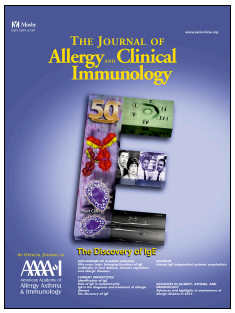
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Maternal house dust mite exposure during pregnancy enhances severity of HDM-induced asthma in murine offspring

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Word Count (Introduction, Results, Discussion, Methods) – 4,111

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Abstract

Background: Atopic status of the mother and maternal exposure to environmental factors is 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 like house dust mite (HDM), in the absence of additional adjuvants, influences offspring asthma development.

Objective: To determine if 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, Th2-associated cytokine synthesis and pulmonary dendritic cell activity was assessed.

Results: Compared to offspring of PBS-exposed mothers, offspring of HDM-exposed mothers demonstrate increased AHR, airway inflammation, Th2 cytokine production, 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 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 under-appreciated influences on the overall prevalence of allergic asthma.

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Key Messages

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

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- Key words – Asthma, Allergen, Pregnancy, Maternal Exposures, Environment

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Abbreviations Used

AF – *Aspergillus fumigatus*

AHR – Airway hyperresponsiveness

ANOVA – Analysis of Variance

BAL – bronchoalveolar lavage

CFA – complete Freund's adjuvant

DC – dendritic cell

HDM – house dust mite

Ig – immunoglobulin

i.p. - intraperitoneal

i.t. – intratracheal

MFI – mean fluorescence intensity

PBS – Phosphate buffered saline

SEM – Standard error of the mean

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Introduction

Allergic asthma is one of the most common immunological 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]. While 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 development of asthma in offspring if pregnancy was initiated during the "Th2 phase" of the anti-parasitic 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 maternal exposure to allergens themselves may alter asthma development in offspring. Supporting this, pre-sensitization of female mice to ovalbumin (OVA) in the presence of alum, and OVA re-exposure during pregnancy increased asthma development in offspring of exposed mothers[29-32]. Prior sensitization of female mice to *Aspergillus fumigatus* extract, and re-exposure during early pregnancy increased airway eosinophilia, and IL-4 promoter hypomethylation in grandoffspring of exposed mothers[33]. Intra-uterine 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 co-administration of alum makes it difficult to determine whether the observed effects are allergen or adjuvant-driven and while 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 as 1) there is a dose-response relationship between HDM exposure and asthma development that does not exist for other common indoor aeroallergens (e.g. cat or dog dander)[37-39]; 2) 50 - 80% of asthmatics demonstrate evidence of a 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 if maternal exposure during pregnancy to a common human allergen, house dust mite (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.

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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, OH). Cincinnati Children's Hospital IACUC approved all animal protocols.

Mating, and allergen treatment protocols

As A/J mice are relatively poor breeders, naïve, age-matched colony reared A/J females were mated, and allowed to rear one litter to gain experience in rearing pups prior to being selected for further study. A/J mice were chosen as we have demonstrated that this strain develops severe AHR associated with a mixed Th2/Th17 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) i.p. 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 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 6, 8 and 9 weeks of age offspring were anaesthetized with ketamine/xylazine and exposed to PBS (40 µl), HDM (200 µg) as described elsewhere[59], or 1 µg *Aspergillus fumigatus* extract (Greer Labs, Lenoir NC) i.t. as indicated. As C57Bl/6 mice (and µMT mice which are on a C57Bl/6 background)

are considered refractory to induction of asthma, a more vigorous regimen of allergen exposure consisting of i.p. HDM at 6 and 7 weeks of life, and i.t. 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 DC recruitment. Where indicated, AlexaFluor405 (Invitrogen, Carlsbad, CA) labeled HDM (AF405-HDM) was used.

Isolation of fetal tisue, 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 and 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 online repository). Single cell suspensions were generated for flow cytometric analysis.

Statistical Analysis

To determine differences between multiple groups, analysis of variance (ANOVA) was used with *post hoc* comparisons using Tukey's test. Significance was assumed at $p < 0.05$. Titers of HDM-specific IgG_1 and IgG_{2a} were Log10 transformed prior to analysis.

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Results

Maternal exposure to house dust mite 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 if maternal exposure to HDM during pregnancy impacted offspring asthma. Female A/J mice (a strain that develops severe AHR associated with a mixed Th2/Th17 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 6, 8 and 9 weeks of age, offspring were exposed to 200 μg of intratracheal (i.t.) 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 1A). As maternal asthma status was recently suggested to differentially influence asthma development in male and female offspring[45], 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 to offspring from PBS-exposed mothers (Supplementary Figure 1). Increased AHR in offspring of HDM-exposed animals was accompanied by increased recruitment of inflammatory cells in BAL fluid (Fig 1B). Significantly increased numbers of lymphocytes (Fig 1C) and eosinophils (Fig 1D) were observed in offspring of HDM-exposed mothers. While we also observed a trend towards increased the numbers of neutrophils (Fig 1E) and macrophages (Fig 1F) in offspring of HDM-exposed mothers this did not reach statistical significance.

Increased AHR in offspring of HDM-exposed mothers is associated with increased Th2 cytokine production and HDM-specific immunoglobulin production.

As development of asthma is the result of excessive Th2 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 Th2 cytokines IL-4, IL-5, IL-13, as well as the Th17-associated cytokine IL-17A (Fig 2A-D). There was no appreciable production of IFNγ (data not shown). Consistent with *in vivo* data, elevated levels of IL-4, IL-5, and IL-13 (Fig 2A, 2B, 2C) were observed in cells isolated from HDM-exposed offspring of HDM-exposed mothers compared to cultures of cells from HDM-exposed offspring of PBS-exposed mothers. In contrast, production IL-17A was comparable in HDM-exposed offspring of PBS- and HDM-exposed mothers (Fig 2D). Moreover accumulation of pulmonary CD4+ T cells expressing IL-13 (Fig 2E) and IL-17A (Fig 2F) was observed in HDM-challenged offspring, and maternal HDM exposure significantly increased the frequency of IL-13+ CD4+ T cells (Fig 2E) but did not influence the frequency of IL-17A+ CD4+ T (Fig 2F) following HDM exposure of offspring. MFI of IL-13 or IL-17A in cytokine expressing cells was not elevated (Supplementary Figure 2), 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.

Finally, as development of allergy is also associated with an allergen-specific humoral response, we also examined Ig levels in animals from the various groups. HDM exposure induced substantial increases in levels of total IgE (a reliable surrogate for HDM-specific IgE)[46], 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 3A) although this did not reach statistical significance. HDM-specific IgG_1 (Fig 3D) and HDM-specific IgG_{2a} levels (Fig 3E) were significantly elevated in HDM-exposed offspring of HDM-exposed mothers compared to 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, while 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 two 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, Th2 cytokine production, pulmonary inflammation and AHR following HDM exposure of offspring.

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

As 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 numbers of most DC subsets in the lung (with the exception of pDCs), but there was no difference in the numbers of DCs recruited to the lungs in offspring of control, or HDM-exposed mothers (Supplementary Fig 3). 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 pDCs (data not shown). A significant decrease in phagocytic capacity of mDCs (measured by HDM mean fluorescence intensity (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 co-stimulatory 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 dendritic cells (BMDCs) 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 (Supplementary Fig 4), suggesting that the observed decrease in pulmonary mDC 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 Aspergillus fumigatus-induced airway inflammation

Prior maternal sensitization with OVA (alum), and subsequent OVA challenge in pregnancy has 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 6, 8 and 9 weeks of age, offspring (housed according to maternal exposure) were exposed to either intratracheal HDM or *Aspergillus fumigatus* (AF) extract. As expected, prior maternal HDM exposure increased the magnitude of HDM-induced airway inflammation (Fig. 5A). The effect was particularly striking for airway eosinophilia (Fig. 5B), although strong trends were observed for airway lymphocytes, neutrophils and macrophages (Fig 5C – E). In contrast, maternal HDM exposure had no impact on the severity of AF-induced airway inflammation as levels of total airway infiltrating cells, eosinophils, lymphocytes, neutrophils, and macrophages were all comparable in AF-exposed offspring of PBS-exposed, and HDM-exposed mothers.

To further characterize the asthmatic response in AF-exposed offspring, we also examined production of Th2-associated cytokines in AF-exposed offspring. In these studies cells were stimulated with ConA to directly compare the changes in capacity for cytokine production induced by prior HDM or AF exposure. In HDM-exposed offspring prior maternal HDM exposure resulted in a trend towards increased production of the Th2 cytokines IL-4, IL-5 and IL-13, an increased frequency of IL-13^+ T cells (Fig 6D), and increased levels of total IgE (Fig 6E). In contrast, we could discern no significant impact of maternal HDM exposure on ConA-induced cytokine production, the frequency of pulmonary IL-13+ CD4+ T cells, or IgE levels in AF-exposed offspring, (Fig 6A – 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.

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 (d10 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- cell populations, although appreciable AF405 signals were not detected in any cell population in tissues harvested from HDM-exposed mothers (Supplementary Figure 5A – B). 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 (Supplementary Figure 5C). 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 there is not a strong transfer of HDM-derived proteins following maternal HDM exposure.

Finally, to determine if 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[47]. 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 which 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.

As C57Bl/6 mice are more resistant to the development of AHR, a more robust protocol of HDM-induced AHR was utilized wherein mice are given 10 μg HDM i.p. (in the absence of additional adjuvant) at 6 and 7 weeks of life, and then challenged with 100 μg of HDM i.t. 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 significantly greater AHR in C57Bl/6 offspring (Fig 7A), suggesting that maternal HDM exposure-mediated exacerbation of asthma is not limited to A/J mice. Moreover, while 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 BAL fluid (Fig 7B – F), as well as an increased recruitment of IL-13+ CD4+ T cells, but not IL-17A+ CD4+ T cells (Supplementary Figure 6). Additionally, 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 (Supplementary Figure 7)(IgG_{2a} is not expressed in C57Bl/6 mice[48], and as such, was not measured). As expected, PBS-treated offspring of HDM-exposed C57Bl/6 mothers displayed readily detectable levels of HDM-specific IgG_1, while PBS-treated offspring of HDM-treated μMT mothers had undetectable levels of HDM-specific IgG_1, suggesting that this immunoglobulin is likely maternally derived (Supplementary Figure 7). 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, house dust mite (HDM), in the absence of powerful Th2-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 Th2 cytokine production and frequency of CD4+IL-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 *Aspergillus fumigatus*-induced airway inflammation.

As 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 systemic Th1 phase of the infection, offspring were found to be protected from development of asthma[21]. Anti-IFNγ abrogated this protective effect of maternal *Schistosoma mansoni* infection, suggesting that maternal cytokines can influence asthma development in offspring[21]. Feeding female mice OVA prior to pregnancy to induce tolerance protects from the development of OVA-induced allergic airway disease in an FcRn and IFNγ-dependent manner[49]. Similarly, sensitization with OVA (CFA), and re-exposure to OVA during pregnancy limited the development of OVA-specific IgE and pulmonary eosinophilia in offspring of exposed mothers[50]. 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], maternal *Aspergillus fumigatus*-exposure[33], and in *Schistosoma mansoni*-infected mothers impregnated during the Th2 phase of the anti-helminthic response[21]. Blockade of IL-4 during pregnancy completely abrogated the increased sensitivity of offspring of following maternal OVA (alum) sensitization and challenge[30], again suggesting 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, Th2 cytokine production or immunoglobulin production following *Aspergillus fumigatus* exposure in offspring of HDM-exposed mothers. Thus, while 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 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 to OVA (alum) sensitization and challenge performed by Hamada *et al*[30]. Alternatively, while HDM exposures in offspring of HDM-exposed mothers began at 6 weeks of age 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 Th2 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 dendritic cell (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 co-stimulatory 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 to splenic DCs from offspring of control mothers[32]. These results suggest that splenic DCs from offspring of allergen-exposed mothers were more "pro-asthmatic". In contrast to these reports, we find that the natural allergen, HDM, in the absence of additional immune-activating adjuvants drives limited change in co-stimulatory molecule expression or function of DCs in offspring of HDM-exposed mothers, while actually decreasing phagocytic capacity of various pulmonary DC subsets. Interestingly, the observed decrease in phagocytic capacity was not observed in HDM-pulsed BMDCs 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. While 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 AF-specific immune responses are impacted following maternal HDM-exposure is not consistent with the induction of an intrinsically more "pro-asthmatic" population of pulmonary dendritic cells.

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 (Supplementary Figure 8), 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 WT C57Bl/6 animals still drives significantly enhanced HDM-induced AHR, airway inflammation and pulmonary Th2 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 non-specific IgE and IgG_1, IgG_{2c} following allergen challenge[51]. As B-1B cell-derived Abs typically recognize polysaccharide antigens with low affinity[52], and HDM contains glycosylated proteins[53], it is conceivable that B1-derived maternal Igs are

455 either sufficient to transfer increased HDM-reactivity to offspring, or they facilitate the
456 transplacental passage of antigens in Ig:antigen complexes[54-57], allowing for priming of
457 HDM-specific T cells in offspring of exposed mothers. Subsequent inhalational exposure
458 in offspring of HDM-exposed mothers may then represent additional "boosts" not present
459 in offspring of PBS-exposed mothers, and drive the development of a more robust
460 response. Finally, it is possible that vertical transmission of maternal T cells (either
461 through transplacental passage or via breast milk) passively sensitizes the fetus to HDM
462 through a maternal microchimerism-dependent mechanism as has been observed in
463 model of maternal tuberculosis of Candida infection[58]. These possibilities are currently
464 being considered in our laboratory.
465
466 In conclusion, we report that maternal HDM exposure during pregnancy is sufficient to
467 enhance neonatal sensitivity to HDM. This is associated with increased AHR, airway
468 inflammation, Th2 cytokine production, immunoglobulin levels and a modest decrease in
469 the phagocytic capacity of pulmonary macrophage populations following HDM exposure
470 in offspring. Interestingly, in contrast to previous reports of allergen-independent
471 influence of maternal OVA (alum) sensitization and challenge on offspring asthma, these
472 effects are wholly allergen dependent, as exposure of offspring to an unrelated allergen,
473 *Aspergillus fumigatus*, does not result in the development of more severe airway
474 inflammation, Th2 cytokine production or immunoglobulin synthesis. These findings
475 have important implication for regulation of asthma risk, and suggest that increased
476 HDM burden in the developed world may have under-appreciated influences on the
477 overall prevalence of allergic asthma.
478

Figure Legends

Figure 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 6, 8 and 9 weeks of age and development of AHR was assessed (A). After sacrifice, BAL fluid was collected to assess (B) total BAL cellularity, and the number of (C) lymphocytes, (D) eosinophils, (E) neutrophils and (F) macrophages. Mean + SEM shown. Data represent 7 – 18 mice pooled from 4 experiments.

Figure 2: Increased Th2 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 Methods. Single cell suspensions of lung cells were restimulated with HDM, and production of (A) IL-4, (B) IL-5, (C) IL-13 and (D) IL-17A were assessed by ELISA. The frequency of CD4+ cells producing (E) IL-13 and (F) IL-17A was assessed by flow cytometry. Mean + SEM shown. * indicates $p < 0.05$. Data represent 7 – 18 mice pooled from 4 experiments.

Figure 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 Methods. Serum isolated at time of sacrifice was assayed for (A) total IgE, (B) HDM-specific IgG1 and (C) HDM-specific IgG2a by ELISA. Lowest dilution on plate indicated. Mean + SEM shown. * and *** indicate $p < 0.05$ and $p < 0.001$. Data represent 7 – 18 mice pooled from 4 experiments.

Figure 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 Methods. Amount of HDM phagocytosed (MFI) and frequency of HDM-bearing (% of total lung cells) (A) myeloid DCs, and (B) alveolar macrophages were assessed by flow cytometry.

510 Mean + SEM shown. * and *** indicate $p < 0.05$ and $p < 0.001$. Data represent 7 – 18
511 mice pooled from 4 experiments.
512
513 Figure 5: Maternal HDM exposure does not increase the severity of AHR and airway
514 inflammation in *Aspergillus fumigatus*-exposed offspring. Offspring of PBS- and HDM-
515 exposed mothers were exposed to PBS, HDM or AF extract at 6, 8 and 9 weeks of age.
516 After sacrifice, BAL fluid was collected to assess (A) total BAL cellularity, and the
517 number of (B) eosinophils, (C) lymphocytes, (D) neutrophils and (E) macrophages.
518 Mean + SEM shown. Data represent 2 – 5 mice pooled from 2 experiments.
519
520 Figure 6: Maternal HDM exposure does not increased the magnitude of the Th2 response
521 or magnitude of the Ig response following exposure of offspring to *Aspergillus fumigatus*.
522 Offspring of PBS- and HDM-exposed mothers were exposed to PBS, HDM or AF extract
523 at 6, 8 and 9 weeks of age. Single cell suspensions of lung cells were restimulated with
524 ConA, and production of (A) IL-4, (B) IL-5, (C) IL-13 were assessed by ELISA. (D) The
525 frequency of CD4+ cells producing IL-13 was assessed by flow cytometry. (E) Serum
526 isolated at time of sacrifice was assayed for total IgE. Mean + SEM shown. Data
527 represent 2 – 5 mice pooled from 2 experiments.
528
529 Figure 7: Offspring of HDM-exposed μMT dams demonstrate increased severity of AHR
530 and airway inflammation. Male C57Bl/6 mice were mated with female C57Bl/6 dams
531 (left column) or B cell deficient μMT dams (right columns) and pregnant female mice
532 were exposed to HDM or PBS on days 0, 4 and 10 of pregnancy. Offspring of PBS- and
533 HDM-exposed mothers were exposed to i.p. PBS or HDM at 6 and 7 weeks of age, and
534 challenged with i.t. PBS or HDM at 8 and 9 weeks of age and development of AHR was
535 assessed 72 hours later. (A). After sacrifice, BAL fluid was collected to assess (B) total
536 BAL cellularity, and the number of (C) macrophages, (D) eosinophils, (E) lymphocytes
537 and (F) neutrophils. Mean + SEM shown. Data represent 3 – 13 mice pooled from 3
538 experiments.
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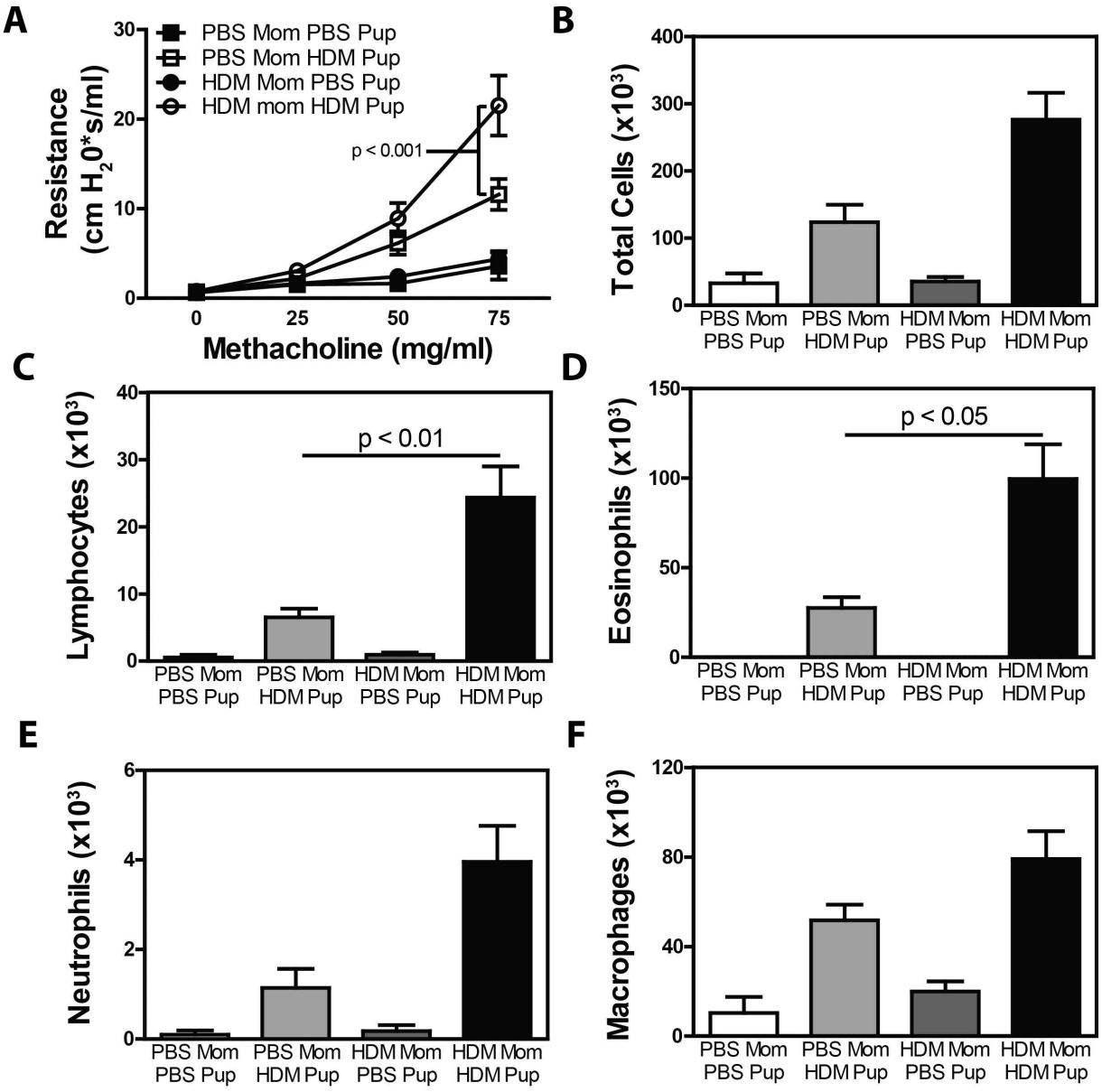
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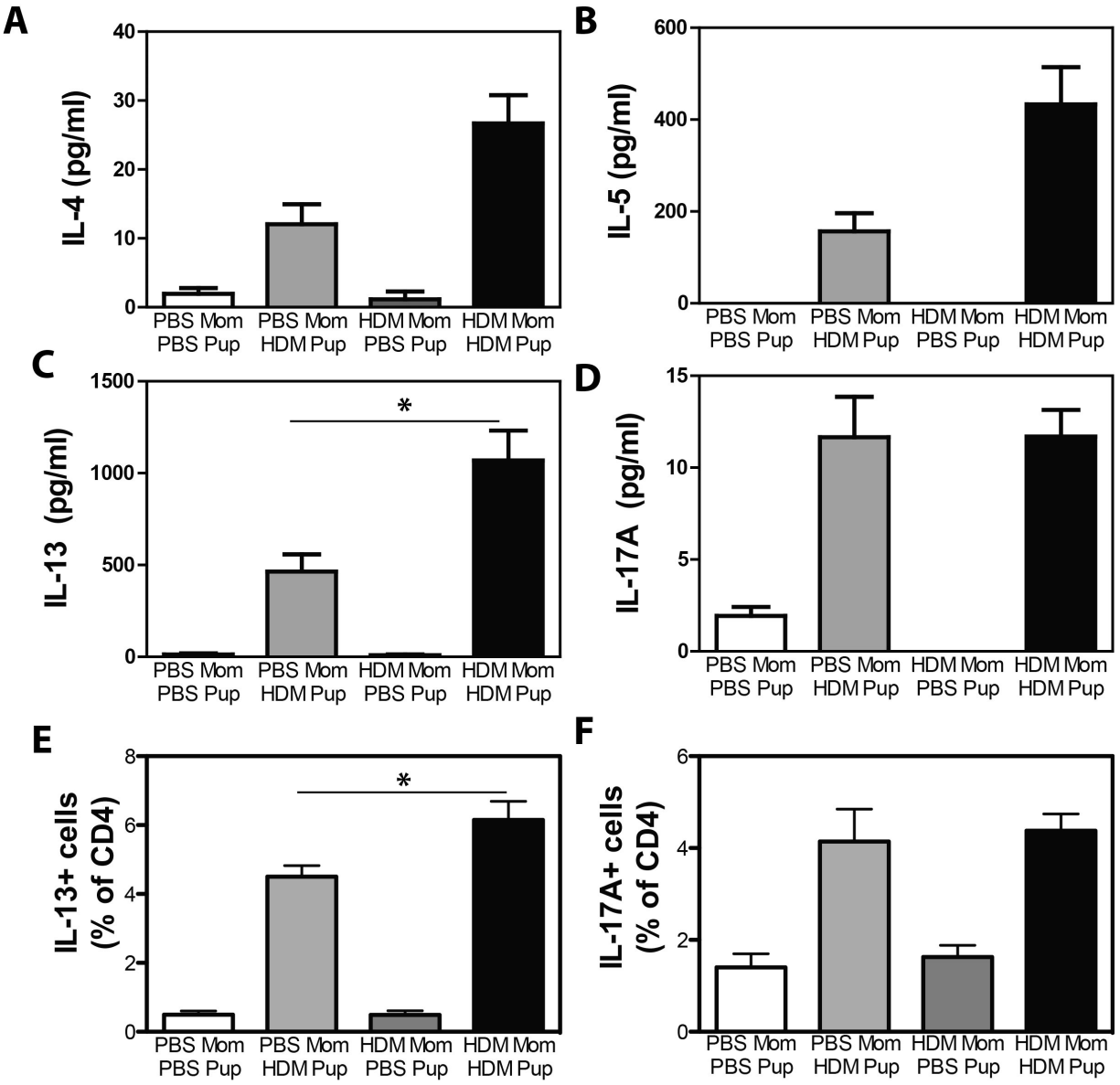
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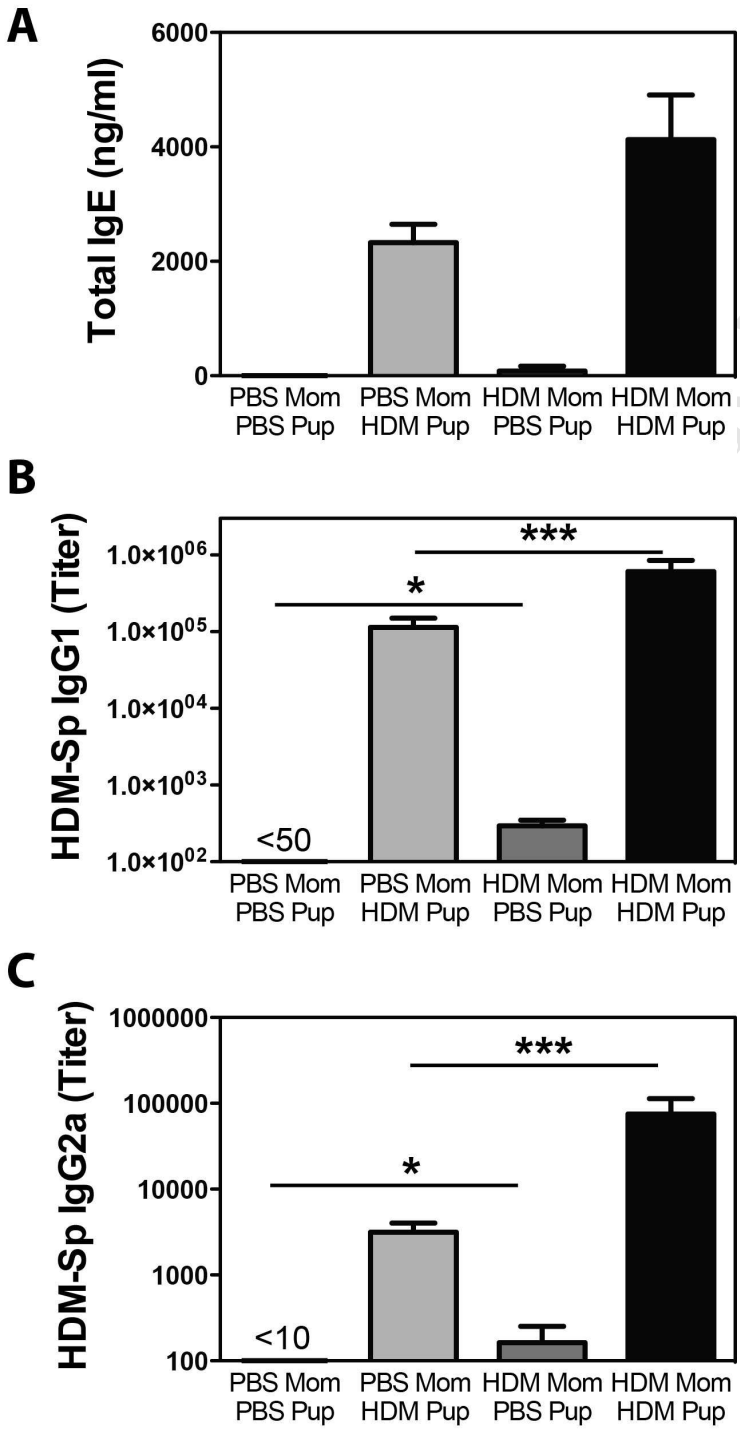


Richgels et al Fig 1

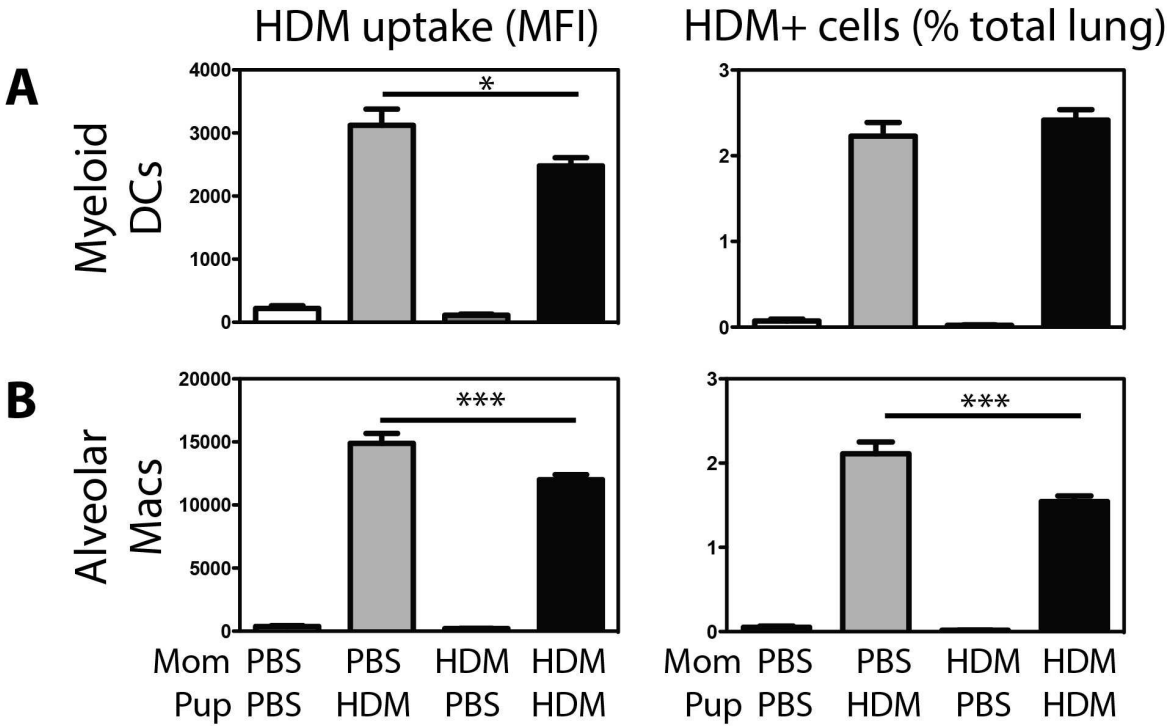


Richgels et al Fig 2

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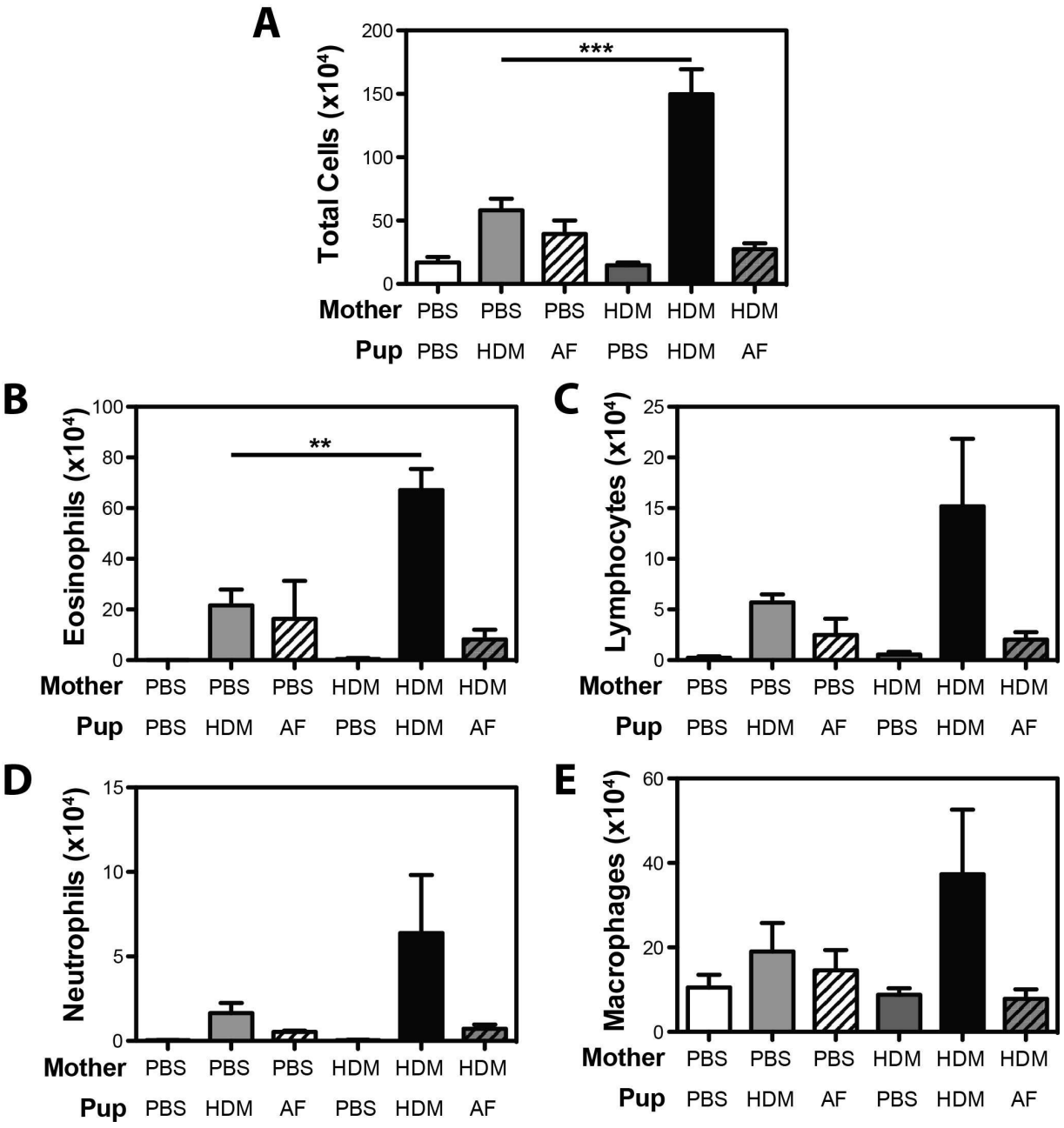


Richgels et al Fig 3

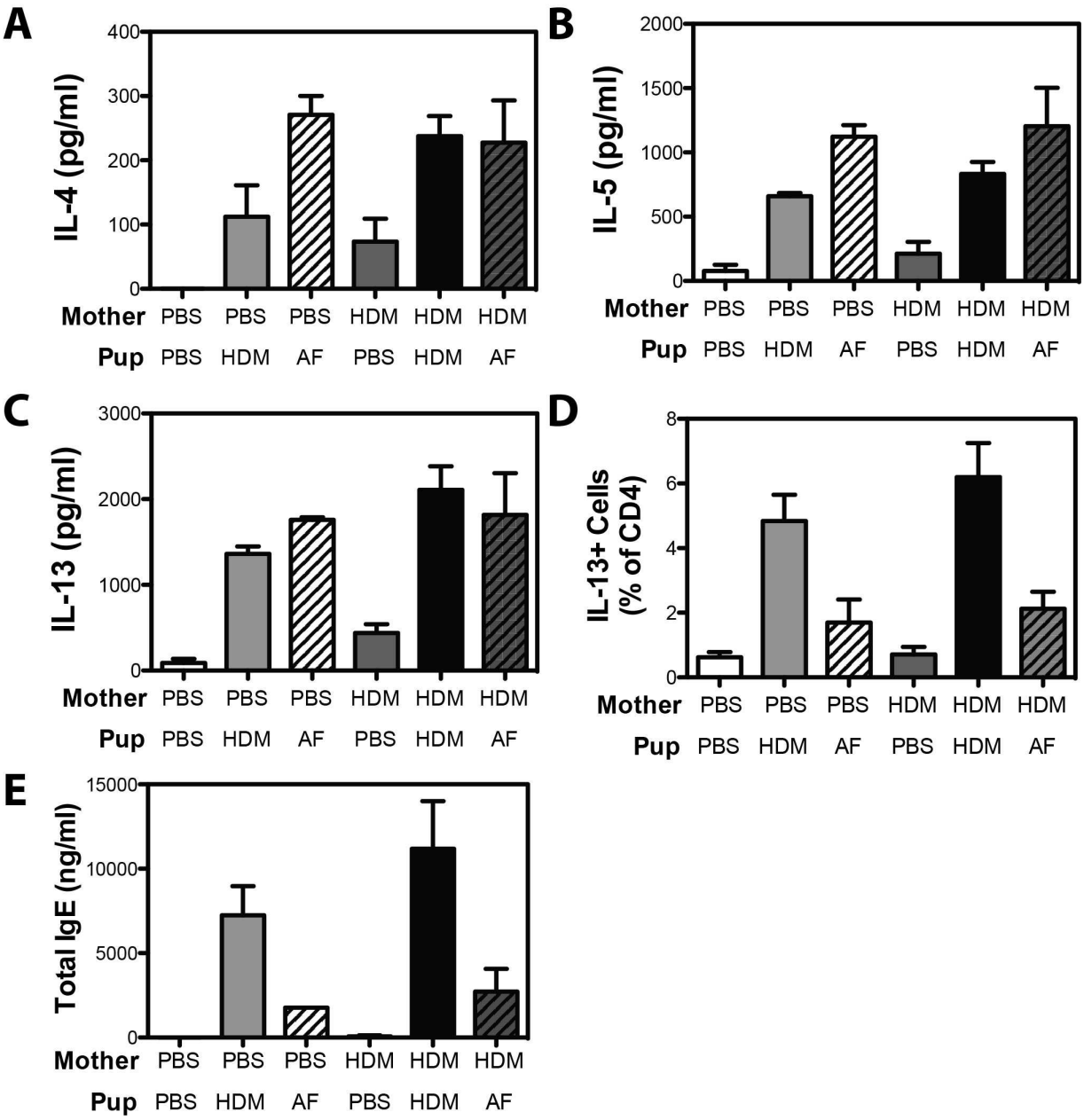


Richgels et al Fig 4

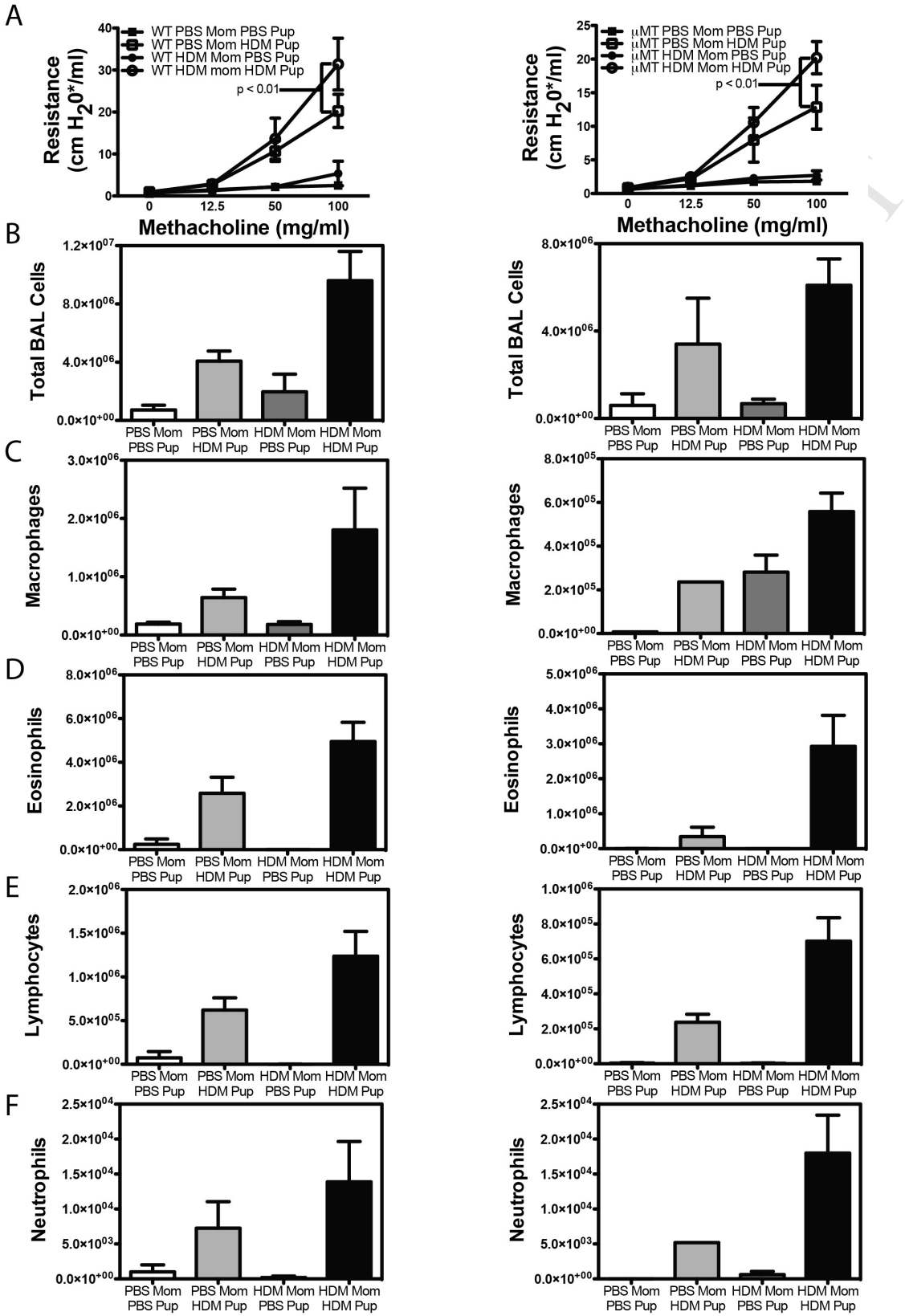
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Richgels et al Fig 5



Richgels et al Fig 6



Richgels et al Fig 7

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

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Assessment of allergen induced allergic responses

Airway responses were evaluated using a flexiVent system (SCIREQ Scientific Respiratory Equipment Inc, Montreal, PQ). Mice were anaesthetized 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 15s with an Aeroneb ultrasonic nebulizer, and ventilated for an additional 10s. 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 Abs from Pharmingen (San Diego, CA). To collect BALF, lungs were lavaged three times with 1.0-ml of cold Hanks' balanced salt solution (Invitrogen, Carlsbad, CA). Recovered lavage fluid (70 to 80%) was centrifuged (300*g* for 6 min, then 5000g for 30 seconds) and the cell pellet resuspended in 150 ul of ACK for 2 minutes to lyse erythrocytes. 500 ul of 5% FBS in PBS was added and cells were centrifuged (300*g* for 6 min, then 5000g for 30 seconds). Cells were resuspended in 200 ul of 5% FBS in PBS and counted with a hemocytometer. Slides were prepared by cytocentrifugation (Cytospin 4; Thermo Scientific, Waltham, MA), and stained with Camco Stain Pak (Cambridge Diagnostic Products, Fort Lauderdale, FL). Bronchoalveolar lavage (BAL) cell differential counts were determined using morphologic criteria under a light microscope with evaluation of ≥ 500 cells/slide. 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, IN) 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, and 10% FBS, and 0.1% BME. 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 x 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 CA), 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, $1x10^6$ lung cells were stimulated with PMA (100 ng/ml; Sigma) and ionomycin (1 ug/ml; Sigma). After overnight stimulation, Brefeldin A and monensin (eBioscience) 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 BMDC 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 (San Diego, CA) unless otherwise indicated. Data were acquired with an LSRII flow cytometer (BD Biosciences, San Jose, CA) equipped with lasers tuned to 355nm, 405nm, 488nm, 561nm, and 640nm. Spectral overlap was compensated using the FACSDiVa software (BD Biosciences) and analyzed using FlowJo software (Treestar Inc., Ashland, OR).

Generation and culture of bone marrow derived dendritic cells

Bone marrow cells (3 x 10^5 cells/ml) were cultured in complete RPMI supplemented with GM-CSF (10 ng/ml, Peprotech, Rocky Hill, NJ, USA). Medium was changed on day 3. On day 6, cells were harvested and counted. 100,000 DCs were cultured with medium, or AF405-HDM at 0.1 μg/ml, 1.0 μg/ml 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 manusfacturer's directions. Tissue culture supernatants were frozen at -80°C and thawed immediately prior to use. Total IgE, IgG_1, and IgG_{2a} 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 degrees overnight in 0.1 M Na_2HPO_4. After blocking with 10% FBS in PBS at room temperature for 2 hours, 10-fold serial dilutions of serum (starting at 1:10 for HDM-specific IgG2a, 1:50 for HDM-specific IgG1) 8 times down the plate and the plates were incubated at 4 degrees overnight. Biotinylated detection antibodies (BD bioscience) were

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112 added, followed by Avidin Peroxidase, and color was developed with Super Aqua Blue
113 (eBioscience, San Diego, CA). The reciprocal of the dilution factor resulting in an O.D.
114 of 0.6 (HDM-specific IgG2a) or 1.5 (HDM-specific IgG1) was determined. All HDM-
115 specific Ig assays from all experiments were run at the same time to facilitate
116 comparisons between experiments.
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Supplementary figure legends

Supplementary Figure 1: 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 6, 8 and 9 weeks of age and development of AHR was assessed in (A) female and (B) male offspring. * indicates $p < 0.05$ between PBS Mom HDM Pup, and HDM Mom HDM Pup groups. Mean ± SEM shown. Data represent 7 – 18 mice pooled from 4 experiments.

Supplementary Figure 2: Similar magnitude of cytokine production in Th2 and Th17 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 Methods. 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. Mean + SEM shown. Data represent 2 – 7 mice in a single representative experiment of 3 performed.

Supplementary Figure 3: 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 Methods. Total numbers (MFI) and % of HDM-bearing (A) CD103+ DCs, (B) inflammatory DCs, (C) myeloid DCs, (D) alveolar macrophages, and (E) plasmacytoid DCs were assessed by flow cytometry. Mean + SEM shown. * and *** indicate $p < 0.05$ and $p < 0.001$. Data represent 7 – 18 mice pooled from 4 experiments.

Supplementary Figure 4: 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), expression of CD80 (C), CD86

(D) and MHC Class II (E) were assessed. Plots show representative analysis from 1 of three cultures performed.

Supplementary Figure 5: 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 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 A and B show strategy to identify CD11c and CD11b-expressing cells, and lower panels show AF405 histograms. C represents cells pooled from all control offspring and offspring of HDM-exposed mothers.

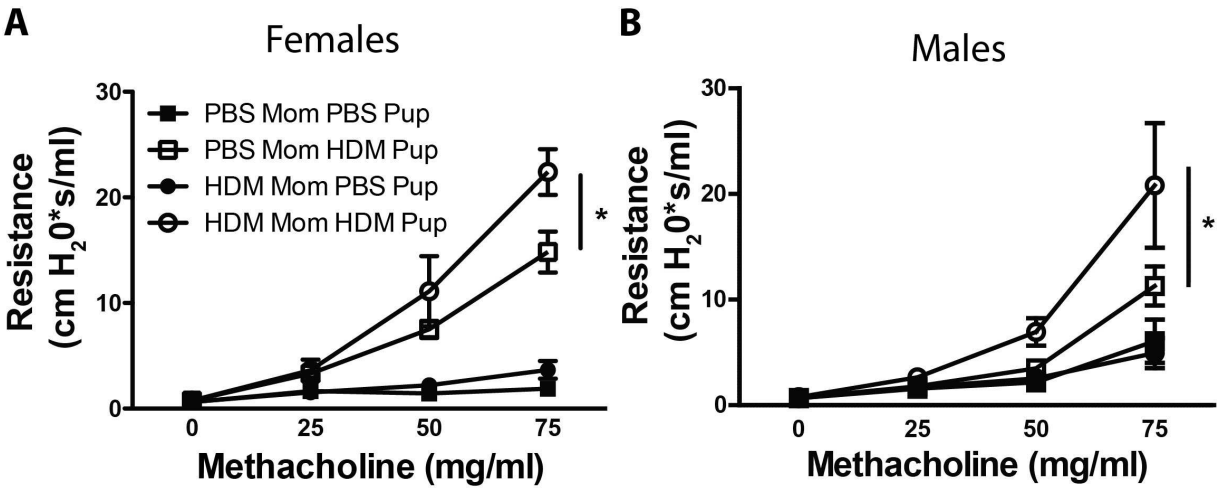
Supplementary Figure 6: Offspring of HDM-exposed μMT dams demonstrate increased Th2 cell recruitment. Naïve μMT dams (right graphs) or WT C57Bl/6 dams (left graphs) were mated with naïve C57Bl/6 sires and pregnant female mice were exposed to HDM or PBS on days 0, 4 and 10 of pregnancy. Offspring of PBS- and HDM-exposed mothers were exposed to i.p. PBS or HDM at 6 and 7 weeks of age, and challenged with i.t. PBS or HDM at 8 and 9 weeks of age and sacrificed 72 hours later. Single cell suspensions of lung cells were restimulated with HDM, and the frequency of CD4+ cells producing (A) IL-13 and (B) IL-17A was assessed by flow cytometry. Mean + SEM shown. * indicates $p < 0.05$. Data represent 3 – 13 mice pooled from 3 experiments.

Supplementary Figure 7: 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 i.p. PBS or HDM at 6 and 7 weeks of age, and challenged with i.t. PBS or HDM at 8 and 9 weeks of age and sacrificed 72 hours later. Levels of HDM-specific IgG1 (A) and Total IgE (B) were assessed in the serum by

ELISA. Mean + SEM shown. * and *** indicates $p < 0.05$ and $p < 0001$ respectively. Data represent 3 – 13 mice pooled from 3 experiments.

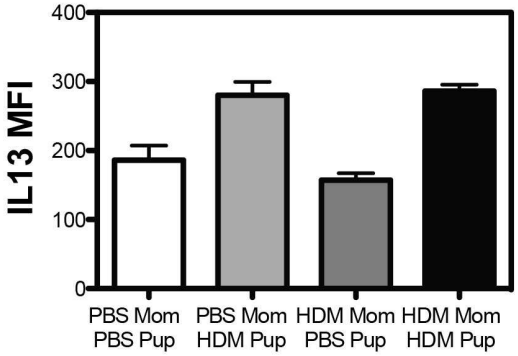
Supplementary Figure 8: Kinetics of HDM-specific immunoglobulin levels in offspring of HDM-exposed mothers. Naïve A/J offspring of HDM-exposed dams were bled at the indicated times, and the levels of HDM-specific IgG_1 (A) and IgG_{2a} (B) present in the serum were assessed by ELISA. Dotted line indicates background titers present in naïve animal run at the same time as serum from offspring of HDM-exposed mother. Each time point consists of 2 or 3 independent animals

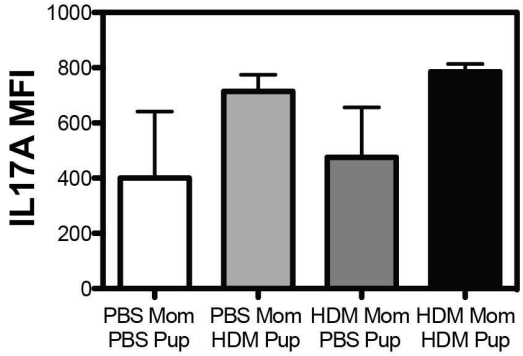
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Richgels et al Supp Fig 1

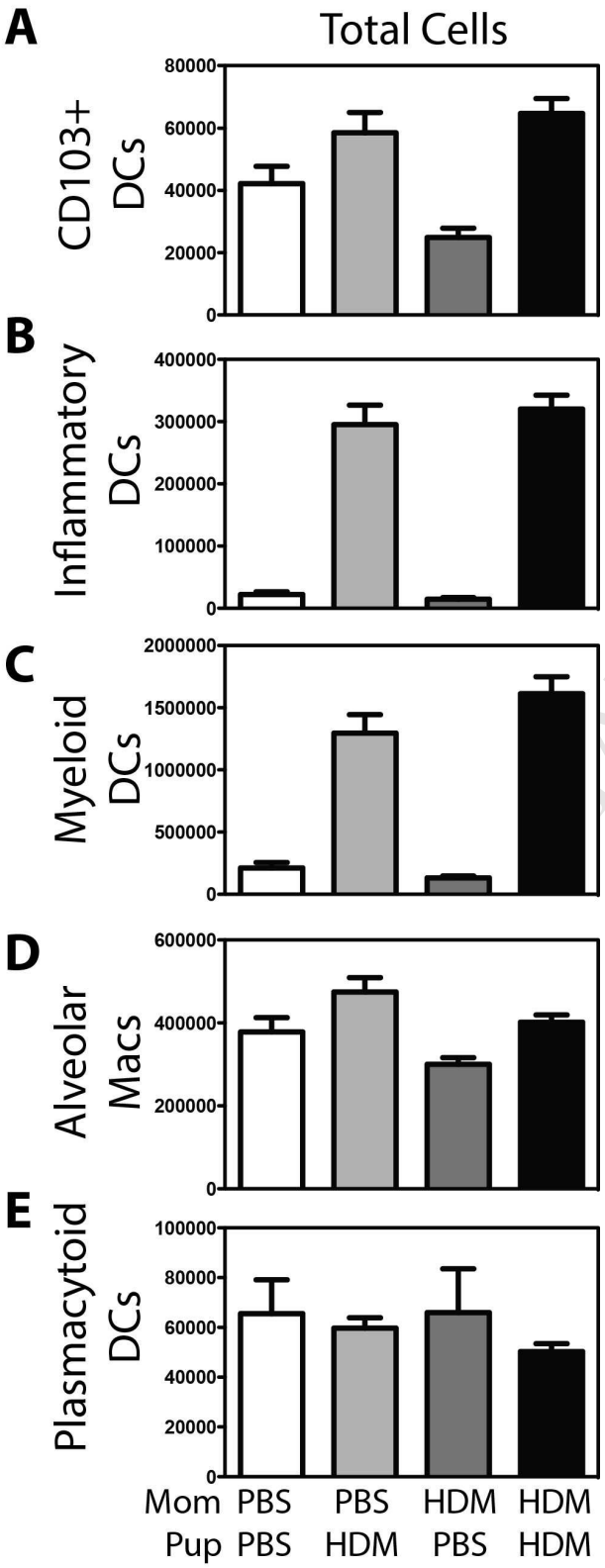
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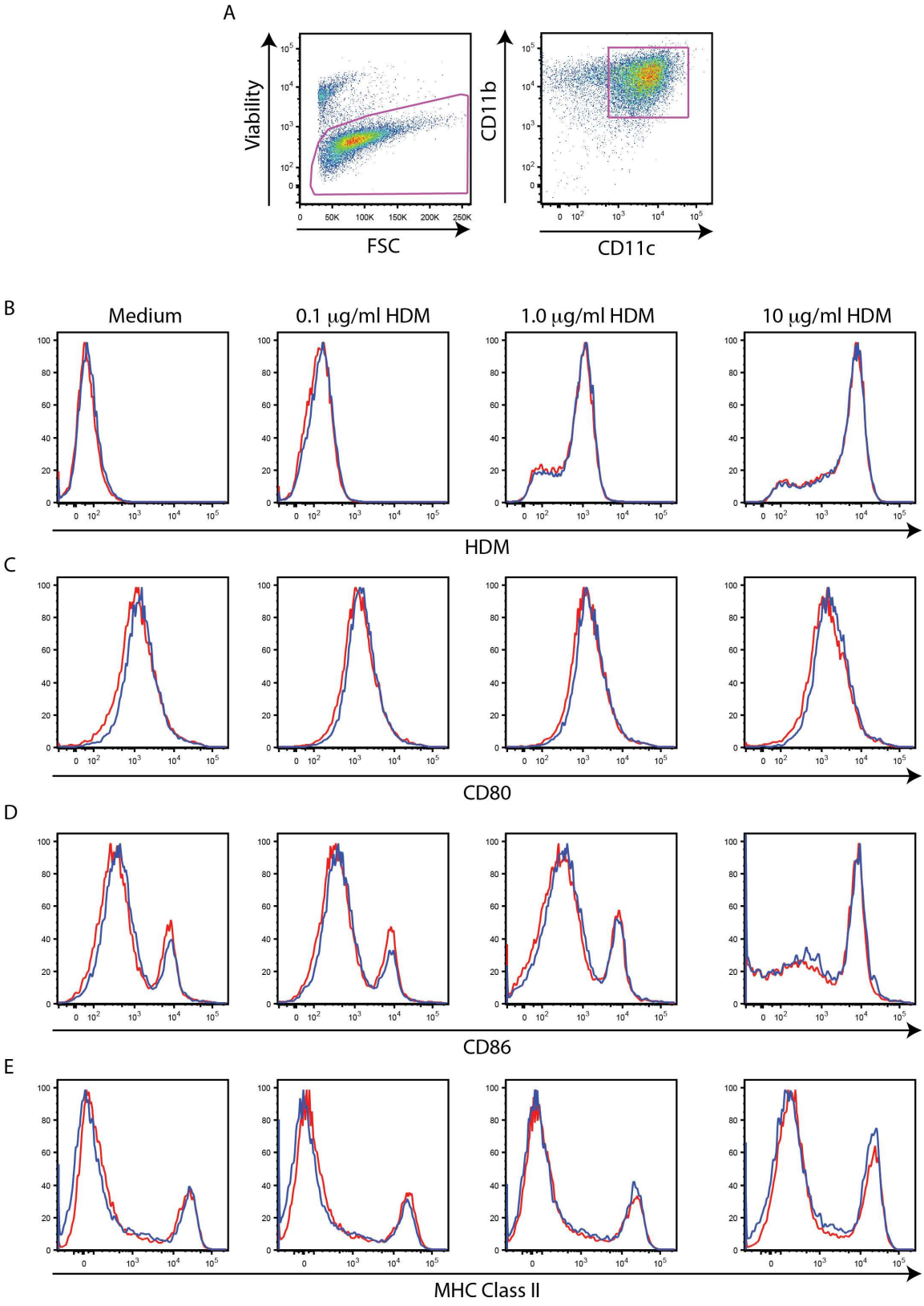
Richgels et al Supplemental Figure 2

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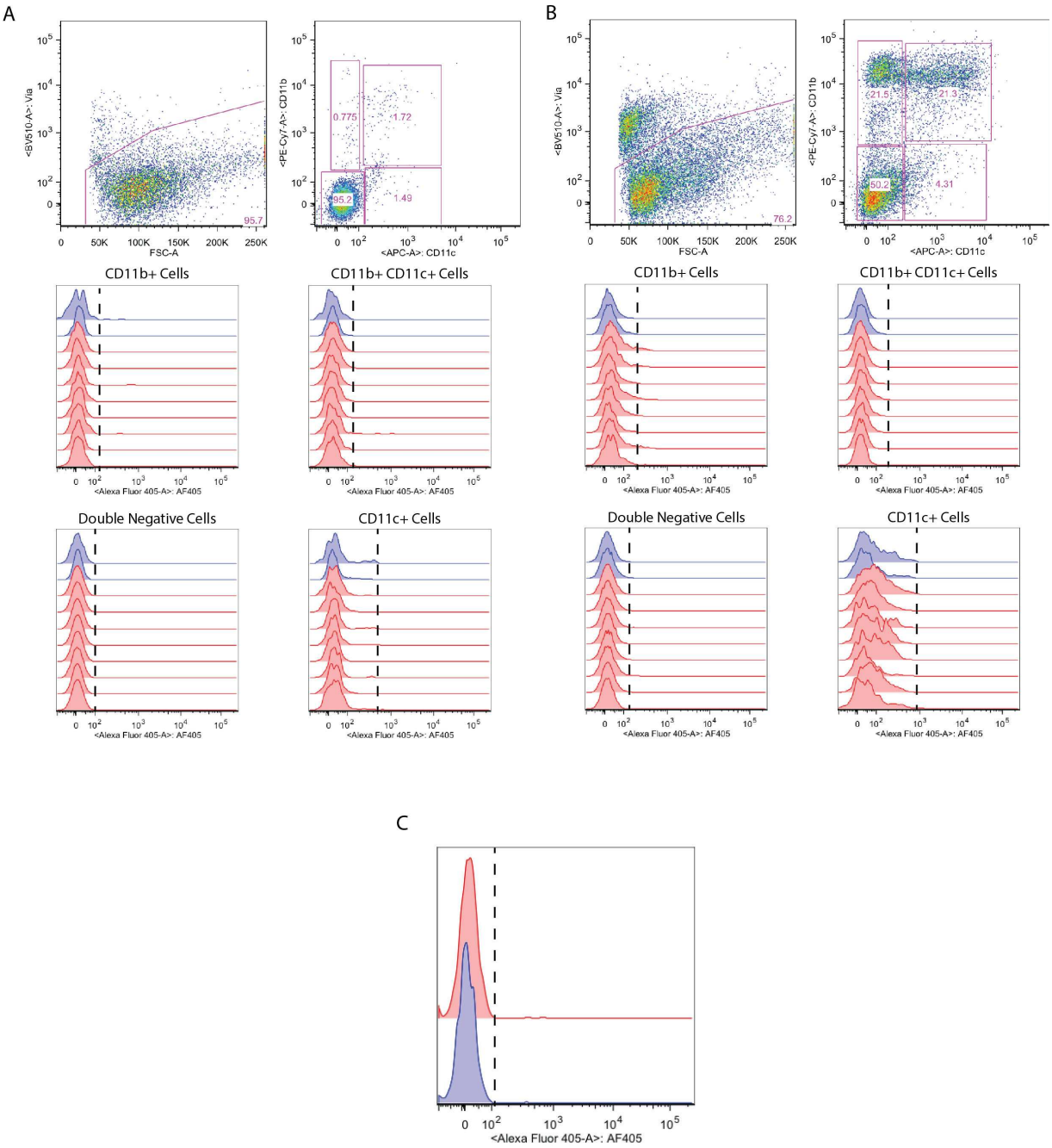


Richgels et al Supp Fig 3

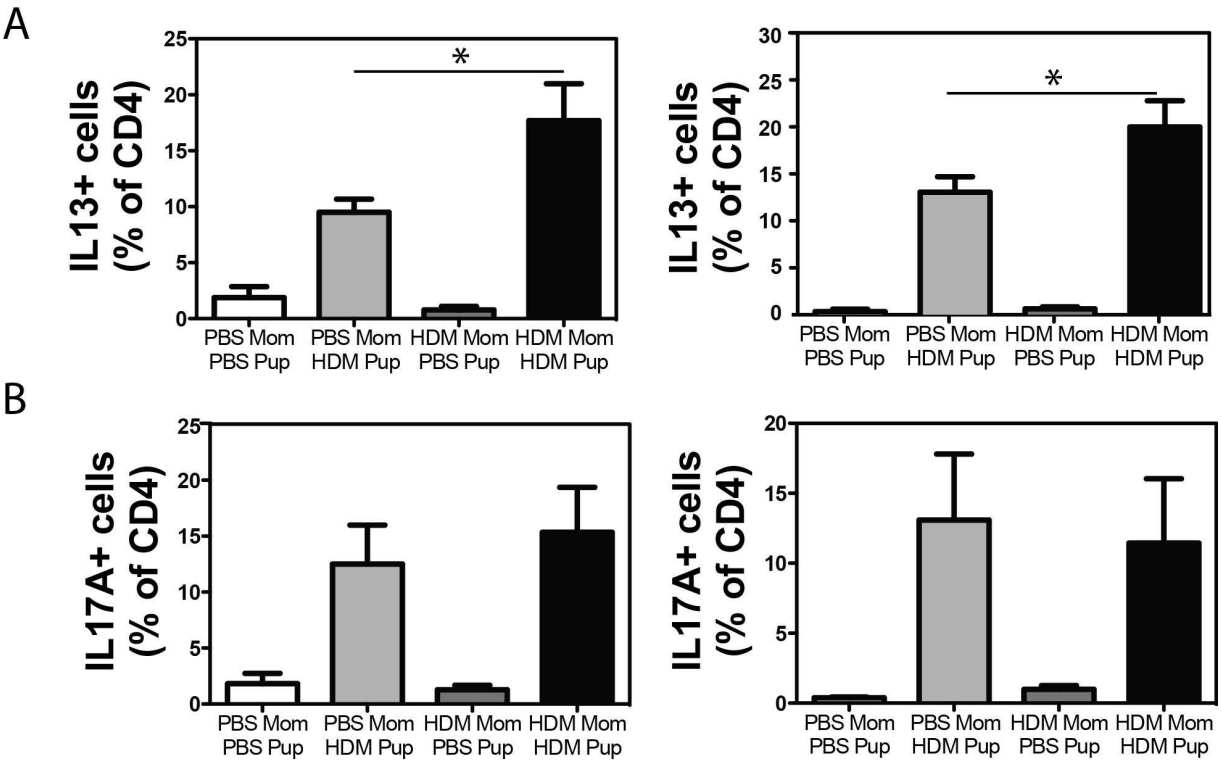
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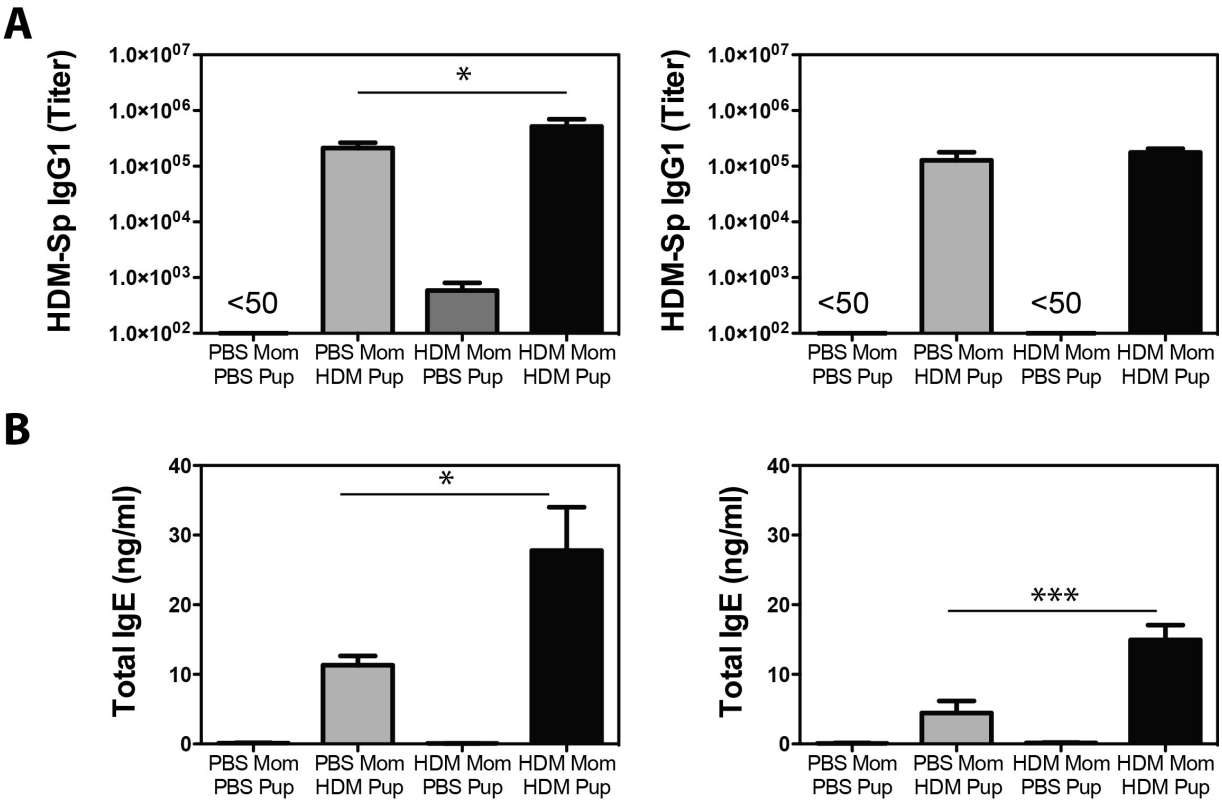


Richgels et al Supplemental Figure 5



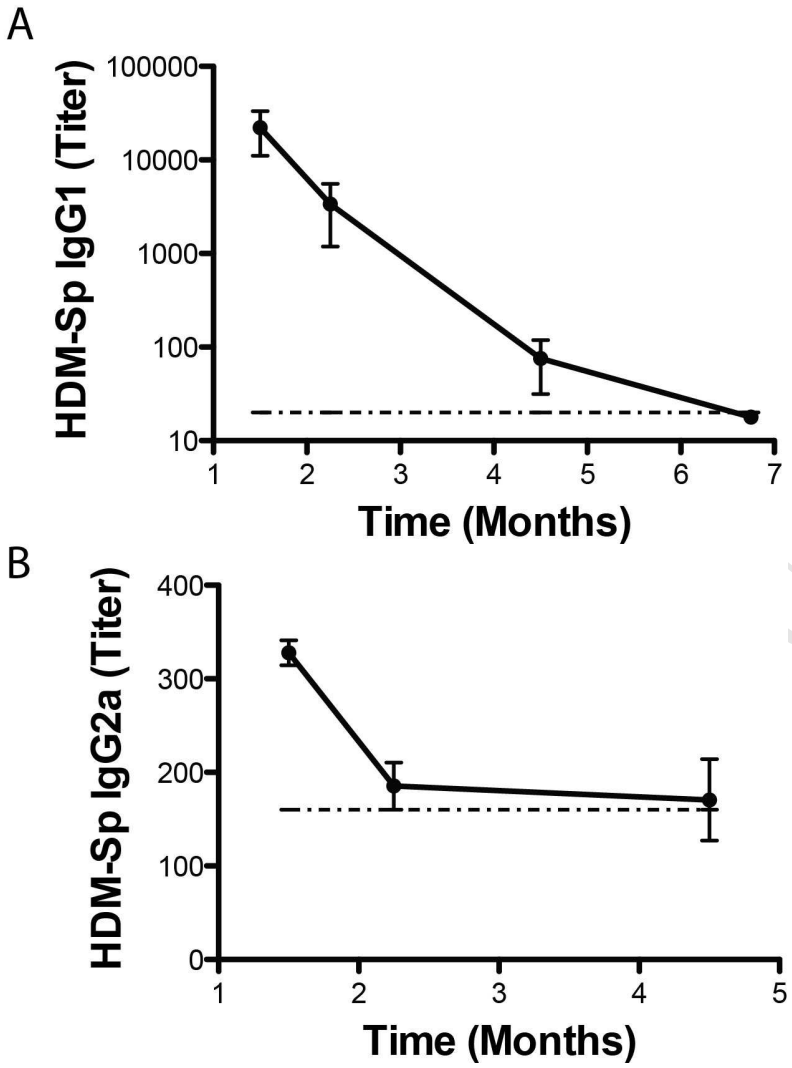
Richgels et al Supplemental Figure 6

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