Maternal house dust mite exposure during pregnancy enhances severity of house dust mite–induced asthma in murine offspring

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Background: Atopic status of the mother and maternal exposure to environmental factors are associated with increased asthma risk. Moreover, animal models demonstrate that exposure to allergens in strongly sensitized mothers influences offspring asthma development, suggesting that *in utero* exposures can influence offspring asthma. However, it is unclear whether maternal exposure to common human allergens such as house dust mite (HDM), in the absence of additional adjuvants, influences offspring asthma development.

Objective: We sought to determine whether maternal HDM exposure influences asthma development in offspring.

Methods: Pregnant female mice were exposed to PBS or HDM during pregnancy. Using offspring of PBS- or HDM-exposed mothers, the magnitude of HDM or *Aspergillus fumigatus* (AF) extract–induced airway hyperresponsiveness (AHR), airway inflammation, immunoglobulin production, T_H2-associated cytokine synthesis, and pulmonary dendritic cell activity was assessed.

Results: Compared with offspring of PBS-exposed mothers, offspring of HDM-exposed mothers demonstrate increased AHR, airway inflammation, T_H2 cytokine production, and immunoglobulin levels and a modest decrease in the phagocytic capacity of pulmonary macrophage populations following HDM exposure. Increased sensitivity to AF-induced airway disease was not observed. Offspring of HDM-exposed B-cell–deficient mothers also demonstrated increased HDM-induced AHR, suggesting that transfer of maternal immunoglobulins is not required.

Conclusions: Our data demonstrate that maternal exposure to HDM during pregnancy increases asthma sensitivity in offspring in an HDM-specific manner, suggesting that vertical transmission of maternal immune responses may be involved. These findings have important implications for regulation of asthma risk, and suggest that exposure to HDM in the developed world may have underappreciated influences on the overall prevalence of allergic asthma. (J Allergy Clin Immunol 2017;140:1404-15.)

Key words: *Asthma, allergen, pregnancy, maternal exposures, environment*

Allergic asthma is one of the most common immunologic disorders of children in developed nations[1] and more than 300 million people suffer from allergic asthma worldwide.[2] The incidence of asthma has increased over the past few decades.[3] Although there is a genetic component to asthma heritability,[4-7] the rise in asthma incidence is too rapid to be ascribed a purely genetic basis, suggesting that changes in the environment are influencing disease development in genetically susceptible populations.[8-10] Recent studies suggest that exposure to specific environmental stimuli during critical early life periods influence the development of asthma later in life. For example, childhood exposure to microbial products,[11] cigarette smoke,[12,13] or environmental pollutants[14] influences asthma development later in life. This critical developmental window appears to extend into the prenatal period as well, as *in utero* exposures to these factors also influence asthma risk,[13,15-18] as do alterations in maternal diet.[19,20] Similarly, infection with the helminthic parasite *Schistosoma mansoni* increases the development of asthma in offspring if pregnancy was initiated during the "T_H2 phase" of the antiparasitic immune response.[21] Consistent with the existence of such a "prenatal window," maternal asthma is a risk factor for the development of asthma, whereas paternal asthma does not confer as great a risk,[22-26] suggesting that maternal exposures/factors can contribute to asthma development. However, the precise nature of these factors remains unclear.

One important risk factor for the development of asthma is allergen sensitization.[27,28] Given that asthma development can be influenced by maternal asthmatic status,[22-26] and exposures occurring in the prenatal period exert a profound influence on asthma development, we speculate that maternal exposure to allergens themselves may alter asthma development in offspring. Supporting this, presensitization of female mice to ovalbumin (OVA) in the presence of alum and OVA reexposure during pregnancy increased asthma development in offspring of exposed mothers.[29-32] Previous sensitization of female mice to *Aspergillus fumigatus* extract and reexposure during early pregnancy increased airway eosinophilia and IL-4 promoter

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Abbreviations used
AHR: Airway hyperresponsiveness
DC: Dendritic cell
HDM: House dust mite
OVA: Ovalbumin

hypomethylation in grandoffspring of exposed mothers.[33] Intrauterine injection of OVA or house dust mite (HDM)-derived related proteins (Der P2) at very high concentrations (>5 μg) triggered the development of fatal anaphylaxis and pronounced airway dysfunction in exposed fetuses.[34] However, OVA is not an aeroallergen, *A fumigatus* exposure is not predictive of asthma development in humans,[35] the coadministration of alum makes it difficult to determine whether the observed effects are allergen or adjuvant-driven, and although allergen has been detected in the amniotic fluid in humans, it is typically present at ng/mL concentrations.[36] As such, it remains unclear whether maternal exposure to common allergens can also influence asthma development in offspring.

In humans, exposure to HDM is regarded as a powerful driver of asthma because (1) there is a dose-response relationship between HDM exposure and asthma development that does not exist for other common indoor aeroallergens (eg, cat or dog dander),[37-39] (2) 50% to 80% of patients with asthma demonstrate evidence of an HDM-specific immune response,[40] and (3) locales where HDM responses are not strongly linked to the development of allergic asthma are regions with low relative humidity (New Mexico, Northern Sweden[41,42]), which naturally limits the growth of dust mite species. Given the strong association between HDM and allergic sensitization in humans, we sought to determine whether maternal exposure during pregnancy to a common human allergen, HDM, in the absence of additional adjuvants could influence the development of asthma in offspring of exposed mothers using a mouse model of experimental asthma.

METHODS

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

Mice

Male and female A/J mice (5-6 weeks old) were bred and housed in a specific pathogen-free facility at Cincinnati Children's Hospital (Cincinnati, Ohio). Cincinnati Children's Hospital Institutional Animal Care and Use Committee approved all animal protocols.

Mating and allergen treatment protocols

Because A/J mice are relatively poor breeders, naive, age-matched colony reared A/J females were mated, and allowed to rear 1 litter to gain experience in rearing pups before being selected for further study. A/J mice were chosen because we have demonstrated that this strain develops severe airway hyperresponsiveness (AHR) associated with a mixed T_H2/T_H17 profile.[43,44] Age-matched, experienced mothers were subsequently mated in pairs or trios. For delivery, and during the nursing period, mothers were individually housed. After setting up mating cages, females were checked for vaginal plugs daily. The day a mating plug was first observed was considered day 0. At this time mothers were randomized into PBS- or HDM-exposed groups. Mothers were exposed to 100 μL PBS or 20 μg HDM (Greer Labs, Lenoir, NC) intraperitonealy on days 0, 4, and 10 of pregnancy. PBS- and HDM-exposed mothers were housed singly, or in pairs, by group. These days were selected to ensure that a robust HDM-specific immune response was ongoing during initial lung and immune (thymic) development (days 9-12 of gestation). Offspring of exposed mothers were weaned at 28 days of life, and subsequently housed by maternal exposure. At age 6, 8, and 9 weeks, offspring were anesthetized with ketamine/xylazine and exposed to PBS (40 μL), HDM (200 μg) as described elsewhere,[45] or 1 μg *Aspergillus fumigatus* extract (Greer Labs) intratrachealy as indicated. Because C57Bl/6 mice (and μMT mice that are on a C57Bl/6 background) are considered refractory to induction of asthma, a more vigorous regimen of allergen exposure consisting of intraperitoneal HDM at 6 and 7 weeks of life and intratracheal HDM at 8 and 9 weeks of life was used. Mice were sacrificed 72 hours after final allergen/PBS exposure for assessment of airway function and dendritic cell (DC) recruitment. Where indicated, AlexaFluor405-labeled (Invitrogen, Carlsbad, Calif) HDM (AF405-HDM) was used.

Isolation of fetal tissue, placental tissue, and amniotic fluid

To isolate fetal tissue, placental tissue, and amniotic fluid, pregnant females were euthanized with sodium pentobarbital, the fetuses and placentas were individually excised, amniotic fluid was carefully extracted through a 28-gauge needle, and samples were pooled by maternal exposure. Placentas and fetuses were digested and minced as described for the lung (see this article's Online Repository at www.jacionline.org). Single-cell suspensions were generated for flow cytometric analysis.

Statistical analysis

To determine differences between multiple groups, ANOVA was used with *post hoc* comparisons using Tukey test. Significance was assumed at a P value of less than .05. Titers of HDM-specific IgG_1 and IgG_{2a} were log10 transformed before analysis.

RESULTS

Maternal exposure to HDM allergen exacerbates asthma in offspring

Given that prenatal exposures to environmental stimuli can influence offspring asthma, and HDM sensitization is a powerful risk factor for asthma development, we sought to determine whether maternal exposure to HDM during pregnancy impacted offspring asthma. Female A/J mice (a strain that develops severe AHR associated with a mixed T_H2/T_H17 response[43,44]) were exposed to 20 μg HDM extract on days 0, 4, and 10 of pregnancy to ensure a strong HDM-specific immune response was ongoing during initial pulmonary and immune (thymic) development (days 9-12 of gestation). At age 6, 8, and 9 weeks, offspring were exposed to 200 μg of intratracheal HDM. AHR was assessed 72 hours after the final allergen exposure. Pooled male and female offspring of HDM-exposed mothers demonstrated a marked increase in AHR (~90% increase over offspring of PBS-exposed mothers; Fig 1, *A*). Because maternal asthma status was recently suggested to differentially influence asthma development in male and female offspring,[46] we also compared HDM-induced AHR in male and female offspring of HDM-exposed mothers. Both male and female offspring of HDM-exposed mothers demonstrated significantly increased AHR compared with offspring from PBS-exposed mothers (see Fig E1 in this article's Online Repository at www.jacionline.org). Increased AHR in offspring of HDM-exposed

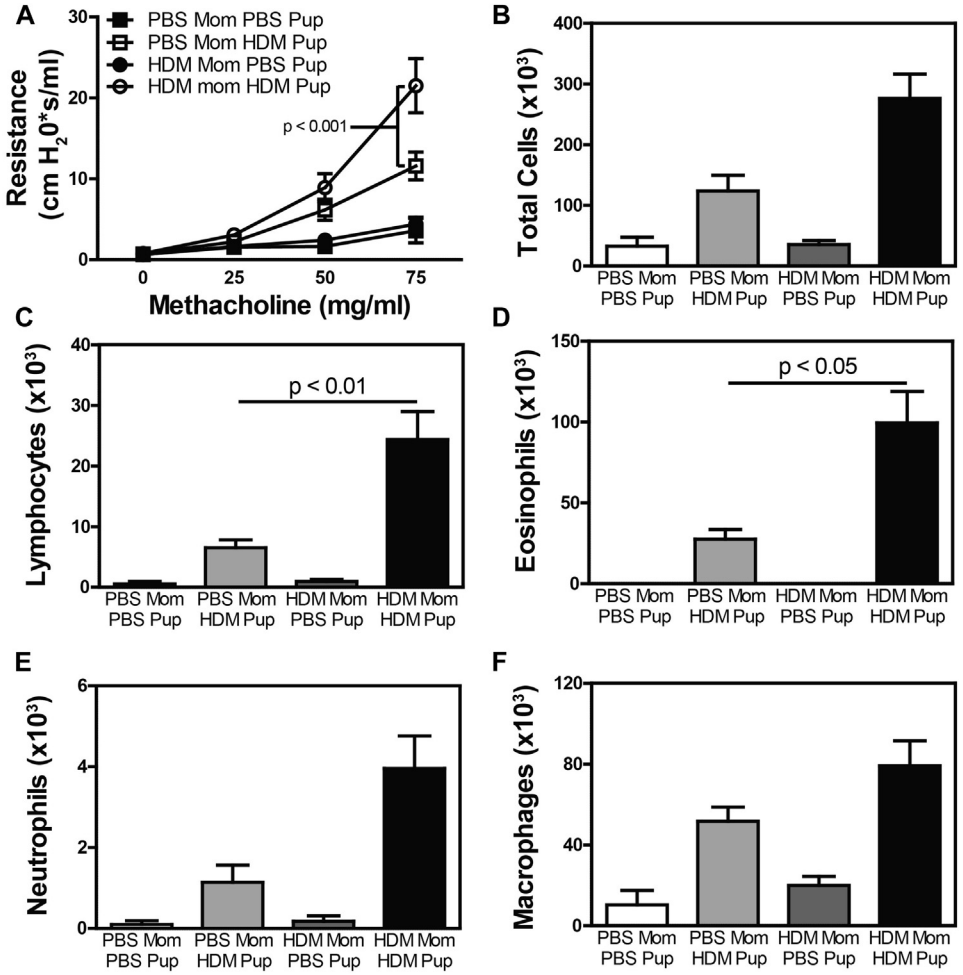

FIG 1. Maternal HDM exposure increases the severity of AHR and airway inflammation in HDM-exposed offspring. Pregnant female mice were exposed to HDM or PBS on days 0, 4, and 10 of pregnancy. Offspring of PBS- and HDM-exposed mothers were exposed to PBS or HDM at age 6, 8, and 9 weeks and development of AHR was assessed **(A)**. After sacrifice, BAL fluid was collected to assess total BAL cellularity **(B)** and the number of lymphocytes **(C)**, eosinophils **(D)**, neutrophils **(E)**, and macrophages **(F)**. *BAL*, Bronchoalveolar lavage. Mean + SEM shown. Data represent 7 to 18 mice pooled from 4 experiments.

animals was accompanied by increased recruitment of inflammatory cells in bronchoalveolar lavage fluid (Fig 1, *B*). Significantly increased numbers of lymphocytes (Fig 1, *C*) and eosinophils (Fig 1, *D*) were observed in offspring of HDM-exposed mothers. Although we also observed a trend toward increased numbers of neutrophils (Fig 1, *E*) and macrophages (Fig 1, *F*) in offspring of HDM-exposed mothers, this did not reach statistical significance.

Increased AHR in offspring of HDM-exposed mothers is associated with increased T_H2 cytokine production and HDM-specific immunoglobulin production

As development of asthma is the result of excessive T_H2 cytokine production, we also assessed cytokine production in HDM-restimulated lung cell cultures from PBS- or HDM-exposed offspring of PBS- or HDM-exposed mothers. HDM exposure induced production of the T_H2 cytokines IL-4, IL-5, IL-13, as well as the T_H17-associated cytokine IL-17A (Fig 2, *A-D*). There was no appreciable production of IFN-γ (data not shown). Consistent with *in vivo* data, elevated levels of IL-4, IL-5, and IL-13 (Fig 2, *A-C*) were observed in cells isolated from HDM-exposed offspring of HDM-exposed mothers compared with cultures of cells from HDM-exposed offspring of PBS-exposed mothers. In contrast, production of IL-17A was comparable in HDM-exposed offspring of PBS- and HDM-exposed mothers (Fig 2, *D*). Moreover, accumulation of pulmonary $CD4^+$ T cells expressing IL-13 (Fig 2, *E*) and IL-17A (Fig 2, *F*) was observed in HDM-challenged offspring, and maternal HDM exposure significantly increased the frequency of $IL\text{-}13^+$ $CD4^+$ T cells (Fig 2, *E*) but did not influence the frequency of $IL\text{-}17A^+$ $CD4^+$ T cells (Fig 2, *F*) following HDM exposure of offspring. The mean fluorescence intensity of IL-13 or IL-17A in cytokine-expressing cells was not elevated (see Fig E2 in this article's Online Repository at www.jacionline.org), suggesting that the increase in cytokine production observed in HDM-stimulated lung cell cultures was due to increased frequency of cytokine-producing cells, and not an increased capacity for cytokine production in individual cells.

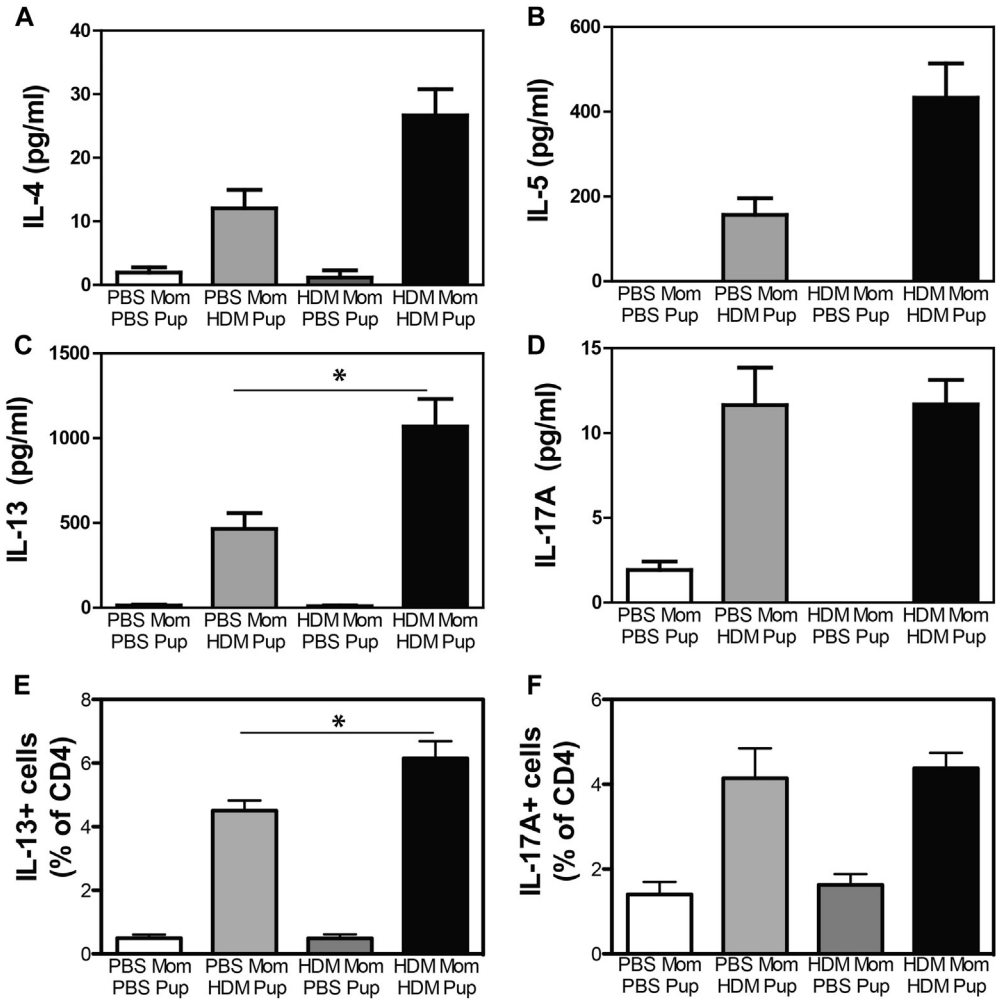

FIG 2. Increased T_H2 cytokine production in HDM-exposed offspring of HDM-exposed mothers. Offspring of PBS- and HDM-exposed mothers were treated with PBS or HDM as described in the Methods section. Single-cell suspensions of lung cells were restimulated with HDM, and production of IL-4 **(A)**, IL-5 **(B)**, IL-13 **(C)**, and IL-17A **(D)** was assessed by ELISA. The frequency of $CD4^+$ cells producing IL-13 **(E)** and IL-17A **(F)** was assessed by flow cytometry. Mean + SEM shown. Data represent 7 to 18 mice pooled from 4 experiments. $*P < .05$.

Finally, because development of allergy is also associated with an allergen-specific humoral response, we also examined immunoglobulin levels in animals from the various groups. HDM exposure induced substantial increases in levels of total IgE (a reliable surrogate for HDM-specific IgE),[47] HDM-specific IgG_1, and HDM-specific IgG_{2a} (Fig 3). Offspring of HDM-exposed mothers tended to have higher levels of total IgE (Fig 3, *A*) although this did not reach statistical significance. HDM-specific IgG_1 (Fig 3, *D*) and HDM-specific IgG_{2a} levels (Fig 3, *E*) were significantly elevated in HDM-exposed offspring of HDM-exposed mothers compared with HDM-exposed offspring of PBS-exposed mothers. Interestingly, we observed significant levels of HDM-specific IgG_1 and IgG_{2a} in PBS-exposed offspring of HDM-exposed mothers, presumably a result of vertical transmission of immunoglobulins from mother to child. Moreover, although differences in the amounts of Der p allergens, HDM, and serine protease activity in different batches of commercially available HDM can contribute to differential capacity to induce airway inflammation and AHR, we observed similar results when using 2 separate batches of HDM (one providing 1.5 μg Der P1 and 12.9 EU LPS per maternal exposure and another providing 0.65 μg Der p1 and 1.6 EU LPS per maternal exposure). As such, it is unlikely that the observed results were HDM lot-specific. Collectively, these data demonstrate that HDM exposure of mothers during pregnancy is sufficient to drive the development of more intense humoral responses, T_H2 cytokine production, pulmonary inflammation, and AHR following HDM exposure of offspring.

Offspring of HDM-exposed mothers display decreased allergen uptake by pulmonary macrophages

Because maternal exposure to allergens in the context of adjuvants globally alters DC function in offspring,[29] we also examined the impact of maternal HDM exposure on *in vivo* pulmonary DC function in offspring. HDM exposure increased the number of most DC subsets in the lung (with the exception of plasmacytoid DCs), but there was no difference in the

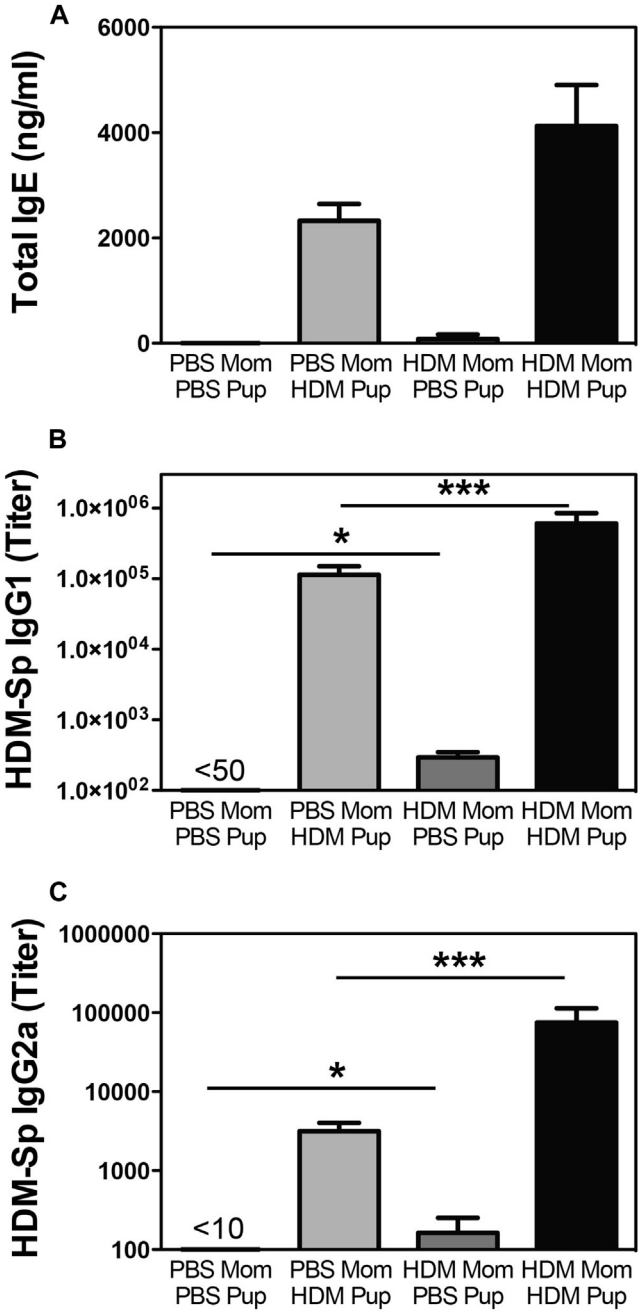

FIG 3. Increased total and HDM-specific IgG production in HDM-exposed offspring of HDM-exposed mothers. Offspring of PBS- and HDM-exposed mothers were treated with PBS or HDM as described in the Methods section. Serum isolated at time of sacrifice was assayed for total IgE **(A)**, HDM-sp IgG_1 **(B)**, and HDM-sp IgG_{2a} **(C)** by ELISA. Lowest dilution on plate indicated. *sp*, Specific. Mean + SEM shown. Data represent 7 to 18 mice pooled from 4 experiments. $*P < .05$ and $***P < .001$.

numbers of DCs recruited to the lungs in offspring of control, or HDM-exposed mothers (see Fig E3 in this article's Online Repository at www.jacionline.org). To assess the functional capacity of different DC subsets to take up exogenous allergen, mice were exposed to fluorescently labeled HDM on the final allergen exposure, and allergen uptake was monitored through gating on HDM^+ cells. Maternal HDM exposure did not alter the phagocytic capacity of pulmonary CD103+ DCs, inflammatory DCs, or plasmacytoid DCs (data not shown). A significant decrease in phagocytic capacity of myeloid DCs (measured by HDM MFI) and a decreased phagocytic capacity and frequency of HDM-bearing alveolar macrophages was observed in offspring of HDM-exposed mothers (Fig 4). We observed no significant differences in costimulatory molecule expression in pulmonary DC subsets (CD80, CD86, PD-L1, PD-L2, MHC class II) between HDM-exposed offspring of HDM-exposed mothers and HDM-exposed offspring of PBS-exposed mothers (data not shown). Interestingly, when bone marrow–derived DCs generated from control and offspring of HDM-exposed dams were stimulated with HDM *in vitro*, we observed no differences in either allergen uptake or costimulatory molecule expression (see Fig E4 in this article's Online Repository at www.jacionline.org), suggesting that the observed decrease in pulmonary myeloid DC phagocytic activity may be related to alteration in the local inflammatory milieu in the lungs of offspring of HDM-exposed mothers and not intrinsic differences in DC populations following maternal HDM exposure.

Maternal HDM exposure does not influence the magnitude of *A fumigatus*–induced airway inflammation

Previous maternal sensitization with OVA (alum) and subsequent OVA challenge in pregnancy have been demonstrated to drive the development of asthma to unrelated allergens in offspring of exposed mothers.[30] To assess the influence of maternal HDM exposure on the development of asthma to an unrelated allergen, mothers were exposed to HDM on days 0, 4, and 10 of pregnancy. At age 6, 8, and 9 weeks, offspring (housed according to maternal exposure) were exposed to either intratracheal HDM or *A fumigatus* extract. As expected, previous maternal HDM exposure increased the magnitude of HDM-induced airway inflammation (Fig 5, *A*). The effect was particularly striking for airway eosinophilia (Fig 5, *B*), although strong trends were observed for airway lymphocytes, neutrophils, and macrophages (Fig 5, *C-E*). In contrast, maternal HDM exposure had no impact on the severity of *A fumigatus*–induced airway inflammation as levels of total airway infiltrating cells, eosinophils, lymphocytes, neutrophils, and macrophages were all comparable in *A fumigatus*–exposed offspring of PBS-exposed and HDM-exposed mothers.

To further characterize the asthmatic response in *A fumigatus*–exposed offspring, we also examined the production of T_H2-associated cytokines in *A fumigatus*–exposed offspring. In these studies, cells were stimulated with ConA to directly compare the changes in capacity for cytokine production induced by previous HDM or AF exposure. In HDM-exposed offspring, previous maternal HDM exposure resulted in a trend toward increased production of the T_H2 cytokines IL-4, IL-5, and IL-13, an increased frequency of $IL\text{-}13^+$ T cells (Fig 6, *D*), and increased levels of total IgE (Fig 6, *E*). In contrast, we could discern no significant impact of maternal HDM exposure on ConA-induced cytokine production, the frequency of pulmonary $IL\text{-}13^+$ $CD4^+$ T cells, or IgE levels in *A fumigatus*–exposed offspring (Fig 6, *A-E*). Collectively, these data suggest that the impact of maternal exposure to allergens in the absence of strong immune-skewing adjuvants is limited to the allergen to which the mother is exposed.

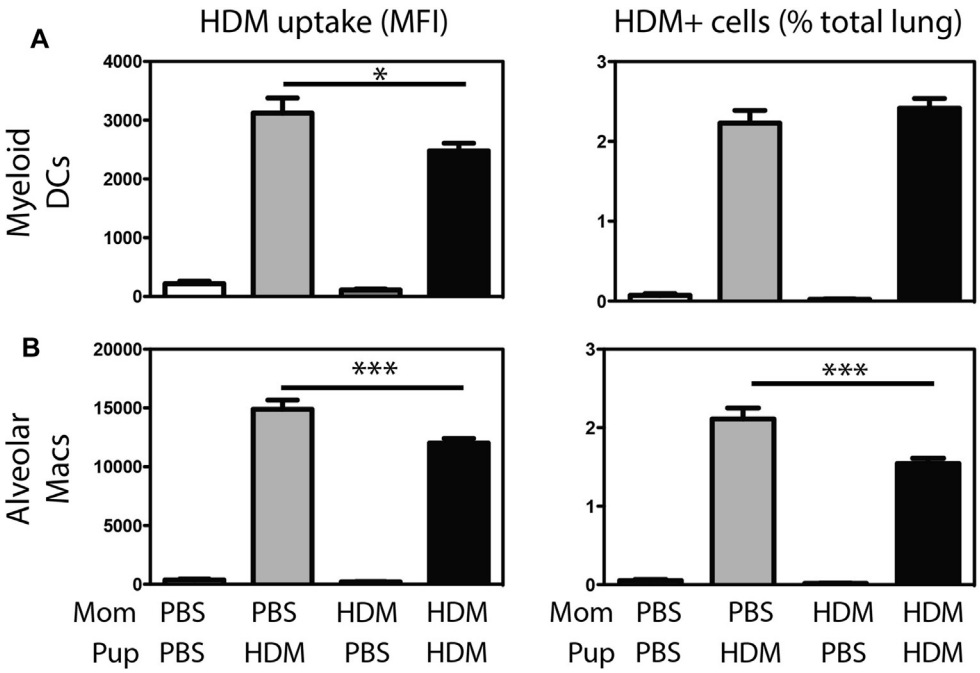

FIG 4. Minimal changes in allergen uptake by pulmonary DC populations were observed in HDM-exposed offspring of HDM-exposed mothers. Offspring of PBS- and HDM-exposed mothers were treated with PBS or HDM as described in the Methods section. Amount of HDM phagocytosed (MFI) and frequency of HDM-bearing (% of total lung cells) myeloid DCs **(A)** and alveolar macrophages **(B)** were assessed by flow cytometry. *Macs*, Macrophages; *MFI*, mean fluorescence intensity. Mean + SEM shown. Data represent 7 to 18 mice pooled from 4 experiments. $*P < .05$ and $***P < .001$.

Exacerbation of offspring asthma following maternal HDM exposure is not associated with detectable allergen present in fetal tissues and does not require maternal immunoglobulins

To begin to explore the mechanisms whereby maternal allergen exposures influence the severity of offspring asthma, the final maternal HDM exposure (day 10 of pregnancy) was replaced with AF405-labeled HDM. Mothers were sacrificed on day 12 of pregnancy, and we isolated the cells from the amniotic fluid and both placental and fetal tissues to investigate the presence of AF405-labeled HDM. Both fetal and placental tissues showed unique populations of $CD11b^+$, $CD11c^+$, $CD11b^+CD11c^+$, and CD11b-$CD11c^-$ cells, although appreciable AF405 signals were not detected in any cell population in tissues harvested from HDM-exposed mothers (see Fig E5, *A* and *B*, in this article's Online Repository at www.jacionline.org). Cell numbers were much lower in samples of amniotic fluid, and analysis of pooled cell samples from HDM-exposed mothers demonstrated no increase in the AF405 channel (Fig E5, *C*). Finally, levels of major HDM allergens, DerP1 and DerP2, were assessed by ELISA in samples of pooled amniotic fluid from PBS- or HDM-exposed mothers. Again, levels of DerP1 and DerP2 were consistently below the level of detection (~1 ng/mL) in amniotic fluid of HDM-exposed mothers (data not shown). These data suggest that there is no strong transfer of HDM-derived proteins following maternal HDM exposure.

Finally, to determine whether transfer of maternal immunoglobulins was required for the increased severity of allergic asthma observed in offspring of HDM-exposed mothers, we made use of immunoglobulin-deficient μMT mice.[48] To this end, we mated female μMT mice with control C57Bl/6 mice to generate offspring in which no transfer of maternal HDM-specific immunoglobulins could occur, but that could themselves mount an immunoglobulin response following HDM sensitization and challenge. Pregnant female μMT mice were treated with HDM on days 0, 4, and 10 of pregnancy, as described above. Because C57Bl/6 mice are more resistant to the development of AHR, a more robust protocol of HDM-induced AHR was used wherein mice are given 10 μg HDM intraperitonealy (in the absence of additional adjuvant) at 6 and 7 weeks of life, and then challenged with 100 μg of HDM intratrachealy at 8 and 9 weeks of life. AHR was assessed 72 hours after the final allergen exposure. As observed in offspring of HDM-exposed A/J mothers, HDM exposure during pregnancy resulted in significantly greater AHR in C57Bl/6 offspring (Fig 7, *A*), suggesting that maternal HDM exposure-mediated exacerbation of asthma is not limited to A/J mice. Moreover, although the overall magnitude of AHR in offspring generated by mating μMT females with C57Bl/6 males was lower than that observed in offspring of pure C57Bl/6 matings, maternal exposure to HDM similarly exacerbated AHR in offspring of HDM-exposed μMT dams. Maternal HDM exposure was also associated with increased recruitment of all cell types into the bronchoalveolar lavage fluid (Fig 7, *B-F*), as well as an increased recruitment of IL-13^+ $CD4^+$ T cells, but not IL-$17A^+$ $CD4^+$ T cells (see Fig E6 in this article's Online Repository at www.jacionline.org). In addition, maternal HDM exposure significantly increased the titer of total IgE and HDM-specific IgG_1 in offspring of C57Bl/6 mothers and total IgE in offspring of HDM-exposed μMT mothers (see Fig E7 in this article's Online Repository at www.jacionline.org) (IgG_{2a} is not expressed in C57Bl/6 mice,[49] and as such, was not measured). As expected, PBS-treated offspring of HDM-exposed C57Bl/6 mothers

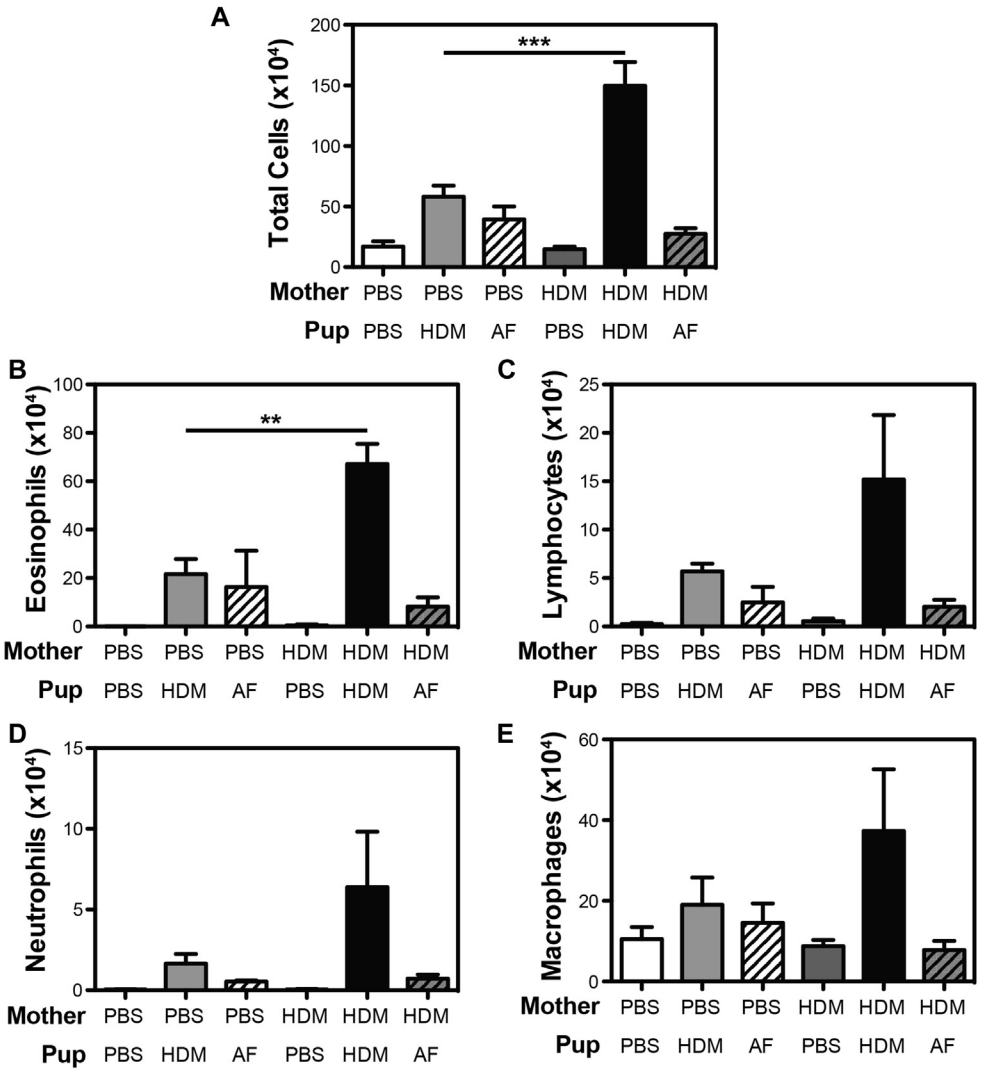

FIG 5. Maternal HDM exposure does not increase the severity of AHR and airway inflammation in *A fumigatus*–exposed offspring. Offspring of PBS- and HDM-exposed mothers were exposed to PBS, HDM, or *A fumigatus* extract at age 6, 8, and 9 weeks. After sacrifice, BAL fluid was collected to assess total bronchoalveolar lavage cellularity **(A)** and the number of eosinophils **(B)**, lymphocytes **(C)**, neutrophils **(D)**, andmacrophages **(E)**. *AF*, *A fumigatus*. $^{**}P < .01$ and $^{***}P < .001$. Mean + SEM shown. Data represent 2 to 5 mice pooled from 2 experiments.

displayed readily detectable levels of HDM-specific IgG_1, whereas PBS-treated offspring of HDM-treated μMT mothers had undetectable levels of HDM-specific IgG_1, suggesting that this immunoglobulin is likely maternally derived (Fig E7). Thus, these data suggest that transfer of maternal HDM-specific immunoglobulins is not absolutely required for the development of more severe AHR after maternal HDM exposure.

DISCUSSION

Herein, we demonstrate that prenatal exposure to the relevant human aeroallergen, HDM, in the absence of powerful T_H2-skewing adjuvants, is sufficient to exacerbate the development of HDM-driven allergic asthma in offspring later in life. Specifically, offspring of allergen-exposed mothers demonstrated more robust AHR and airway inflammation. The increased phenotypic measures of asthma were associated with a greater capacity for T_H2 cytokine production and frequency of $CD4^+IL\text{-}13^+$ cells in the lung, increased immunoglobulin production, and a decreased capacity of allergen uptake by alveolar macrophages. Interestingly, the increased sensitivity to allergen-induced AHR in offspring of exposed mothers appeared to be unique to the allergen the mothers were exposed to, as offspring of HDM-exposed mothers did not demonstrate increased severity of *A fumigatus*–induced airway inflammation.

Because maternal asthma status, moreso than paternal asthma status, is linked to increased risk of asthma, our study focused on the ability of maternal exposures to influence offspring asthma. As such, we cannot ascertain whether paternal allergen exposures might similarly influence asthma development in offspring. However, maternal exposures have been reported to influence, both positively and negatively, the development of asthma in other animal models. For example, if *Schistosoma mansoni*–infected mothers were impregnated during the

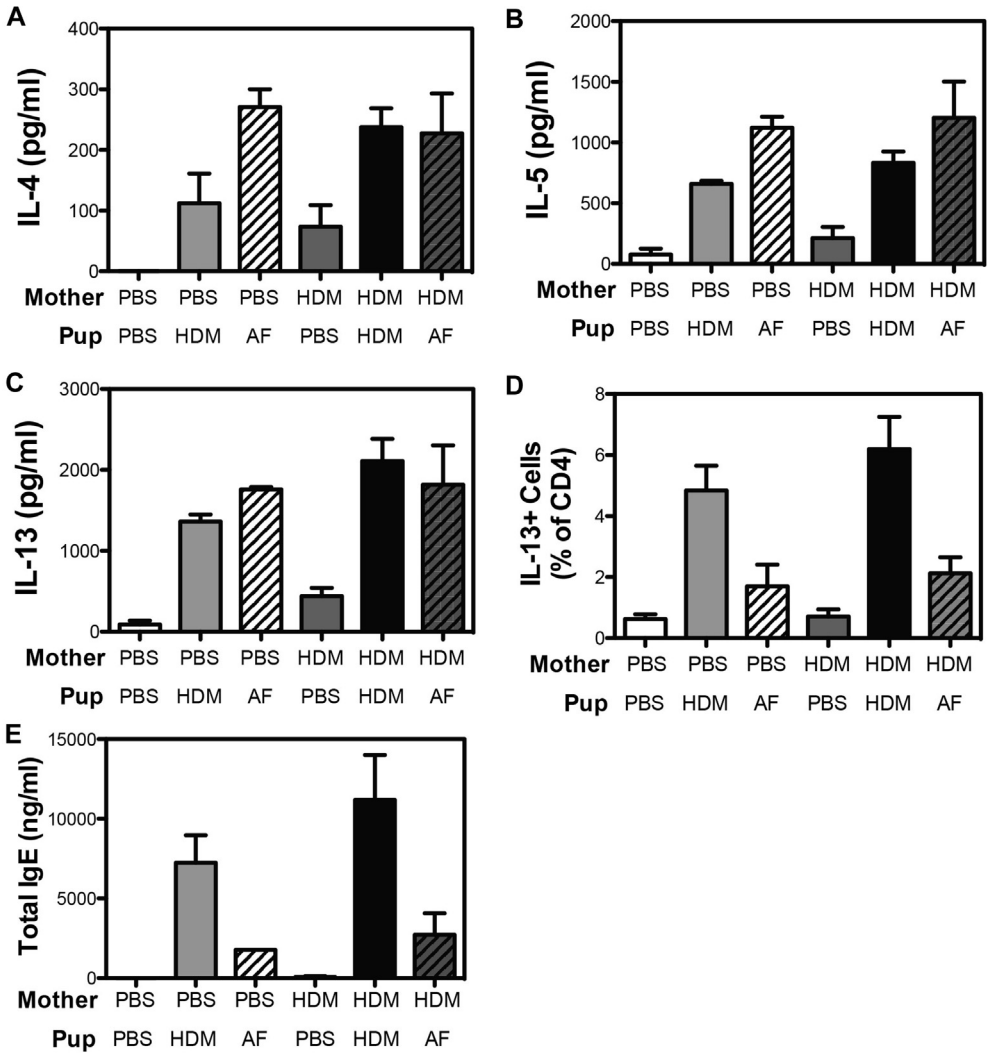

FIG 6. Maternal HDM exposure does not increase the magnitude of the T_H2 response or the magnitude of the immunoglobulin response following exposure of offspring to *A fumigatus*. Offspring of PBS- and HDM-exposed mothers were exposed to PBS, HDM, or *A fumigatus* extract at age 6, 8, and 9 weeks. Single-cell suspensions of lung cells were restimulated with ConA, and production of IL-4 **(A)**, IL-5 **(B)**, and IL-13 **(C)** was assessed by ELISA. **D,** The frequency of $CD4^+$ cells producing IL-13 was assessed by flow cytometry. **E,** Serum isolated at time of sacrifice was assayed for total IgE. *AF, A fumigatus*. Mean + SEM shown. Data represent 2 to 5 mice pooled from 2 experiments.

systemic T_H1 phase of the infection, offspring were found to be protected from the development of asthma.[21] Anti–IFN-γ abrogated this protective effect of maternal *Schistosoma mansoni* infection, suggesting that maternal cytokines can influence asthma development in offspring.[21] Feeding female mice OVA before pregnancy to induce tolerance protects from the development of OVA-induced allergic airway disease in an FcRn- and IFN-γ–dependent manner.[50] Similarly, sensitization with OVA (complete Freund's adjuvant), and reexposure to OVA during pregnancy, limited the development of OVA-specific IgE and pulmonary eosinophilia in offspring of exposed mothers.[51] Contrary to this protective effect, our observations of increased asthma development in offspring of HDM-exposed mothers are consistent with reports showing increased asthma development in offspring following maternal OVA (alum) sensitization and challenge,[29-32] offspring after maternal *A fumigatus* exposure,[33] and in offspring of mothers who were impregnated during the T_H2 phase of the immune response that develops after *Schistosoma mansoni* infection.[21] Blockade of IL-4 during pregnancy completely abrogated the increased sensitivity of offspring following maternal OVA (alum) sensitization and challenge,[30] again suggesting that maternal cytokines can influence offspring asthma. However, in their model of maternal OVA exposure–induced alterations of offspring asthma, Hamada et al also report that offspring are also more sensitive to asthma in a casein-driven model, suggesting that the vertical transmission of asthma sensitivity was not antigen specific. In contrast, we failed to observe increased airway inflammation, T_H2 cytokine production, or immunoglobulin production following *A fumigatus* exposure in offspring of HDM-exposed mothers. Thus, although we do observe increased maternal IL-4 levels following HDM exposure (data not shown), it seems unlikely that circulating maternal IL-4 is responsible for increased development of asthma in offspring of HDM-exposed mothers

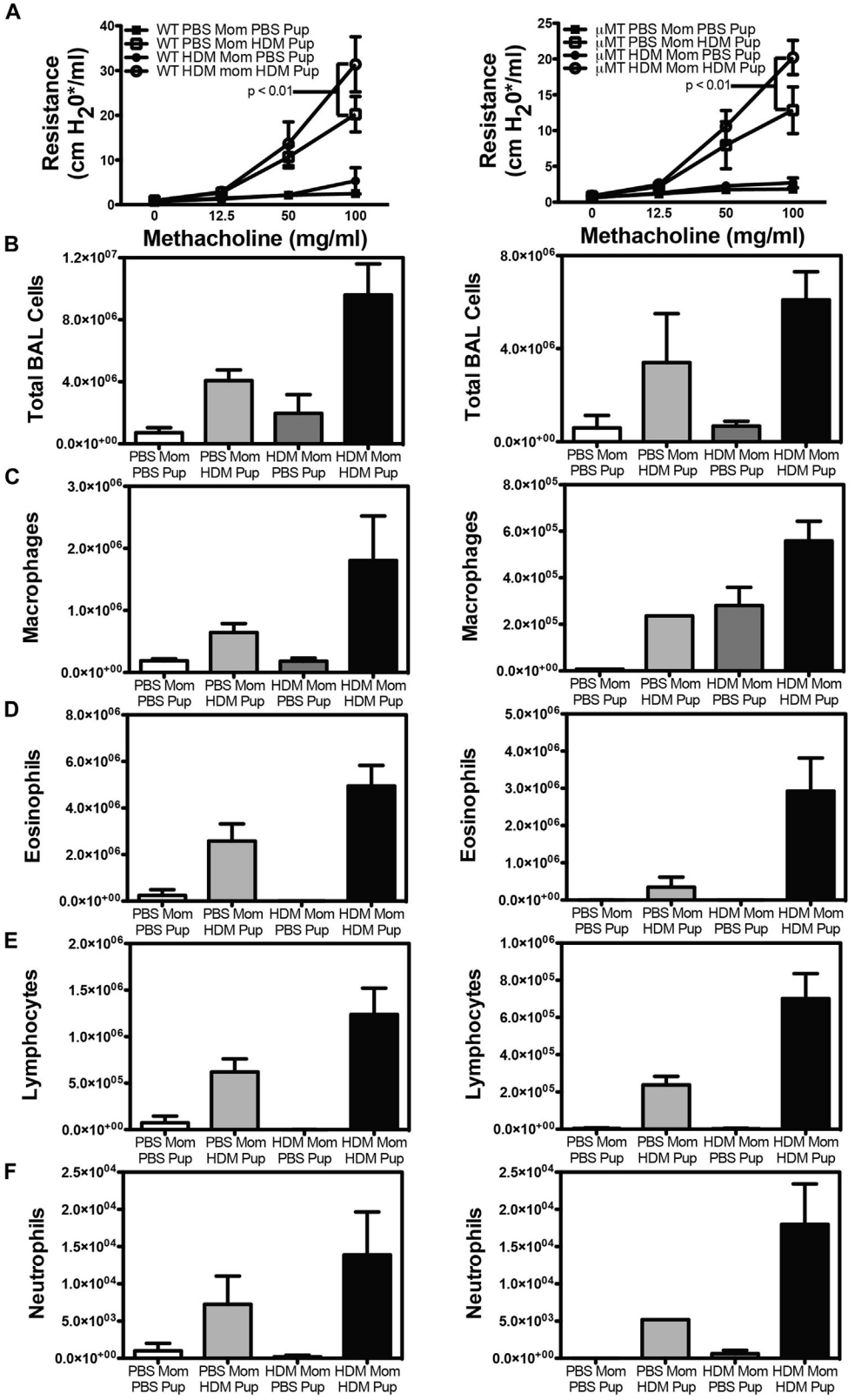

FIG 7. Offspring of HDM-exposed μMT dams demonstrate increased severity of AHR and airway inflammation. Male C57Bl/6 mice were mated with female C57Bl/6 dams *(left column)* or B-cell–deficient μMT dams *(right columns)* and pregnant female mice were exposed to HDM or PBS on days 0, 4, and 10 of pregnancy. Offspring of PBS- and HDM-exposed mothers were exposed to intraperitoneal PBS or HDM at age 6 and 7 weeks, and challenged with intratracheal PBS or HDM at age 8 and 9 weeks and the development of AHR was assessed 72 hours later **(A)**. After sacrifice, BAL fluid was collected to assess total BAL cellularity **(B)** and the number of macrophages **(C)**, eosinophils **(D)**, lymphocytes **(E)**, and neutrophils **(F)**. *BAL*, Bronchoalveolar lavage. Mean + SEM shown. Data represent 3 to 13 mice pooled from 3 experiments.

in our study. It is possible that the failure to induce increased asthma development to an unrelated antigen in our model is due to a lower overall capacity of HDM to induce increased circulating IL-4 levels as compared with OVA (alum) sensitization and challenge performed by Hamada et al.[30] Alternatively, although HDM exposures in offspring of HDM-exposed mothers began at age 6 weeks in our study, initial allergen exposure of offspring from OVA (alum)-sensitized and -challenged animals occurred at day 3 of life.[30] Thus, it is also conceivable that the ability of maternal IL-4 to enhance the development of T_H2 responses to unrelated allergens is lost between day 3 and week 6 of life.

Another mechanism whereby OVA exposure in pregnant OVA (alum)-sensitized mothers influences offspring asthma is through modification of DC activity in offspring. Indeed, DCs isolated from the spleens of offspring of OVA (alum)-sensitized and -challenged mothers demonstrated an increased capacity to induce T-cell proliferation in *in vitro* cultures despite no alterations in overall capacity to express MHC class II or costimulatory molecules and transfer of DCs from offspring of OVA-exposed mothers to control offspring increased the development of both OVA and casein-induced AHR.[29] This increased capacity to drive T-cell proliferation and development of asthma was associated with substantial changes in the DNA-methylation profile of splenic DCs from offspring of OVA (alum)-sensitized and -challenged mothers compared with splenic DCs from offspring of control mothers.[32] These results suggest that splenic DCs from offspring of allergen-exposed mothers were more "proasthmatic." In contrast to these reports, we find that the natural allergen, HDM, in the absence of additional immune-activating adjuvants drives limited change in costimulatory molecule expression or function of DCs in offspring of HDM-exposed mothers, while actually decreasing the phagocytic capacity of various pulmonary DC subsets. Interestingly, the observed decrease in phagocytic capacity was not observed in HDM-pulsed bone marrow–derived DCs generated from offspring of HDM-exposed mothers, suggesting that these alterations were not likely due to epigenetic changes within the DCs themselves, but rather related to differences in the inflammatory milieu of the lung. Although we did not directly compare T-cell stimulatory capacity of DCs from control offspring and offspring of HDM-exposed animals, our observations that HDM-specific, but not *A fumigatus*–specific, immune responses are impacted following maternal HDM exposure is not consistent with the induction of an intrinsically more "proasthmatic" population of pulmonary DCs.

The key distinguishing feature of our model from other published reports is the antigen-specific nature of our observed effects. Interestingly, there are readily detectable levels of HDM-specific IgG_1 and IgG_{2a} in the serum of PBS-exposed offspring of HDM-exposed mothers, and levels can persist up to 7 months after birth (see Fig E8 in this article's Online Repository at www.jacionline.org), suggesting that maternal-derived immunoglublins may drive more severe AHR in offspring of HDM-exposed mothers. However, we find that exposure of B-cell–deficient μMT dams to HDM following mating with wild-type C57Bl/6 animals still drives significantly enhanced HDM-induced AHR, airway inflammation, and pulmonary T_H2 recruitment in offspring. This result suggests that transfer of maternal immunoglobulins is not required for exacerbation of the asthma phenotype following maternal HDM exposure. However, it was recently demonstrated that B-1B cells present in the lungs of μMT mice can produce antigen nonspecific IgE and IgG_1 and IgG_{2c} following allergen challenge.[52] As B-1B cell–derived antibodies typically recognize polysaccharide antigens with low affinity,[53] and HDM contains glycosylated proteins,[54] it is conceivable that B1-derived maternal immunoglobulins are either sufficient to transfer increased HDM reactivity to offspring, or they facilitate the transplacental passage of antigens in immunoglobulin:antigen complexes,[55-58] allowing for priming of HDM-specific T cells in offspring of exposed mothers. Subsequent inhalational exposure in offspring of HDM-exposed mothers may then represent additional "boosts" not present in offspring of PBS-exposed mothers, and drive the development of a more robust response. Finally, it is possible that vertical transmission of maternal T cells (either through transplacental passage or via breast milk) passively sensitizes the fetus to HDM through a maternal microchimerism-dependent mechanism as has been observed in model of maternal tuberculosis of Candida infection.[59] These possibilities are currently being considered in our laboratory.

In conclusion, we report that maternal HDM exposure during pregnancy is sufficient to enhance neonatal sensitivity to HDM. This is associated with increased AHR, airway inflammation, T_H2 cytokine production, and immunoglobulin levels and a modest decrease in the phagocytic capacity of pulmonary macrophage populations following HDM exposure in offspring. Interestingly, in contrast to previous reports of allergen-independent influence of maternal OVA (alum) sensitization and challenge on offspring asthma, these effects are wholly allergen dependent, as exposure of offspring to an unrelated allergen, *A fumigatus*, does not result in the development of more severe airway inflammation, T_H2 cytokine production, or immunoglobulin synthesis. These findings have important implication for regulation of asthma risk, and suggest that increased HDM burden in the developed world may have underappreciated influences on the overall prevalence of allergic asthma.

Key messages

- **Maternal exposure to a common human allergen, HDM, during pregnancy increases the severity of HDM-induced asthma in murine offspring.**
- **Maternal exposure to HDM does not exacerbate *A fumigatus*–induced asthma in murine offspring of HDM-exposed mothers, suggesting that the effect of HDM is allergen-specific.**
- **These findings have important implications for regulation of asthma risk, and suggest that exposure to HDM in the developed world may have underappreciated influences on the overall prevalence of allergic asthma.**

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METHODS

Assessment of allergen-induced allergic responses

Airway responses were evaluated using a flexiVent system (SCIREQ Scientific Respiratory Equipment, Inc, Montreal, Quebec, Canada). Mice were anesthetized with sodium pentobarbital (80 mg/kg) and xylazine (16 mg/kg). Mouse tracheas were cannulated with a 20-gauge blunt needle and the mice ventilated at 150 breaths/min, 3.0 cm water positive end expiratory pressure. Mice were paralyzed with pancuronium bromide (0.8 mg/kg) and allowed to stabilize on the ventilator for 2 minutes. Mice were then exposed to aerosolized methacholine (0, 25, 50, and 75 mg/mL) in PBS for 15 seconds with an Aeroneb ultrasonic nebulizer, and ventilated for an additional 10 seconds. Ventilation cycle measurements were made until resistance peaked, then airways were re-recruited with deep inflation and the next dose was executed.

Immediately after AHR measurements, blood was collected from the inferior vena cava to test for total and HDM-specific immunoglobulins using antibodies from Pharmingen (San Diego, Calif). To collect bronchoalveolar lavage fluid, lungs were lavaged 3 times with 1.0 mL of cold HBSS (Invitrogen). Recovered lavage fluid (70% to 80%) was centrifuged (300g for 6 minutes, then 5000g for 30 seconds) and the cell pellet resuspended in 150 μL of ACK for 2 minutes to lyse erythrocytes. Five hundred microliter of 5% FBS in PBS was added and cells were centrifuged (300g for 6 minutes, then 5000g for 30 seconds). Cells were resuspended in 200 μL of 5% FBS in PBS and counted with a hemocytometer. Slides were prepared by cytocentrifugation (Cytospin 4; Thermo Scientific, Waltham, Mass) and stained with Camco Stain Pak (Cambridge Diagnostic Products, Fort Lauderdale, Fla). Bronchoalveolar lavage cell differential counts were determined using morphologic criteria under a light microscope with evaluation of 500 cells/slide or more. Cells enumerated included macrophages, neutrophils, eosinophils, lymphocytes, and epithelial cells.

Isolation of lung cells

After AHR measurements, lungs were removed, minced, and placed in 6 mL of RPMI 1640 containing 1% Penn/Strep, 1% L-glutamine, Liberase TL (0.25 mg/mL) (Roche Diagnostics, Indianapolis, Ind), and DNase I (0.5 mg/mL) (Sigma, St Louis, Mo) at 37°C for 45 minutes. The tissue was forced through a 70-micron cell strainer, and red blood cells were lysed with ACK lysis buffer (Invitrogen). Cells were washed with RPMI containing 1% Penn/Strep, 1% L-glutamine, and 5% FBS. Viable cells were counted via trypan blue exclusion. Where indicated, lung cells were cultured at 250,000 cells per well in a 96-well plate (250 μL final volume) in RPMI with 1% Penn/Strep, 1% L-glutamine, 10% FBS, and 0.1% Beta-mercaptoethanol. Cells were stimulated with media alone, HDM (30 μg/mL), or ConA (5 μg/mL). Tissue culture supernatants were harvested at 72 hours.

Flow cytometry

Staining reactions were performed at 4°C following incubation with FcBlock (mAb 2.4G2) for 30 minutes. To identify pulmonary DC populations, 1×10^6 lung cells were stained with anti-CD11c-PE-CF594 (N418), anti-CD11b-PE-Cy7 (M1/70), and anti-Gr1-APC-Cy7 (RB6-8C5), anti-CD103 PerCp-Cy5.5 (2E7—Biolegend, San Diego, Calif), and anti-CD317 APC (eBio927). DC subsets were characterized as follows: CD103+ DCs ($CD103^+CD11c^+CD11b^{neg}Gr1^{neg}$), inflammatory DCs ($CD103^{neg}CD11c^+CD11b^+Gr1^+$), myeloid DCs ($CD103^{neg}CD11c^+CD11b^+Gr1^{neg}$), alveolar macrophages ($CD103^{neg}$, $CD11c^+CD11b^{neg}Gr1^{neg}$), and plasmacytoid DCs ($CD11c^{dim}CD11b^{neg}CD317^+$). Dead cells were excluded using Live/Dead Blue. To examine the frequency of cytokine-producing T cells, 1×10^6 lung cells were stimulated with PMA (100 ng/mL; Sigma) and ionomycin (1 μg/mL; Sigma). After overnight stimulation, Brefeldin A and monensin (eBioscience, San Diego, Calif) were added to cell cultures for the final 4 hours. Cells were stained with fixable Viability Dye eFluorE506, fixed, permeabilized, and stained with anti–CD4-PE-Cy7 (RM4-5), anti–IL-17A-PE (eBio17B7), and anti–IL-13 APC (eBio13A). To assess costimulatory molecule expression in bone marrow–derived DC cultures, cells were stained with CD11b-PE-Cy7, CD11c-PE-eFluor610, MHC class II-FITC (clone M5/114.15.2), CD80-PE (clone 16-10A1), and CD86-PeCy5 (Clone GL1). Cells were stained with fixable Viability Dye eFluorE506 to exclude dead cells. To assess HDM uptake in fetal tissue placental tissue and cells from the amniotic fluid, cells were stained with CD11b-PE-Cy7, CD11c-PE-eFluor610, and fixable Viability Dye eFluorE506 to exclude dead cells. All mAbs were from eBioscience unless otherwise indicated. Data were acquired with an LSRII flow cytometer (BD Biosciences, San Jose, Calif) equipped with lasers tuned to 355, 405, 488, 561, and 640 nm. Spectral overlap was compensated using the FACSDiVa software (BD Biosciences) and analyzed using FlowJo software (Treestar Inc, Ashland, Ore).

Generation and culture of bone marrow–derived DCs

Bone marrow cells (3×10^5 cells/mL) were cultured in complete RPMI supplemented with GM-CSF (10 ng/mL, Peprotech, Rocky Hill, NJ). Medium was changed on day 3. On day 6, cells were harvested and counted. A total of 100,000 DCs were cultured with medium, or AF405-HDM at 0.1, 1.0, or 10 μg/mL for 24 hours. Stimulated DCs were harvested, washed extensively in PBS, and used for subsequent studies.

Determination of cytokine, total, and HDM-specific immunoglobulin levels

Cytokine levels in samples were measured by ELISA using matched antibody pairs purchased from eBioscience (IL-4, IL-5, IL-10, IL-13, IL-17A) according to the manufacturer's directions. Tissue culture supernatants were frozen at −80°C and thawed immediately before use. Total IgE, IgG_1, and IgG_{2a} levels were measured by ELISA using matched mAb pairs from BD Biosciences. To determine the titers of HDM-specific immunoglobulins present in the serum, plates were coated with HDM extract (100 μg/mL) at 4°C overnight in 0.1 mol Na_2HPO_4. After blocking with 10% FBS in PBS at room temperature for 2 hours, samples were added in a series of 8, 10-fold serial dilutions (starting at 1:10 for HDM-specific IgG_{2a}, and 1:50 for HDM-specific IgG_1). Plates were incubated at 4°C overnight. Biotinylated detection antibodies (BD Bioscience) were added, followed by Avidin Peroxidase, and color was developed with Super Aqua Blue (eBioscience, San Diego, Calif). The reciprocal of the dilution factor resulting in an OD of 0.6 (HDM-specific IgG_{2a}) or 1.5 (HDM-specific IgG_1) was determined. All HDM-specific immunoglobulin assays from all experiments were run at the same time to facilitate comparisons between experiments.

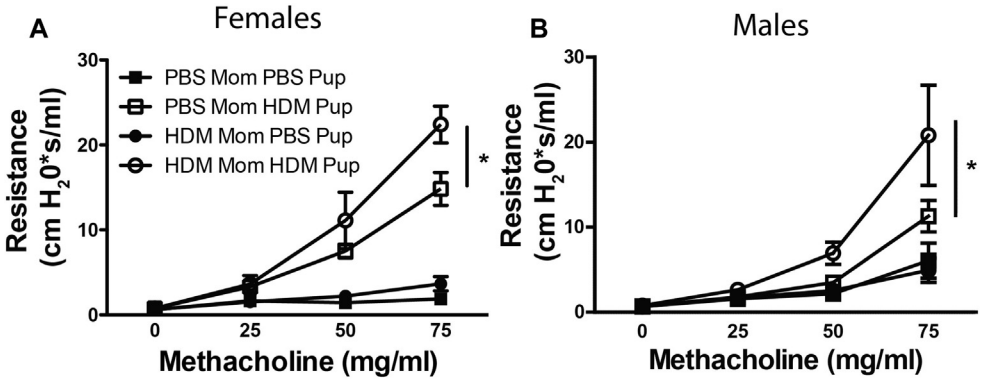

FIG E1. Maternal HDM exposure increases the severity of AHR in male and female offspring of HDM-exposed mothers. Pregnant female mice were exposed to HDM or PBS on days 0, 4, and 10 of pregnancy. Offspring of PBS- and HDM-exposed mothers were exposed to PBS or HDM at age 6, 8, and 9 weeks and development of AHR was assessed in female **(A)** and male **(B)** offspring. Mean ± SEM shown. Data represent 7 to 18 mice pooled from 4 experiments. *$P < .05$ between PBS Mom HDM Pup and HDM Mom HDM Pup groups.

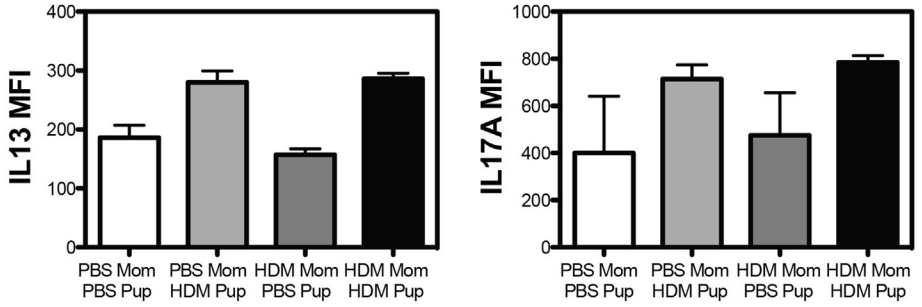

FIG E2. Similar magnitude of cytokine production in T_H2 and T_H17 cells in control offspring and offspring of HDM-exposed dams. Offspring of PBS- and HDM-exposed mothers were treated with PBS or HDM as described in the Methods section. The MFI of IL-13 *(left panel)* and IL-17A *(right panel)* in IL-13^+ $CD4^+$ T cells and IL-$17A^+$ $CD4^+$ T cells was assessed by flow cytometry. *MFI,* Mean fluorescence intensity. Mean + SEM shown. Data represent 2 to 7 mice in a single representative experiment of 3 performed.

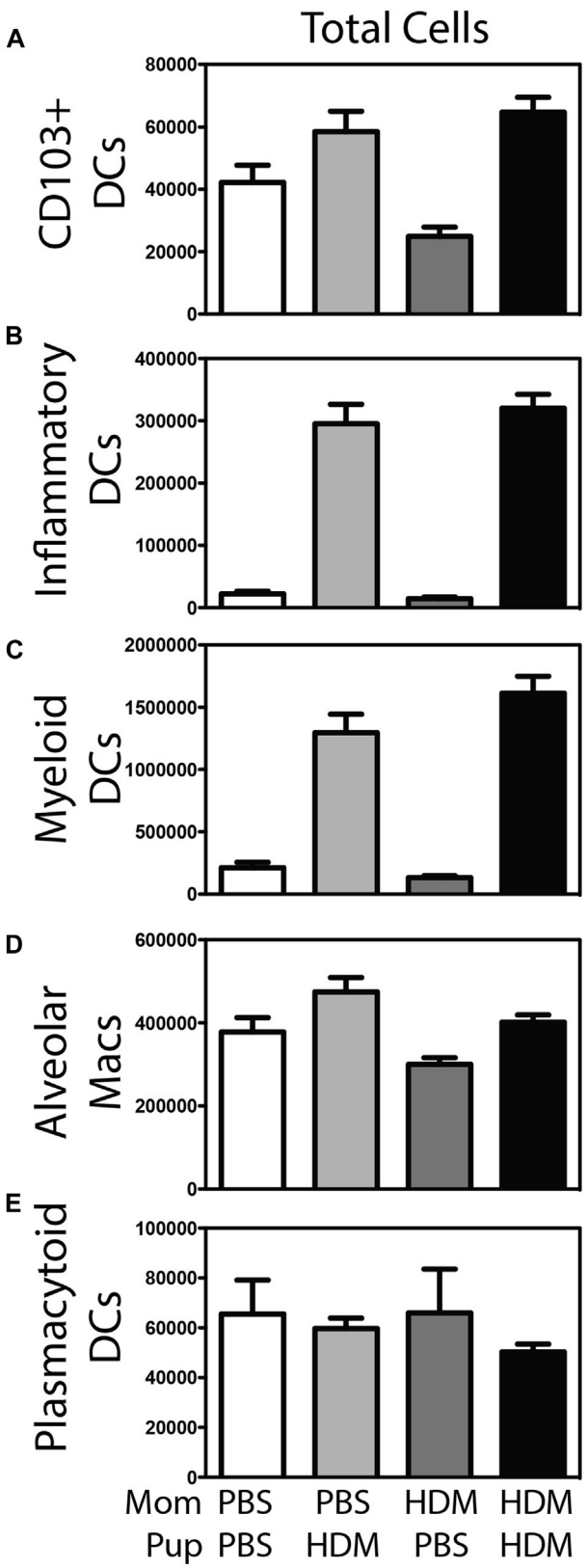

FIG E3. No changes in pulmonary DC numbers in HDM-challenged offspring of HDM-exposed mothers. Offspring of PBS- and HDM-exposed mothers were treated with PBS or HDM as described in the Methods section. Total numbers (mean fluorescence intensity) and % of HDM-bearing $CD103^+$ DCs **(A)**, inflammatory DCs **(B)**, myeloid DCs **(C)**, alveolar macrophages **(D)**, and plasmacytoid DCs **(E)** were assessed by flow cytometry. Mean + SEM shown. Data represent 7 to 18 mice pooled from 4 experiments.

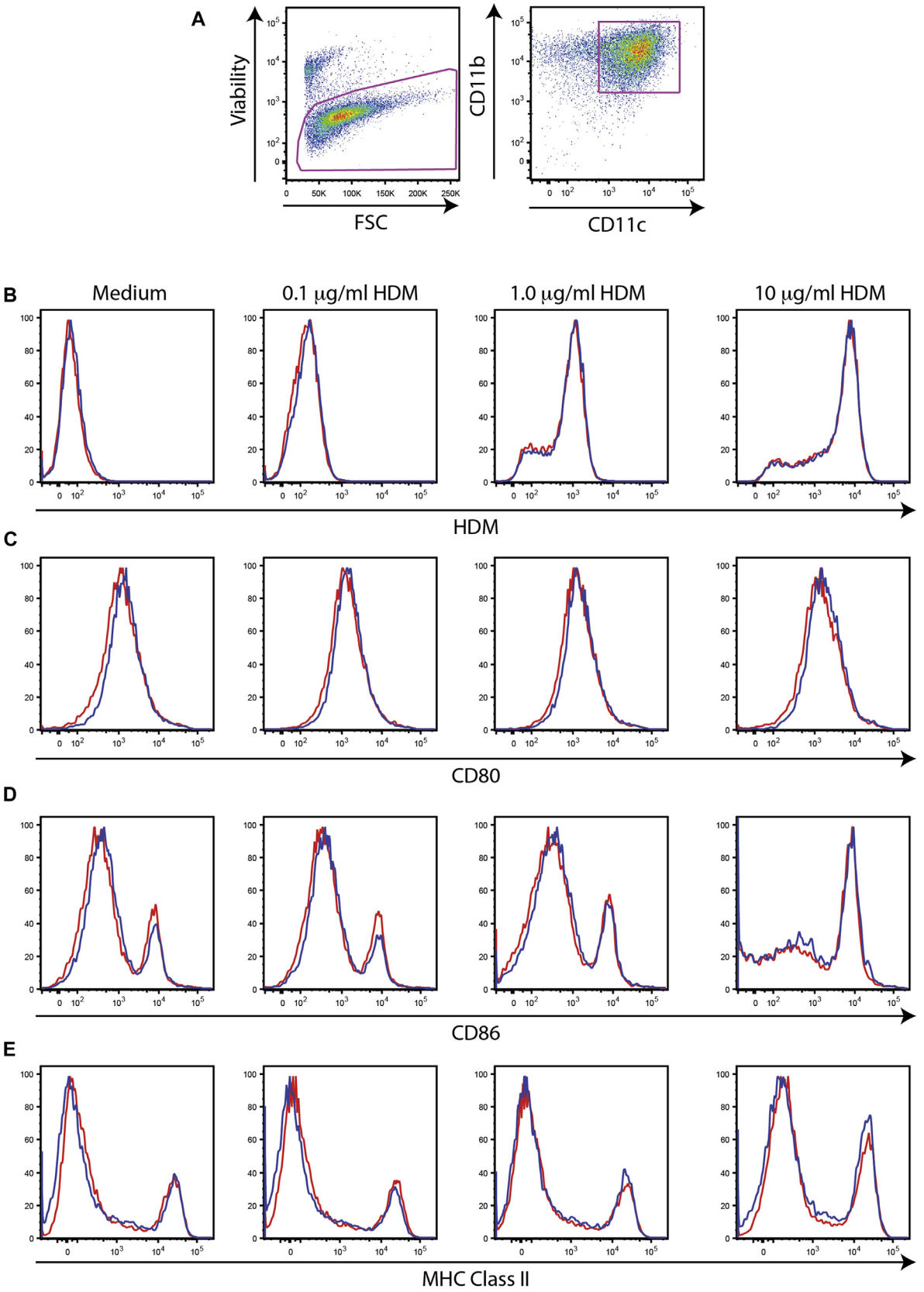

FIG E4. BMDCs from offspring of HDM-exposed mothers do not display altered capacity for allergen uptake or allergen-stimulated costimultory molecule expression. BMDCs were generated from control offspring (blue histograms) or offspring of HDM-exposed mothers (red histrograms). BMDCs were cultured with PBS or HDM at the indicated concentrations and live DCs were identified by expression of CD11c and CD11b **(A)**. Uptake of fluorescently labeled HDM **(B)** and expression of CD80 **(C)**, CD86 **(D)**, and MHC class II **(E)** were assessed. Plots show representative analysis from 1 of 3 cultures performed. *BMDC*, Bone marrow–derived DCs; *FSC*, forward scatter.

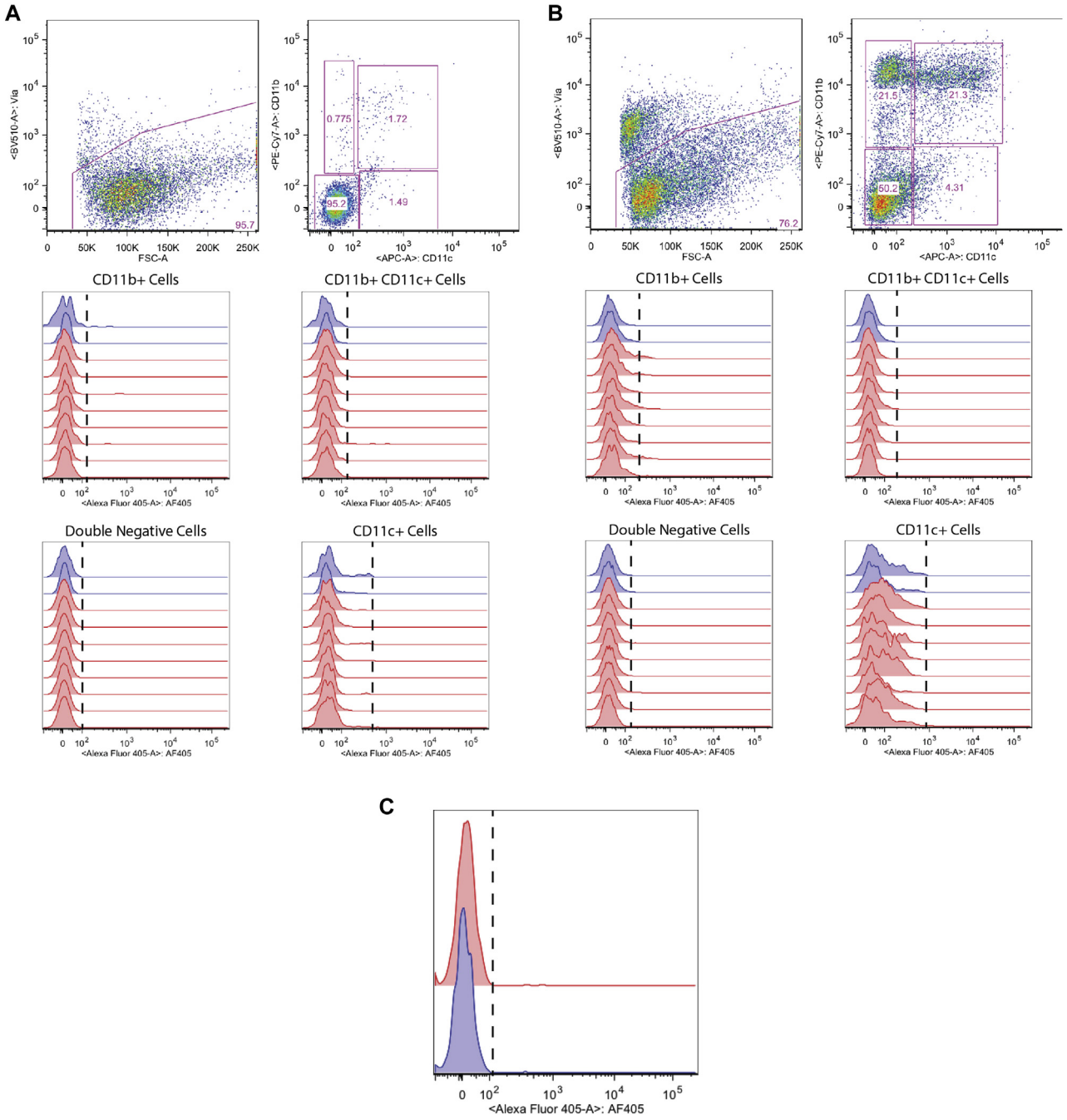

FIG E5. After maternal HDM exposure, HDM is not detectable in fetal tissues, placental tissues, of amniotic fluid cells. Pregnant female mice were exposed to HDM or PBS on days 0 and 4 of pregnancy, and with AF405-labeled HDM on day 10 of pregnancy. Mice were sacrificed on day 12 of pregnancy and the presence of HDM was assessed in fetal tissue **(A)**, placental tissue **(B)**, and cells from the amniotic fluid **(C)** of control offspring (blue histograms) and offspring of HDM-exposed mothers (red histograms). Top panels in Fig E5, *A* and *B*, show strategy to identify CD11c and CD11b-expressing cells, and lower panels show AF405 histograms. Fig E5, *C*, represents cells pooled from all control offspring and offspring of HDM-exposed mothers.

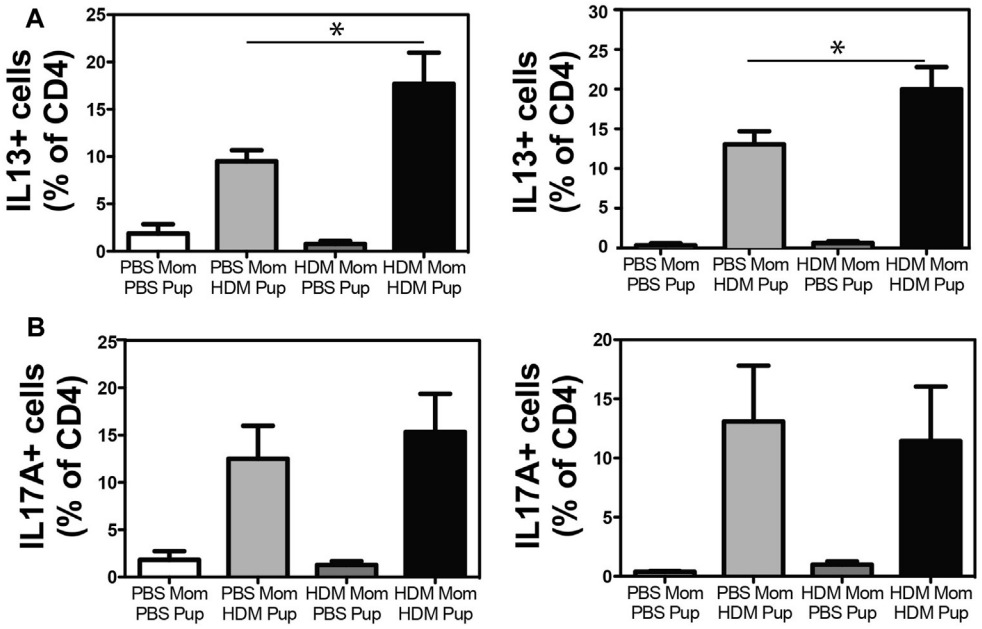

FIG E6. Offspring of HDM-exposed μMT dams demonstrate increased T_H2 cell recruitment. Naive μMT dams *(right graphs)* or wild-type C57Bl/6 dams *(left graphs)* were mated with naive C57Bl/6 sires and pregnant female mice were exposed to HDM or PBS on days 0, 4, and 10 of pregnancy. Offspring of PBS- and HDM-exposed mothers were exposed to intraperitoneal PBS or HDM at age 6 and 7 weeks, and challenged with intratracheal PBS or HDM at age 8 and 9 weeks and sacrificed 72 hours later. Single-cell suspensions of lung cells were restimulated with HDM, and the frequency of $CD4^+$ cells producing IL-13 **(A)** and IL-17A **(B)** was assessed by flow cytometry. Mean + SEM shown. Data represent 3 to 13 mice pooled from 3 experiments. $*P < .05$.

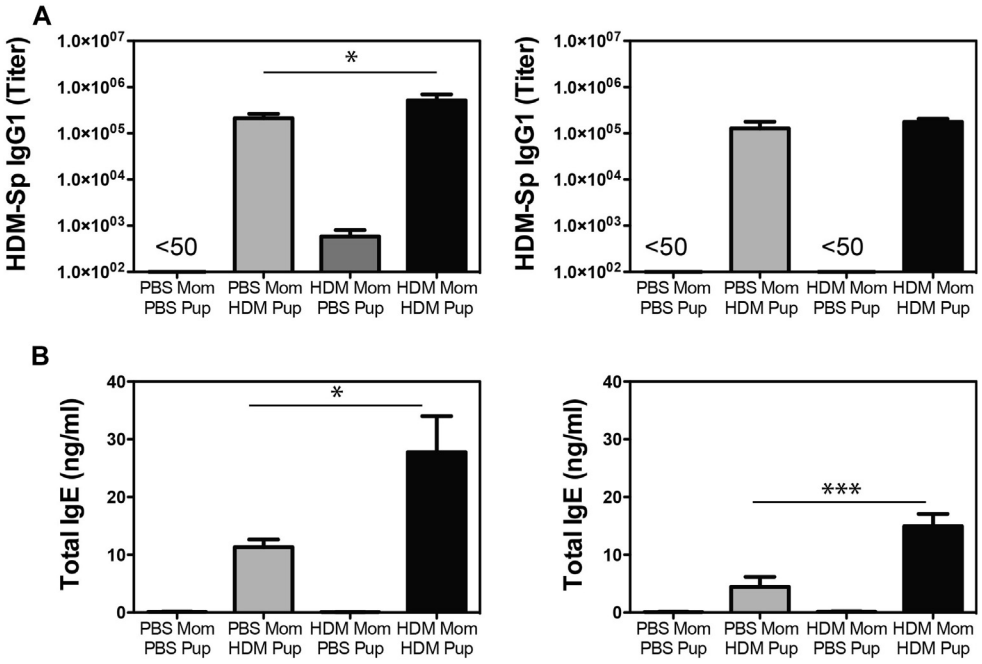

FIG E7. Offspring of HDM-exposed μMT dams demonstrate increased total IgE production. Male C57Bl/6 mice were mated with female C57Bl/6 dams *(left column)* or B-cell–deficient μMT dams *(right columns)* and pregnant female mice were exposed to HDM or PBS on days 0, 4, and 10 of pregnancy. Offspring of PBS- and HDM-exposed mothers were exposed to intraperitoneal PBS or HDM at age 6 and 7 weeks, and challenged with intratracheal PBS or HDM at age 8 and 9 weeks and sacrificed 72 hours later. Levels of HDM-specific IgG_1 **(A)** and total IgE **(B)** were assessed in the serum by ELISA. *Sp*, Specific. Mean + SEM shown. Data represent 3 to 13 mice pooled from 3 experiments. $*P < .05$ and $***P < .001$.

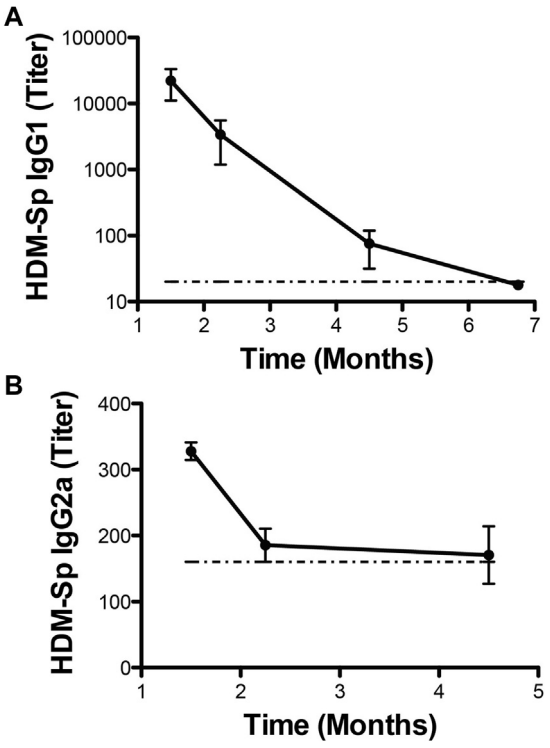

FIG E8. Kinetics of HDM-specific immunoglobulin levels in offspring of HDM-exposed mothers. Naive A/J offspring of HDM-exposed dams were bled at the indicated times, and the levels of HDM-specific IgG_1 **(A)** and IgG_{2a} **(B)** present in the serum were assessed by ELISA. Dotted line indicates background titers present in naive animal run at the same time as serum from offspring of HDM-exposed mother. Each time point consists of 2 or 3 independent animals. *Sp*, Specific.