# Exposure to allergen and diesel exhaust particles potentiates secondary allergen-specific memory responses, promoting asthma susceptibility

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**Background: Exposure to traffic pollution particulate matter, predominantly diesel exhaust particles (DEPs), increases the risk of asthma and asthma exacerbation; however, the underlying mechanisms remain poorly understood.**
**Objective: We sought to examine the effect of DEP exposure on the generation and persistence of allergen-specific memory T cells in asthmatic patients and translate these findings by determining the effect of early DEP exposure on the prevalence of allergic asthma in children.**
**Methods: The effect of DEPs on house dust mite (HDM)–specific memory responses was determined by using an asthma model. Data from children enrolled in the Cincinnati Childhood Allergy and Air Pollution Study birth cohort were analyzed to determine the effect of DEP exposure on asthma outcomes.**
**Results: DEP coexposure with HDM resulted in persistent $T_H2/T_H17$ $CD127^+$ effector/memory cells in the lungs, spleen, and lymph nodes of adult and neonatal mice. After 7 weeks of rest, a single exposure to HDM resulted in airway hyperresponsiveness and increased $T_H2$ cytokine levels in mice that had been previously exposed to both HDM and DEPs versus those exposed to HDM alone. On the basis of these data, we examined whether DEP exposure was similarly associated with increased asthma prevalence in children in the presence or absence of allergen exposure/sensitization in the Cincinnati Childhood Allergy and Air Pollution Study birth cohort. Early-life exposure to high DEP levels was associated with significantly increased asthma prevalence among allergic children but not among nonallergic children.**
**Conclusion: These findings suggest that DEP exposure results in accumulation of allergen-specific $T_H2/T_H17$ cells in the lungs, potentiating secondary allergen recall responses and promoting the development of allergic asthma. (J Allergy Clin Immunol 2015;136:295-303.)**

***Key words:*** *Allergic asthma, traffic pollution, house dust mite, diesel exhaust particle, memory, recall, children*

A recent comprehensive and systematic review of worldwide traffic emissions and health science by a special panel convened by the Health Effects Institute found sufficient evidence that exposure to traffic-related air pollution (TRAP) causes asthma exacerbation in children.[1] Diesel exhaust particles (DEPs) are a key component of traffic-related particulate matter and are the main contributor to TRAP-related asthma exacerbations in children.[2,3] These primary ultrafine DEPs (diameter, <1.0 μm) can reach the small airways, including the alveolar/gas exchange regions of the lung, exacerbating respiratory disease symptoms.[3] This exposure is highly significant because in large cities in North America, up to 45% of the population resides in zones that are most affected by TRAP.[1] Furthermore, more than 30% of schools are located in high TRAP exposure areas.[4] Even short-term exposure to high diesel traffic was able to reduce airway function in asthmatic patients.[5] Similarly, we recently reported that higher DEP exposure is associated with increased asthma severity in allergic children with asthma.[6]

Allergic asthma is generally regarded as a $T_H2$ disease characterized by increased eosinophil and $T_H2$ cytokine (IL-4, IL-5, and IL-13) levels, but severe asthma is often characterized by mixed $T_H2/T_H17$ responses.[7] In mice DEPs alone had no effect on airway hyperresponsiveness (AHR), but coexposure with house dust mite (HDM) exacerbated allergic airway responses, including allergen-specific IgE levels, eosinophilia, and AHR.[8] Transfer of antigen-specific IL-17A and IL-13 double-producing $CD4^+$ effector T 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.[9] Repeated DEP exposure promoted accumulation of $T_H17$ and $T_H2/T_H17$ coproducer cells in the lungs of exposed mice.[6] Neutralization of IL-17A alleviated DEP-mediated exacerbation of HDM-induced AHR, supporting a role for IL-17A in patients with severe asthma.[6]

Here, we examined the effect of DEP exposure on allergen-specific memory and recall responses. Generation and maintenance of memory T cells in the lungs are still poorly understood and have been studied predominantly in the context of

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*Abbreviations used*
AHR: Airway hyperresponsiveness
BALF: Bronchoalveolar lavage fluid
CCAAPS: Cincinnati Childhood Allergy and Air Pollution Study
CD62L: CD62 ligand
CDPM: Combustion-derived particulate matter
CLCA3: Chloride channel, calcium-activated, family member 3
DEP: Diesel exhaust particle
ECAT: Elemental carbon attributable to traffic (a proxy for DEP exposure)
HDM: House dust mite
IL-13R: IL-13 receptor
PE: Phycoerythrin
SPT: Skin prick test
TRAP: Traffic-related air pollution

viral infections.[10] Memory T cells are long-lived antigen-specific T cells that arise during expansion of effector T cells and survive the contraction phase of the effector response. Based primarily on studies focusing on $CD8^+$ memory T cells, 3 subpopulations have been described: central memory, effector memory, and tissue-resident memory T cells.[11] Memory T cells express high surface CD44 levels, and most express CD127, the receptor for IL-7, which plays a central role in $CD4^+$ T-cell homeostatic proliferation.[12] Central memory T cells circulate in secondary lymphoid organs (spleen and lymph nodes) and express CD62 ligand (CD62L) and CCR7. Effector/memory T cells downregulate CD62L and CCR7 to leave lymphoid tissues and then express tissue-specific integrins and chemokine receptors.[11,13] These infiltrating effector memory T cells differ from tissue-resident memory T cells, which are generated locally and express CD69, an early activation marker.[11,14,15]

In the present study we assess the effect of DEP-mediated asthma exacerbations on the generation and persistence of memory T cells. Because the nature and size of the effector response influences the nature and size of the memory T-cell pool,[16-18] we hypothesized that the increased accumulation of effector/memory $T_H2$ cells in the lungs of mice coexposed to HDM plus DEPs will result in the persistence of more HDM-specific memory $T_H2$ cells in the lungs on resolution of the effector $T_H2$ response, potentiating future recall responses. We further hypothesized that if DEP exposure potentiates recall responses to allergen, then early-life DEP exposure might promote the development of allergic asthma in children.

## METHODS

For a complete description of the materials and methods used in the murine experiments, see the Methods section in this article's Online Repository at www.jacionline.org.

### Cincinnati Childhood Allergy and Air Pollution Study cohort

The Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) is an ongoing prospective birth cohort study that has been described previously.[19-22] For this analysis, 578 children from CCAAPS who had at least 1 skin prick test (SPT) response between the ages of 1 and 4 years and an asthma diagnosis at age 7 years were included. DEP exposure levels were estimated from the birth record address; a high DEP level was defined as the top quartile, as described previously.[21] Early-life aeroallergen sensitization and early HDM sensitization were defined as a positive SPT response for any of the 15 aeroallergens or a positive SPT response to HDM, respectively, at one examination between the ages of 1 and 4 years. Asthma diagnosis at age 7 years was based on reported symptoms and spirometric testing based on American Thoracic Society criteria.[23] This study was approved by the Institutional Review Boards of Cincinnati Children's Hospital Medical Center and the University of Cincinnati.

### Statistical analysis

For the human data, statistical analyses were performed with SAS software (SAS Institute, Cary, NC). The differences in the proportion of children with asthma with respect to DEP exposure (low vs high) and early persistent atopy (no vs yes) were evaluated with $\chi^2$ tests. A $P$ value of less than .05 was considered significant. We also evaluated a logistic model adjusted for race, sex, and mother's education level. For the murine studies, 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 post test on the relevant groups.

## RESULTS

### DEP-associated neutrophilia persists after HDM-induced $T_H2$ responses return to baseline

First, we determined how coexposure to DEPs affects the resolution of HDM-induced lung inflammation by assessing eosinophil and $T_H2$ cytokine bronchoalveolar lavage fluid (BALF) levels 8 and 30 days after the last exposure (Fig 1, *A*). As we have previously shown,[6,24] DEP coexposure exacerbates HDM-induced lung inflammation (Fig 1, *B*). One week later, HDM-mediated induction of BALF eosinophilia had largely subsided, and DEP-related neutrophilia represented the major inflammatory cell type in the BALF (Fig 1, *C* and *D*). BALF levels of IL-5 and eotaxin-1 (CCL11) were also significantly ablated, whereas CXCL1 and CXCL5 levels remained increased 8 days after the last exposure (see Fig E1, *B* and *C*, in this article's Online Repository at www.jacionline.org). At 30 days after exposure, neutrophil BALF levels were still significantly increased in DEP-exposed mice (Fig 1, *C*). In contrast, eosinophils and $T_H2$ cytokines were no longer present in the BALF of HDM plus DEP–exposed mice (Fig 1, *D-F*). Exposure to DEPs alone did not induce AHR, but HDM and DEP coexposure significantly exacerbated AHR 24 hours after the last exposure (Fig 1, *G*). By 30 days after exposure, AHR had returned to baseline values (Fig 1, *G*). Consistent with decreasing BALF $T_H2$ cytokine levels and AHR, goblet cell numbers (as assessed based on chloride channel, calcium-activated, family member 3 [CLCA3; also known as gob-5] expression) and mucus production were also diminished (Fig 1, *H*, and see Fig E1, *D*). Taken together, these results suggest that allergic $T_H2$ response resolved within 1 month after the last exposure, even in mice coexposed to HDM and DEPs.

### DEP and HDM coexposure promotes a persistent increase in lung effector/memory $T_H2$ cell numbers

Coexposure to HDM and DEPs significantly increased lung cell numbers compared with those in mice exposed to either DEPs or HDM alone (Fig 2, *A*). This accumulation of inflammatory cells in the lungs lasted for more than a week, but lung cell numbers returned to baseline within a month (Fig 2, *A*). Numbers of effector T cells, which were defined as $CD4^+CD44^+CD62L^-$ T cells, were significantly increased in mice coexposed to HDM and DEPs compared with those exposed to HDM alone (Fig 2,

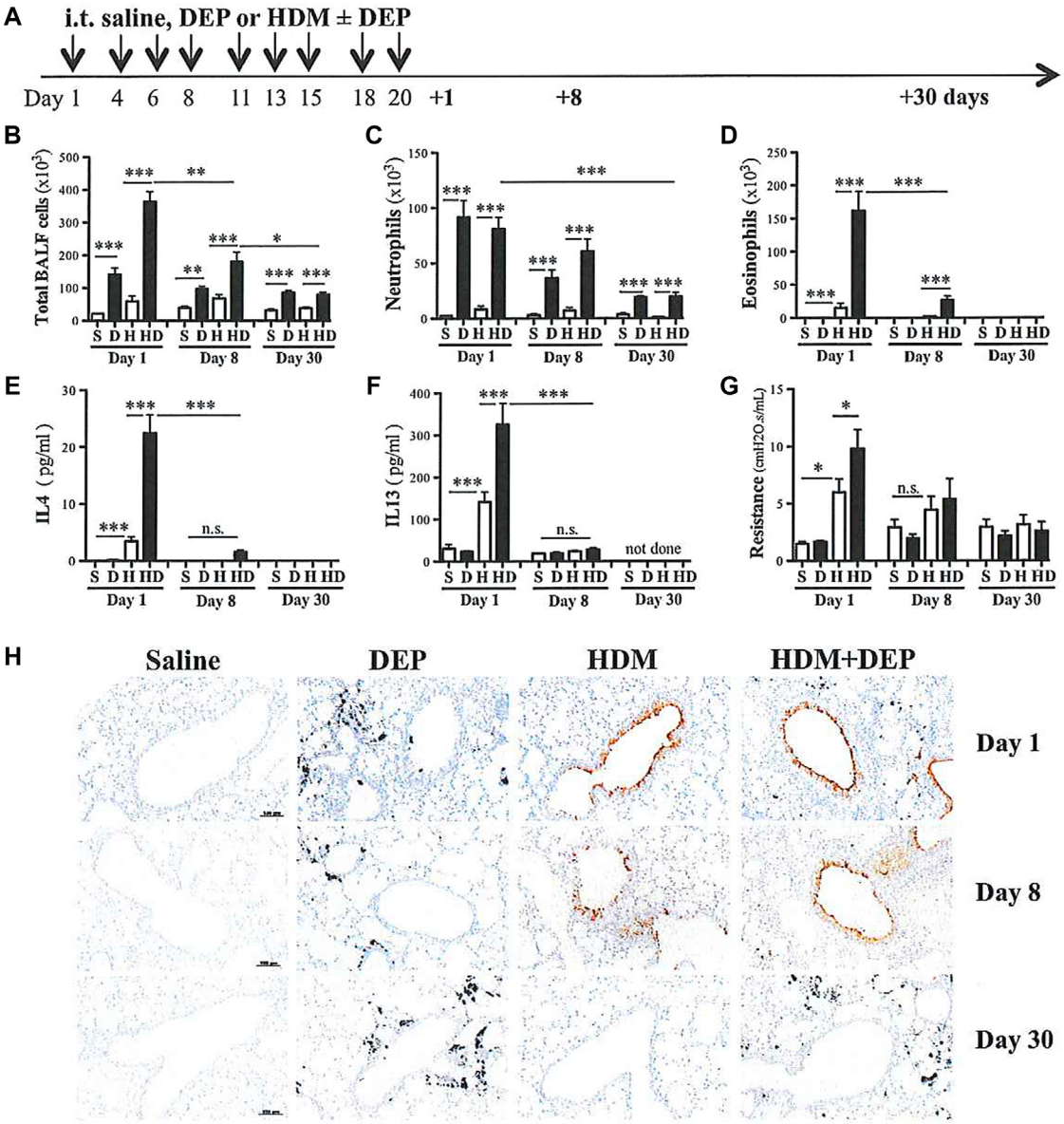


**FIG 1.** DEP exacerbation of HDM-induced airway $T_H2$ responses returns to baseline within a month. **A,** Protocol: BALB/c mice were exposed intratracheally *(i.t.)* to 50 μL of saline, DEP (150 μg), and/or HDM (10 μg) 3 times a week over a 3-week period. **B-D,** Mice were killed 1, 8, and 30 days after the last exposure, and total BALF cell levels (Fig 2, *B*), as well as BALF neutrophil (Fig 2, *C*) and eosinophil (Fig 2, *D*) counts, were assessed (3 separate experiments with a total of n = 11-16 mice per group [day 1], n = 7-12 mice per group [day 8], and n = 5-8 mice per group [day 30]). **E** and **F,** BALF levels of IL-4 (Fig 1, *E*) and IL-13 (Fig 1, *F*) were assessed by using the multiplex assay and IL-13/IL-13Rα2 ELISA, respectively. **G,** Airway resistance to methacholine challenges was assessed by using flexiVent. **H,** Representative photomicrographs of CLCA3-stained lung sections. Scale = 100 μm. *D*, DEP, *H*, HDM; *n.s.*, not significant; *S*, saline. $*P < .05$, $**P < .01$, and $***P < .001$, 1-way ANOVA with the Bonferroni multiple comparison test.

*B*). One month after the last HDM plus DEP exposure, an increased proportion of $CD44^+$ effector/memory $CD4^+$ T cells was still observed in the lungs. A similar expression pattern was observed for CD69 (Fig 2, *C*), which is in accordance with its expression by $CD44^+$ effector cells and tissue memory cells.[14]

To establish the nature of these lung effector/memory T cells, using flow cytometry, we assessed their ability to make IFN-γ, IL-13, and/or IL-17A after *ex vivo* stimulation with phorbol 12-myristate 13-acetate and ionomycin in the presence of Brefeldin A. One month after the last exposure, the proportion of IL-$13^+$ $T_H2$ lung cells was significantly greater in HDM and DEP–coexposed mice versus that seen in mice exposed only to HDM (Fig 3, *A*). The proportion of IL-$17A^+$ $T_H17$ cells was increased in mice exposed to DEPs, either alone or in combination with HDM (Fig 3, *A*). Interestingly, coexposure to HDM and DEPs resulted in higher levels of IL-13 and IL-17A double-positive T cells compared with those seen in mice exposed to HDM alone (Fig 3, *B*). IFN-$\gamma^+$ $T_H1$ cell counts remained unchanged (Fig 3, *A*). The $CD4^+CD44^+$ T cells expressed higher levels of IL-7 receptor (CD127) than the naive

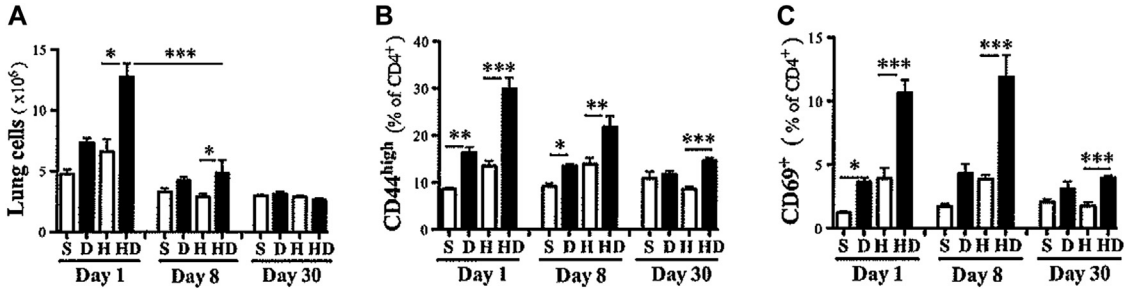


**FIG 2.** Persistent increase in lung effector/memory T-cell counts after HDM plus DEP exposure. Characterization of lung T cells 1, 8, and 30 days after the last exposure to saline *(S)*, DEP *(D)*, HDM *(H)*, or HDM plus DEP *(HD)* by using fluorescence-activated cell sorting. **A,** Total lung cells. **B** and **C,** $CD4^+CD44^+CD62L^-$ T cells (Fig 2, *B*) and CD69 expression (Fig 2, *C*) by effector/memory T cells (2 separate experiments with 2-8 mice per group). $*P < .05$, $**P < .01$, and $***P < .001$, 1-way ANOVA with the Bonferroni multiple comparison test.

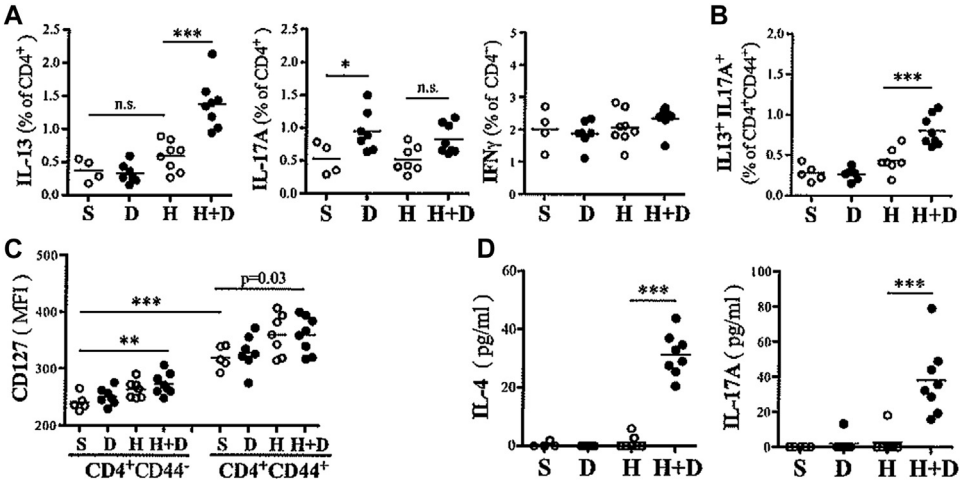


**FIG 3.** Increased presence of memory $T_H2$ and $T_H17$ cells in the lungs 1 month after coexposure to HDM and DEP. **A,** $T_H1$, $T_H2$, and $T_H17$ lung cells were identified by means of fluorescence-activated cell sorting as $CD4^+$ T cells expressing IFN-γ, IL-13, and IL-17A, respectively, after *ex vivo* stimulation with phorbol 12-myristate 13-acetate/ionomycin for 4 hours. **B** and **C,** Percentage of $IL\text{-}13^+IL\text{-}17^+$ double producers among $CD4^+CD44^+$ T cells (Fig 3, *B*) and IL-7 receptor mean fluorescence intensity (*CD127 [MFI]*; Fig 3, *C*) on naive and $CD4^+CD44^+$ T cells. Fig 3, *C*, IL-4 and IL-17A were detected by means of ELISA in supernatants of lung cells stimulated with HDM (30 μg/mL) for 5 days (n = 4-8 mice per group). $*P < .05$, $**P < .01$, and $***P < .001$, 1-way ANOVA with the Bonferroni multiple comparison test (*P* values were calculated by using the *t* test). *D*, DEP, *H*, HDM; *n.s.*, not significant; *S*, saline.

$CD4^+CD44^-$ T cells, pointing to a memory phenotype notably among $CD4^+$ T cells from mice pre-exposed to HDM plus DEPs (Fig 3, *C*). Together, these findings suggest the presence of higher numbers of HDM-specific memory $T_H2$ cells in the lungs of HDM and DEP–coexposed mice versus those seen in mice exposed to HDM alone. To support this, we assessed HDM recall responses *in vitro*. Lung cells from HDM and DEP–coexposed mice secreted significantly more IL-4 and IL-17A compared with levels seen in mice exposed to HDM alone (Fig 3, *D*). Similar findings were made in mice that were killed 45 days after the last allergen exposure (see Fig E2 in this article's Online Repository at www.jacionline.org).

## Increased HDM recall responses in lymphoid organs are not dependent on the continued presence of DEPs

In contrast to HDM, which is rapidly degraded, DEPs, or at least their carbon cores, persist within phagocytic cells for weeks.[25] We assessed how long DEPs persisted in lungs after a single DEP exposure and found numerous DEP-positive cells in the lungs 3 months later. Furthermore, more than half of all BALF macrophages still contained DEPs, although less on a per cell basis (see Fig E3 in this article's Online Repository at www.jacionline.org). Similar observations were made in mice that were killed 45 days after the last allergen exposure (see Fig E2).

Although lymph node cells containing DEPs were observed 45 days after the last DEP exposure, the size and cellular count of these lymph nodes were comparable with those of lymph nodes from saline-exposed mice (see Fig E4, *A* and *B*, in this article's Online Repository at www.jacionline.org). On restimulation with HDM, only mice exposed to both HDM and DEPs secreted large amounts of cytokines, most notably IL-4 and IL-17A (see Fig E4, *C*). Similarly, on HDM restimulation, splenocytes from mice exposed to HDM plus DEPs secreted significantly more IL-4 and IL-17A compared with that seen in mice exposed only to HDM (see Fig E4, *D*).

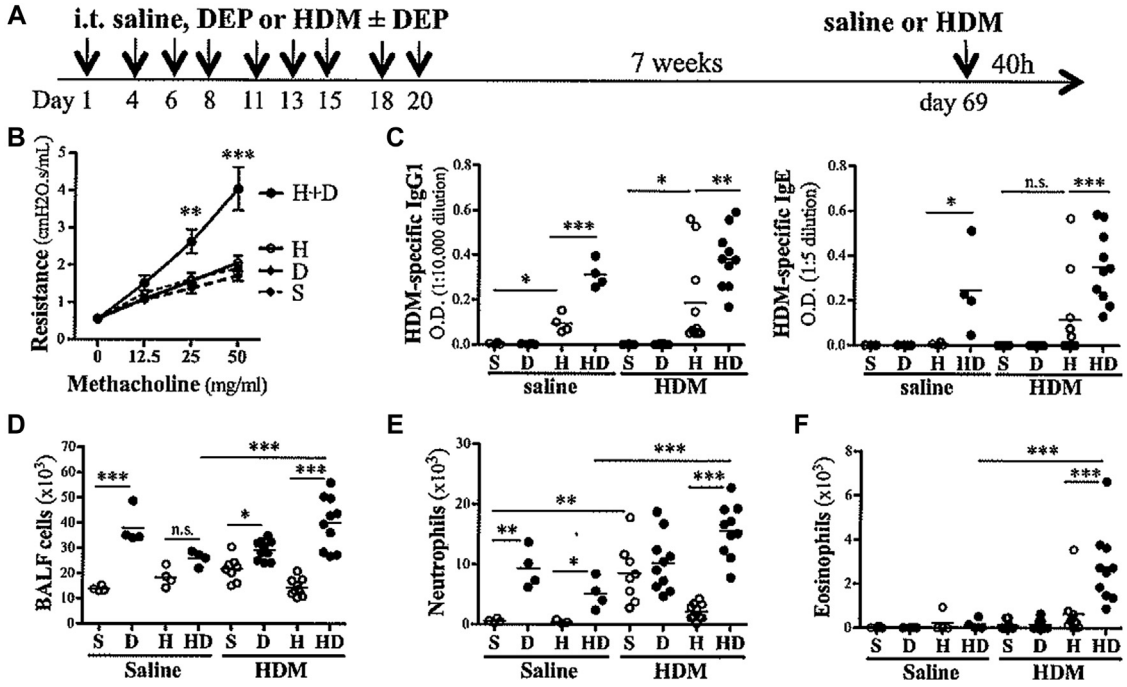


**FIG 4.** Primary exposure to HDM and DEP potentiates secondary HDM-specific responses *in vivo*. **A,** Protocol: A single intratracheal *(i.t.)* challenge with either saline or 10 μg of HDM was performed 7 weeks after the last exposure to either saline, DEP, HDM, or HDM plus DEP. **B,** AHR was assessed 40 hours after HDM recall challenge (2-way ANOVA). **C,** HDM-specific $IgG_1$ and IgE levels 7 weeks after primary exposure. **D-F,** Total BALF cell (Fig 4, *D*), neutrophil (Fig 4, *E*), and eosinophil (Fig 4, *F*) counts (n = 4-10 mice per group). $*P < .05$, $**P < .01$, and $***P < .001$, 1-way ANOVA with the Bonferroni multiple comparison test. *D*, DEP, *H*, HDM; *n.s.*, not significant; *S*, saline.

Because lungs, lymph nodes, and, to a lesser extent, spleens from DEP (± HDM)–exposed mice contain DEP-loaded phagocytic cells, we could not exclude that the presence of these DEP-positive cells within our cultures was responsible for the observed increase in IL-4 and IL-17A cytokine production rather than the presence of higher numbers of $T_H2/T_H17$ effector/memory cells. To address this, we used a highly enriched population of $CD4^+$ splenocytes from mice exposed 45 days before to either saline, DEP, HDM, or HDM plus DEP and restimulated them *in vitro* with plate-bound anti-CD3 (see Fig E4, *E*). After 3 days of culture, significantly more IL-4 and IL-17A was secreted by $CD4^+$ T cells originating from HDM and DEP–coexposed mice compared with that seen in HDM-exposed mice (see Fig E4, *E*), supporting the presence of more $T_H2$ and $T_H17$ cells in the spleens of HDM and DEP–coexposed mice.

## DEP and HDM coexposure promotes increased HDM recall responses *in vivo*

Based on our findings demonstrating that mice previously coexposed to HDM and DEPs have significantly more HDM-specific T cells present both locally and in lymphoid tissues 6 to 7 weeks after the last allergen exposure, we exposed these mice to a single dose of HDM and assessed allergic responses 40 hours later (Fig 4, *A*). Mice previously exposed to saline, DEPs, or HDM alone did not mount an allergic response to this HDM challenge. In contrast, mice previously coexposed to HDM and DEP had increased AHR (Fig 4, *B*).

We previously demonstrated that DEPs exacerbate not only allergic T-cell responses but also B-cell responses, as evidenced by increased HDM-specific $IgG_1$ and IgE plasma levels in mice coexposed to HDM and DEP compared with levels in mice exposed only to DEPs.[6] Similar observations were made 7 weeks after 9 exposures to either HDM or HDM and DEPs (Fig 4, *C*), suggesting continued production of HDM-specific IgE and $IgG_1$ antibodies.

In addition to the chronic neutrophilic inflammation observed in the lungs of mice exposed to DEPs, secondary HDM challenge induced additional cellular recruitment into the airways (Fig 4, *D* and *E*). In mice previously coexposed to HDM and DEPs, this secondary HDM challenge also induced a significant recruitment of eosinophils into the BALF (Fig 4, *F*). Accordingly, these HDM and DEP–pre-exposed mice had increased eotaxin-1 BALF levels after HDM recall compared with those seen in mice only exposed to HDM (Fig 5, *A*).

BALF $T_H2$ cytokine (IL-4, IL-5, and IL-13) levels after secondary HDM challenge were increased in mice with primary exposure to HDM and DEPs compared with those exposed only to HDM (Fig 5, *B*). Similarly, BALF IFN-γ levels were also increased in mice previously exposed to HDM and DEPs (Fig 5, *C*). IL-17A BALF levels were mostly less than detection levels, but levels of the pro-$T_H17$ cytokine IL-6 were also increased in mice previously exposed to both HDM and DEPs (data not shown and Fig 5, *D*). IFN-$\gamma^+$ cells were not predominantly $CD4^+$ T cells, whereas IL-$13^+$ and IL-$17A^+$ cells were mostly $CD4^+CD44^+$ effector T cells (data not shown). $T_H2$ and $T_H17$ cell numbers were increased in the lungs of mice previously exposed to both HDM and DEPs (Fig 5, *E*).

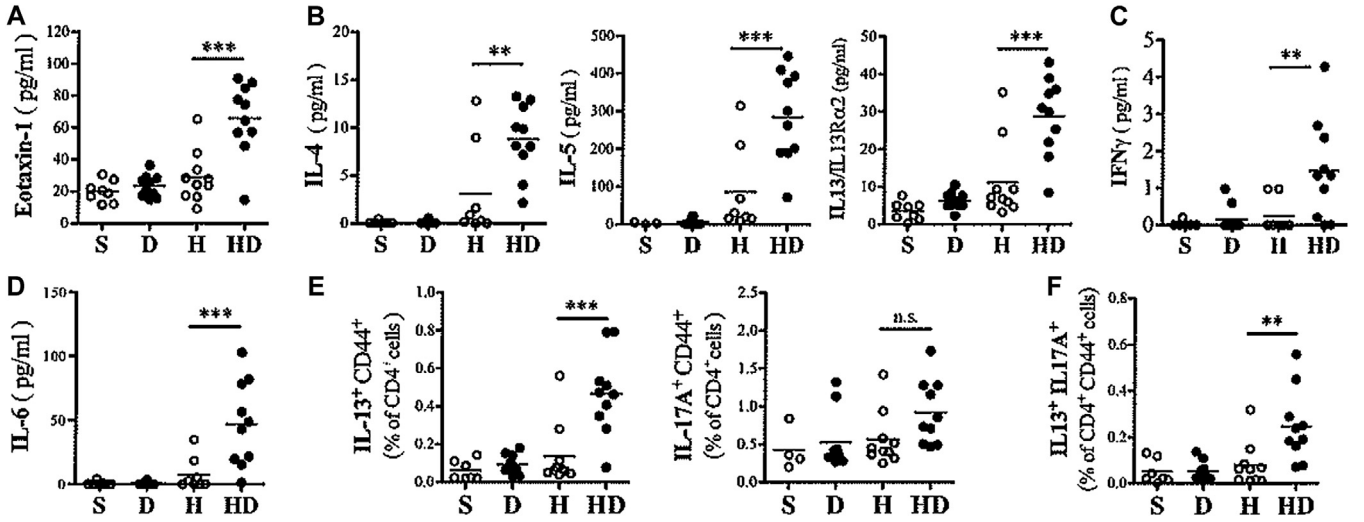


**FIG 5.** Increased presence of HDM-specific memory cells 7 weeks after coexposure to HDM plus DEP results in increased $T_H2/T_H17$ responses on secondary HDM challenge. **A-D,** BALF levels of eotaxin-1 (Fig 5, *A*), $T_H2$ cytokines (IL-4, IL-5, and IL-13; Fig 5, *B*), the $T_H1$ cytokine IFN-γ (Fig 5, *C*), and the $T_H17$-related cytokine IL6 (Fig 5, *D*; n = 8-10 mice per group). **E,** $T_H2$ and $T_H17$ lungs cells were identified by means of fluorescence-activated cell sorting as $CD4^+CD44^+$ T cells expressing IL-13 and IL-17A, respectively, after *ex vivo* stimulation with phorbol 12-myristate 13-acetate/ionomycin for 4 hours. **F,** Percentage of $IL\text{-}13^+IL\text{-}17^+$ double-producing $CD4^+CD44^+$ T cells (n = 4-6 mice per group). $^{**}P < .01$ and $^{***}P < .001$, 1-way ANOVA with the Bonferroni multiple comparison test. *D*, DEP, *H*, HDM; *n.s.*, not significant; *S*, saline.

Accordingly, effector/memory T cells coexpressing IL-13 and IL-17A were more numerous in the lungs of mice previously exposed to both HDM and DEPs compared with mice previously exposed to HDM only (Fig 5, *F*). Taken together, these results demonstrate that the increased accumulation of HDM-specific memory cells in the lungs of mice that had been previously exposed to both HDM and DEPs versus those exposed to HDM only, promotes increased HDM recall responses *in vivo*.

## Primary neonatal coexposure to HDM and DEPs promotes increased HDM recall responses

Because exposure to DEPs increases the severity of allergic asthma in young mice and prenatal exposure increases asthma susceptibility,[24,26] we next examined the effect of DEP coexposure on recall responses in neonatal mice. Three-day-old neonatal mice were subjected to 9 daily intranasal exposures to either saline, DEPs, HDM, or HDM plus DEPs. Then, after a 4-week rest period, the mice were challenged twice with HDM (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org). Airway resistance was significantly increased in mice previously exposed to both HDM and DEPs compared with those exposed to HDM alone (Fig E5, *B*). Total BALF cell counts were similarly increased after HDM recall exposures in mice previously exposed to HDM or HDM plus DEPs, including similar eosinophil BALF levels between the 2 groups, despite increased eotaxin-1 BALF levels in the HDM plus DEP–pre-exposed mice (data not shown and Fig E5, *C*). In accordance with increased AHR, mice previously exposed to HDM and DEPs had higher BALF levels of $T_H2$ cytokines (IL-4, IL-5, and IL-13; see Fig E5, *D*). IL-6 and IL-17A BALF levels were also significantly increased in mice pre-exposed to HDM and DEPs compared with those in mice with primary exposure to HDM alone (Fig E5, *E*). HDM sensitization in neonatal mice appeared weaker than that in adult mice and did not demonstrate increased HDM-specific $IgG_1$ levels in neonates coexposed to HDM and DEPs (see Fig E5, *F*). Overall, the results in neonatal mice paralleled our observations in adult mice supporting that exposure to DEPs promotes asthma development in the context of allergen exposure and allergic sensitization.

## Exposure to high DEP levels in infants contributed to sensitization earlier in life

We then hypothesized that if DEP exposure potentiates recall responses to allergen, early-life DEP exposure might promote development of allergic asthma in children. To test this hypothesis, we used the CCAAPS birth cohort, an existing birth cohort of 762 children, 578 with known exposure levels to traffic-related pollution, notably elemental carbon attributable to traffic (ECAT); sensitization to aeroallergens; and development of asthma.

First, we examined whether ECAT exposure affected allergic sensitization. Overall, birth ECAT levels were not associated with aeroallergen sensitization during the first 4 years of life (70% vs 68%, $P = .66$). However, when each year was analyzed independently, children exposed to high ECAT levels at birth were more likely to be aeroallergen sensitized at age 2 years (47% vs 35%, $P = .011$) and maintained this high level of sensitization at ages 3 (47%) and 4 (48%) years, whereas the proportion of children sensitized to an aeroallergen and exposed to low ECAT levels increased gradually from age 1 to 4 years (18%, 35%, 42%, and 51%, respectively; Fig 6, *A*). This suggests that although high ECAT exposure at birth does not contribute to overall higher rates of sensitization, it does contribute to sensitization earlier in life.

## Exposure to high DEP levels in infants contributed to significantly greater asthma prevalence at age 7 years

We next examined children coexposed to aeroallergen and ECAT versus children exposed to allergen alone to determine

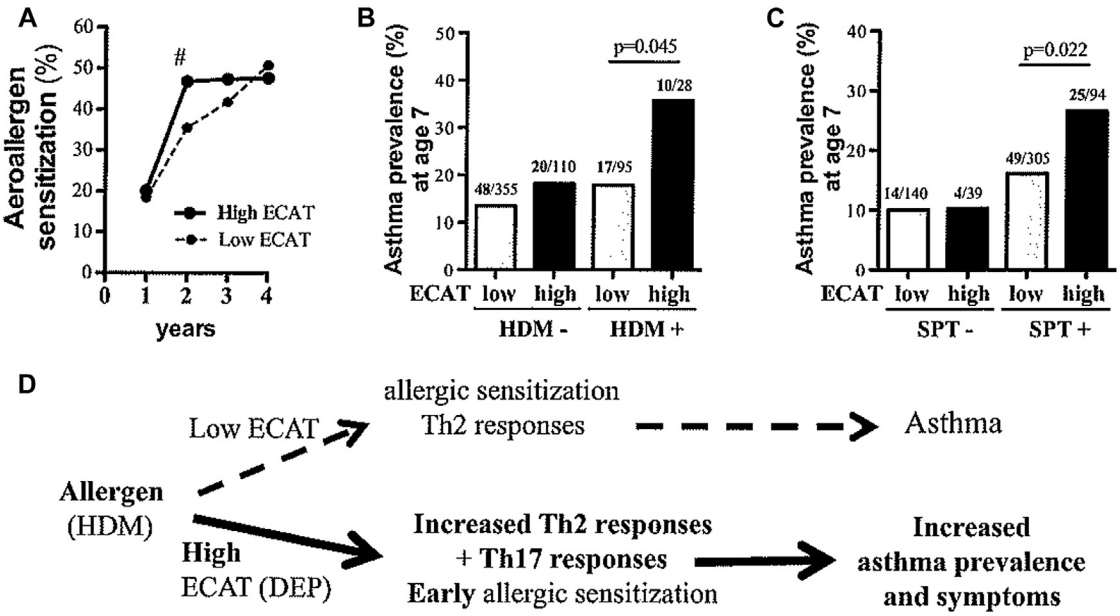


**FIG 6.** Exposure to high birth ECAT levels contributed to significantly higher asthma prevalence at age 7 years. **A,** Children exposed to high ECAT levels are more likely to be sensitized to an aeroallergen at age 2 years compared with children exposed to low ECAT levels (35% vs 47%). #$P = .011$. **B** and **C,** Children exposed to high ECAT levels (top quartile) that were sensitized to HDM (Fig 6, *B*) or had positive SPT responses (Fig 6, *C*) to any of the 15 aeroallergens tested in the first 4 years of life were significantly more likely to have asthma by age 7 years ($P = .045$ and $P = .022$, respectively). All *P* values were calculated by using $\chi^2$ tests and SAS software; numbers of asthmatic patients/total cases in each group are presented above asthma prevalence bars. **D,** Proposed model.

whether coexposure to high ECAT levels during the first year of life (similar to DEP plus HDM coexposure in our mouse model) would result in increased asthma prevalence at age 7 years. First, we specifically examined HDM sensitization (proxy for HDM exposure) and ECAT coexposure (Fig 6, *B*). Among children sensitized to HDM by age 4 years, coexposure to high ECAT levels at birth (defined as the top 25%) doubled the risk of asthma development by age 7 years compared with that seen in those exposed to low ECAT levels (defined as the lower 75%; 36% vs 18%, $P = .045$). HDM sensitization did not significantly increase the risk of asthma in children exposed to low DEP levels (14% vs 18%, $P = .277$). To assess whether our findings with HDM sensitization could be extended to other allergens, we included sensitization to any aeroallergen at age 1 through 4 years. We found a significant increase in asthma prevalence at age 7 years (16% vs 29%, $P = .02$) in children coexposed to high ECAT levels at birth compared with that in children coexposed to low ECAT levels (Fig 6, *C*).

In our logistic model adjusted for race, sex, and mother's education level, we observed a significant increasing prevalence of asthma moving from low ECAT levels and negative SPT responses to high ECAT levels and positive SPT responses (odds ratio, 1.4; 95% CI, 1.1-1.8; $P = .009$). When we restricted this adjusted analysis to just those subjects with positive SPT responses, we still observed a trend of increased asthma prevalence with high ECAT exposure (odds ratio, 1.5; 95% CI, 0.8-2.7; $P = .18$), even though the sample size was reduced by 31% in this restricted analysis. Although early atopy and high birth ECAT exposure are independent risk factors for asthma development at age 7 years (10% vs 19% [$P = .01$] and 14% vs 22% [$P = .04$], respectively), coexposure of aeroallergen sensitization at ages 1 to 4 years and high ECAT levels at birth results in an almost 3-fold higher risk of asthma compared with that seen in those with negative SPT responses (10% vs 27%, $P = .038$; Fig 6, *C*). The results were similar when average ECAT levels for the first 7 years of life were used and when HDM was excluded from the definition of early atopy (data not shown). However, there was no difference in asthma prevalence when we evaluated current ECAT exposure levels, indicating that high birth ECAT levels are driving this association. Taken together, these findings support that the effect of ECAT exposure is not specific to HDM coexposure but rather to any environmental allergen and suggest that exposure to high levels of ECAT at birth increases susceptibility to allergic asthma.

## DISCUSSION

In this study we demonstrate that DEP exposure promotes increased numbers and persistence of allergen-specific memory T cells in murine lungs. These memory T cells, which are poised to produce increased quantities of $T_H2$ cytokines (IL-4 and IL-13) and IL-17A, the prototypical $T_H17$ cytokine, generate a strong and rapid response on secondary exposure to allergen in adult and neonatal mice. In allergen-exposed and sensitized children in the CCAAPS birth cohort, coexposure to high ECAT levels in the first year of life was associated with earlier allergen sensitization and increased asthma prevalence. Collectively, these data support that exposure to DEP/ECAT results in early sensitization and accumulation of allergen-specific $T_H2/T_H17$ memory/effector cells in the lungs, thereby potentiating secondary allergen recall responses and development of allergic asthma (Fig 6, *D*).

These effects of DEPs/ECAT at different points in the allergic inflammatory cascade act to amplify the adverse health effects of DEP/ECAT exposure. Indeed, we have previously demonstrated that increased exposure to traffic pollution in the CCAAPS birth cohort increases wheezing at age 3 years.[21] We now report that early-life exposure to high DEP/ECAT levels is associated with increased prevalence of asthma at age 7 years in the context of aeroallergen sensitivity. Although allergic sensitization is a known risk factor for asthma, it alone does not necessarily lead to allergic disease.[27] After adjusting for sex, race, and mother's education, we observed a 50% increasing trend in asthma risk in children with positive SPT responses who were exposed to high compared with low ECAT levels, further supporting our results. Taken together with the limited effect of DEP exposure on humoral immunity in children and neonatal mice, we propose that coexposure to a common aeroallergen (HDM) and traffic-related ultrafine particulate matter (DEPs) early in life potentiates primary and secondary effector/memory $T_H2$ and $T_H17$ responses that promote the development of childhood asthma (Fig 6, *D*). We did not observe an increase in allergic sensitization by age 4 years among children exposed to high ECAT levels. It is likely that other exposures negate the effect of DEPs on sensitization as time ensues. However, earlier sensitization indicates that children exposed to high DEP/ECAT levels are likely to have memory effector cells parked in their lungs earlier, specifically during the critical first year of life, during which exposures might be most harmful. Indeed, these children had the highest prevalence of asthma, suggesting that the timing of exposure and sensitization are critical to the overall outcome of asthma. In this study the most relevant DEP/ECAT exposure was in the first year of life; there was no difference in asthma prevalence when current ECAT exposure level was used.

Repeated coexposure to both HDM and DEPs induced a significantly stronger effector $T_H2$ response characterized by increased BALF levels of $T_H2$ cytokines and eosinophils than exposure to HDM alone, resulting in increased AHR (Fig 1). When these mice are no longer exposed to HDM, this HDM-specific response is no longer needed, and most effector T cells die. Indeed, 8 days after the last exposure, $T_H2$ cytokines are only detectable in HDM and DEP–coexposed mice, reflecting the time needed to return to baseline for $T_H2$ effector responses of different amplitude (Fig 1). It is well established that after the contraction phase, memory $CD8^+$ T-cell populations are maintained, whereas memory $CD4^+$ T-cell counts slowly decrease over time.[10,28] The evidence linking a stronger effector T-cell response to the formation of a larger memory T-cell pool has been mostly limited to $CD8^+$ T cells.[17,29] However, the nature and size of the $CD4^+$ primary response also influences the nature and size of the memory $CD4^+$ T-cell pool.[18,30] Because HDM and DEP coexposure results in a greater accumulation of $CD4^+$ effector T cells in the lungs compared with exposure to HDM alone and assuming a similar rate of contraction of the effector pool, it is not surprising that the contraction phase would result in higher levels of HDM-specific memory T cells in the lungs. Indeed, a month after the last exposure, significantly more $T_H2$ cells were present in the lungs, resulting in a stronger HDM recall responses (Fig 3). Similar results were observed 45 days after the last exposure, and thus the observations are not likely to be due to a delayed contraction phase (see Fig E2).

We extended our findings to neonatal mice because early-life exposures to DEPs have been postulated to be most relevant. Similar to our observations in adult mice, AHR and airway inflammation (BALF cell counts and $T_H2$ cytokine levels) were significantly increased in mice coexposed to HDM and DEPs compared with those exposed to HDM alone. In a previous report neonatal exposure to nebulized OVA resulted in tolerance (absence of AHR), whereas coexposure to the air pollutant residual oil fly ash, a component of ambient particulate matter derived from oil-burning power plants, resulted in increased AHR both during primary challenge and after secondary exposure 10 days later.[31] In contrast, when neonatal mice were repeatedly pre-exposed to combustion-derived particulate matter (CDPM) before being coexposed to CDPM and HDM or CDPM and OVA in OT-II mice, tolerance was observed in these young C57Bl/6 mice.[32] However, when the CDPM and OVA–coexposed OT-II neonates were allowed to reach adulthood, a secondary OVA recall response generated AHR only in mice previously coexposed to CDPM and OVA as neonates.[32] This was associated with accumulation of $CD4^+$ T cells expressing IL-4 or IL-17A in the lungs, which is similar to our findings.[32]

We have previously demonstrated that IL-17A contributes to asthma exacerbation and that coexposure to HDM and DEPs increases IL-17A and IL-13 double-producing T-cell numbers.[6] These double-producing cells have been shown to be more pathogenic than classic $T_H2$ cells and exacerbate chronic allergic asthma.[9] A recent study revealed that asthmatic patients have a higher frequency of dual $T_H2/T_H17$ cells in their BALF compared with healthy control subjects and that these cells are more resistant to steroids *in vitro*.[33] Additionally, asthmatic patients with a predominant $T_H2/T_H17$ phenotype had more severe airway hyperreactivity than asthmatic patients with a $T_H2$ phenotype.[33] In the present study we demonstrate that HDM and DEP coexposure results in persistent accumulation of memory $T_H2$ cells in the lungs, as well as increased $IL\text{-}17A^+IL\text{-}13^+CD44^+CD4^+$ T-cell counts. The increased presence of these double-producing $CD4^+$ T cells 6 weeks after the last HDM and DEP coexposure taken together with the data demonstrating increased HDM-induced IL-17A and IL-4 secretion in lung cells from mice previously exposed to both HDM and DEPs suggest that these double-producing memory cells might play a significant role in the increased AHR observed after secondary HDM challenge (Fig 4).

In summary, our data demonstrate that DEP and HDM coexposure results in increased accumulation of HDM-specific memory $T_H2$ and $T_H17$ cells in the lungs, potentiating secondary recall responses and promoting asthma development. This might contribute to the increased prevalence of allergic asthma observed in children with early exposure to high levels of traffic-related DEPs. This raises the possibility of asthma prevention by minimizing DEP exposure in young children or by counteracting the effect of DEP exposure with early intervention (perhaps by targeting IL-17A) in high-risk sensitized and DEP-exposed children.

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**Key messages**

- **DEPs persist in murine lungs months after exposure and are associated with increased BALF IL-17A levels and chronic pulmonary neutrophilia.**
- **DEP exposure exacerbates HDM-induced allergic airway responses in neonatal and adult mice, resulting in increased effector/memory T-cell accumulation in the lungs and potentiating HDM recall responses *in vitro* and *in vivo*.**
- **Exposure to high DEP levels in early life and coexposure to aeroallergen sensitization by age 4 years promotes development of allergic asthma at age 7 years.**
- **Exposure to DEPs at birth is associated with earlier sensitization in young children.**

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# METHODS

## Murine asthma model

Wild-type 5- to 7-week-old BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, Me). HDM extract *(Dermatophagoides pteronyssinus)* was purchased from Greer Laboratories (Lenoir, NC). DEPs were generated from a 4-cylinder Deutz diesel engine at the US Environmental Protection Agency (Research Triangle Park, NC); detailed characterization of this compressor DEP has been described elsewhere and compared with other sources of DEPs.[E1] Mice received either 50 μL of saline, 150 μg of DEPs, 10 to 13 μg of HDM extract (representing 3.3 μg of protein, 1.1 μg of Der p 1, or 0.5 EU of endotoxin) or both intratracheally 3 times a week for 3 weeks and were killed 1, 8, 30, or 45 to 50 days after the last exposure (Figs 1, *A*, and 4, *A*). The mice were maintained and handled under Institutional Animal Care and Use Committee–approved procedures (Cincinnati Children's Hospital Medical Center) and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council).

## AHR

Mice were anesthetized with ketamine, xylazine, and acepromazine (100, 20, and 10 mg/mL, respectively, mixed at a ratio of 4:1:1). Invasive measurements of airway responsiveness were made with the flexiVent apparatus (SCIREQ, Montreal, Quebec, Canada). Mouse tracheas were cannulated with a 19-guage blunt needle, and the mice were ventilated at 150 breaths/min and 3.0 cm $H_2O$ 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 was assessed after exposure to increasing concentrations of aerosolized methacholine (0, 12.5, 25, and 50 mg/mL). The average of the 3 highest dynamic resistance values with a coefficient of determination of 0.9 or greater (as determined by using flexiVent software) was used to determine the dose-response curve.

## BALF cell collection and analysis

Bronchoalveolar lavage was performed by means of tracheal cannulation. Lungs were lavaged with 1 mL of PBS plus 0.5% BSA plus 2 mmol/L EDTA. The collected BALF was centrifuged, and total cell numbers counted with a hemocytometer. Cells were spun onto slides and stained with the HEMA3 stain set (Fisher Scientific, Kalamazoo, Mich). After the slides were cover slipped and deidentified, 200 cells were counted, and the total number of each cell type was calculated. To estimate the amount of DEPs present in each alveolar macrophages, we established a scale from 0 to 4, where 0 is no visible DEP particles, 1 is less than 10 visible DEP aggregates within the cell, 2 is less than one third of the cytoplasm filled with DEPs, 3 is between 33% and 75% loaded with DEPs, and 4 is more than 75% of the cell surface covered with DEPs (an example is presented in Fig E3).

## BALF cytokine measurements

BALF cytokine levels were assessed by using Luminex xMAP technology (Millipore, Billerica, Mass) with Cytokine/Chemokine Panel I, according to the manufacturer's instructions. Total BALF IL-13 levels (free and IL-13 receptor [IL-13R] α2 bound) were measured, as previously described.[E2] Briefly, wells were coated with 50 μL of anti–IL-13 (4 μg/mL; AF-413-NA; R&D Systems, Minneapolis, Minn) overnight at 4°C. Afterward, free IL-13 present in BALF samples was complexed to recombinant IL-13Rα2/Fc chimera (10 ng/mL; 539-IR, R&D Systems) after 1 hour's incubation at 37°C, and samples are wedded to wells and incubated for 2 hours at room temperature, followed by a 1-hour incubation with biotinylated IL-13Rα2 (100 ng/mL; BAF539, R&D Systems) and a 30-minute incubation with streptavidin–horseradish peroxidase (1:200; DY998, R&D Systems). Finally, TMB substrate (OptEIA; BD Biosciences, Franklin Lakes, NJ) was added to each well and incubated in the dark for 15 to 25 minutes. The reaction was stopped with 2N $H_2SO_4$, and changes in OD were measured at 450 nm.

## Histology and immunohistochemistry

The left lobe of the lung was fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Sections were stained by means of a periodic acid–Schiff reaction, according to the manufacturer's recommendations (Poly Scientific R&D Corp, Bay Shore, NY). Goblet cells were identified by using CLCA3 (gob5) immunostaining with a rabbit polyclonal anti-mouse CLCA3 antibody (ab46512 1:10,000; Abcam, Cambridge, Mass), as previously described.[E3]

## HDM-specific $IgG_1$ and IgE ELISAs

Blood was collected in K2EDTA microtainer tubes (BD) and centrifuged for 6 minutes at 3000 rpm, and plasma was transferred to Eppendorf tubes and stored at −20°C until use. Plasma HDM-specific IgE and $IgG_1$ levels were measured by means of ELISA. Briefly, an ELISA plate was coated overnight with 0.01% HDM in PBS. After washing 3 times with 0.05% Tween 20, wells were blocked for 1 to 2 hours with 200 μL of PBS/BSA 1%. For HDM-specific IgE, plasma was diluted one fifth in 100 μL of PBA/BSA 1%, whereas for HDM-specific $IgG_1$, plasma samples needed to be diluted at least 1:2,000 and 1:10,000 before being added to wells and incubated for 2 hours. After washing, biotin–anti-mouse IgE (2 μg/mL R35-118) or biotin–anti-mouse $IgG_1$ (2 μg/mL A85-1; BD PharMingen, San Diego, Calif) was added for 1 hour, followed by washing and addition of streptavidin–horseradish peroxidase (1:100, R&D Systems) for 30 minutes. After washing 5 times, TMB substrate was added, the reaction was stopped with 2N $H_2SO_4$ after 5 to 10 minutes, and changes in OD were measured at 450 nm.

## 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 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 1 mL of PBS plus 0.5% BSA plus 2 mmol/L EDTA before being counted with the Z2 Coulter particle count and size analyzer. Between $5 \times 10^5$ and $5 \times 10^6$ 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). T cells were stained with combinations of CD4–fluorescein isothiocyanate, CD62L–phycoerythrin (PE), CD69-PE, CD127-PE/Cy7, γδ T-cell receptor–PE/Cy7, CD25-AF647, CD3-AF700, and CD44–Pacific Blue (BioLegend, San Diego, Calif). 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–PerCP5.5 were 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).

## *In vitro* HDM restimulation

Lung cells were isolated, as described above. Spleen cells were passed through a 70-μm cell strainer with a syringe rubber. Lymph nodes were crushed between 2 slides. After red blood cell lysis, cells were counted and plated in round-bottom 96-well plates at a density of 100,000 cells per well in duplicate or triplicate. The cells were then cultured in the presence of HDM (30 μg/mL) for 5 to 6 days. Supernatants were collected, and replicates were pooled before being stored at −20°C.

## $CD4^+$ T-cell isolation, *in vitro* culture, and cytokine measurements

$CD4^+$ T cells were isolated with AutoMACS, according to the manufacturer's instructions. Purity was greater than 90%. The $CD4^+$ T cells were cultured in anti-CD3–coated round-bottom 96-well plates at a density of 100,000 cells per well for 3 days. Supernatants were collected and stored at −20°C until use. IL-4 and IL-17A levels in supernatants were assessed by means of ELISA, according to manufacturer's instructions (ELISA MAX Standard, BioLegend).

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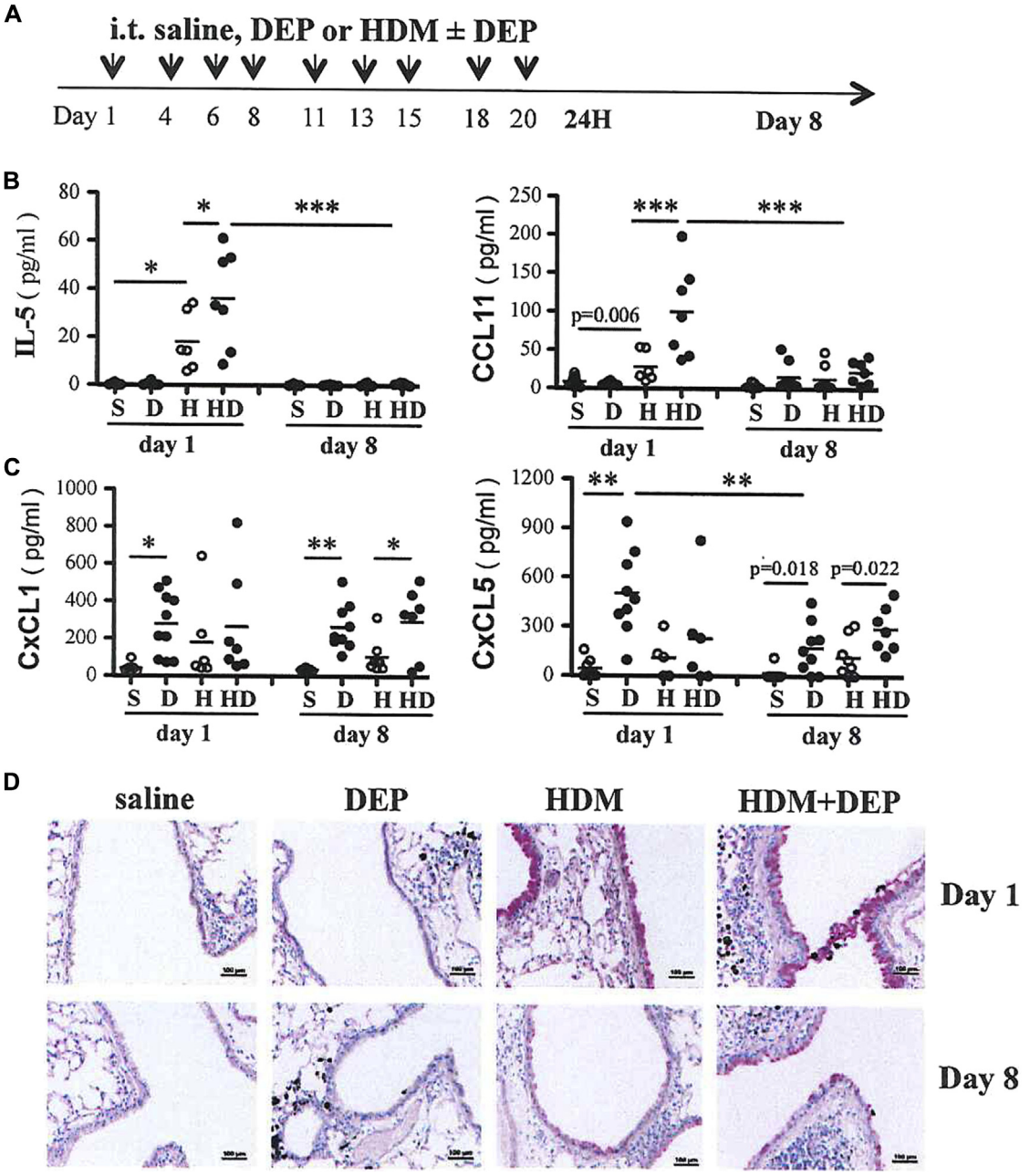


**FIG E1.** HDM-induced eosinophil cytokine levels return to baseline, whereas DEP-induced neutrophil chemokine levels remain increased 8 days after exposure. **A,** Protocol: BALB/c mice were exposed intratracheally *(i.t.)* to saline, DEP (150 μg), and/or HDM (10 μg) 3 times a week over a 3-week period. Mice were killed 1 and 8 days after the last exposure. **B** and **C,** BALF levels of the eosinophil-related cytokines IL-5 and eotaxin-1, as well as BALF levels of the neutrophil chemokines CXCL1 and CXCL5, were assessed by using the multiplex assay (n = 6-10 mice per group). **P* < .05, ***P* < .01, and ****P* < .001, 1-way ANOVA. *D,* DEP, *H,* HDM; *S,* saline. **D,** Representative photomicrographs of lung section stained with periodic acid–Schiff. *Scale* = 100 μm.

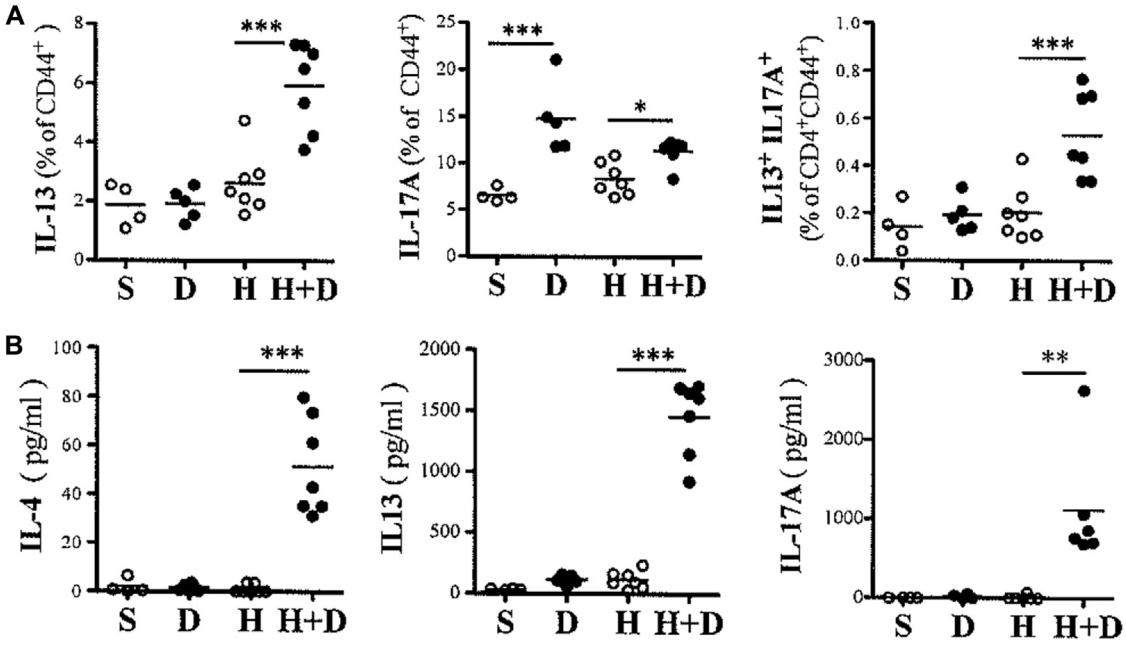


**FIG E2.** Increased presence of HDM-specific memory cells in the lungs 6 to 7 weeks after coexposure to HDM and DEP. **A,** IL-13$^+$ and IL-17$^+$ lung cells were identified by means of fluorescence-activated cell sorting among CD3$^+$CD4$^+$CD44$^+$ T cells after *ex vivo* stimulation with phorbol 12-myristate 13-acetate/ionomycin for 4 hours. **B,** IL-4, IL-13, and IL-17A were detected by using ELISAs in supernatants of lung cells stimulated with HDM (30 μg/mL) for 5 days (n = 4-7 mice per group). **P* < .05, ***P* < .01, and ****P* < .001, 1-way ANOVA with the Bonferroni multiple comparison test.

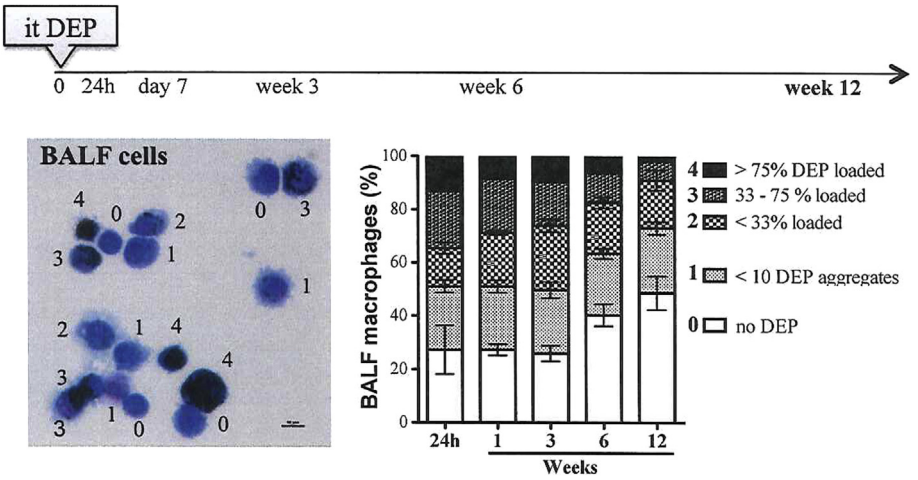


**FIG E3.** DEPs persist in lung phagocytic cells over 3 months after a single DEP exposure. BALB/c mice were exposed intratracheally *(it)* to 50 μL of saline or DEPs (150 μg) once and killed 1 day or 1, 3, 6, or 12 weeks after exposure (n = 4 mice per group). The amount of DEPs present in alveolar macrophages was estimated by using a scale of 0 to 4.

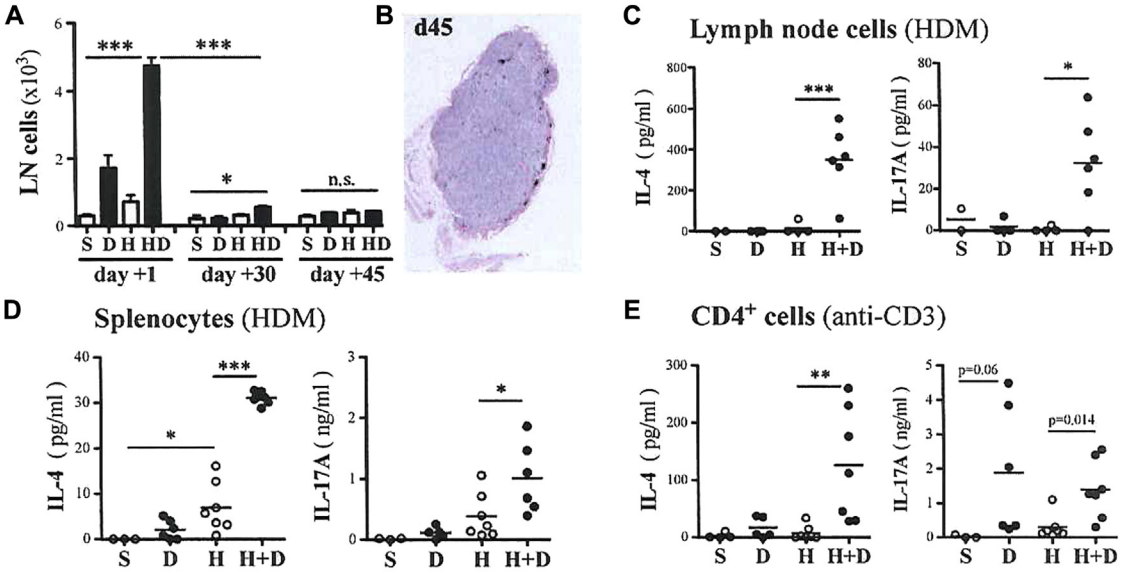


**FIG E4.** Increased presence of HDM-specific cells in draining lymph nodes and spleens 6 to 7 weeks after coexposures to HDM and DEPs. **A,** Lymph node cell numbers 1, 30, and 45 days after exposure to saline, DEP, HDM, or both. **B,** Photomicrograph of lymph nodes 45 days after the last HDM plus DEP exposure. **C** and **D,** IL-4 and IL-17A levels in supernatants of lymph node cells (n = 2-6 mice per group; Fig E4, *C*) or spleen cells (n = 3-7 mice per group; Fig E4, *D*) stimulated with HDM (30 μg/mL) for 6 days. **E,** IL-4 and IL-17A secreted by highly enriched $CD4^+$ T cells from spleen cells stimulated with plate bound anti-CD3 for 3 days (n = 3-7). $*P < .05$, $**P < .01$, and $***P < .001$, 1-way ANOVA with the Bonferroni multiple comparison test; *P* values were calculated by using the *t* test. *D*, DEP, *H*, HDM; *n.s.*, not significant; *S*, saline.

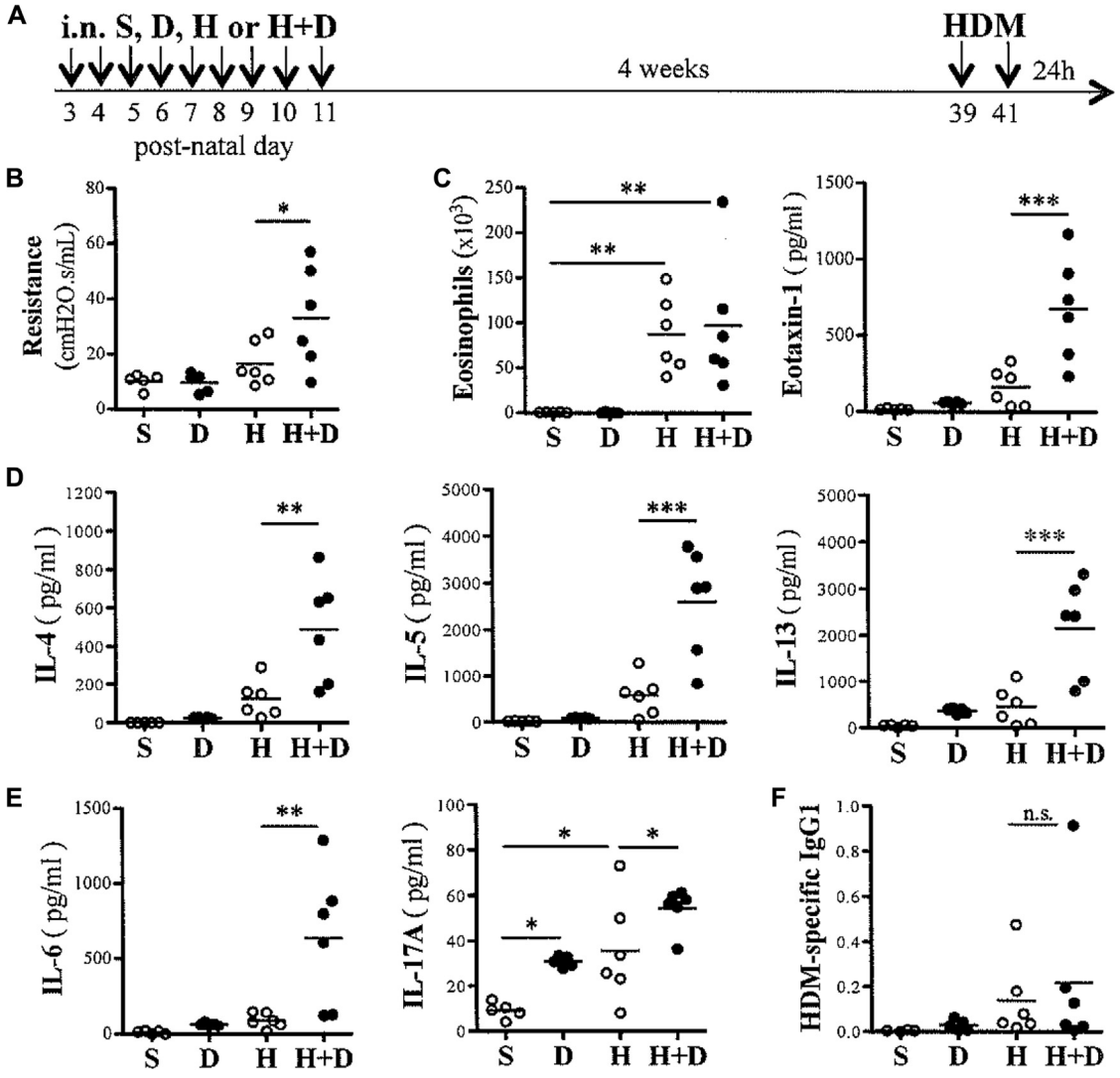


**FIG E5.** Early-life exposure to DEPs and HDM potentiates HDM-specific recall responses in adult mice. **A,** Protocol: Postnatal day 3 pups were exposed intranasally *(i.n.)* for 9 days to either saline, DEP (6 mg/kg), HDM (2 mg/kg), or HDM plus DEP and then rested for 4 weeks before all groups were rechallenged with HDM. **B,** Airway resistance was measured by using flexiVent 24 hours after the last HDM rechallenge. **C,** BALF eosinophil numbers and eotaxin-1 levels. **D** and **E,** BALF $T_H2$ cytokines (IL-4, IL-5, and IL-13; Fig E5, *D*) and $T_H17$-related cytokines (IL-6 and IL-17A; Fig E5, *E*) were assessed by using the multiplex assay (n = 5-6 mice per group). **F,** HDM-specific serum $IgG_1$ levels. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$, 1-way ANOVA with the Bonferroni multiple comparison test. *D*, DEP, *H*, HDM; *n.s.*, not significant; *S*, saline.