Diesel exhaust particle induction of IL-17A contributes to severe asthma

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Background: IL-17A has been implicated in severe forms of asthma. However, the factors that promote IL-17A production during the pathogenesis of severe asthma remain undefined. Diesel exhaust particles (DEPs) are a major component of traffic-related air pollution and are implicated in asthma pathogenesis and exacerbation.

Objective: We sought to determine the mechanism by which DEP exposure affects asthma severity using human and mouse studies.

Methods: BALB/c mice were challenged with DEPs with or without house dust mite (HDM) extract. Airway inflammation and function, bronchoalveolar lavage fluid cytokine levels, and flow cytometry of lung T cells were assessed. The effect of DEP exposure on the frequency of asthma symptoms and serum cytokine levels was determined in children with allergic asthma.

Results: In mice exposure to DEPs alone did not induce asthma. DEP and HDM coexposure markedly enhanced airway hyperresponsiveness compared with HDM exposure alone and generated a mixed T_H2 and T_H17 response, including IL-13$^+$IL-17A$^+$ double-producing T cells. IL-17A neutralization prevented DEP-induced exacerbation of airway hyperresponsiveness. Among 235 high DEP–exposed children with allergic asthma, 32.2% had more frequent asthma symptoms over a 12-month period compared with only 14.2% in the low DEP–exposed group ($P = .002$). Additionally, high DEP–exposed children with allergic asthma had nearly 6 times higher serum IL-17A levels compared with low DEP–exposed children.

Conclusions: Expansion of T_H17 cells contributes to DEP-mediated exacerbation of allergic asthma. Neutralization of IL-17A might be a useful potential therapeutic strategy to counteract the asthma-promoting effects of traffic-related air pollution, especially in highly exposed patients with severe allergic asthma. (J Allergy Clin Immunol 2013;132:1194-204.)

Key words: *Allergic asthma, house dust mite, diesel exhaust particle, IL-17A, regulatory T cell*

Asthma is characterized by reversible allergen-induced chronic airway inflammation and mucus production, resulting in airway obstruction and airway hyperresponsiveness (AHR). Allergic asthma is generally regarded as a T_H2 disease because increased levels of eosinophils and T_H2 cytokines (IL-4, IL-5, and IL-13) are often observed[1]; however, different patterns of airway inflammation are observed in the lungs of asthmatic patients. Several asthma subgroups have been identified based on different inflammatory profiles, including eosinophilic asthma, neutrophilic asthma, and mixed neutrophilic/eosinophilic asthma.[2,3] Asthmatic patients with mixed inflammatory phenotypes including both eosinophils and neutrophils have the lowest lung function and the worst asthma control.[3,4]

Neutrophilic inflammation is a hallmark of T_H17 responses.[5] The importance of T_H17 cells and their cytokines, IL-17A and IL-17F, which exist as homodimers and heterodimers, was first demonstrated in patients with autoimmune diseases, such as experimental autoimmune encephalomyelitis.[6] However, mounting evidence suggests a role for T_H17 cells in asthmatic patients.[6,7] Immunohistochemistry on bronchial biopsy specimens from patients with either mild, moderate, or severe asthma (American Thoracic Society criteria) demonstrates a marked increase in the number of IL-17A$^+$ and IL-17F$^+$ cells in patients with severe asthma compared with numbers seen in either patients with mild asthma or healthy control subjects.[4] In mice deficient for either IL-17A or its receptor (formed by the IL-17RA/IL-17RC complex), airway neutrophilia and AHR are attenuated after allergen exposure, further supporting a key role for IL-17A in asthmatic patients. However, IL-17A delivery or transfer of T_H17 cells is not sufficient to induce AHR in these mice.[8,9] Thus T_H17 cells appear to promote disease in the context of allergen-induced T_H2 responses. Hence it is important to delineate the signals that promote T_H17 responses in the context of allergic asthma.

The association between air pollution, including traffic emissions, and increased risk for respiratory disease is now widely accepted and might contribute to the dramatic increase in the incidence of asthma in industrialized countries.[10,11] Recently, particulate matter has been shown to induce IL-17A in the lungs of exposed mice,[12,13] prompting us to investigate the role of diesel exhaust particles (DEPs), a major component of particulate matter in traffic-related air pollution, on T_H17 responses in patients with allergic asthma.

The effects of DEP exposure have been examined in both healthy subjects and asthmatic patients.[14-16] Fine and ultrafine

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Abbreviations used
AHR: Airway hyperresponsiveness
BALF: Bronchoalveolar lavage fluid
DEP: Diesel exhaust particle
Foxp3: Forkhead box protein 3
GCPCR: Greater Cincinnati Pediatric Clinic Repository
HDM: House dust mite
IL-13R: IL-13 receptor
OR: Odds ratio
PE: Phycoerythrin
PEES: Pediatric Environmental Exposures Study
SPT: Skin prick test
Treg: Regulatory T

DEP particles (diameter <2.5 μm) can reach the small airways, including the alveolar/gas exchange regions of the lung, exacerbating asthma symptoms.[14,15] Bronchial inflammation and airway resistance increased in healthy subjects after exposure to DEPs. Even short-term exposure to streets with high diesel traffic reduced airway function (FEV_1) in patients with mild-to-moderate asthma compared with that seen in subjects who walked for a similar time in an area not exposed to traffic.[17] However, the mechanisms by which DEPs contribute to asthma exacerbations remain poorly understood.

Experimental studies in mice and rats have shown that DEP exposure can exacerbate allergic airway responses, including allergen-specific IgE, eosinophilia, and AHR.[18] The adjuvant effect of DEPs has been attributed to their ability to serve as an allergen carrier, to the different components of the DEP itself, or both. Indeed, DEPs derived from different sources generate distinct immune responses *in vivo*.[19] Coexposure to allergen and DEPs is known to increase allergen-specific IgE levels in human subjects and mice.[15,18] Coexposure has also been reported to enhance local and systemic T_H2 cytokine release compared with allergen alone.[15,18,19] However, little is known about the effects of DEP exposure on T_H17 responses in the context of allergic asthma.

Here we demonstrate that exposure of mice to DEPs induces regulatory T (Treg) and T_H17 cell accumulation in their lungs. Coexposure to DEPs and a common allergen (house dust mite [HDM]) caused a mixed T_H2/T_H17 response and increased asthma severity characterized by more severe inflammation, mucus production, and AHR. IL-17A blockade with a neutralizing antibody alleviated DEP-induced enhancement of AHR, supporting a role for T_H17 cells in severe asthma exacerbation in the context of DEP exposure. In children with allergic asthma, we also found DEP exposure to be associated with more frequent asthma symptoms and increased IL-17A serum levels, suggesting our findings can be extrapolated to human disease.

METHODS

Subjects

The 235 children included in this study are enrolled in the Greater Cincinnati Pediatric Clinic Repository (GCPCR), which is a clinical repository of more than 6500 participants, including more than 2300 asthmatic children.[20] The 46 children with IL-17A serum data were also enrolled in the Pediatric Environmental Exposures Study (PEES), a case-control study with nearly 400 total children seeking to better understand the effects of DEP exposure on childhood asthma. Children were invited to participate in PEES from the GCPCR and public advertising. For both populations, informed consent was obtained by a trained clinical research coordinator from participants and their parents/guardians by using a protocol approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board. Children ages 5 to 18 years with confirmed allergic asthma (diagnosed according to American Thoracic Society criteria[21]) who had either positive skin prick test (SPT) responses to 1 or more aeroallergens or had a doctor's diagnosis of allergic rhinitis were included in our analysis. Skin prick testing was performed with the GreerPick system (Greer Laboratories, Lenoir, NC), as ordered during participants' clinical visits. Tests were read 15 minutes after placement, and results were considered positive when the wheal around the allergen tested was greater than 3 mm and was also greater than that with the saline negative control. Histatrol (Center Laboratories, Port Washington, NY), 1 mg/mL, was used as the positive control. Eleven aeroallergens prevalent in the Ohio River Valley were evaluated. Panels included mold mixes (n = 2), grass mix, ragweed (giant and short), tree pollen mixes (n = 2), weed mix, dust mite mix (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), cat (Fel d 1), dog (hair and dander), and cockroach mix (*Periplaneta americana* and *Blattella germanica*). One hundred nineteen children were defined as allergic based on both a physician's diagnosis and SPT responses. Forty-eight children were defined as allergic based on SPT responses only, and the remaining 68 children were considered allergic based on a physician's diagnosis of allergic rhinitis.

The ArcView Geographic Information System software (Esri, Redlands, Calif) was used to obtain longitude and latitude coordinates from each child's primary residential address. Estimates of elemental carbon attributable to traffic exposure was derived by using a land-use regression model from the resulting coordinates with previously established methods.[22] Single estimates of DEP exposure were determined for each child. The range of estimates was 0.25 to 0.85 $\mu g/m^3$ across the entire study population. Because previous studies have indicated that the highest-risk group includes those children in the highest quartile of exposure,[23] high DEP exposure was defined as those with values of greater than 0.46 $\mu g/m^3$. The frequency of asthma symptoms was determined by using a parent-reported questionnaire over the last 12 months before their children's visits. Children must have had at least 1 respiratory symptom frequency score to be included. For children with multiple symptom scores (from multiple questionnaires completed over time), the highest symptom score was included in this analysis. The symptom frequency scores were determined from symptom frequency questions for wheezing, coughing, shortness of breath, and chest tightness. Possible frequency answer choices included never having symptoms on average over the last 12 months (score 0), having symptoms less than 1 time a week on average over the last 12 months (score 0), or having symptoms on average 1 to 2 (score 1), 3 to 5 (score 2), or 6 to 7 (score 3) times a week over the past 12 months. For the purposes of our study, children with severe asthma are those with 1 or more symptoms that occur on average at least 6 to 7 days a week over the past 12 months (maximum score of 4 for ≥1 symptoms). IL-17A levels were determined from serum samples collected from PEES participants. Of the nearly 400 total PEES participants, only the first 120 have cytokine data available, and only 46 of 82 children with asthma met our inclusion criteria for this study. There were no significant differences in demographic or exposure variables among the 46 children included in our analysis and the other children.

Briefly, 25 μL of serum was tested by using the premixed 39-Plex MILLIPLEX MAP Human Cytokine/Chemokine assay (Millipore, Billerica, Mass), including IL-17A, and analyzed on a Luminex 100 (Bio-Rad Laboratories, Hercules, Calif). Concentrations were determined with a 5 parametric curve–fitting algorithm by using MasterPlex QT Quantitation Software (MiraiBio, Alameda, Calif). IL-17A values ranged from 0.69 to 646.56 pg/mL. The lower limit for detection was 0.5 pg/mL, and 5 of the 46 children had undetectable values. Because we observed a nonnormal distribution for IL-17A that was not remedied by typical transformation, we used an unbiased approach and deemed those children with levels in the top quartile (>24 pg/mL) as having "high" IL-17A levels. Because several children had equal IL-17A levels at this level, we chose to include all of them in the higher category, resulting in 30% of the children having high IL-17A levels. Similarly, for IL-4, IL-5, and IL-13, binary variables were made defining the top quartile as high and the bottom 3 quartiles as low. Correlations

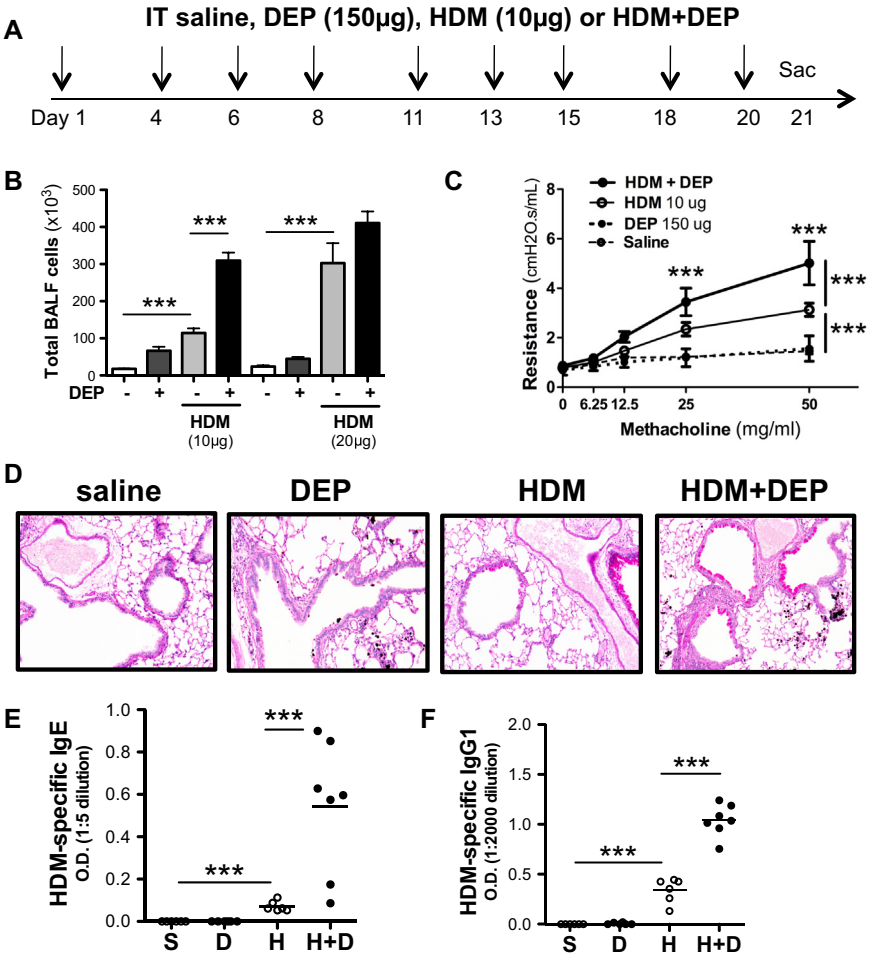

FIG 1. DEPs exacerbate HDM-induced airway responses. **A,** Protocol. *IT,* Intratracheal. **B,** Total BALF cell counts. **C,** Airway resistance. Data were from 2 separate experiments (n = 10-18 mice per group). ***P < .001 (1-way ANOVA). **D,** Representative periodic acid–Schiff–stained airways. **E** and **F,** HDM-specific IgG_1 (Fig 1, *E*) and IgE (Fig 1, *F*) titers. *D,* DEPs; *H,* HDM extract; *S,* saline.

(Pearson correlation coefficients) were determined, and univariate and multivariate logistic regression were performed by using these binary variables, as well as continuous variables, to examine associations between these cytokines and DEP exposure. Only the analyses of binary variables are provided.

Murine asthma model and anti–IL-17A treatment

Wild-type 6- to 8-week-old BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, Me). HDM extract *(D pteronyssinus)* was purchased from Greer Laboratories. DEPs were generated from a 4-cylinder Deutz diesel engine at the Environmental Protection Agency (Research Triangle Park, NC); detailed characterization of this compressor DEP has been described previously and compared with other sources of DEPs.[19] Mice received either 50 μL of saline or 150 μg of DEPs, 10 μg of HDM extract (representing 3.3 μg of protein, 1.1 μg of Der p 1, and 0.5 EU of endotoxin), or both administered intratracheally 3 times a week for 3 weeks and were killed 1 day after the last exposure. In some experiments mice were given a rat anti-murine IL-17A antibody (100-200 μg; M210; Amgen, Seattle, Wash) or an IgG_1 control antibody administered intratracheally during HDM with or without DEP challenges in a final volume of 50 μL. All animal protocols were approved by the Institutional Animal Care and Use Committee.

AHR

Invasive measurements of airway responsiveness were made on a flexiVent apparatus (Scireq, Montreal, Quebec, Canada). Mice were anesthetized with ketamine, xylazine, and acepromazine (respectively 100, 20, and 10 mg/mL mixed at a ratio of 4:1:1). Mouse tracheas were cannulated with a 20-gauge blunt needle, and the mice were ventilated at 150 breaths/min and a 3.0 cm of water positive end-expiratory pressure. Two total lung capacity perturbations were then performed for airway recruitment before baseline measurement and subsequent methacholine challenges were performed. Dynamic resistance (R) and compliance (C) were determined by fitting the data to a single compartment model of airway mechanics, where Ptr is defined as RV + EV + Po and Ptr is defined as tracheal pressure, V is volume, E is elastance, Po is a constant, and C is defined as 1/E. Measurements were made with a 1.25-second, 2.5-Hz volume-driven oscillation applied to the airways by using a computer-controlled piston (SnapShot perturbation). PBS or methacholine was aerosolized for 15 seconds (Aeroneb ultrasonic nebulizer; Aerogen, Galway, Ireland), which was followed by 15 seconds of ventilation, and a SnapShot perturbation was performed, which was followed by 10 seconds of ventilation. Twelve SnapShot/ventilation cycle measurements were made. The procedure was repeated for 0, 6.25, 12.5, 25, and 50 mg/mL concentrations of methacholine. The maximum resistance and minimum compliance values with a coefficient of determination of 0.9 or greater (as determined by the flexiVent software) was used to determine the dose-response curve.

Bronchoalveolar lavage fluid collection and analysis

Bronchoalveolar lavage was performed by means of cannulation of the trachea. The lungs underwent lavage with 1 mL of PBS plus 0.5% BSA plus 2

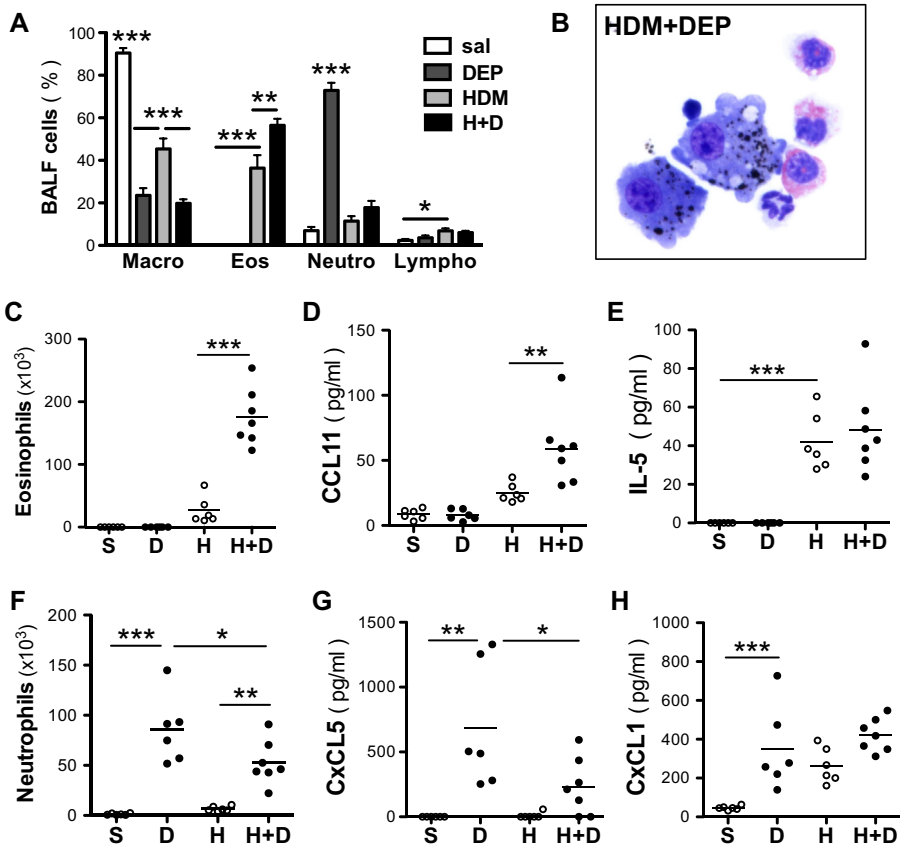

FIG 2. DEPs induce neutrophilia and exacerbate HDM-induced eosinophilia. **A,** Differential counts. **B,** BALF cell counts. **C** and **F,** Total BALF eosinophil (Fig 2, *C*) and neutrophil (Fig 2, *F*) counts assessed 24 hours after the last intratracheal exposure. **D, E, G,** and **H,** BALF levels of CCL11 (eotaxin-1; Fig 2, *D*), IL-5 (Fig 2, *E*), CXCL5 (ENA78; Fig 2, *G*), and CXCL1 (KC; Fig 2, *H*) were assessed by using ELISA (representative experiment with 6-7 mice per group). ***$P < .001$, **$P < .01$, and *$P < .05$ (1-way ANOVA). *D*, DEPs; *H*, HDM extract; *S*, saline.

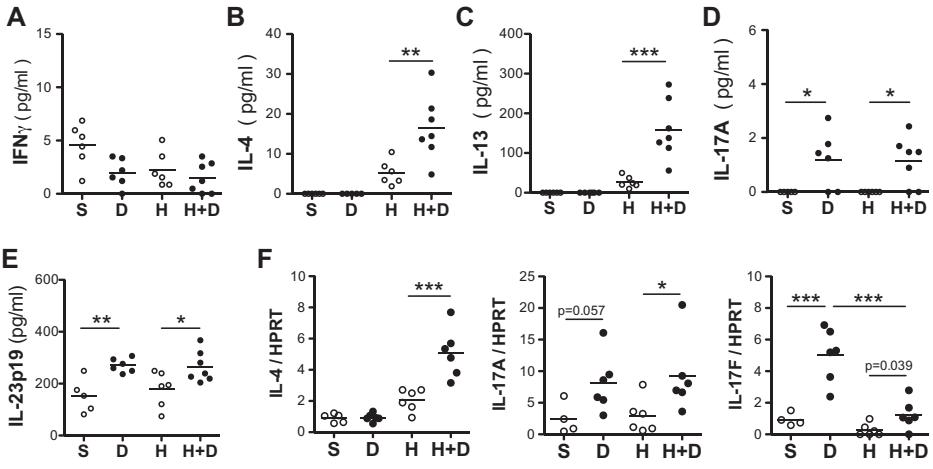

FIG 3. DEPs induce IL-17A and exacerbate HDM-induced T_H2 responses. **A-D,** IFN-γ (Fig 3, *A*), IL-4 (Fig 3, *B*), IL-13 (Fig 3, *C*), and IL-17A (Fig 3, *D*) BALF levels assessed 24 hours after the last exposure. **E,** IL-23p19 in lung homogenates. **F**, IL-4, IL-17A, and IL-17F mRNA lung levels (representative experiment with 5-7 mice per group). ***$P < .001$, **$P < .01$, and *$P < .05$ (1-way ANOVA). *D*, DEPs; *H*, HDM extract; *S*, saline.

mmol/L EDTA. The collected bronchoalveolar lavage fluid (BALF) was centrifuged, and the total cell numbers were counted with a hemocytometer. Cells were spun onto slides and stained with the HEMA3 stain set (Fisher Scientific, Kalamazoo, Mich). Four hundred cells were counted, and the total number of each cell type was calculated.

Histology and immunohistochemistry

The left lobe of the lung was fixed in formalin, paraffin embedded, and cut into 5-μm sections. Sections were stained with hematoxylin and eosin or periodic acid–Schiff, according to the manufacturer's recommendations (PolyScientific, Bay Shore, NY).

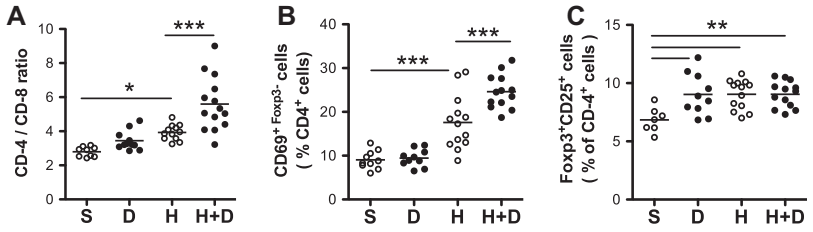

FIG 4. HDM plus DEP exposures exacerbate HDM-induced T-cell activation. **A,** Representative fluorescence-activated cell sorting dot plots of the proportion of $CD4^+$ cells versus $CD8^+$ cells among $CD3^+$ lung T cells. **B,** Percentages of $CD69^+Foxp3^-$ cells among $CD4^+$ lung T cells. **C,** Percentages of $Foxp3^+CD25^+$ cells among $CD4^+$ lung T cells (n = 10-14 mice per group, 2 separate experiments). *$P < .05$, **$P < .01$, and ***$P < .001$ (1-way ANOVA). *D*, DEPs; *H*, HDM extract; *S*, saline.

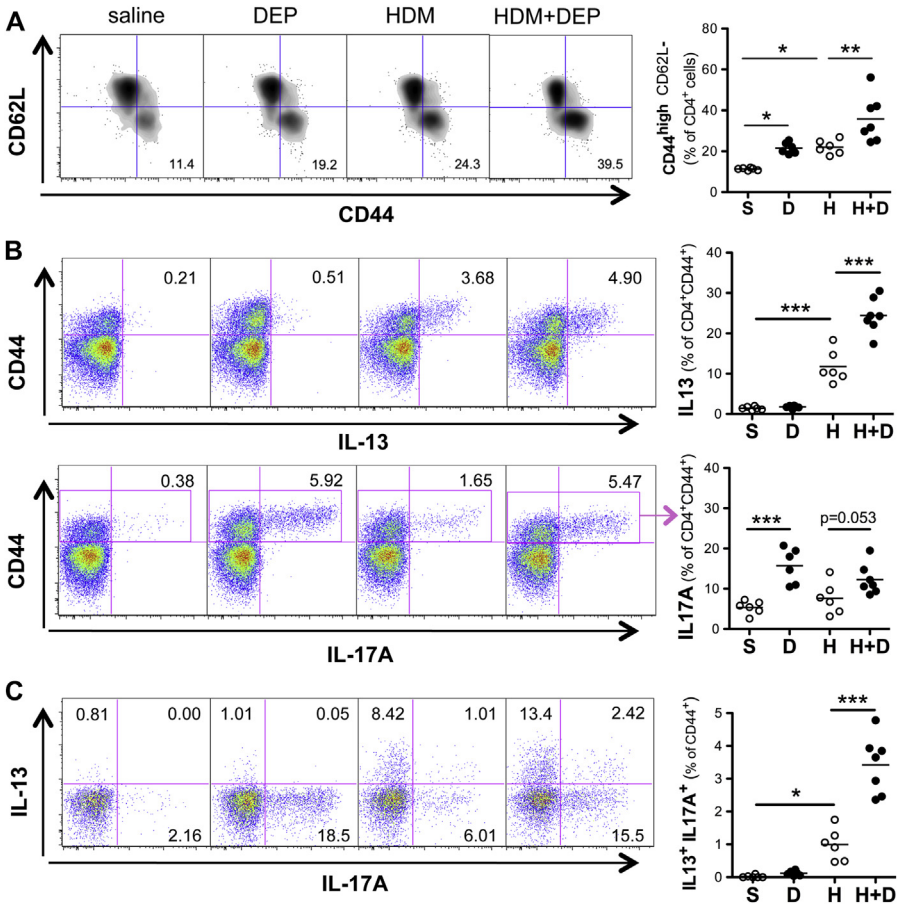

FIG 5. Exposure to HDM plus DEPs generates $IL\text{-}17A^+IL\text{-}13^+CD4^+$ effector T cells. **A,** Representative fluorescence-activated cell sorting dot plots of lung $CD44^{high}CD62^{low}$ effector T cells. **B,** Intracellular staining for $CD4^+$ T cells producing IL-13 and IL-17A after *ex vivo* restimulation with phorbol 12-myristate 13-acetate plus ionomycin (n = 5-7 mice per group). **C,** Representative fluorescence-activated cell sorting dot plots of cytokine expression by $CD4^+CD44^+$ effector T cells and frequency of $IL\text{-}13^+IL\text{-}17A^+$ double producers (n = 5-7 mice per group). *$P < .05$, **$P < .01$, and ***$P < .001$ (1-way ANOVA). *D*, DEPs; *H*, HDM extract; *S*, saline.

ELISA

For measurement of HDM-specific IgE and IgG_1 levels, wells were coated with 0.01% HDM (Greer Laboratories) overnight. Plasma was diluted 1:5 for IgE and 1:2000 for IgG_1. After 2 hours of incubation, plates were washed, and either horseradish peroxidase–conjugated anti-mouse IgG_1 (X56; 1:1000; BD Biosciences PharMingen, San Jose, Calif) or biotin–anti-mouse IgE (R35-118; 1:250; PharMingen) was added for 1 hour, followed by an incubation with streptavidin–horseradish peroxidase (R&D DY998; 1:200) in the case of IgE. BALF cytokine levels were assessed by using Luminex xMAP technology (Millipore) with the Cytokine/Chemokine Panel I and according to the manufacturer's instructions. IL-23p19 was assessed in lung lysates (homogenized in 1% Triton X) by using ELISA, according to the manufacturer's instructions (BioLegend, San Diego, Calif). To assess total BALF IL-13 levels (free and IL-13 receptor [IL-13R] α2 bound), the IL-13 standard and samples were preincubated with 10 ng/mL murine IL-13Rα2–Fc (R&D Systems, Minneapolis, Minn) at 37°C for 1 hour. Anti-mouse IL-13 polyclonal antibody (1 mg/mL) was used for capture, and

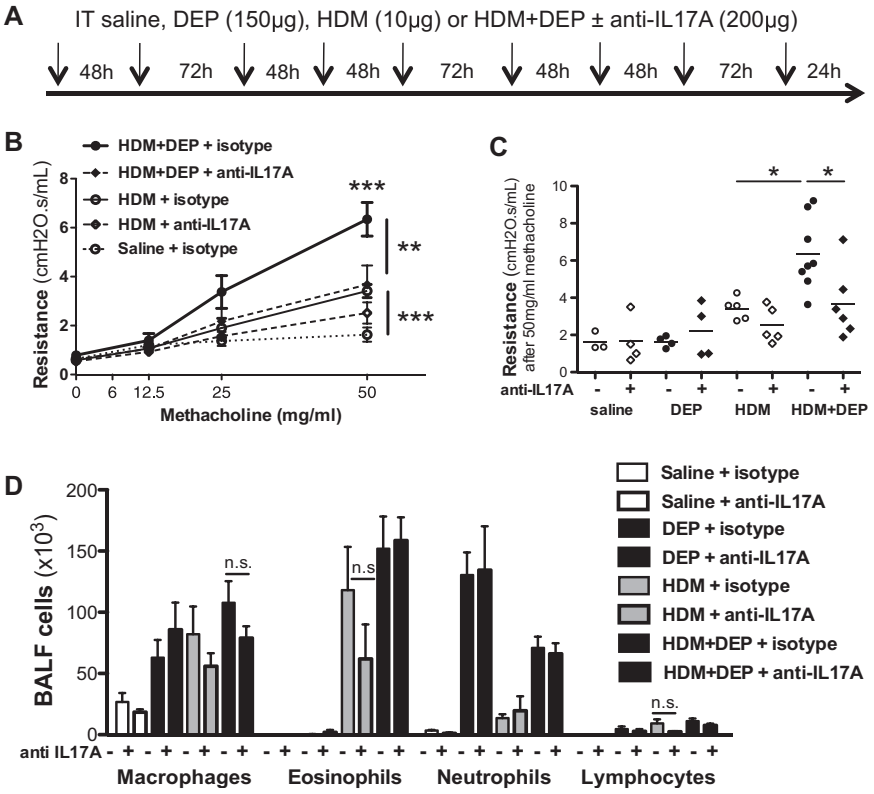

FIG 6. IL-17A neutralization alleviated DEP exacerbation of HDM-induced airway responses. **A,** Mice were administered a rat anti-murine IL-17A (200 μg; M210) or an IgG_1 control antibody intratracheally during HDM with or without DEP challenge. *IT,* Intratracheal. **B,** AHR was assessed 24 hours after the last exposure to HDM with or without DEPs. **C,** AHR after the last methacholine dose (50 mg/mL). **D,** Differential counts. ***$P < .001$, **$P < .01$, and *$P < .05$ (2-way ANOVA for Fig 6, *B* and *D*, and 1-way ANOVA for Fig 6, *C*). *n.s.*, Not significant.

biotinylated anti-mouse IL-13Rα2 polyclonal antibody (0.5 mg/mL) was used for detection (R&D Systems).

Real-time PCR

Total RNA was isolated from homogenized mouse skin by using Trizol (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions, and DNase treated (Qiagen, Valencia, Calif) before being reverse transcribed with the First Strand Superscript Synthesis kit (Invitrogen). Quantitative real-time PCR analysis of murine skin was done with LightCycler FastStart DNA master SYBR green I as a ready-to-use reaction mixture (Roche, Mannheim, Germany). cDNAs were amplified by using the primers listed in Table E1 in this article's Online Repository at www.jacionline.org, and gene expression was normalized to hypoxanthine guanine phosphoribosyltransferase.

Isolation of lung cells and staining for flow cytometry

Lungs were removed, and the upper right lobe was minced and incubated at 37°C for 25 to 30 minutes in 2 mL of RPMI 1640 containing Liberase DL (0.5 mg/mL; Roche Diagnostics, Indianapolis, Ind) and DNAse I (0.5 mg/mL; Sigma, St Louis, Mo). Lung cells were passed through a 70-μm cell strainer with a syringe rubber, and the strainer was washed with 5 mL of RPMI plus DNAse I media. Cells were centrifuged and resuspended in 2 mL of RPMI before counting with a hemocytometer, and cell viability was confirmed by means of trypan blue exclusion. Approximately 500,000 lung cells were transferred to a 96-well plate with V-shaped wells on ice, centrifuged, and resuspended in PBS containing Fc Block (2.4G2 mAb; BD Biosciences). T cells were stained with CD4–fluorescein isothiocyanate, CD3–phycoerythrin (PE)/Cy7, CD69-PE, CD25-AF647, CD8–allophycocyanin/Cy7, and CD44–Pacific Blue (BioLegend). Cells were labeled with the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, according to the manufacturer's instructions (Invitrogen by Life Technologies, Carlsbad, Calif). Intracellular staining for IL-13–PE, IL-17A–AF647, IFN-γ–PerCP5.5, and forkhead box protein 3 (Foxp3)–PerCP5.5 was conducted according to the manufacturer's instructions (eBioscience, San Diego, Calif). Acquisition was done on a FACSCanto III (Becton Dickinson, Mountain View, Calif) and analyzed with FlowJo software (Tree Star, Ashland, Ore).

Statistical analysis

For our studies of childhood allergic asthma, a χ^2 test and logistic regression were used to evaluate differences in proportions of more severe asthma symptoms between children with low and high DEP exposure. Secondhand smoke exposure (yes/no), age, race (African American, white, and other/mixed race), sex, annual family income (<$40,000/≥$40,000), type of health insurance (public/private/self-pay), maternal education (less than high school or GED/high school or GED or greater), being prescribed asthma controllers (yes/no), and parent-reported imperfect adherence over the previous 2 weeks (yes/no) were evaluated as possible covariates in our models and fitted by using backwards elimination. Pearson correlation coefficients were used to examine correlations between IL-17A levels and levels of other T_H2 cytokines. Analyses were performed with SAS software (version 9.2; SAS Institute, Cary, NC). All mouse-related statistical analyses were done with Prism software (GraphPad Software, La Jolla, Calif). Statistical significance was assessed by using 1-way ANOVA, followed by a Bonferroni posttest, except when described otherwise in the figure legend.

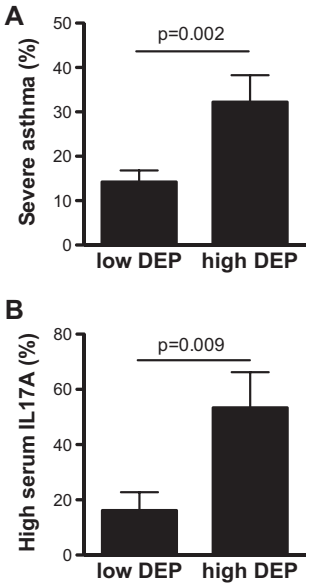

FIG 7. DEP exposure exacerbates asthma severity and increases IL-17A levels in childhood allergic asthma. **A,** Among 235 GCPCR participants with allergic asthma, those with high DEP exposure (n = 44) had more frequent asthma symptoms compared with those with low exposure. The observed association remained significant, even after adjusting for age, race, sex, annual family income, type of health insurance, maternal education, being prescribed asthma controllers, adherence to asthma medication over the prior 2 weeks, and secondhand smoke exposure in multivariate logistic models ($P = .01$). **B,** Among 46 PEES asthmatic patients, 53% of children with high DEP exposure also had high IL-17A levels. The association remained significant after adjustment for the above covariates and asthma symptom frequency.

RESULTS

DEP exposure exacerbates HDM-induced AHR, inflammation, and allergen sensitization

To determine the mechanisms underlying DEP-induced exacerbation of asthma, we used an experimental asthma model that did not rely on intraperitoneal sensitization in the presence of an adjuvant; BALB/c mice were exposed intratracheally to either saline, HDM, DEPs, or HDM plus DEPs 9 times over a 3-week period (Fig 1, *A*). At low doses of allergen (10 μg of total HDM extract per dose), a marked synergistic increase in BALF inflammation was observed in mice exposed to HDM plus DEPs compared with that seen in either DEP- or HDM-exposed mice (Fig 1, *B*). This synergy was no longer observed in mice exposed to higher doses of DEPs. Thus to study DEP exacerbation of HDM-induced asthma, we used a submaximal dose of HDM extract and a dose of DEPs that had previously been shown to exacerbate ovalbumin-induced airway responses.[19] Chronic allergen exposure of BALB/c mice to low doses of HDM over a 3-week period resulted in AHR, airway inflammation, goblet cell hyperplasia, and mucus production (Fig 1, *C* and *D*). DEP exposure alone also induced airway inflammation but did not promote AHR or mucus production (Fig 1, *B-D*). Coexposure to DEPs significantly increased HDM-induced inflammation and AHR (Fig 1, *B-D*).

DEP and HDM coexposure exacerbates allergen sensitization

Repeated DEP exposures over a 3-week period did not result in a significant increase in total IgE or IgG_1 plasma levels compared with those seen in saline-treated mice (data not shown). However, exposure to both HDM and DEPs significantly increased total IgE and IgG_1 levels over exposure to HDM alone (data not shown). Accordingly, repeated exposures to HDM plus DEPs also significantly increased HDM-specific IgG_1 and HDM-specific IgE levels compared with those seen after HDM exposure alone (Fig 1, *E* and *F*), suggesting increased allergic sensitization in the presence of DEPs.

DEP and HDM coexposure results in a mixed T_H17 and T_H2 response

The nature of the inflammatory response differed markedly between HDM and DEPs (Fig 2, *A*). DEPs were predominantly taken up by alveolar macrophages and occasionally by neutrophils (Fig 2, *B*). Repeated HDM exposure resulted in BALF eosinophilia and increased levels of IL-5 and CCL11 (eotaxin-1; Fig 2, *C-E*). In contrast, exposure to DEPs alone was characterized by a marked neutrophilia and increased CXCL1 (KC) and CXCL5 (ENA-78 and LIX) levels in the BALF (Fig 2, *F-H*). CXCL5 was specifically induced by DEPs but not HDM, whereas increased BALF levels of CXCL1 were observed after exposure to either HDM or DEPs. Coexposure to HDM and DEPs did not increase neutrophilia or neutrophil chemokine levels to greater than those observed with DEP exposure alone (Fig 2, *F-H*). However, HDM and DEP coexposure increased BALF eosinophilia and eotaxin levels compared with that seen after HDM exposure alone (Fig 2, *C* and *D*). BALF T-cell levels trended higher after HDM and DEP exposures and were further increased after coexposures to HDM and DEP compared with those after HDM alone (Fig 2, *A*).

Next we assessed BALF levels of T_H1, T_H2, and T_H17 cytokines after DEP and HDM exposure. Levels of IFN-γ were unaffected after challenge with HDM, DEP, or both, whereas T_H2 cytokine levels (IL-4, IL-5, and IL-13) were significantly increased after exposure to HDM alone but not to DEPs alone (Figs 2, *E*, and 3, *A-C*). Coexposure to HDM and DEPs induced a further increase in IL-4 and IL-13 levels compared with HDM exposure alone (Fig 3, *B* and *C*). Exposure to DEPs alone induced IL-17A and IL-17F but not T_H2 cytokines (Fig 3). Additionally, the pro-T_H17 cytokine IL-23 was also increased by DEP exposure (Fig 3, *E*). In the presence of HDM, DEPs induced IL-17A, IL-17F, and IL-23, but the levels were not enhanced over those observed with DEPs alone. Notably, coexposure to HDM and DEPs induced a mixed T_H2 and T_H17 response (Fig 3).

DEP and HDM coexposure results in increased $CD44^+CD62L^-$ lung effector T_H2 and T_H17 cell numbers

Lung cells were isolated and analyzed by means of flow cytometry to determine the effect of HDM and DEP exposures on pulmonary T cells. An increase in $CD4^+$ T-cell numbers over $CD8^+$ T-cell numbers was observed in HDM-exposed mice but not after exposure to DEP alone (Fig 4, *A*). HDM and DEP coexposure further increased the proportion of $CD4^+$ T cells (Fig 4, *A*) compared with HDM alone. Among $CD4^+$ T cells, expression of the early activation marker CD69 was increased after HDM exposure (Fig 4, *B*). HDM and DEP coexposure resulted in further increases in CD69 surface expression compared with HDM exposure alone (Fig 4, *B*). The percentage

of lung $CD4^+$ T cells positive for Foxp3 and CD25 was similarly increased by DEP, HDM, and HDM plus DEP exposures (Fig 4, *C*).

Consistent with the observed enhancement in T-cell activation (Fig 4, *B*), HDM and DEP coexposure increased the proportion of $CD44^+CD62L^-$ lung effector T cells compared with HDM alone (Fig 5, *A*). To determine the phenotype of these effector T cells, lung $CD4^+$ T cells were stimulated *ex vivo* with phorbol 12-myristate 13-acetate and ionomycin, and then intracellular cytokine staining was performed for IL-13, IL-17A, and IFN-γ (Fig 5). DEP exposure alone induced IL-$17A^+$, but not IL-13^+ or IFN-γ^+, effector T cells (Fig 5 and see Fig E1 in this article's Online Repository at www.jacionline.org). In contrast, HDM exposure alone resulted in increased IL-13–producing $CD44^+$ effector T cells. HDM and DEP coexposure resulted in a marked increase in IL-13–producing lung effector T-cell numbers compared with those seen after HDM exposure alone (Fig 5, *B*). The number of IL-17A–producing lung effector cells was not further enhanced with coexposure over DEP exposure alone. However, an increase in the number of IL-13^+/IL-$17A^+$ coproducing effector T cells was observed in the DEP and HDM exposure group compared with the group undergoing HDM exposure alone (Fig 5, *C*). Thus DEPs enhance the generation of effector T cells capable of producing both IL-13 and IL-17A in the presence of allergen.

DEP exacerbation of HDM-induced allergic inflammation is partially dependent on IL-17A

It has been recently demonstrated that IL-17A can exacerbate IL-13–induced AHR.[8] Our findings suggest a contribution of IL-17A to DEP exacerbation of HDM-induced allergic responses. To directly address this possibility, we used an anti–IL-17A antibody (Fig 6, *A*). IL-17A neutralization in the lungs counteracted the synergistic effect of DEPs on HDM-induced AHR (Fig 6, *B* and *C*). Surprisingly, BALF inflammation and, most notably, BALF neutrophil levels were not significantly altered by anti–IL-17A treatment (Fig 6, *D*), suggesting that IL-17A can exacerbate AHR independently of neutrophils.

DEP exposure exacerbates asthma symptoms and increases IL-17A serum levels in childhood allergic asthma

To translate our mouse model findings and determine whether asthma severity (based on asthma symptom frequency) is altered in DEP-exposed children with allergic asthma, we identified 235 children with allergic asthma in the GCPCR between the ages of 5 and 18 years. We compared the proportion of children with 1 or more symptoms of wheeze, cough, shortness of breath, or chest tightness 6 to 7 days per week over the past 12 months exposed to low versus high levels of DEPs (>0.464 $\mu g/m^3$).[23] Our analysis revealed a significant association between asthma symptom frequency and DEP exposure (odds ratio [OR], 2.9; 95% CI, 1.4-5.7). Indeed, among the highly exposed group, 32.2% of the children had more frequent asthma symptoms compared with only 14.2% in the low-exposure group (Fig 7, *A*). To account for potential confounders, we fit a multivariate logistic model using backward elimination, adjusting for age, race, sex, annual family income, type of health insurance, maternal education, being prescribed asthma controllers, adherence to asthma medication over the prior 2 weeks, and secondhand smoke exposure. With only race and annual family income remaining in the final model, we continued to observe a significant association between severe asthma and high DEP exposure (OR, 2.9; 95% CI, 1.3-6.9).

To assess whether DEP exposure was similarly associated with IL-17A production in children with allergic asthma, we evaluated IL-17A levels, as well as T_H2 cytokine levels, in a subset of 46 PEES participants with available serum (Table I). We examined the association between DEP exposure and serum IL-17A levels. Serum IL-17A levels in the children ranged from 0 to 646.56 pg/mL (with a 0.5 pg/mL lower limit of detection) and were not normally distributed. Children in the top quartile (30% after considering children with equal levels at the 25% level) of IL-17A values were defined as having high levels (>24 pg/mL). Using this binary variable, we found that high DEP exposure was associated with high IL-17A levels (OR, 5.9; 95% CI, 1.5-24.0; Fig 7, *B*). After using backwards elimination to fit a logistic model, only DEP exposure, sex, and being prescribed asthma controllers remained in the model, and the association between DEP exposure and IL-17A was strengthened as a result (OR, 12.1; 95% CI, 1.2-125.6). Taken together with the data presented in Fig 7, *A*, our findings support that among children with allergic asthma, high DEP exposure is associated with both greater asthma symptoms and increased serum IL-17A levels. Importantly, no other cytokine evaluated (IL-4, IL-5, and IL-13) was associated with DEP exposure (Table II). IL-17A was the only cytokine significantly associated with DEP exposure. No other covariate was associated with IL-17A levels or significantly modified the association between IL-17A and DEP exposure (Fig 7, *B*).

DISCUSSION

The present study demonstrates that DEP exposure is associated with increased asthma symptoms and increased IL-17A levels in human and murine asthma. Furthermore, IL-17A blockade in mice prevented DEP-induced exacerbation of allergic asthma. IL-17A has been implicated in patients with severe forms of asthma; however, the factors that promote IL-17A production during the pathogenesis of severe asthma have remained undefined. Our data provide the first evidence that DEP exposure is a factor that contributes to severe asthma by enhancing IL-17A production. In addition, our data demonstrate that increased serum IL-17A levels, which have been associated with severe adult allergic asthma, might also be an important marker of asthma severity in asthmatic children. Current asthma therapies are largely focused on decreasing T_H2 responses; however, approaches targeting neutralization of IL-17A might be useful therapeutic strategies to counteract the asthma-promoting effects of traffic-related air pollution, especially among high DEP–exposed patients with severe asthma.

Exposure of mice to DEPs induced accumulation of T_H17 cells and neutrophils in the lungs. Coexposure to HDM and DEPs resulted in a mixed neutrophil and eosinophil lung infiltrate, a mixed T_H2 and T_H17 response, and enhanced AHR. The synergy was not evident when large doses of DEPs or allergen were used, likely because the effect of the single high-dose exposure masked the synergy response. Similarly, among children with allergic asthma, those exposed to high DEP levels had increased asthma severity and increased serum IL-17A levels compared with those

TABLE I. Population characteristics of study participants

Variables	GCPCR (n = 235)		PEEPS (n = 46)	
	No.	% of total	No.	% of total
Higher annual asthma symptom frequency	44	18.7	7	15.2
Black/African American	116	49.4	34	73.9
Caucasian/white	98	41.7	12	26.0
Other/mixed race	21	8.9	0	0.0
Male sex	141	60.0	31	67.3
High DEP exposure (>0.464 μg/m^3)	59	25.1	15	32.6
Secondhand smoke exposure	114	48.5	34	73.9
Diagnosis of allergic rhinitis	187	79.6	35	76.1
Any positive SPT response	166	70.6	37	92.5
Public insurance	114	48.5	27	58.7
Annual family income <$40,000	135	57.4	34	75.6
Maternal education less than high school	21	8.9	7	15.6
Currently prescribed ICSs or leukotrienes	166	70.6	33	71.4
Imperfect adherence over last 2 wk	86	44.8	21	53.9
	Mean	SD	Mean	SD
Age (range, 5-18 y)	10.0	3.1	9.8	2.7

Children with allergic asthma from the GCPCR and PEES are included. Diagnosis of allergic rhinitis (yes/no), SPT response (positive/negative), age at study consent, race, sex, annual family income, type of health insurance (public vs private/self-pay), maternal education, being prescribed asthma controllers, adherence to asthma medication over the prior 2 weeks, and secondhand smoke exposure were determined by means of questionnaire or electronic medical records. Severe asthma was defined as having 1 or more symptoms that occur at least 6 to 7 days per week compared with others with less frequent symptoms. High DEP exposure is based on the highest quartile of elemental carbon attributable to traffic (0.464 μg/m^3) estimated at the children's current home address. Children with greater than 24 pg/mL serum IL-17A were considered to have high IL-17A levels.

TABLE II. DEP exposure is associated with serum IL-17A but not T_H2 cytokine levels (n = 46)

	DEPs	
	OR	95% CI
IL-4	0.86	0.19-3.92
IL-5	1.44	0.38-5.50
IL-13	1.25	0.30-5.16
IL-17A	5.94	1.47-23.97

Cytokine levels were determined from serum samples collected from 46 PEES participants by using the premixed 39-Plex MILLIPLEX MAP Human Cytokine/Chemokine assay. Presented are binary variables, with the top quartile indicating high cytokine levels and the bottom 3 quartiles indicating low cytokine levels. High DEP exposure was defined as the quartile of values greater than 0.46 μg/m^3. Logistic regression was performed, and unadjusted ORs and 95% CIs are presented. IL-17A was the only significant association observed, and inclusion of potential confounders in the models only strengthened the magnitude of the association between IL-17A levels and DEPs (OR, 12.1; 95% CI, 1.2-125.6).

with low DEP exposure. Unlike the mouse model, in which absent, low, and high DEP exposure can be defined and controlled, there are no children with absent DEP exposure. It is not possible to directly compare the low- and high-exposure groups between the human and mouse studies. We defined the low- and high-exposure groups of children based on published data from the Cincinnati Childhood Allergy and Air Pollution Study birth cohort.[22] All of the children were atopic (ie, allergen exposed). The association between high DEP exposure and high serum IL-17A levels was not observed with T_H2 cytokines and remained significant, even after adjusting for age, race, sex, annual family income, type of health insurance, maternal education, being prescribed asthma controllers, self-reported adherence to asthma medication over the prior 2 weeks, and secondhand smoke exposure in multivariate logistic models. These findings suggest that children with allergic asthma and concomitant exposure to DEPs might have a T_H17-driven component to their airways disease.

The murine findings are further supported by previous studies that have found that exposure to DEPs induces proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and neutrophil chemokines *in vivo*.[24,25] We have observed that IL-1β and IL-6, which induce T_H17 differentiation,[26] are secreted in BALF 3 hours after DEP exposure and return to baseline levels within 24 hours (data not shown). IL-23 is not necessary for T_H17 differentiation but is required to maintain and expand T_H17 cell numbers.[26] Accordingly, our data demonstrated that DEP induction of IL-17A was associated with increased lung levels of IL-23.

Finally, we show that DEPs induce release of neutrophil chemokines (CXCL1 and CXCL5) into the lungs of exposed mice. Although IL-17A promotes secretion of neutrophil chemokines by epithelial cells,[27] DEPs can directly induce secretion of T_H17-related cytokines (IL-1β and IL-6) and chemokines (IL-8) by human bronchial epithelial cells.[28,29] Thus DEPs induce neutrophilia not only by promoting T_H17 responses but also by directly inducing neutrophil chemokines in exposed lung epithelial cells.

Several studies have proposed that members of the aryl hydrocarbon family, some of which are present on DEPs, can promote the differentiation of Treg cells through the aryl hydrocarbon receptor.[30,31] DEPs induced an increase in the proportion of $CD25^+Foxp3^+CD4^+$ T cells similar to that induced by HDM. We did not access the functionality of these Treg cells. However, the increase in effector T-cell numbers, specifically those of HDM-induced T_H2 cells and DEP-induced Th17 cells, suggest that these Treg cells have a limited ability to control T_H2 and T_H17 responses. Indeed, exposure to high levels of air pollution has been associated with impaired Treg cell function.[32] One of the mechanisms by which Treg cells control T-cell proliferation is by depleting the pool of IL-2 available to effector T cells to sustain proliferation. Because IL-2 is a negative regulator of T_H17 differentiation,[33] this mechanism could favor T_H17 differentiation in DEP-exposed mice.

There is mounting evidence that DEP exposure plays a significant role in the development of asthma and expression of asthma symptoms. However, the relationship is complex and depends on the timing and duration of the exposure, as well as the dose and coexposures.[34] In one study coexposure of asthmatic patients to cat allergens and DEPs resulted in only mild increases in inflammation.[35] The observed effect of DEPs might have been much greater with a different DEP dose or in children. These investigators did not assess IL-17A production.

Two recent studies have shown that particulate matter induces IL-17A in the lungs of exposed mice.[12,13] We extend this finding by demonstrating that DEPs, a component of traffic-related particulate matter, induce IL-17A (mRNA and protein) in exposed lungs. Furthermore, we demonstrated that exposure to DEPs alone was able to promote accumulation of T_H17 cells in the lungs of exposed mice. Finally, we demonstrated that neutralization of IL-17A alleviated DEP

exacerbation of HDM-induced AHR. As is the case for DEPs, IL-17A is unable to induce AHR by itself.[8,36] However, IL-17A can exacerbate IL-13–induced AHR and partially mediates HDM-induced AHR.[8] Importantly, T_H17-derived IL-17A has recently been demonstrated to exacerbate AHR by directly promoting airway smooth muscle contraction.[37] In this study mice lacking αvβ8 integrin on $CD11c^+$ cells do not mount a significant T_H17 response in a classic murine asthma model. Despite normal allergen-induced inflammation, T_H2 responses, and mucus production, the absence of IL-17A production by T_H17 cells was enough to alleviate allergen-induced AHR.[37] This is consistent with our finding that anti–IL-17A treatment decreased AHR without significantly affecting BALF inflammation.

It has been recently demonstrated that IL-17A levels in bronchial biopsy specimens from asthmatic patients are associated with disease severity.[4] Importantly, severe asthma characterized by neutrophilia is often resistant to steroid treatment.[38,39] In an experimental murine model T_H17 cells were demonstrated to mediate steroid-resistant airway inflammation and AHR.[40] Our data suggest that repeated DEP exposure contributes to the development of severe asthma by promoting a mixed T_H2/T_H17 response. Indeed, among children with allergic asthma, we found that DEP exposure was associated with increased asthma symptoms regardless of age, race, sex, annual family income, type of health insurance, maternal education, being prescribed asthma controllers, self-reported adherence to asthma medication over the prior 2 weeks, and secondhand smoke exposure in multivariate logistic models. It is important to note that patients exposed to DEPs are likely exposed to other components of air pollution that might be contributing to the overall phenotype as well.

It is tempting to speculate that DEPs promote severe asthma by inducing the development of IL-13/IL-17 double-producing $CD4^+$ T cells. We have identified a population of IL-17A and IL-13 double-producing $CD4^+$ effector T cells in HDM- and DEP-coexposed mice similar to the recently described IL-17A– and IL-13–producing T cells.[41,42] Wang et al[41] demonstrated that the T_H17-polarizing cytokines IL-1β, IL-6, and IL-21 are capable of inducing classical T_H2 cells to produce significant amounts of IL-17. Conversely, murine and human T_H17 cells have the potential to become IL-4/IL-17 dual-producing cells.[42,43] Transfer of antigen-specific, dual-producing cells into BALB/c mice triggered more severe inflammation on allergen challenge compared with transfer of conventional T_H2 or T_H17 cells, highlighting the potential role of this novel cell subset in allergic asthma severity.[41]

In conclusion, DEP exposure induces a mixed T_H2 and T_H17 response and increased AHR and lung inflammation in a mouse model of allergen-induced asthma. Similarly, in allergic children with asthma, higher DEP exposure is associated with increased asthma symptoms and higher serum IL-17A levels.

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Key messages

- **In children with allergic asthma, DEP exposure was associated with more frequent asthma symptoms and increased IL-17A blood levels.**
- **Similarly, DEP exposure worsened the allergic asthma phenotype in an experimental asthma model, resulting in increased AHR, allergen sensitization, BALF T_H2 and T_H17 cytokines levels, and pulmonary eosinophilia compared with HDM alone.**
- **Exposure to DEPs alone induced a T_H17 response associated with neutrophilia but did not result in AHR, eosinophilia, T_H2 cytokines, or mucus production.**
- **IL-17A neutralization alleviated DEP-induced AHR in mice.**

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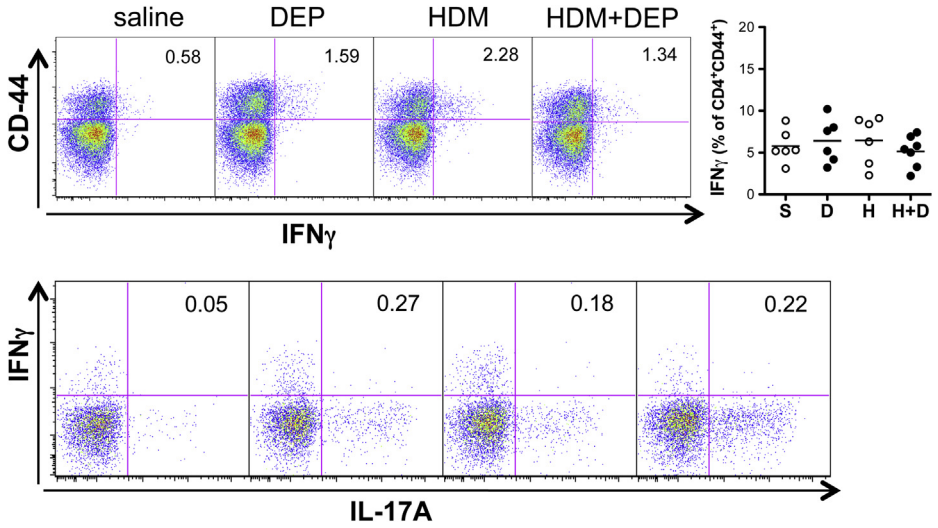

FIG E1. Exposure to HDM with or without DEPs does not promote a T_H1 response. Representative fluorescence-activated cell sorting dot plots of IFN-γ expression by $CD4^+CD44^+$ effector T cells and frequency of IFN-γ^+IL-$17A^+$ double producers (n = 5-7 mice per group). *D*, DEPs; *H*, HDM extract; *S*, saline.

TABLE E1. Sequences of primers used for real-time PCR

	Forward primer	bp	Reverse primer	bp
mHPRT	TGCCGAGGATTTGGAAAAAG	20	CCCCCCTTGAGCACACAG	18
mIFNG	CAGCAACAGCAAGGCGAAAAAGG	23	TTTCCGCTTCCTGAGGCTGGAT	22
mIL4	CTGTAGGGCTTCCAAGGTGCTTCG	24	CCATTTGCATGATGCTCTTTAGGC	24
mIL17A	ACTACCTCAACCGTTCCACG	20	AGAATTCATGTGGTGGTCCA	20
mIL17F	TGGAGAAACCAGCATGAAGTG	21	AGTCCCAACATCAACAGTAGC	21

All amplicons span at least 1 intron.
HPRT, Hypoxanthine guanine phosphoribosyltransferase.