

A mouse model links asthma susceptibility to prenatal exposure to diesel exhaust

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Background: Most asthma begins in the first years of life. This early onset cannot be attributed merely to genetic factors because the prevalence of asthma is increasing. Epidemiologic studies have indicated roles for prenatal and early childhood exposures, including exposure to diesel exhaust. However, little is known about the mechanisms. This is largely due to a paucity of animal models.

Objective: We aimed to develop a mouse model of asthma susceptibility through prenatal exposure to diesel exhaust.

Methods: Pregnant C57BL/6 female mice were given repeated intranasal applications of diesel exhaust particles (DEPs) or PBS. Offspring underwent suboptimal immunization and challenge with ovalbumin (OVA) or received PBS. Pups were examined for features of asthma; lung and liver tissues were analyzed for transcription of DEP-regulated genes.

Results: Offspring of mice exposed to DEPs were hypersensitive to OVA, as indicated by airway inflammation and hyperresponsiveness, increased serum OVA-specific IgE levels, and increased pulmonary and systemic T_H2 and T_H17 cytokine levels. These cytokines were primarily produced by natural killer (NK) cells. Antibody-mediated depletion of NK cells prevented airway inflammation. Asthma susceptibility was

associated with increased transcription of genes known to be specifically regulated by the aryl hydrocarbon receptor and oxidative stress. Features of asthma were either marginal or absent in OVA-treated pups of PBS-exposed mice.

Conclusion: We created a mouse model that linked maternal exposure to DEPs with asthma susceptibility in offspring. Development of asthma was dependent on NK cells and associated with increased transcription from aryl hydrocarbon receptor- and oxidative stress-regulated genes. (*J Allergy Clin Immunol* 2013;■■■■:■■■-■■■.)

Key words: Prenatal exposure, diesel exhaust particles, asthma, mouse model, natural killer cells, aryl hydrocarbon receptor, IL-5, IL-13, IL-17

Over the last several decades, the prevalence of asthma has continuously increased. In the last 10 years, the prevalence of asthma in the United States has increased from 7.3% (20.3 million persons in 2001) to 8.4% (25.7 million persons in 2010).¹ This recent increase in prevalence implicates industrialization- and urbanization-generated environmental exposures in disease pathogenesis. Prenatal and early childhood exposures are likely to have the highest effect because they occur in periods of intense developmental programming and thereby have the potential to induce long-term memory in cells, systems, and organs. The role of early programming is underscored by the fact that asthma symptoms in most cases start in the first years of childhood. The argument for prenatal/maternal influences is provided by the observation of a strong association of childhood asthma with maternal asthma.²⁻⁴ Among children less than 5 years old, the risk of asthma is more than 3-fold greater for those with mothers with asthma than those with fathers with asthma.² One of the plausible explanations is that an offspring's predisposition to asthma is shaped prenatally by an altered intrauterine environment. This alteration of the intrauterine environment is imposed by maternal disease, disease-triggering maternal exposures, or both.

In support of the latter hypothesis, there are many epidemiologic studies showing an association between various *in utero* exposures and asthma susceptibility.⁵⁻¹² Among prenatal insults with linkage to asthma, solid and consistent epidemiologic evidence has been provided for exposure to traffic-related pollution, including diesel exhaust.⁵⁻⁸ Mothers who lived near highways during pregnancy are more likely to have children with asthma.⁵ Prenatal exposure to polycyclic aromatic hydrocarbons (PAHs), which are diesel exhaust-derived toxins, is associated with increased risk of allergic sensitization and early childhood wheeze.^{6,8}

Although epidemiologic data support the hypothesis on the prenatal origins of asthma, the mechanistic understanding is still very poor. There are many obstacles to conducting mechanistic studies during pregnancy and infancy. Intentional exposures of pregnant women are unethical. Studies of infants have been

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Abbreviations used

AhR:	Aryl hydrocarbon receptor
AhRR:	Aryl hydrocarbon receptor repressor
BALF:	Bronchoalveolar lavage fluid
Cyp1a1:	Cytochrome P450, family 1, subfamily A, polypeptide 1
Cyp1b1:	Cytochrome P450, family 1, subfamily B, polypeptide 1
DEP:	Diesel exhaust particle
Hmox1:	Heme oxygenase 1
NK:	Natural killer
Nrf2:	Nuclear factor (erythroid-derived 2)-like 2
OVA:	Ovalbumin
PAH:	Polycyclic aromatic hydrocarbon
PND:	Postnatal day
ROS:	Reactive oxygen species

limited by the scant size of biological samples and ethical concerns. Finally, only a few animal models are available. In regard to prenatal diesel exhaust exposures, the positive link to asthma has been established by 3 earlier models.¹³⁻¹⁵ We have created a complementary mouse model and provided new mechanistic insights.

METHODS

Time-mated C57BL/6 female mice were anaesthetized with isoflurane and given intranasal applications of diesel exhaust particles (DEPs; 50 μ g, National Institute of Standards and Technology, Gaithersburg, Md; SRM 2975)¹⁵⁻¹⁸ in 50 μ L of PBS (15 mice) or 50 μ L of PBS alone (9 mice) on gestation days 3, 6, 9, 12, 15, and 18. The volume was delivered through 2 sequential injections of 25 μ L administered 15 minutes apart, each into a different nostril.

On postnatal day (PND) 5, pups of 10 DEP-exposed mice and 5 PBS-exposed mice (5-8 pups from each mother) were given intraperitoneal injections of 50 μ L of the immunizing mixture, which contained ovalbumin (OVA; 5 μ g) and Imject Alum (0.5 mg of aluminum hydroxide and 0.5 mg of magnesium hydroxide; Thermo Scientific, Rockford, Ill) in PBS. Pups from another 5 DEP-exposed mice and 4 PBS-exposed mice were given injections of 50 μ L of PBS. On PNDs 20, 21, and 22, OVA-immunized offspring of 3 DEP-exposed mice were given injections of either the anti-NK1.1 antibody or mouse IgG_{2a} isotype control (natural killer [NK] cell depletion experiment; see the detailed [Methods](#) section in this article's Online Repository at www.jacionline.org). On PNDs 23, 24, and 25, all pups underwent pulmonary challenges. OVA-immunized pups were given intranasal applications of 50 μ g of OVA in 15 μ L of PBS, and PBS-injected pups were given intranasal applications of 15 μ L of PBS without OVA, all after isoflurane-induced anesthesia. On PND 27 (22 days after immunization and 2 days after final pulmonary challenge), blood samples were collected (by means of tail nick), and serum was isolated. FlexiVent studies were conducted on PND 28 (3 days after the final pulmonary challenge). A separate set of mice was used to obtain bronchoalveolar lavage fluid (BALF) and lung and liver tissues.^{19,20} For each measured parameter, offspring of 3 to 7 mice per group were analyzed. All experiments were approved by the Institutional Animal Care and Use Committee at National Jewish Health. Other methods can be found in the [Methods](#) section and [Table E1](#) in this article's Online Repository at www.jacionline.org.

RESULTS**Mouse model of asthma susceptibility through prenatal exposure to DEPs**

To develop our mouse model of prenatally induced asthma susceptibility, we selected the most valuable elements from existing models (protocols by Fedulov et al,¹³ Auten et al,¹⁴ and Reiprich et al¹⁵; see [Table E2](#) in this article's Online Repository at www.jacionline.org). Similar to Auten et al,¹⁴ we used

C57BL/6 mice. This approach allows an immediate use of genetically modified mice that are typically on the C57BL/6 background. We also incorporated the principle of repeated maternal exposure, which is similar to the studies of Auten et al¹⁴ and Reiprich et al,¹⁵ because human exposure to diesel exhaust is chronic. For maternal challenges, we used DEPs because their proasthma activity was proved by all 3 prenatal exposure protocols and by other studies in adult mice and human subjects.^{13-15,21-31} The inflammatory activity of DEPs is attributed to components such as PAH, quinones, sulfuric acid, and metal oxides.³²

In our model 50 μ g of DEPs was repeatedly applied to pregnant C57BL/6 mice through the intranasal route ([Fig 1](#)). Control mice received PBS. The DEP dose of 50 μ g is commonly used in mouse models, including those by Auten et al and Fedulov et al.^{13,14,16} We considered using exposure to titanium dioxide or carbon black particles because they were used in some studies as "control" particles.^{13,33} We chose not to do so because these particles have specific biologic effects, including effects on fetal development.^{13,34,35}

From the model of Fedulov et al,¹³ we took the idea of suboptimal immunization of offspring. The rationale was that standard immunization protocols produce vigorous inflammation, and therefore effects of maternal DEP exposure might become masked. The selected protocol of suboptimal sensitization included a single injection of a low dose of OVA during the neonatal period, when antigen exposure elicits a weak immune response or even immune tolerance.^{36,37} Intranasal challenge with OVA or PBS was done on PNDs 23, 24, and 25. Mice were analyzed 72 hours after the final challenge.

Prenatal exposure to DEPs reduces body weight

Exposures had no effect on litter size (5-8 pups from mothers in each group). At the age of 4 weeks, mice exposed prenatally to DEPs and then challenged with PBS after birth (DEP-PBS mice) or OVA after birth (DEP-OVA mice) had significantly reduced body weight (12.49 ± 0.34 and 12.27 ± 0.29 g, respectively) compared with mice exposed to PBS prenatally and after birth (PBS-PBS mice; 13.74 ± 0.37 g, $P < .01$ for both comparisons) but not to mice exposed to PBS prenatally and OVA after birth (PBS-OVA mice; 12.82 ± 0.46 g, $P > .05$ for both comparisons). There was no significant difference in body weight between DEP-OVA and DEP-PBS mice and between PBS-OVA and PBS-PBS mice. Thus postnatal exposure to OVA did not have a significant effect on body weight.

In utero exposure to DEPs facilitates induction of airway inflammation

DEP-OVA pups had peribronchial inflammatory infiltrates ([Fig 2, A and B](#)). BALF from these mice had increased numbers of cells, including eosinophils, neutrophils, and lymphocytes, compared with BALF from pups of other groups ([Fig 2, C-G](#)). Airway inflammation was absent in PBS-PBS, PBS-OVA, and DEP-PBS mice.

Prenatal exposure to DEPs facilitates development of airway hyperreactivity

Airway response to methacholine is generally weak in pups/young mice compared with that seen in adult mice. Furthermore, baseline airway resistance in pups is high. The baseline airway

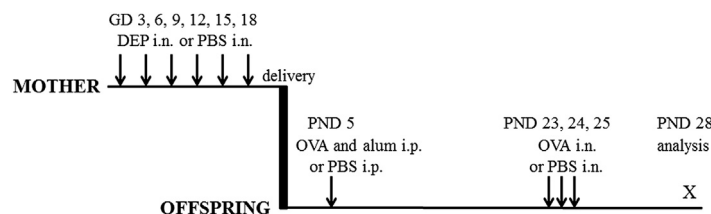


FIG 1. Experimental protocol. Timed pregnant C57BL/6 mice were given intranasal (*i.n.*) applications of either DEPs or PBS on indicated gestation days (*GD*). Offspring were given intraperitoneal (*i.p.*) injections of PBS or a mixture of OVA and alum in PBS and then intranasal applications of OVA or PBS on indicated postnatal days (*PND*). These mice were then analyzed 3 days after the final intranasal application.

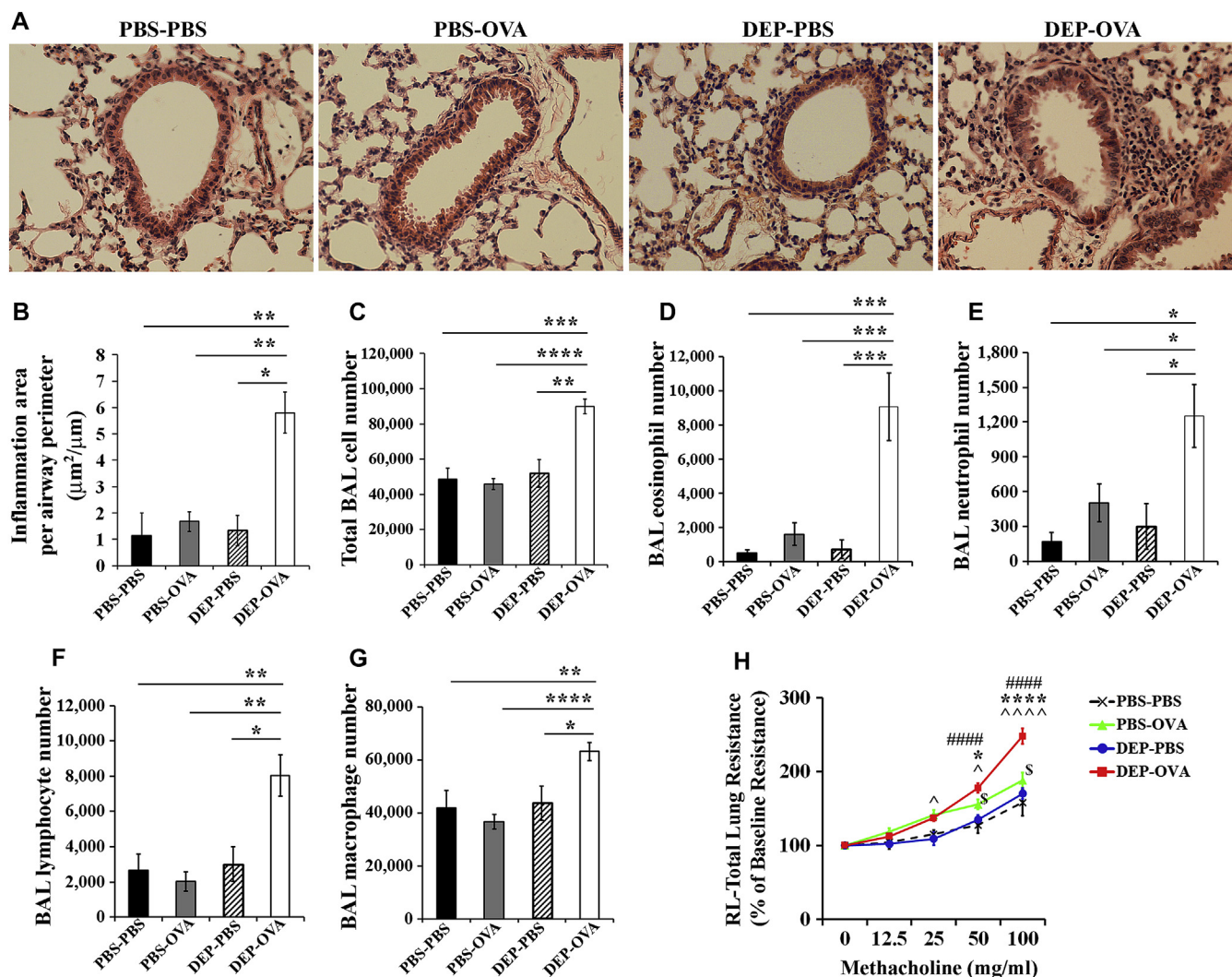


FIG 2. Airway inflammation and resistance. Experiments included PBS-PBS (prenatal and postnatal PBS), PBS-OVA (prenatal PBS and postnatal OVA), DEP-PBS (prenatal DEP and postnatal PBS), and DEP-OVA (prenatal DEP and postnatal OVA) mice. **A** and **B**, Peribronchial inflammation (histologic analysis). Mean $\pm$ SEM values are shown from 3 to 6 mice per group. **C-G**, Mean $\pm$ SEM BALF cell counts are shown from 10 to 21 mice per group. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$. **H**, Total lung resistance. Mean $\pm$ SEM values are shown from 9 to 18 mice per group. #####DEP-OVA versus PBS-PBS mice; ****DEP-OVA versus PBS-OVA mice; ***DEP-OVA versus DEP-PBS mice; and \$PBS-OVA versus PBS-PBS mice.

resistance values for PBS-PBS, PBS-OVA, DEP-PBS, and DEP-OVA mice were high and comparable (2.22 ± 0.22 , 2.15 ± 0.14 , 2.11 ± 0.17 , and 2.02 ± 0.15 cm H₂O.s/mL, respectively; $P > .05$ for all comparisons). Nonetheless, DEP-OVA mice showed augmented airway resistance after exposure to methacholine (Fig 2, H). In addition, a less pronounced but significant increase

in airway resistance was observed in PBS-OVA mice. Airway hyperresponsiveness can occur in the absence of inflammation.³⁸ This hyperresponsiveness might be due to activation of airway cells, such as mast cells. PBS-OVA mice had some OVA-specific IgE (as discussed below), which might activate resident mast cells on OVA challenge.

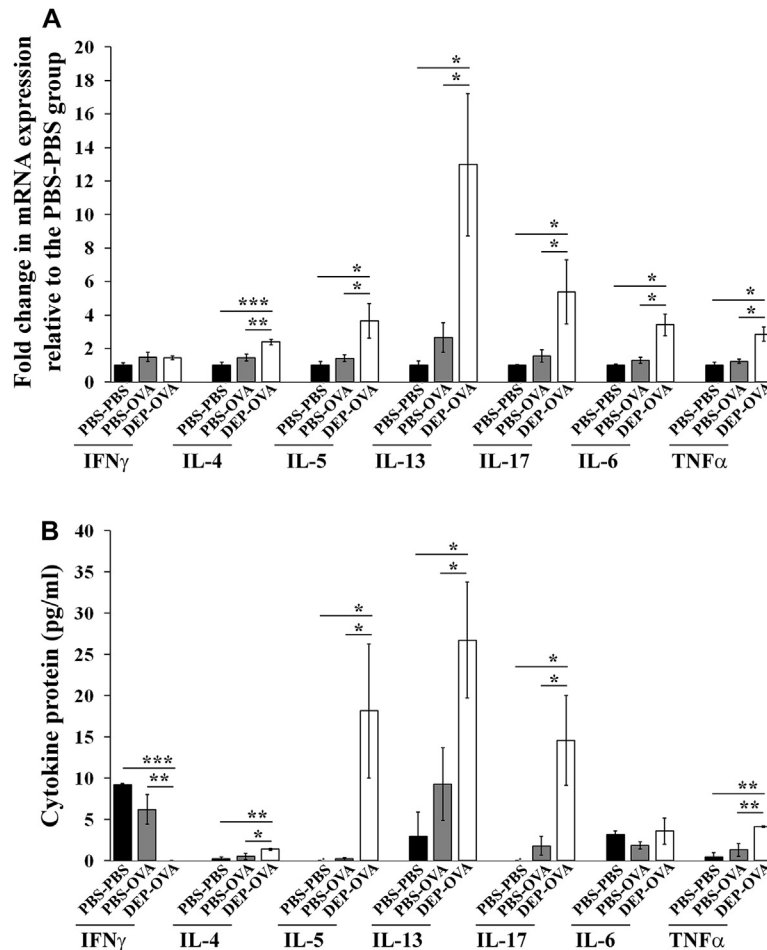


FIG 3. Expression of cytokines in lungs. PBS-PBS, PBS-OVA, and DEP-OVA mice were analyzed for relative expression of cytokine transcripts in lung tissue (**A**; mean $\pm$ SEM values of 6-13 mice per group) and absolute level of cytokine proteins in BALF (**B**; mean $\pm$ SEM values of 4 to 6 mice per group). Levels of each cytokine transcript were normalized to 18S rRNA and expressed as the fold change in expression relative to the PBS-PBS group. * $P < .05$, ** $P < .01$, and *** $P < .001$.

Prenatal exposure to DEPs primes pulmonary T_H2 - and T_H17 -type responses

Lung tissues from DEP-OVA mice had higher levels of mRNAs encoding the T_H2 cytokines IL-4, IL-5, and IL-13; the T_H17 cytokine IL-17; and the proinflammatory cytokines IL-6 and TNF- α compared with PBS-PBS and PBS-OVA mice (Fig 3, A, and see Fig E1 in this article's Online Repository at www.jacionline.org). Levels of *Il4*, *Il5*, *Il13*, *Il17*, and *Tnf α* transcripts corresponded with levels of proteins in BALF (Fig 3, B). DEP-OVA mice had reduced expression of the T_H1 cytokine IFN- γ at the protein level (Fig 3, B). PBS-OVA mice did not have any significant increases in cytokine production compared with PBS-PBS mice.

Prenatal exposure to DEPs increases production of allergen-specific IgE

OVA-specific IgE was detected in sera of all mice immunized with OVA, regardless of prenatal exposure (Fig 4). Thus our OVA exposure protocol is sufficient to induce OVA-specific IgE but insufficient to produce pulmonary inflammation in the absence of the prenatal stimulus. Sera from DEP-OVA mice had significantly higher concentrations of OVA-specific IgE than sera from all other groups of mice.

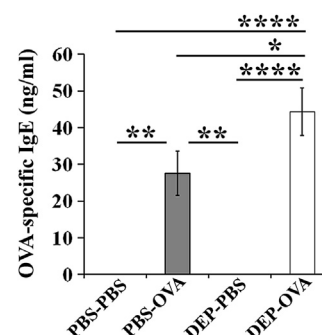


FIG 4. OVA-specific IgE in serum. Sera from PBS-PBS, PBS-OVA, DEP-PBS, and DEP-OVA mice were analyzed for concentrations of OVA-specific IgE. Mean $\pm$ SEM values are shown from 20 to 27 mice per group. * $P < .05$, ** $P < .01$, and **** $P < .0001$.

In utero exposure to DEPs promotes production of cytokines by NK cells

Production of IL-5, IL-13, and IL-17 was higher in splenocytes from DEP-OVA mice compared with that seen in mice from other studied groups (Fig 5, A-C). In DEP-OVA mice the majority (>60%) of cytokine-positive cells were NK cells (Fig 5, D-F).

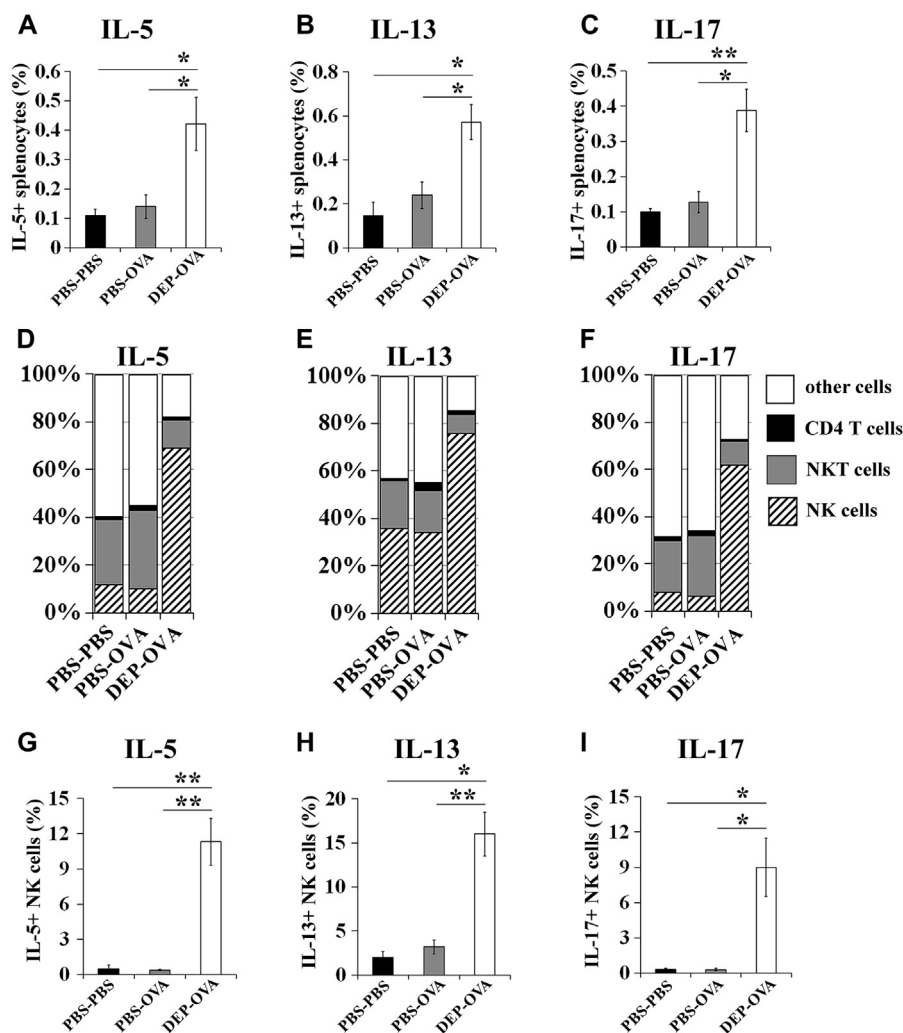


FIG 5. Cytokine expression in splenocyte populations. OVA-stimulated and immunostained splenocytes (CD3, CD4, NK1.1, NKp46, and a cytokine) from PBS-PBS, PBS-OVA, and DEP-OVA mice were analyzed by means of flow cytometry. CD4 T, NK, and NKT cells were defined as CD3⁺CD4⁺NK1.1[−]NKp46[−], CD3⁺NK1.1⁺NKp46⁺, and CD3⁺NK1.1⁺ cells, respectively. **A–C**, Cytokine-positive splenocytes expressed as a percentage of all splenocytes. **D–F**, Percentages of cytokine-positive cells attributable to individual cell populations. **G–I**, Cytokine-positive NK cells expressed as a percentage of all NK cells. Mean $\pm$ SEM values are shown from 6 mice per group. * $P < .05$ and ** $P < .01$.

In these pups CD4⁺ T cells accounted for only 0.9% to 1.5% of cytokine-positive cells (Fig 6, D–F). The percentages of NK cells expressing IL-5, IL-13, and IL-17 were highest in DEP-OVA mice and equal to 11%, 16%, and 9%, respectively (Fig 6, G–I). Also, DEP-OVA mice had the highest percentage of NK cells expressing the activation marker CD69 (27%, see Fig E2 in this article's Online Repository at www.jacionline.org). Frequencies of NK cells in spleens of mice from studied treatment groups were similar (PBS-PBS mice, 2.65% $\pm$ 0.33%; PBS-OVA mice, 2.55% $\pm$ 0.42%; and DEP-OVA mice, 2.49% $\pm$ 0.39%).

Depletion of NK cells prevents development of airway inflammation

To study the role of NK cells in our model, OVA-immunized offspring of DEP-exposed mice were given injections of the NK cell-depleting antibody anti-NK1.1^{39–41} (or the isotype control IgG) 72, 48, or 24 hours before the first intranasal OVA challenge.

The anti-NK1.1 antibody efficiently depleted NK cells. In spleens of mice injected with the anti-NK1.1 antibody, only 0.11% $\pm$ 0.03% of cells were NK cells (NKp46⁺CD3[−]), whereas in the spleens of mice injected with the isotype control IgG, 2.53% $\pm$ 0.34% of cells were NKp46⁺CD3[−] cells ($P < .01$). NK cell depletion significantly reduced airway inflammation (Fig 6).

Prenatal exposure to DEPs affects expression of aryl hydrocarbon receptor signature transcripts

From a toxicological standpoint, PAHs are important components of DEPs.^{42,43} PAHs cross the placenta.⁴⁴ Prenatal exposure to PAHs increases the risk of allergic sensitization and wheezing in children.^{6–8} PAHs activate the transcriptional factor aryl hydrocarbon receptor (AhR).⁴⁵ Activated AhR regulates several components of immune response, including the production of IL-17,^{46–48} which we found to be increased in DEP-OVA mice. Therefore we investigated AhR-dependent transcription in mice

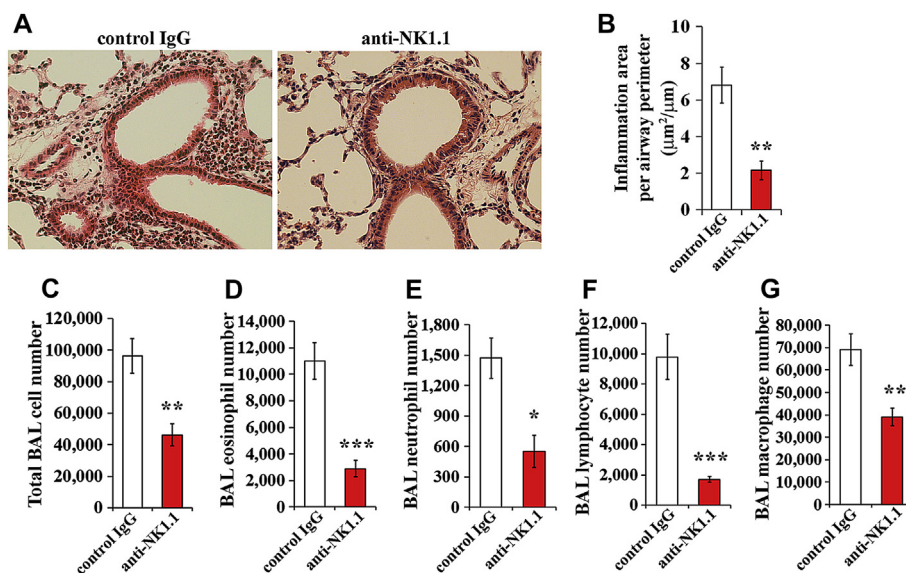


FIG 6. Effect of NK cell depletion. Pregnant mice were exposed to DEPs, and offspring were immunized and challenged with OVA (see Fig 1). Anti-NK1.1 or isotype control IgG were injected 72, 48, and 24 hours before the first OVA challenge. **A** and **B**, Peribronchial inflammation (histologic analysis). **C–G**, BALF cell numbers. Mean $\pm$ SEM values are shown from 8 mice per group. * $P < .05$, ** $P < .01$, and *** $P < .001$.

exposed to DEPs *in utero*. We measured levels of AhR signature transcripts encoding the AhR repressor (*AhRR*); cytochrome P450, family 1, subfamily A, polypeptide 1 (*Cyp1a1*); and cytochrome P450, family 1, subfamily B, polypeptide 1 (*Cyp1b1*).^{45,49} We analyzed liver in addition to lung tissue because the liver is an important target for AhR ligands.⁵⁰ Levels of all 3 transcripts were increased in lungs of 4-week-old DEP-PBS and DEP-OVA pups (Fig 7, A, and see Fig E3, A–C, in this article's Online Repository at www.jacionline.org). These pups also demonstrated augmented levels of *AhRR* and *Cyp1b1* in liver tissue (Fig 7, B, and see Fig E3, D and F). Interestingly, PBS-OVA pups had increased levels of *Cyp1a1* mRNA in lung tissue compared with that seen in PBS-PBS mice (Fig 7, A, and see Fig E3, B). This might be due to activation of the *Cyp1a1* promoter by non-AhR transcription factors in response to OVA exposure. In summary, prenatal exposure to DEPs was associated with increased transcription from AhR-regulated genes in the lung and liver. Interestingly, this result was obtained by using 4-week-old offspring, thus occurring roughly 4 weeks after final maternal exposure to DEPs.

Prenatal exposure to DEPs results in upregulation of oxidative stress signature transcripts

PAHs are metabolized through AhR-inducible CYP1A1 and CYP1B1, which leads to generation of reactive oxygen species (ROS).⁵¹ ROS are also produced on exposure to DEP-derived quinones or allergens.⁵² We measured levels of the ROS-sensitive transcripts heme oxygenase 1 (*Hmox1*) mRNA and nuclear factor (erythroid-derived 2)–like 2 (*Nrf2*) mRNA.⁵² We observed moderate upregulation of *Hmox1* mRNA in lungs and livers of DEP-OVA pups and, to a lesser extent, in lungs but not livers of PBS-OVA pups compared with that seen in PBS-PBS mice (Fig 8 and see Fig E4 in this article's Online Repository at www.jacionline.org).

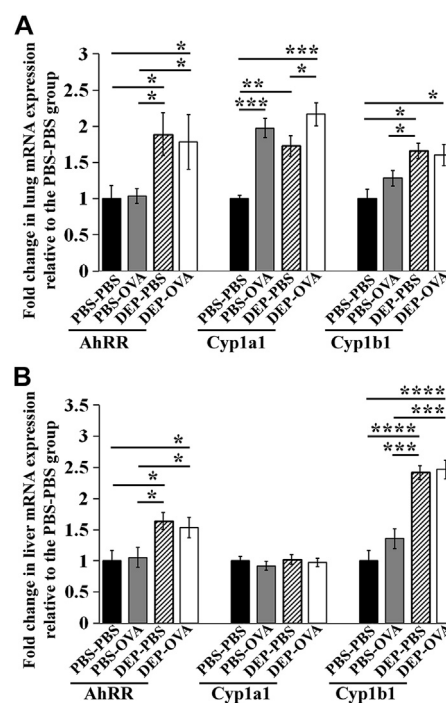


FIG 7. Expression of AhR signature transcripts. Lungs (**A**) and livers (**B**) from PBS-PBS, PBS-OVA, DEP-PBS, and DEP-OVA mice were analyzed for relative levels of *AhRR*, *Cyp1a1*, and *Cyp1b1* mRNA, as in Fig 3. Mean $\pm$ SEM values are shown from 6 to 13 mice per group. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

DISCUSSION

We developed a new mouse model of maternal transmission of asthma susceptibility. In this model suboptimal OVA immunization and challenge of pups born to DEP-exposed mice resulted in airway hyperresponsiveness and eosinophilic inflammation, with

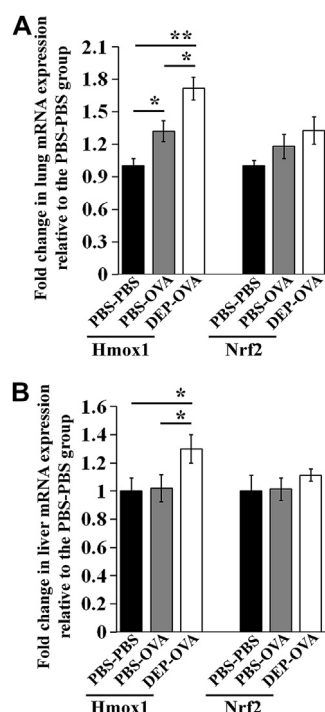


FIG 8. Levels of oxidative stress-regulated transcripts. Lungs (A) and livers (B) from PBS-PBS, PBS-OVA, and DEP-OVA mice were analyzed for relative levels of *Hmox1* and *Nrf2* mRNAs, as in Fig 3. Mean $\pm$ SEM values are shown from 6 to 13 mice per group. * $P < .05$ and ** $P < .01$.

increased expression of T_H2 - and T_H17 -type and inflammatory cytokines. Features of asthma were either marginal or absent in OVA-treated pups of vehicle-exposed mice. Therefore prenatal exposure to DEPs decreases the threshold for induction of asthma in response to allergens. The T_H2 - and T_H17 -type cytokines were mainly produced by NK cells; depletion of these cells blocked development of airway eosinophilic inflammation. Prenatal exposure to DEPs upregulated expression of genes known to be controlled by AhR and oxidative stress; the upregulation persisted 1 month after birth, even though mice were no longer exposed to DEPs.

We chose to expose prenatal mice to DEPs because the link between DEPs and asthma is well established.^{5-8,21-31,53-58} Epidemiologic studies associated exposure to diesel exhaust with development of new asthma and exacerbation of pre-existing asthma.^{5-8,53-58} The causality was determined in controlled exposure studies in human subjects and adult mice.²¹⁻³¹ DEP exposure increased production of IL-17, T_H2 -type cytokines, and specific IgEs; increased eosinophilic inflammation in the upper and lower respiratory tracts; and increased vascular permeability and airway resistance.^{21-31,59} Although there is some understanding of how postnatal exposure to diesel exhaust triggers asthma, the mechanisms underlying asthma induction after *in utero* exposure have not been characterized. Through development of this mouse model, we hope to provide a tool for mechanistic studies.

Our model incorporated the most valuable elements of earlier models. Table E2 contains experimental details of these models. The field was pioneered by Fedulov et al.¹³ In their model pregnant BALB/c mice were given a single intranasal dose of DEPs on gestation day 14. Pups were immunized through a single intraperitoneal injection of low-dose OVA in alum during the neonatal

period and challenged with aerosolized OVA when they were 2 weeks old. Asthma developed only in DEP-exposed pups. We followed this immunization strategy.

The model by Auten et al.¹⁴ used C57BL/6 mice and combined repeated prenatal exposure to diesel exhaust (inhalation) or DEPs (intranasal application) with chronic postnatal ozone exposure. Prenatal exposures increased ozone-induced airway hyperreactivity.

A model created by Reiprich et al.¹⁵ used BALB/c mice and incorporated repeated prenatal coexposure to DEPs and LPS, postnatal exposure to LPS, and standard (optimal) postnatal immunization and challenge with OVA. Mice were intraperitoneally immunized with an optimal dose of OVA in alum at 6 and 8 weeks of age and then underwent intranasal challenge with OVA 14 to 16 days and 21 to 23 days after the initial immunization. This OVA treatment induced asthma in control pups (offspring of mice given injections of vehicle). The DEPs inhibited LPS-mediated protection against asthma. As discussed earlier, we incorporated the repeated DEP exposure principle from protocols by Auten et al.¹⁴ and Reiprich et al.¹⁵ and, as described by Auten et al.,¹⁴ used C57BL/6 mice.

The link between prenatal diesel exhaust exposure and asthma was examined in 2 additional studies that reached different conclusions.^{60,61} In the model of Sharkhuu et al.,⁶⁰ pregnant mice repeatedly inhaled diesel exhaust. Their pups were immunized (PNDs 42 and 43) and challenged (PNDs 54, 55, and 56) with intranasal OVA. The authors reported no asthma-promoting effects of prenatal exposure to diesel exhaust. In the model of Corson et al.,⁶¹ pregnant mice repeatedly inhaled diesel exhaust and received intranasal applications of *Aspergillus fumigatus* extract. Their pups then received several doses of *A. fumigatus* extract beginning at 9 to 10 weeks of age. In this model the combined prenatal exposure to diesel exhaust and *A. fumigatus* extract protected against airway eosinophilia. The discrepancies in outcomes between positive (Auten et al.,¹⁴ Fedulov et al.,¹³ and Reiprich et al.¹⁵) and negative (Sharkhuu et al.⁶⁰ and Corson et al.⁶¹) studies could result from differences in methods used for prenatal exposure (instillation of the DEP suspension vs inhalation of diesel exhaust) and postnatal exposure (allergen vs ozone; differences in dose, route, timing, and type of postnatal allergen).

Pathogenesis of asthma involves T_H2 - and T_H17 -type cytokines. In our model these cytokines were produced mainly by NK cells. This is in contrast to conventional mouse models of asthma, in which $CD4^+$ T cells are a primary source of T_H2 - and T_H17 -type cytokines. NK cells are believed to secrete IFN- γ , TNF- α , granzymes, and perforins to kill cells infected with viruses and tumor cells.⁶² Less is understood about IL-17 $^+$ / T_H2 -type cytokine-positive NK cells, although studies have reported that these cells emerge during development of specific disorders.⁶³⁻⁶⁸ T_H2 -type cytokine-positive NK cells have been detected in patients with atopic asthma, atopic dermatitis, parasitic infections, and autoimmune diseases.⁶³⁻⁶⁶ IL-17 $^+$ NK cells have been observed in mouse models of toxoplasmosis and hepatic ischemia-reperfusion injury,^{67,68} but there are no reports of their association with asthma or allergy.

Our data strongly indicated that NK cells were responsible for the development of airway inflammation in our model. Antibody-mediated depletion of these cells before allergen challenge prevented airway inflammation. Antibody-based strategies for depletion of NK cells were previously tested in conventional models of asthma. By using these approaches, 4 research groups showed that NK cells positively contribute to the initiation

allergic inflammation,⁶⁹⁻⁷² whereas 1 group reported that NK cells suppress the resolution of pulmonary inflammation.^{73,74} The studies might have reached different conclusions because different experimental conditions or different phases of allergic response are associated with activation of distinct NK cell subsets. Alternatively, NK cells dynamically change phenotype during allergic response. A third possibility involves deletion of asthma-relevant non-NK cell subsets by injected antibodies.

Two antibodies are commonly used to deplete NK cells *in vivo*: anti-NK1.1 and anti- α sialo-GM1.^{39-41,73} We chose anti-NK1.1 because it is more specific for NK cells. One limitation of this approach is that anti-NK1.1 also depletes NK T cells. However, the role of NK T cells in airway inflammation in our model is probably not significant; in DEP-OVA mice these cells accounted for only 8% to 12% of cytokine-producing cells.

The AhR might provide a molecular link between maternal exposure to diesel exhaust and asthma in offspring. This receptor is activated by PAHs, major components of DEPs that pass through the placenta.⁴⁴ PAH-bound AhR activates transcription of many genes, including those that regulate the immune response.⁴⁵⁻⁴⁸ Many studies have shown that AhR induces transcription of the *Il17* gene in T cells and type 3 innate lymphoid cells.⁴⁶⁻⁴⁸ Accordingly, DEPs were shown to stimulate IL-17 production by splenocytes.⁷⁵ The AhR is expressed in NK cells,^{76,77} and it is likely that AhR regulates *Il17* also in NK cells.

The effect of the AhR on T_H2 -type cytokines is less studied. AhR deficiency impairs production of IL-5 and IL-13 by splenocytes and mast cells, respectively.^{78,79} Conversely, the AhR ligand 6-formylindolo[3,2-b]carbazole increases production of IL-13 by mast cells.⁸⁰ DEP-derived PAHs induce IL-13 production by PBMCs.⁸¹ AhR-deficient $CD4^+$ T cells increase secretion of IL-4 on stimulation through CD3 and CD28.⁸² In contrast, other studies have shown that PAHs synergize with concanavalin A and antigen to stimulate IL-4 production by T cells and basophils, respectively.^{83,84}

The AhR also regulates the synthesis of inflammatory mediators, including IL-6 and TNF- α .^{85,86} These cytokines are also induced by ROS; upregulation of transcripts that are characteristic of the oxidative stress response (*Hmox1* mRNA) was observed in our DEP-OVA mice. Therefore inflammatory responses in these mice might be the result of the combined effect of AhR and ROS signaling.

The AhR- and ROS-associated transcriptional response in DEP-OVA mice was systemic because it was observed in 2 different organs (ie, the lung and liver). Additional evidence for the systemic effects of DEPs included the lower body weight of DEP-PBS and DEP-OVA mice compared with PBS-PBS mice. One likely scenario is that in fetuses exposed to DEPs, activation of the AhR and ROS pathways led to systemic priming of proinflammatory responses and systemic NK cell activation that ultimately facilitated postnatal induction of asthma upon lung-targeted allergen challenge.

There is evidence that AhR mediates allergic inflammation. AhR expression is increased in PBMCs from patients with allergic asthma.⁸⁷ The putative AhR ligand 4-nonylphenol enhances allergic inflammation in the lung.⁸⁸ The inflammation is reduced in mice carrying the AhR^d allele, which is defective in ligand binding. Mice that express a constitutively active form of AhR in keratinocytes, spontaneously develop skin lesions that resemble atopic dermatitis.⁸⁹ Repeated intranasal administration of PAHs aggravates allergic rhinitis in the guinea pig model.⁹⁰

PAHs also increase production of allergen-specific IgE.^{91,92} We recognize that certain AhR ligands might be anti-inflammatory. Immunotoxic 2,3,7,8-tetrachlorodibenzo-p-dioxin promotes development of regulatory T cells and thereby suppresses allergic sensitization.^{93,94} It is now believed that 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity results from inappropriate activation of AhR, leading to deregulated physiologic functions.⁹⁵

In summary, we have developed a model of asthma susceptibility through prenatal exposure of mice to DEPs. We provided mechanistic insight into this process, delineating the role of NK cells in asthma development, along with activation of AhR- and oxidative stress-regulated genes.

Key messages

- Mice exposed *in utero* to diesel particulate matter have increased susceptibility to asthma.
- Asthma in these mice is mediated by NK cells and is associated with enhanced transcription of genes known to be specifically regulated by AhR and oxidative stress.

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METHODS

NK cell depletion *in vivo*

The experiment involved OVA-immunized offspring of 3 DEP-exposed mice. Each litter was divided into 2 groups. Pups from the first group were intraperitoneally injected with 200 μ g of the anti-NK1.1 antibody (clone PK136; BioLegend, San Diego, Calif).^{E1-E3} Pups from the second group received 200 μ g of the mouse IgG_{2a} isotype control antibody (BioLegend). The injections were performed on PNDs 20, 21, and 22. All pups were intranasally challenged with OVA on PNDs 23, 24, and 25, as described in the parent protocol.

Measurement of airway hyperresponsiveness

Lung resistance was measured with the FlexiVent apparatus (SCIREQ, Montreal, Quebec, Canada), as previously described.^{E4-E7} Resistance values for each methacholine dose were expressed as a percentage of baseline (saline inhalation) resistance.

Histology and morphometric analysis

Paraffin-embedded lungs were sectioned and stained with hematoxylin and eosin.^{E4-E7} The MetaMorph software (Molecular Devices, Sunnyvale, Calif) was used to measure areas of the peribronchial cellular infiltrate and a perimeter of the bronchial epithelial basement membrane (proxy for airway perimeter).^{E4-E8} Results are expressed as an inflammation area per 1 μ m of airway perimeter.

OVA-specific IgE ELISA

OVA-specific IgE was measured in serum, as previously described.^{E4,E9} Purified OVA-specific mouse IgE (AbD Serotec, Raleigh, NC) was used for the standard curve.

Cytokine ELISA

The cytokine concentration was measured in cell-free supernatants of BALF.^{E5} For IFN- γ , IL-4, IL-5, IL-6, and TNF- α , multiplex ELISA was used (Quansys Biosciences, Logan, Utah). Concentrations of IL-13 and IL-17 were determined by using traditional ELISA (eBioscience, San Diego, Calif, and R&D Systems, Minneapolis, Minn, respectively).

Real-time PCR

RNA was extracted from Trizol lysates by using the PureLink RNA Mini Kit (Life Technologies, Grand Island, NY). cDNA was synthesized with ImProm-II reverse transcriptase and random primers (all from Promega, Madison, Wis). cDNA was amplified and quantified by using transcript-specific primers (Table E1), the Absolute qPCR SYBR Green mix (Thermo Scientific), and the ABI Prism 7000 Sequence Detection System (Life Technologies).^{E4}

Flow cytometry

Splenocytes were treated with OVA at 100 μ g/mL for 30 hours (and monensin at 2 μ mol/L for a final 6 hours) and stained with fluorescently labeled antibodies against CD3, CD4, NK1.1, NKp46, and a cytokine (IL-5, IL-13, or IL-17). Separate splenocyte samples were treated with OVA without

monensin and immunostained for CD3, NK1.1, NKp46, and CD69. Cells were analyzed with an CyAn ADP cytometer (Beckman Coulter, Fullerton, Calif).

Statistical analyses

Statistical analyses were done with GraphPad Prism 6 software (GraphPad, La Jolla, Calif). After tests were performed: 1-way ANOVA, 2-way repeated-measures ANOVA, and the Holm-Sidak multiple comparison test. Results are shown as means $\pm$ SEMs. A *P* value of less than .05 was considered statistically significant.

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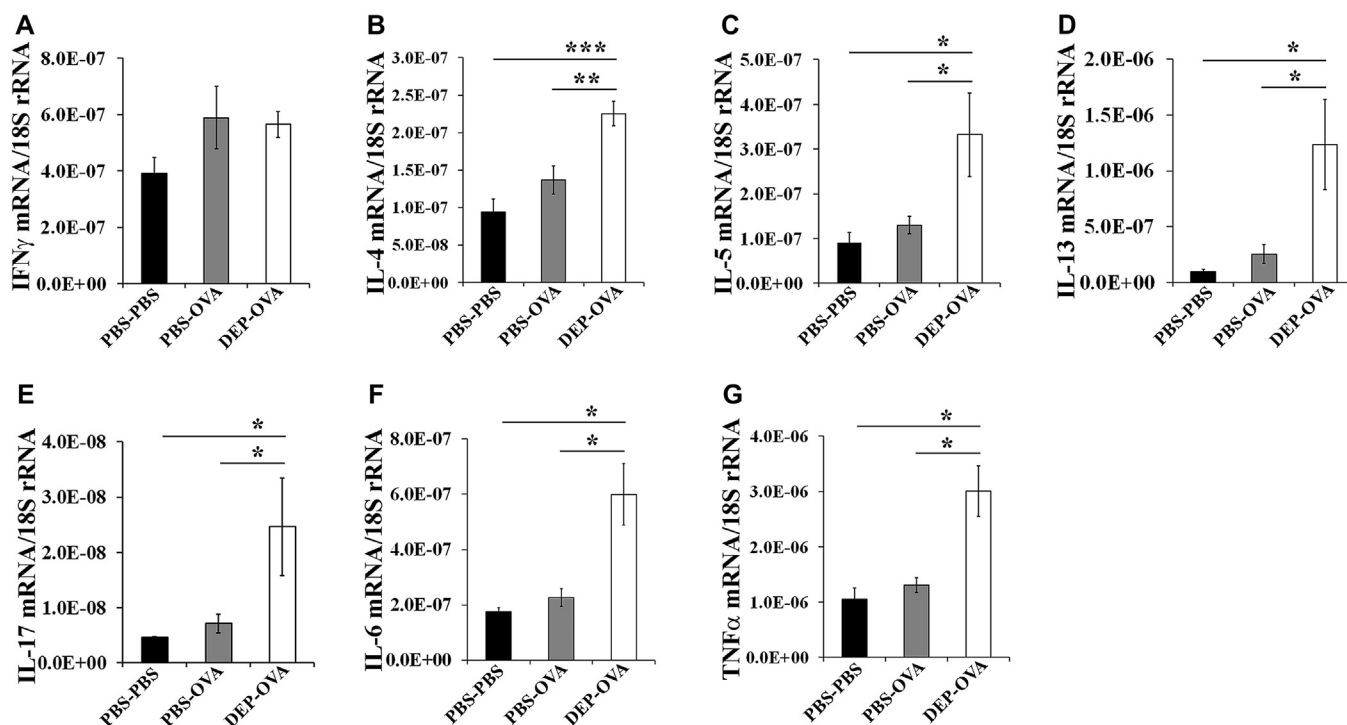


FIG E1. Expression of cytokine mRNA in the lung. Three offspring groups (PBS-PBS, PBS-OVA, and DEP-OVA mice) were studied. Values for each cytokine transcript were normalized to housekeeping 18S rRNA and expressed as a fraction of this rRNA. Mean $\pm$ SEM values are shown from 6 to 13 mice per group. * P < .05, ** P < .01, and *** P < .001.

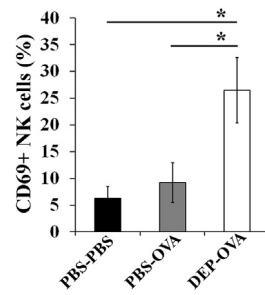


FIG E2. CD69 expression on NK cells. OVA-stimulated and immunostained (CD3, NK1.1, NKp46, and CD69) splenocytes were analyzed by means of flow cytometry. CD69⁺ NK cells (CD3⁻NK1.1⁺NKp46⁺) are expressed as a percentage of all NK cells. Mean $\pm$ SEM values are shown from 6 mice per group. * $P < .05$.

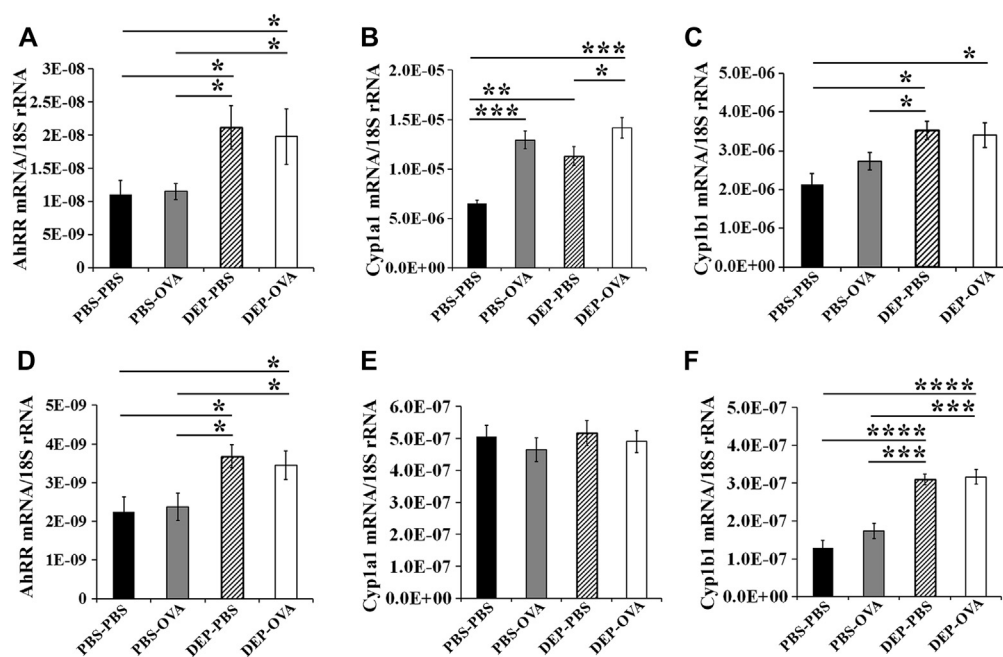


FIG E3. Expression of AhR-regulated transcripts. Lungs (**A-C**) and livers (**D-F**) from 4 offspring groups (as in Fig 2) were analyzed for relative expression of *AhRR*, *Cyp1a1*, and *Cyp1b1* mRNA. Values for each AhR-regulated transcript were normalized and expressed as in Fig E1. Mean $\pm$ SEM values are shown from 6 to 13 mice per group. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

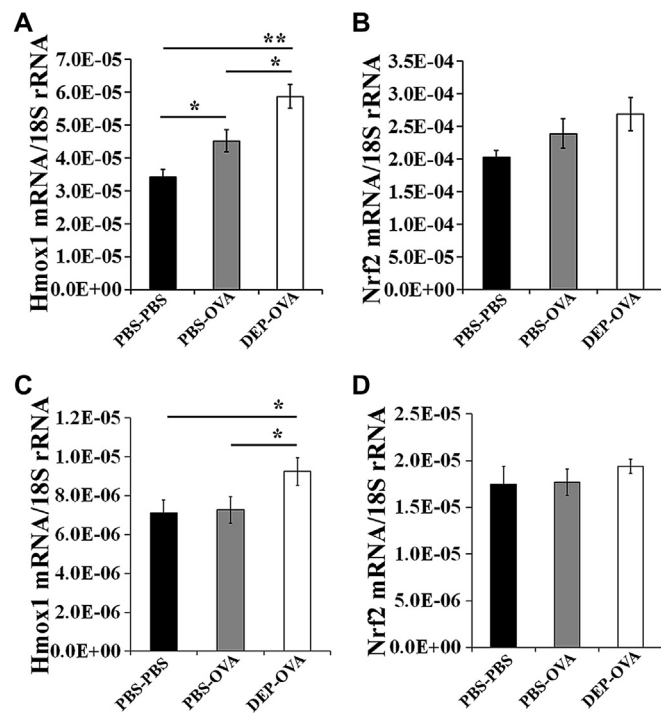


FIG E4. Expression of oxidative stress-regulated transcripts. Lungs (**A** and **B**) and livers (**C** and **D**) from 3 offspring groups (as in [Figs 3 and E1](#)) were analyzed for relative expression of *Hmox1* and *Nrf2* mRNA. Values for each oxidative stress-regulated transcript were normalized and expressed as in [Fig E1](#). Mean $\pm$ SEM values are shown from 6 to 13 mice per group. * $P < .05$ and ** $P < .01$.

TABLE E1. Sequences of transcript-specific primers

Transcript name	Forward primer	Reverse primer
AhRR	GTGTACATACGCCGGTAGGA	GGTGCCGTTTGGAAGGATTT
Cyp1a1	GACCTTCCGGCATTCATCCT	GCCATTCAGACTTGTATCTCTTGTG
Cyp1b1	AGGATGTGCCTGCCACTATT	CACAACCTGGTCCAACCTCAG
Hmox1	GAGCAGAACCAGCCTGAACT	TCAAGGCCTCAGACAAATCCT
IFN- γ	GCGTCATTGAATCACACCTGA	CTGGACCTGTGGGTTGTTGA
IL-4	CCATATCCACGGATGCGACA	GACGAGCTCACTCTCTGTGG
IL-5	GAGGCTTCCTGTCCCTACTC	CCCACGGACAGTTTGATTCTT
IL-6	TGTTCTCTGGGAAATCGTGGA	TTCTGCAAGTGCATCATCGT
IL-13	GAGCAACATCACACAAGACCA	GATGTTGGTCAGGGAATCCAG
IL-17	AGACTACCTCAACCGTTCCAC	CACTGAGCTTCCCAGATCACA
Nrf2	CTCAGCATGATGGACTTGGAG	GCCTCCAAAGGATGTCAATCAA
TNF- α	CTCTTCTCATTCTGCTTGTGG	GATGATCTGAGTGTGAGGGTCT
18S rRNA	CGGCGACGACCCATTCTGAAC	GAATCGAACCTGATTCCCCGT

TABLE E2. Previous prenatal DEP/diesel exhaust exposure models

		Model by Fedulov et al ^{E10}	Model by Auten et al ^{E11}	Model by Reiprich et al ^{E12}	Model by Sharkhuu et al ^{E13}	Model by Corson et al ^{E14}
Mouse strain		BALB/c	C57BL/6	BALB/c	BALB/c	BALB/c
Maternal exposure	DEP/DE	<i>Agent:</i> DEP	1) <i>Agent:</i> DEP	<i>Agent:</i> DEP	<i>Agent:</i> DE	<i>Agent:</i> DE
		<i>Dose:</i> 50 µg	<i>Dose:</i> 50 µg	<i>Dose:</i> 20 µg (30 min before LPS exposure)	<i>Dose:</i> 0.8 or 3.1 mg/m ³ , 4 h/d	<i>Dose:</i> 1.09 mg/m ³ , 5 h/d
		<i>Route:</i> i.n.	<i>Route:</i> o.p.	<i>Route:</i> i.n.	<i>Route:</i> inhalation	<i>Route:</i> inhalation
		<i>Frequency:</i> once (GD 14)	<i>Frequency:</i> twice weekly for 3 weeks of pregnancy	<i>Frequency:</i> 3 times per week from GD 7 until delivery	<i>Frequency:</i> daily (from GD 9 to GD 18)	<i>Frequency:</i> daily, Monday to Friday during second and third week of pregnancy
			2) <i>Agent:</i> DE			
			<i>Dose:</i> 0.5-2 mg/m ³ 4 h/d			
			<i>Route:</i> inhalation			
			<i>Frequency:</i> daily (from GD 9 to GD 17)			
	Other treatments			<i>Agent:</i> LPS		<i>Agent:</i> <i>A fumigatus</i> extract
				<i>Dose:</i> aerosolized solution at 1 µg/10 mL, 10 min/d (30 min after DEP exposure)		<i>Dose:</i> 62.5 µg
				<i>Route:</i> inhalation		<i>Route:</i> i.n.
				<i>Frequency:</i> 3 times per week from GD 7 until delivery		<i>Frequency:</i> 7 times (days -20, -16, -12, -8, and -4 before mating, GD 7, GD 14)
Offspring exposure	Immunization with allergen	<i>Agent:</i> OVA and alum		<i>Agent:</i> OVA and alum	<i>Agent:</i> OVA	<i>Agent:</i> <i>A fumigatus</i> extract
		<i>Dose:</i> OVA: 5 µg; alum: 1 mg		<i>Dose:</i> OVA: 20 µg; alum: 2 mg	<i>Dose:</i> 50 µg	<i>Dose:</i> 62.5 µg
		<i>Route:</i> i.p.		<i>Route:</i> i.p.	<i>Route:</i> i.n.	<i>Route:</i> i.n.
		<i>Frequency:</i> once (PND 4)		<i>Frequency:</i> twice (postnatal weeks 6 and 8)	<i>Frequency:</i> twice (PNDs 42 and 43)	<i>Frequency:</i> 5-6 times (4 d apart, beginning at week 9-10 of age)
	Airway challenge with allergen	<i>Agent:</i> OVA		<i>Agent:</i> OVA	<i>Agent:</i> OVA	
		<i>Dose:</i> aerosolized 3% solution		<i>Dose:</i> 20 µg	<i>Dose:</i> 5 µg	
		<i>Route:</i> inhalation		<i>Route:</i> i.n.	<i>Route:</i> i.n.	
		<i>Frequency:</i> daily for 3 d (PNDs 13, 14, and 15)		<i>Frequency:</i> 6 times (days 14-16 and 21-23 after initial immunization)	<i>Frequency:</i> 3 times (PNDs 54, 55, and 56)	
	Other treatments		<i>Agent:</i> ozone	<i>Agent:</i> LPS		
			<i>Dose:</i> 1 ppm, 3 h/d	<i>Dose:</i> aerosolized solution at 1 µg/10 mL, 10 min/d		
			<i>Route:</i> inhalation	<i>Route:</i> inhalation		
			<i>Frequency:</i> 3 d/wk for 4 wk	<i>Frequency:</i> 3 times per week for first 4 wk of life		
Effect of maternal DEP/DE exposures		Eosinophilic inflammation of airways, airway hyperreactivity	Augmentation of ozone-induced airway hyperreactivity	Inhibition of LPS-mediated protection against asthma	No effect	Protection from airway eosinophilia

DE, Diesel exhaust; GD, gestational day; i.n., intranasal; i.p., intraperitoneal; o.p., oropharyngeal.