heightened inflammatory response in PM-exposed C57BL/6 offspring (19–21, 24), they are congruent with the data from a model employing subchronic HDM treatment over a 4-wk period (1–5 wk of age) following early life exposure to combustion-derived particulate matter (CDPM with a mean diameter of 0.2 μm) from 0 to 5 wk of age in C57BL/6 mice (25). Analogous to our results, mice coexposed to CDPM-HDM exhibited reduced Th2-driven pulmonary inflammation in comparison with neonates exposed to FA before and throughout HDM challenge. Airway mucus production did not vary between these groups, consistent with our results. Attenuated allergic airway inflammation has also been shown in cigarette-smoke–exposed mice challenged with OVA (32). On the basis of these findings and our data, an initial suppressive effect may be inferred in regard to early asthma symptoms; however, we conclude that an overall adverse effect from UFP exposure as attenuated immune response likely predisposes offspring to respiratory infection and leads to an exacerbated allergic response later in life. In our model, pulmonary inflammation returned to baseline 6 wk after the last allergen challenge when offspring were 12 wk of age. It is possible that, under our conditions, if rechallenged with HDM as adults, offspring may mount an elevated inflammatory response as in prior models (24, 25), following an early window of immunosuppression.

Our study also evaluated the impact of genetic background (i.e., strain difference) on offspring airway inflammatory response. Several previous models demonstrate that mouse strain impacts airway inflammatory response following sensitization and challenge with OVA and HDM (33–35). Furthermore, two commonly employed inbred strains in asthma models, C57BL/6 and BALB/c, have shown differing airway inflammatory responses following prolonged exposure to DEPM (27, 28). In our model, C57BL/6 offspring from both HDM-exposed groups showed greater airway eosinophilia than BALB/c offspring, in agreement with the previous data showing that eosinophil counts in BAL fluid were greater in C57BL/6 (vs. BALB/c) adult mice exposed to diesel exhaust PM from birth to 6 mo (27). Notably, in our model, airway inflammation was significantly attenuated in the C57BL/6 strain, but not in the BALB/c strain; this response likely corresponds to the C57BL/6 strain’s susceptibility to oxidative stress. Numerous previous models highlight that C57BL/6 mice exhibit higher levels of oxidative stress markers in response to inhalation exposure than BALB/c mice. For example, levels of GST mRNA and protein in lung tissue were significantly lower in C57BL/6 than in BALB/c mice, and 8-hydroxy-2′-deoxyguanosine levels in the lung tissues were significantly greater in the C57BL/6 strain following chronic DEPM exposure (27). Similarly, in models of cigarette smoke or water pipe smoke (a form of tobacco smoke) exposure, C57BL/6 mice exhibit decreased levels of glutathione and increased levels of lipid peroxidation products compared with resistant strains, including BALB/c mice (36–38).

The Nrf2-antioxidant response pathway plays an important role in responding to PM-induced oxidative stress (39). Nrf2 is a redox-sensitive basic leucine zipper transcription factor involved in the regulation of many antioxidant genes (40). This signaling pathway represents an adaptive response to environmental stresses, and extensive research has confirmed the importance of Nrf2 in protection against lung pathologies, including asthma (41) and hyperoxia-induced acute lung injury (42). Disruption of Nrf2 has been shown to enhance susceptibility to allergic airway inflammatory responses induced by chronic exposure DEPM (43). Previous findings from a birth cohort in Korea demonstrating increased susceptibility to lower respiratory tract infections in infants exposed in utero to PM₂.₅ were significantly modified by polymorphisms in maternal genes related to oxidative stress response pathways, especially Nrf2 (44). Likewise, in inbred strains of mice, including C57BL/6 mice, Nrf2 polymorphisms have been shown to influence hyperoxia susceptibility (42), indicating that the differences in Nrf2 response across strains underscores differences in host response to oxidative stress. The mechanisms responsible for the effects of in utero UFP exposure on lung and immune system development are not fully understood, but likely involve maternal systemic inflammation and oxidative stress on the placenta and fetus (2); future work is needed to probe oxidative stress pathways, including Nrf2.

In our model, systemic inflammation data from BALB/c mice indicated increased markers of inflammation, including IFNγ, IL-1β, IL-4, and IL-5, yet decreased IL-3, consistent with a previous study showing that, while neonates dosed with CDPM had a reduced pulmonary immune response to HDM, the total circulating IgE was twofold higher than in air-exposed mice. Conversely, C57BL/6 PM-HDM offspring showed significantly lower IL-9, MCP-1 (i.e., CCL2), and MIP-1α (i.e., CCL3) levels than PM-PBS offspring. Circulating IL-10 levels were significantly higher in the PM-HDM group than in FA-HDM. IL-10 is an important antiinflammatory cytokine secreted by Treg cells. A transient increase in Treg cells likely underlies a decreased inflammatory response. In other models, an increase in pulmonary Treg cells after neonatal CDPM exposure, driven by IL-10 signaling, correlated with increased susceptibility to viral infection (45, 46). We were unable to distinguish Treg cells (i.e., Foxp3+ cells) between FA- and PM-exposed groups using immunohistochemistry in our model; future investigation using flow cytometry may shed light on the role of specific immune subpopulations in the observed response. Another limitation in our study was the small number of BALB/c offspring, particularly in the FA-HDM group (n = 3), leading to high variability in the phenotypic responses. Previous studies have shown that males are more resistant to HDM than females in BALB/c mice (35). Our analysis included offspring of both sexes, and the low numbers rendered the sex-specific analysis difficult in some treatment groups. Future studies evaluating the sex differences in BALB/c offspring are warranted.

In summary, using an in utero exposure model as described in SI Appendix (47, 48), we show that C57BL/6 offspring exposed to UFP in utero and challenged with HDM do not develop a robust airway inflammatory response compared with filtered-air exposure.
HDM-exposed mice. This indicates an early immunosuppressive environment in the lung and provides a platform to explore the mechanisms of immunosuppression and host defense response to oxidative stress. Our work corroborates the limited earlier evidence that in utero UFP exposure adversely affects offspring pulmonary immune responses and provides insight into the impacts of UFP exposure on infant susceptibility to respiratory infection and overall long-term pulmonary health, highlighting the necessity to reduce UFP exposure and develop strategies to protect the fetus during this vulnerable period.

Materials and Methods

Described here is a summary; additional details are provided in SI Appendix. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Texas A&M University.

Mice were housed in a climate-controlled room with a 12/12-h light/dark cycle. Females received a 9% fat breeder diet (Harlan), and males received standard rodent chow (4%) (LabDiet). The exposure chambers consisted of a 12 × 8 × 32 stainless steel box with separated inner compartments and a 1/4″ clear acrylic lid (SI Appendix, Figs. 51 and 52). Air was continuously pumped through the chamber by stainless steel aerosol distribution lines attached to the lid and out exhaust lines on the bottom. UFPs were generated utilizing a commercial constant output atomizer, and the number concentration and size distribution of UFPs in the chambers were constantly monitored. To determine offspring response to allergen challenge following in utero UFP exposure, we followed a well-characterized protocol entailing chronic dosing with HDM (15).

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