

REVIEW ARTICLE

Maternal respiratory sensitization and gestational allergen exposure does not affect subsequent pup responses to homologous or heterologous allergen

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Abstract

Evidence suggests that the predisposition towards atopy begins early in life. Maternal allergy has been associated with an increased risk of the development of allergic disease in offspring. Some studies suggest that the development of childhood atopy may also be influenced by prenatal allergen exposure. In this study, a respiratory allergen exposure model was used to determine the impact of maternal sensitization (with or without additional exposures during pregnancy) on subsequent pup responses to homologous or heterologous allergen. Female BALB/c mice received two intratracheal aspiration (IA) exposures to *Metarhizium anisopliae* crude antigen (MACA) or Hank's buffered salt solution (HBSS) prior to breeding. Some mice also received three additional exposures during pregnancy. Control mothers did not receive treatment. Young adult offspring received three IA exposures to MACA, house dust mite extract (HDM) or HBSS. Offspring sensitized as young adults to either HDM or MACA developed an airway inflammatory response, including increased bronchoalveolar lavage fluid lactate dehydrogenase activity, total protein and total and differential cell counts compared to offspring exposed to HBSS. Increased airway responsiveness to methacholine was observed in pups treated with HDM but not with MACA. Maternal sensitization status (with or without gestational allergen exposure) had no effect on offspring response to either MACA or HDM. In conclusion, this study demonstrates that IA administration of MACA or HDM extract to young adult BALB/c mice induces the development of an inflammatory airway response. In contrast to previous reports, neither maternal sensitization nor gestational allergen exposure could be demonstrated to have a clear effect on offspring sensitization. This discrepancy may be a function of the respiratory sensitization and exposure protocol used in this study, which mimics natural sensitization more closely than do parenteral routes of exposure.

Keywords: Asthma; maternal asthma; gestational exposure; pregnancy; mouse; *Metarhizium anisopliae*

Introduction

Evidence suggests that the predisposition towards atopic disease begins early in life. Maternal allergy is associated with an increased risk of development of allergic diseases (including asthma) in offspring. This finding has been noted in both humans (Martinez et al., 1995; Brown and Halonen, 1999) and domestic animals, such as dogs (Barrett et al., 2003). Furthermore, several studies have suggested a possible link between gestational exposure to certain allergens and the subsequent development of atopic disease in children (Kihlstrom et al., 2003; Hagendorens et al., 2004). It has been suggested that allergic sensitization may occur as a direct result of fetal in utero allergen exposure, as evidenced

by increased cord blood mononuclear cell proliferation and antigen-specific IgE levels in children (Jones et al., 1996; Miller et al., 2001). This suggestion remains controversial, as other investigators have failed to demonstrate persistence of these responses past the neonatal period (Smillie et al., 2001; Bønnelykke et al., 2008). More information regarding the relative influence of maternal sensitization and gestational allergen exposure on the development of offspring allergen-specific sensitization would help clarify these discrepancies and provide greater insight into the mechanisms involved in the induction and perpetuation of allergic disease.

The entomopathogenic fungus *Metarhizium anisopliae* has been used for the biological control of insect and arachnid

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pests. This agent is typically used in an outdoor environment, and the fungus is a normal soil resident (St. Leger, 2008). However, a recent study demonstrated positive skin-prick reactions in sugarcane workers with allergic asthma and rhinitis. These workers had been occupationally exposed to *Metarhizium*. These findings were substantiated by the confirmation of serum IgE reactivity to *Metarhizium* extract by western blotting. In contrast, asymptomatic individuals did not demonstrate skin-prick reactivity to *Metarhizium* (Barbieri et al., 2005). The association of allergic respiratory disease with exposure to this fungus may be of particular concern in tropical and subtropical areas, where *Metarhizium* has recently been proposed for use in the control of mosquito vectors of malaria (Scholte et al., 2005).

Our laboratory has previously demonstrated that multiple exposures to extracts of *M. anisopliae* can induce airway responses characteristic of human allergic asthma in adult BALB/c mice (Ward et al., 1998, 2000). These responses can be obtained following intratracheal aspiration (IA) exposure to *M. anisopliae* crude antigen (MACA). Neither intraperitoneal priming nor adjuvant administration is required for sensitization (Ward et al., 2000).

The objectives of this study were three-fold: to determine (1) the effects of maternal sensitization prior to breeding (with or without additional exposure during pregnancy) on pup susceptibility to sensitization; (2) whether maternal treatment/exposure would be associated with different effects in offspring sensitized in young adulthood to homologous versus heterologous allergen; and, (3) whether maternal sensitization/exposure would induce differential effects by gender in the offspring.

Materials and methods

Animals

Eight-week-old BALB/c mice were obtained from Charles River Laboratories (Raleigh, NC). Female mice were group housed prior to breeding with male BALB/c mice. Females were removed after a 48 hr co-habitation with males and housed singly until pup weaning, after which they were regrouped. All studies conformed to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

All mice were housed in polycarbonate cages with hardwood chip bedding in an environmentally-controlled, Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited animal facility. Environmental enrichment was provided in the form of mouse nestlets (Ancare Corporation, Bellmore, NY) and mouse igloos (Bio-Serv, Frenchtown, NJ). Sentinel mice were monitored serologically and were found to be free of Sendai, mouse pneumonia, mouse hepatitis, other murine viruses, and mycoplasma. Mice also were monitored for, and found to be free of, ectoparasites and endoparasites. The animal holding room temperature ($22.2^{\circ}\text{C} \pm 1.1^{\circ}\text{C}$) and relative humidity ($50\% \pm 10\%$) were maintained at the recommended levels.

Breeding female mice were maintained on a diet suitable for use in pregnant mice (Lab Diet 5008, Purina Mills, St. Louis, MO), while breeding male mice and weaned pups received standard rodent chow (Lab Diet 5P00, Purina Mills). All mice received water *ad libitum*. Mice were acclimated one week at this facility prior to the start of the experiment.

Antigen preparation

M. anisopliae strain 1080 was obtained from the USDA-ARS Entomopathogenic Fungus Collection (Ithaca, NY). Extracts of *M. anisopliae* mycelium and spores/conidia as well as inducible enzymes filtrate were produced as previously described (Ward et al., 1998). Proteins >3000 Da in molecular weight were concentrated using a stirred-cell concentrator with a YM-3 (MWCO 3000) filter (Amicon, Beverly, MA). Concentrated extracts were assayed for total protein concentration as described below. These component extracts/filtrates were combined in equal protein amounts to form the *M. anisopliae* crude antigen (MACA) (Ward et al., 1998). Dosing aliquots were stored at -20°C until use.

Lyophilized whole body extracts of the house dust mites (HDM) *Dermatophagoides farinae* and *D. pteronyssinus* were purchased from Greer Laboratories (Lenoir, NC) and re-hydrated in Hank's Buffered Salt Solution (HBSS; Gibco-Invitrogen, Carlsbad, CA) to 2 mg/mL and mixed equally. Dosing aliquots were stored at -20°C until use.

Experimental design

Maternal sensitization and gestational allergen exposure

At 14 and 7 days before breeding (gestation day-14 [GD-14] and GD-7), female BALB/c mice were exposed to MACA (40 μg) in 50 μL of HBSS or to HBSS alone (50 μL) by IA as previously described (Ward et al., 2000). Briefly, mice were anesthetized by inhalation of a mixture of isoflurane and oxygen, and then vertically suspended by their incisors to facilitate treatment administration. Swallowing was prevented by gently pulling the tongue out of the mouth in an upward direction. Antigen extract was deposited into the oropharynx while the nose was briefly occluded, inducing aspiration of the extract. Selection of the dose used for maternal exposure was based upon previous work in our laboratory, in which 40 μg per IA was the highest dose well-tolerated by young adult BALB/c mice (e.g., which did not produce weight loss or failure to thrive, etc.; Ward et al., unpublished data). For this reason, this dose was chosen to provide maximal maternal allergen exposure with a low risk of significant gestational pup stress and death.

After breeding, some mice received additional IA exposures to MACA or HBSS on GD11, GD15, and GD19. These times correspond to the initial colonization of the thymus with T-cell precursors, to initial T-cell responsiveness to mitogen stimulation and to expression of surface-bound antibodies and mature T-cell receptors (TCR), respectively (Holsapple et al., 2003). Mice receiving only pre-breeding exposures to MACA or HBSS were designated MA-2 and HB-2, respectively, while mice that also received post-breeding exposures were designated MA-5 and HB-5 (Figure 1A). An additional

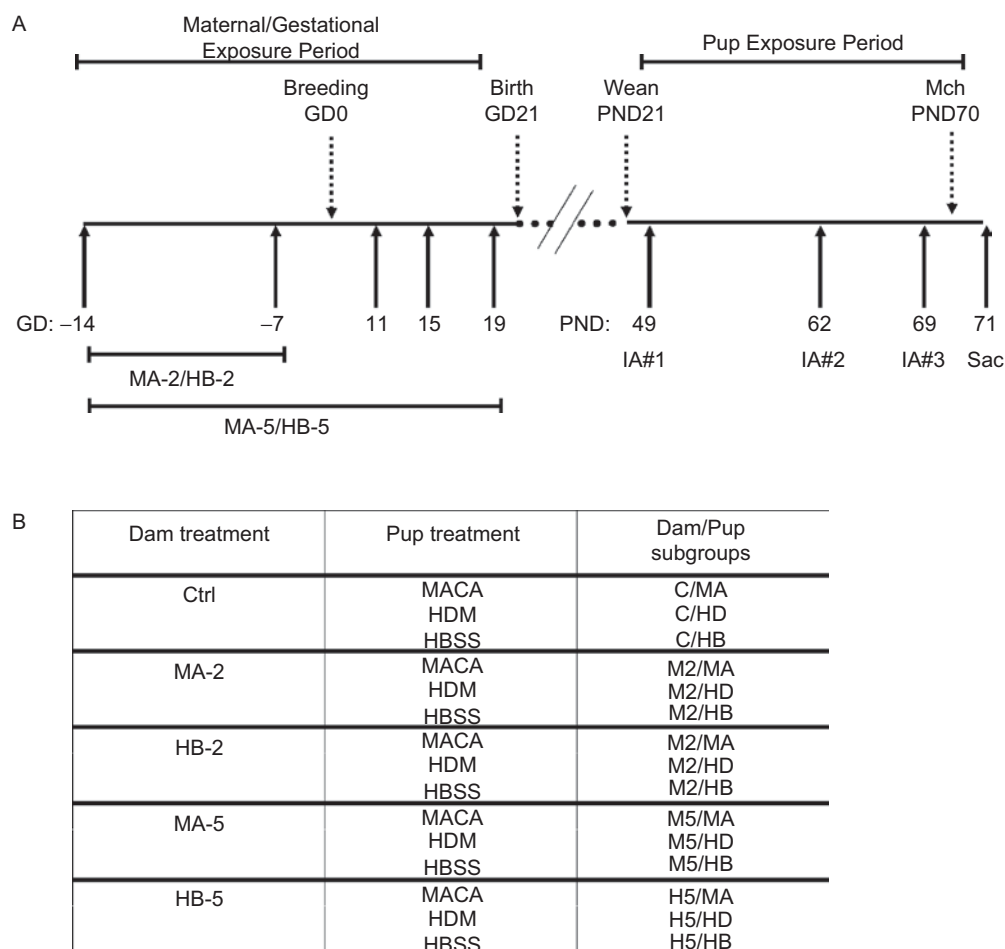


Figure 1. Experimental design. (A) Adult female BALB/c mice received two IA exposures to either MACA or HBSS prior to breeding. Some mice received additional exposures post-breeding (MA-5; HB-5) while others did not (MA-2; HB-2). An additional group of mice were not treated either pre- or post-breeding (Ctrl). Offspring mice received three IA exposures to MACA, HDM or HBSS after weaning. (B) Pups were assigned to treatment groups by maternal treatment and by gender ($n = 49$ – 50 per group; $n = 7$ – 12 per subgroup).

group of control mice received no treatment during these times (Ctrl). Offspring mice were left with their dams until weaning on post-natal day 21 (PND21).

Offspring sensitization

Weanling pups were distributed by maternal treatment and gender into three treatment groups. Each pup treatment group contained both male and female offspring from all dam treatment groups (Figure 1B). As young adults, offspring mice were exposed three times to MACA ($20 \mu\text{g}$ in $50 \mu\text{L}$ HBSS), HDM ($20 \mu\text{g}$ in $50 \mu\text{L}$ HBSS), or to HBSS alone ($50 \mu\text{L}$) by IA on PND49 (as 7-week-olds), PND62, and PND69 (Figure 1A). This lower exposure dose (i.e., $20 \mu\text{g}$ per IA as compared to $40 \mu\text{g}$ for the dams) was chosen to avoid the possibility of “overwhelming” any possible maternal exposure effects.

Airway responsiveness to methacholine was evaluated the day after the final dose (PND70). Mice were sacrificed 2 days after the final dose (PND71) by intraperitoneal injection of pentobarbital sodium and phenytoin sodium (Euthasol euthanasia solution, Virbac Animal Health, Fort Worth, TX). Offspring responses were evaluated both by pup

treatment groups as well as by dam treatment/pup treatment subgroups.

Examination and ammonium sulfide staining of uteri

All bred female mice were sacrificed either on PND 65 or PND 75. At sacrifice, uteri from all mice were examined under a dissecting microscope to count placental implantation sites for determination of the frequency of complete or partial litter death. Subsequently, uteri were stained with ammonium sulfide to enhance detection of fetal resorption sites (Narotsky and Kavlock, 2003). Briefly, uteri were placed into individual wells of a 6-well tissue culture plate, each of which contained 5 mL of an ammonium sulfide solution (diluted to 2% in deionized water; Sigma-Aldrich, St. Louis, MO). After 1 hr, uteri were re-examined and darkly stained implantation sites were counted. Representative examples of uteri from mice (demonstrating unstained and stained uteri, and the presence of countable implantation sites at these timepoints) are provided in Figure 2.

The percentage of pregnancy success per group was calculated using the formula $100 * [(\# \text{ mice bearing live pups}) / (\text{total } \# \text{ mice per group})]$. The percentage of viable pups per

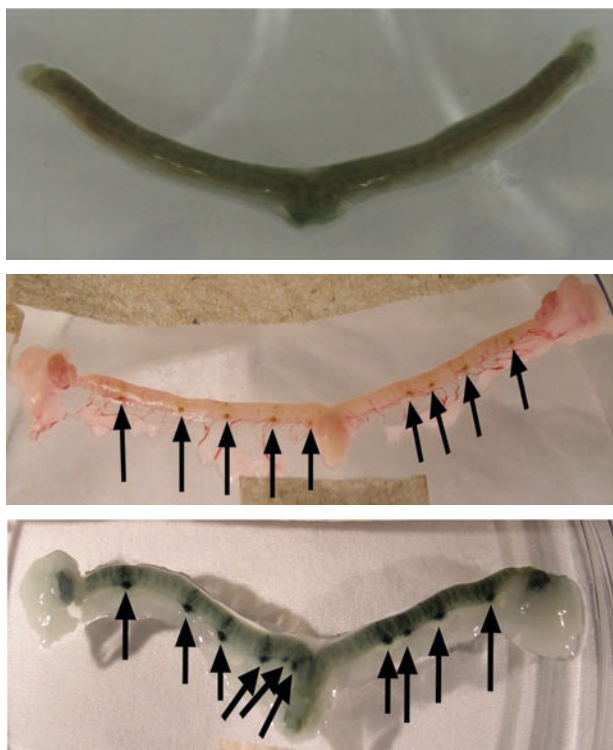


Figure 2. Representative uteri used for determination of implantation sites in female PND65 or PND75 BALB/c mice. Top to Bottom Photos: Stained uterus (PND65) with no implantation sites present (from a mouse that either did not conceive at all or who lost her pups before implantation ($\approx$ GD-3-4); unstained uterus (PND75) with 9 implantation sites present (arrows); and, stained uterus (PND75) containing 10 implantation sites (arrows). These photographs were not altered in any fashion and were taken with a standard hand-held digital camera. (See colour version of this figure online at www.informahealthcare.com/imt)

litter was calculated using the formula $100 * [(\# \text{ live pups}) / (\text{total } \# \text{ implantation sites})]$.

Measurements of airway responsiveness

Airway Responsiveness to Methacholine Aerosol: Airway responsiveness to challenge with the cholinergic agent methacholine (Mch) was measured in unrestrained mice using whole body plethysmography (Buxco Electronics, Troy, NY). Briefly, BioSystem XA software (SFT3812, version 2.0.2.48, Buxco Electronics) was used to calculate enhanced pause (PenH), a unit-less index of airway hyperreactivity that strongly correlates with lung resistance (Hamelmann et al., 1997). PenH is derived from whole body flow parameters [respiratory rate, tidal volume, inspiratory and expiratory times (T_i , T_e), peak inspiratory and expiratory flows (PIF, PEF), and relaxation time (RT)]. PenH reflects changes in pulmonary resistance during bronchoconstriction according to the following equation: $\text{PenH} = ((T_e - RT) \div RT) \times (PEF \div PIF)$. Baseline PenH was measured for 10 min, after which saline and then increasing concentrations of Mch (6.25, 12.5, and 25 mg/mL) were nebulized and delivered through an inlet of the chamber. Measurements of PenH were obtained for 10 min following each nebulization. "Adjusted" PenH values were obtained by subtracting

baseline PenH results from subsequent post-nebulization readings for each mouse.

Bronchoalveolar lavage (BAL) and blood collection

Two days after the final exposure, blood and bronchoalveolar lavage fluid (BALF) samples were collected from all mice as previously described (Ward et al., 1998). Blood samples were collected by cardiac puncture and kept at room temperature for at least 30 min prior to centrifugation. Serum was stored at -20°C . The lungs were lavaged twice with 0.7 mL aliquots of HBSS. BALF aliquots for each animal were pooled and stored on ice. The BALF was centrifuged at $100 \times g$ for 15 min at 4°C . Aliquots of BALF supernatant were assayed for total protein and lactate dehydrogenase (LDH) activity (as described below), and the remainder was stored at -20°C for IgE quantification by ELISA. The cell pellet was resuspended in 1 mL HBSS and cytospin preparations of 200 μL BALF were made by centrifugation onto glass slides (200 rpm, 10 min on a Shandon Cytospin; Shandon Inc., Pittsburgh, PA). Following Wright-Giemsa staining (Fisher Scientific, Fairlawn, NJ), cells were differentially counted at 200 cells per slide (one slide per animal). Total BALF cell counts were obtained from the re-suspended cells using a Coulter counter (Coulter Corp., Miami, FL).

Total IgE ELISA

The BALF and serum total IgE ELISAs were performed as previously described (Ward et al., 2000). Briefly, microtiter plates were coated with rat anti-mouse IgE (Pharmingen-BD, San Diego, CA) in phosphate-buffered saline (PBS, pH 7.3; Pharmingen-BD) and incubated overnight at 4°C . Following blocking with blocking buffer (PBS plus 1% bovine serum albumin [BSA]), serum samples and the mouse IgE standard (monoclonal anti-trinitrophenol (TNP); Pharmingen-BD)-ranging from 800 to 0.7 ng/mL diluted in blocking buffer-were applied to separate dedicated wells and incubated for 1 hr. The plates were then washed three times with wash buffer (PBS plus 0.05% Tween 20; Fisher Scientific), biotinylated detection antibody (rat anti-mouse IgE; Pharmingen; diluted to 1.5 $\mu\text{g/mL}$ in blocking buffer) was then added. The plates were incubated for 1 hr and then washed six times with wash buffer. Streptavidin-horseradish peroxidase (diluted 1:10,000 in blocking buffer; Zymed-Invitrogen, Carlsbad, CA) was then applied and the plates were incubated for 30 min. After this final incubation, the plates were washed six times with wash buffer, and then (3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate solution (Dako, Carpinteria, CA) was applied. Optical density was then read on a SpectraMax 340PC Plate Reader (Molecular Devices Corp., Menlo Park, CA) at a wavelength of 650 nm. Softmax ProR version 2.6.1 (Molecular Devices) software was used for data collection and conversion from optical density to protein concentrations. The limit of detection for this assay was 6.25 ng/mL.

Antigen-specific IgE assay

A rat basophilic leukemia (RBL) cell β -hexosaminidase release assay was performed as an indirect measure of

antigen-specific IgE in serum. The procedure has been previously described in Chung et al. (2005). Briefly, RBL-2H3 cells (ATCC, Rockville, MD) were maintained in a growth medium [71% (v/v) Eagle's minimal essential medium (EMEM, Gibco BRL, Gaithersburg, MD), 23% (v/v) RPMI-1640 (Gibco BRL), 5% (v/v) fetal calf serum (FCS; Gibco BRL), 100 U penicillin/mL, 100 µg streptomycin/mL (Gibco BRL), and 1% (v/v) 0.2 M L-glutamine] at 37°C in a 5% CO₂ humidified incubator. Cells were detached and re-suspended in assay medium [93% (v/v) EMEM, 5% (v/v) FCS, 100 U penicillin/mL, 100 µg streptomycin/mL, and 1% (v/v) 0.2 M L-glutamine] then placed in 96-well flat bottom tissue culture plates (10⁵ cells/100 µL/well). After 18 hr, individual mouse sera (diluted 1:5 in assay medium) were incubated with the RBL cells for 2 hr, which allowed binding of any IgE present. Control cells for spontaneous and total release were sensitized with normal BALB/c mouse serum.

Following incubation, cells were washed three times in Tyrode's buffer (Sigma-Aldrich). Subsequently, allergen extract (100 µL; diluted to 10 µg/mL in Tyrode's buffer) was added and incubated 1 hr at 37°C. Spontaneous release cells were incubated with Tyrode's buffer alone. Total release cells were incubated with Tyrode's buffer containing 1% (v/v) Triton X-100 (Sigma). Cell supernatant (40 µL) from each well was transferred to a fresh 96-well plate. β-Hexosaminidase activity was measured by adding 80 µL of substrate (*p*-nitrophenyl-*N*-acetyl β-D-glucosaminide 1.3 mg/mL in 0.1 M citric buffer [pH 4.5]) and incubating for 1 hr at room temperature. To detect endogenous β-hexosaminidase activity, fungal extract alone in Tyrode's buffer was also incubated with substrate. The enzyme reaction was stopped by adding 100 µL of 0.2 M glycine. Absorbance at 405 nm was measured 30 min later using a SpectraMax 340 PC Plate Reader (Molecular Devices) and Softmax Pro® version 2.6.1 software (Molecular Devices). Data are presented as an adjusted percent total release following subtraction of spontaneous release, calculated using the following formula:

Adjusted % total release

$$= 100 * \left(\frac{(\text{Sample activity}) - (\text{Extract activity})}{(\text{Total activity}) - (\text{Spontaneous activity})} \right)$$

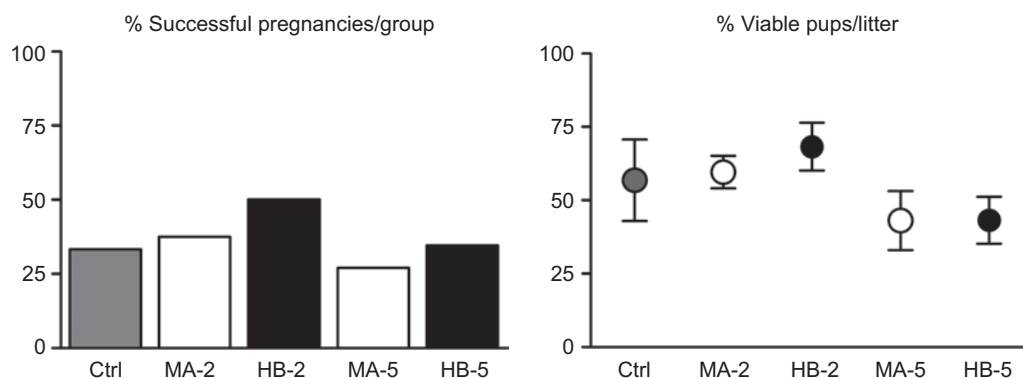


Figure 3. Effects of maternal treatment on pregnancy success and pup viability. Ctrl n = 16; MA-2 n = 16; HB-2 n = 16; MA-5 n = 26; HB-5 n = 26.

Total protein and lactate dehydrogenase (LDH) assays

BALF samples were assayed for total protein using Pierce Coomassie Plus Protein Assay Reagent (Pierce/Thermo Fisher Scientific, Rockford, IL). Concentrations were determined from a standard curve using BSA standards obtained from Sigma Chemical Co. (St. Louis, MO). Additionally, BALF samples were assayed for LDH activity using a commercially prepared kit and controls from Sigma Chemical Co. The assays were modified for use on a KONELAB 30 clinical chemistry Spectrophotometer analyzer (Thermo Clinical Labsystems, Espoo, Finland).

Statistical analysis

The statistical analyses for all the data in this study were performed using SAS version 9.1.3 software (SAS Institute Inc, Cary, NC) to examine the main effects of each model (maternal and pup sensitization) and the effects of gender on exposure outcomes. All biochemical and differential cell count data were analyzed using an analysis of variance (Factorial—ANOVA) procedure. In the case of methacholine hyperresponsiveness, Factorial—ANOVA with repeated measures analysis was performed. Both analyses were performed using a linear mixed model with restricted maximum-likelihood estimation analysis and least squares means. Significant main effects were analyzed using a *post-hoc* test (Tukey's) to determine statistical differences between individual treatment groups and to adjust significance levels to account for multiple comparisons. For all parameters, the litter was considered the experimental unit of analysis. Reported values represent mean ± standard error (SE) and P < 0.05 was considered as statistically significant.

Results

Pregnancy outcome

Pregnancy is associated with profound alterations in maternal respiratory function, such as decreased pulmonary reserve and functional residual capacity (Gluck and Gluck, 2006). Although these alterations are normally well compensated for, additional stressors such as general anesthesia have the potential to tip the balance towards hypoxia, especially for

asthmatic patients. These conditions may result in increased fetal stress and possibly death. We conducted an analysis of pregnancy success and pup survival percentages to determine whether our anesthesia protocol or our maternal sensitizing agent had a significant impact on pup viability. No significant decrease in pregnancy success or pup viability (relative to untreated control mice; Ctrl) was seen regardless of maternal treatment (Figure 3).

IA administration of HDM or MACA induces the development of an airway inflammatory response

To determine the overall effect of the three pup treatment agents (MACA, HDM, and HBSS), we performed the initial evaluation of offspring responses by pooling pup data across all maternal treatments (Figure 4A–4H). The inflammatory response to challenge with HDM was more pronounced than that associated with MACA challenge; BALF total protein concentration, total cells recovered, and numbers of macrophages, neutrophils, and lymphocytes were greater than the same responses in mice challenged with MACA. However, all of these measures were greater in mice challenged with MACA compared to HBSS, with the exception of neutrophil counts, indicating that MACA challenge did result in significant inflammation. Eosinophil counts, a measure of the allergic response to antigen challenge, were likewise higher in mice challenged with HDM compared to MACA, although MACA challenge did result in elevated eosinophil counts compared to HBSS controls. Visual inspection of cohort total serum IgE levels suggests that total IgE levels were also increased by challenge with either antigen, although the significant gender effect (females > males, $P < 0.001$) contributed to greater cohort variability and possibly masked statistically significant differences in challenged females that may be detectable with larger group sizes. A significant gender effect (females > males, $P < 0.01$) was also detected in LDH activity in BALF of control (HBSS) mice, indicating a greater level of cellular damage or leakage in females. The reason for this difference is unclear.

Offspring airway responsiveness to aerosolized methacholine (Mch) was examined as a function of pup treatment and sex (Figure 5). There was an apparent tendency for male mice to demonstrate higher PenH values than did female mice for any given concentration of Mch. However, this gender difference was only significant for HDM-treated mice at the 25 mg/mL concentration. Male HDM-treated mice also displayed significantly greater PenH responses relative to male HBSS- and MACA-treated mice at the 12.5 and 25 mg/mL concentrations. Female response differed less between groups, with a significant difference seen only between female HDM- and HBSS-treated mice at the 25 mg/mL concentration. In contrast, PenH values in MACA-treated mice did not differ significantly from those of HBSS-treated mice for any combination of dose and/or sex (Figure 5).

Gestational exposure to MACA extract has no clear effect on subsequent offspring inflammatory response to homologous allergen

In order to determine the effects of maternal sensitization and/or gestational allergen exposure on subsequent pup responses to homologous allergen, we evaluated the responses of MACA-treated offspring of dams that were sensitized/exposed (or not) to MACA. The inflammatory responses were then compared between pups of different maternal treatment groups.

The responses of offspring of MACA-sensitized dams (with or without additional gestational exposure; i.e., M5/MA and M2/MA mice, respectively) are shown in Figure 5. The responses of mice exposed gestationally to MACA (i.e., M5/MA mice) did not differ significantly from those of identically-treated offspring of untreated dams (C/MA mice; Figure 6). Offspring of dams treated before and after conception with HBSS (H5/MA) also displayed an overall response very similar to that seen in C/MA mice. There was an apparent tendency for female (but not male) offspring of mice treated with HBSS before breeding only (H2/MA) to demonstrate a greater inflammatory response than any of the other offspring, regardless of maternal treatment group, although these differences were not statistically significant (Figure 6A–H) and there is no apparent reason for this trend.

With the exception of female H2/MA pups, the effects of maternal treatment on subsequent offspring inflammatory response did not appear to differ by pup gender. Although male and female offspring rarely had identical responses for any given parameter, few consistent differences were seen between maternal treatment cohorts and gender-matched C/MA mice. In other words, neither sex appeared to be more or less likely to have their response to sensitization affected by maternal sensitization or gestational exposure to homologous allergen. Male mice tended to have lower levels of serum and BALF total IgE than did females (Figure 6H, data not shown), but these differences were not significant. Furthermore, the relative gender differences for these variables also did not appear to be significantly altered by maternal treatment.

Offspring responses to aerosolized Mch were evaluated to determine whether maternal treatment had any effect on subsequent offspring airway reactivity. No significant differences could be demonstrated relative to C/MA offspring regardless of maternal treatment group (data not shown).

Gestational exposure to MACA extract has no clear effect on subsequent offspring inflammatory response to heterologous allergen

In order to determine whether maternal sensitization and/or gestational allergen exposure would have an effect on subsequent pup responses to heterologous allergen, we evaluated the responses of HDM-treated offspring of dams that were sensitized/exposed (or not) to MACA. The inflammatory

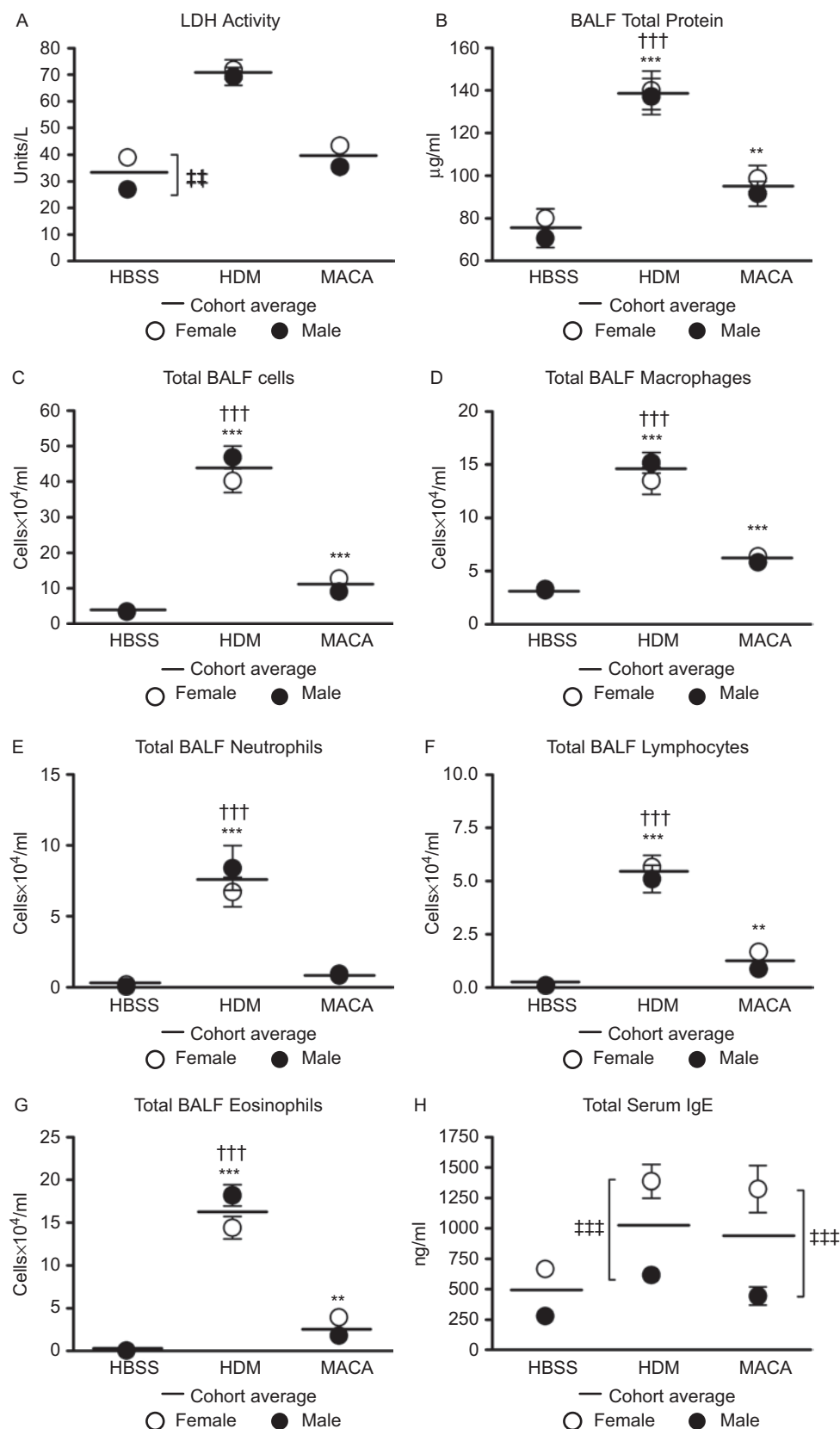


Figure 4. Offspring sensitization response by pup treatment group and gender. (A) BALF LDH activity, (B) BALF total protein, (C) total BALF cells, (D) BALF macrophages, (E) BALF neutrophils, (F) BALF lymphocytes, (G) BALF eosinophils, and (H) total serum IgE. $\ddagger\ddagger$ p < 0.01 male average relative to female average, $\ddagger\ddagger\ddagger$ p < 0.001 male average relative to female average, $**$ p < 0.01 relative to HBSS cohort average, $***$ p < 0.001 relative to HBSS cohort average, $\ddagger\ddagger\ddagger$ p < 0.001 relative to MACA cohort average; n = 49–50 per group; n = 22–26 per sex per group. Values are displayed as mean $\pm$ SE.

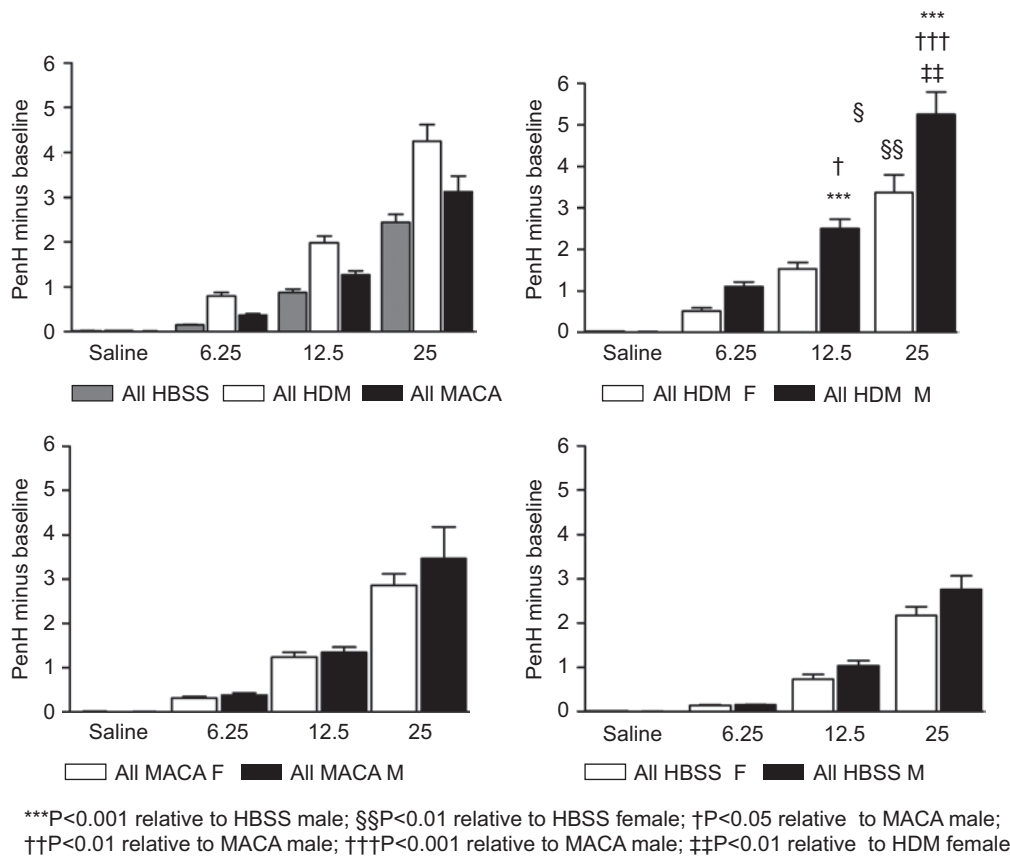


Figure 5. Respiratory responses to increasing concentrations of methacholine aerosol in offspring mice exposed as young adults to HBSS, HDM, or MACA. "Adjusted" PenH values were obtained by subtracting baseline PenH results from subsequent post-nebulization readings for individual mice. ***P < 0.001 relative to HBSS male; §§P < 0.01 relative to HBSS female; †P < 0.05 relative to MACA male; ††P < 0.01 relative to MACA male; †††P < 0.001 relative to MACA male; ‡‡P < 0.01 relative to HDM female. Data are expressed as group mean adjusted PenH ± SE.

responses were then compared between pups of different maternal treatment groups.

As was seen for MACA-treated offspring, no clear difference was seen between the groups overall regardless of maternal treatment or gestational exposure status (data not shown). Furthermore, neither sex appeared to be more or less likely to have their response to sensitization affected by maternal sensitization or gestational exposure to heterologous allergen.

Offspring responses to aerosolized Mch were evaluated to determine whether maternal treatment had any effect on subsequent offspring airway reactivity. However, no significant differences were seen from the responses of control mice, regardless of whether the groups were considered as a whole or subdivided by sex (data not shown).

Discussion and conclusions

In this study, we examined the effects of maternal sensitization and gestational exposure to an allergenic fungal extract upon subsequent offspring sensitization to homologous or heterologous allergen. Our data demonstrated that offspring mice sensitized in young adulthood to either HDM or MACA extract developed a notable airway inflammatory response. In addition, our data show that maternal sensitization prior

to breeding (either with or without allergen exposure during pregnancy) had no effect on the subsequent response of offspring to homologous or heterologous antigen. In general, the allergic responses of males and females to both antigens were similar.

In this study, offspring sensitized in young adulthood by multiple exposures to either HDM or MACA extract developed an airway inflammatory response, including increased BALF LDH activity, total protein, and total and differential cell numbers, relative to offspring mice exposed multiple times to HBSS. HDM-sensitized mice also displayed enhanced airway reactivity relative to both MACA- and HBSS-treated mice. However, serum total IgE levels were similar between the two groups.

These offspring inflammatory responses were then evaluated to determine whether maternal sensitization and/or gestational exposure to MACA extract would be associated with an exacerbation or inhibition of pup sensitization. In this study, the impact of maternal sensitization and gestational exposure to MACA on subsequent pup sensitization to homologous allergen was limited. There was some tendency for MACA-sensitized offspring from both maternal MACA treatment groups (i.e., M2/MA and M5/MA mice) to demonstrate a decreased response compared to MACA-sensitized offspring of untreated dams

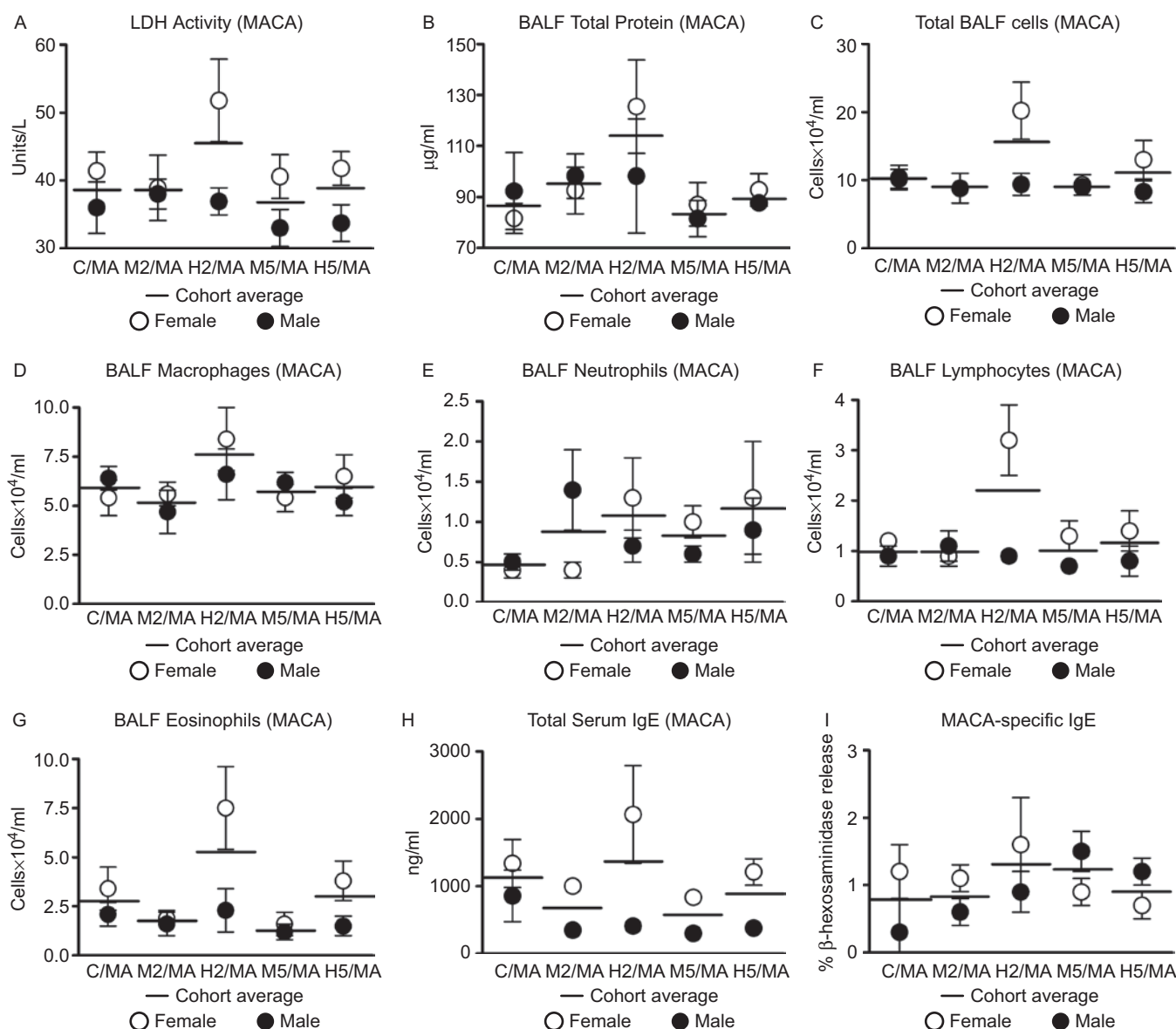


Figure 6. Effects of maternal sensitization/gestational exposure to MACA on subsequent pup sensitization to MACA. (A) BALF LDH activity, (B) BALF total protein, (C) total BALF cells, (D) BALF macrophages, (E) BALF neutrophils, (F) BALF lymphocytes, (G) BALF eosinophils, (H) total serum IgE, and (I) MACA-specific IgE; $n = 7-12$ per subgroup; $n = 4-7$ per sex per subgroup. Values are displayed as mean $\pm$ SE.

(C/MA), but this effect was neither consistently observed nor statistically significant. With one exception, there was no evidence to suggest a gender-based differential susceptibility to inhibition or exacerbation of pup response following maternal sensitization or exposure. Female H2/MA mice displayed the most pronounced inflammatory response of all of the MACA-treated mice, including their male treatment cohorts. The reason for this effect is not clear.

Maternal sensitization and/or gestational exposure to MACA also did not have a clear effect on subsequent pup sensitization to the heterologous allergen HDM. Overall, offspring from both maternal MACA treatment cohorts (i.e., M2/HD and M5/HD mice) exhibited an inflammatory response very similar to that displayed by C/HD mice, and by either group of maternal HBSS treatment cohorts. Susceptibility to

HDM sensitization also did not appear to be affected by pup gender.

The inability to demonstrate any consistent maternal treatment effect on subsequent pup sensitization to either homologous or heterologous allergen is somewhat surprising given the spectrum of published observations. Previous studies in mice investigating the effects of maternal sensitization and/or gestational exposure on subsequent pup responses to homologous allergen have produced conflicting results. Maternal sensitization prior to pregnancy has been reported to provide a degree of protection against subsequent pup sensitization to homologous allergen (Fusaro et al., 2002, 2007; Victor et al., 2003). Gestational allergen exposure was demonstrated to confer similar protection, especially if dams had also been sensitized prior to breeding (Melkild et al., 2002; Uthoff et al., 2003; Fusaro et al., 2007). In some cases, this

protection was attributed to maternal antibody "interference" with neonatal immune responses, as the effects disappeared if pup sensitization was delayed until after maternal antibodies were cleared (Uthoff et al., 2003; Victor et al., 2003). However, other studies have failed to demonstrate maternal antibody interference, as pup responses to homologous allergen were enhanced even if sensitization was initiated shortly after birth (Hamada et al., 2003; Fedulov et al., 2007). In contrast to the conflicting results regarding homologous sensitization, maternal sensitization (with or without gestational allergen exposure) has been consistently reported to enhance pup response to heterologous allergen (Herz et al., 2001; Hamada et al., 2003; Uthoff et al., 2003; Victor et al., 2003).

In this study, maternal treatment/exposure did not appear to have a significant impact on subsequent pup responses to either homologous or heterologous allergen. The reasons for this lack of effect are unclear. One possible explanation may relate to pup age at sensitization. The pups in this study received their first post-natal exposure to allergen at 7 weeks-of-age (PND49), and the last exposure at 10 weeks. Maternal antibody levels would be expected to be intermediate during this time, as they have been demonstrated to persist until 12 weeks in mice (Uthoff et al., 2003; Victor et al., 2003). It is possible that these antibody levels were insufficient to strongly impede sensitization yet remained high enough to inhibit any enhancing effects. However, another study was able to demonstrate clear suppression of offspring IgE responses to homologous allergen in pups beginning sensitization at 7.5 weeks (Melkild et al., 2002). Furthermore, maternal antibody levels would not be expected to affect the response to heterologous allergen.

A second possibility may relate to the choice of maternal exposure agent. Our laboratory has previously demonstrated that multiple respiratory exposures to MACA will induce the development of airway inflammatory responses characteristic of allergic human asthma in BALB/c mice (Ward et al., 2000). We have also determined that this specific maternal exposure protocol is sufficient to induce the development of airway inflammation in the dams themselves (Pucheu-Haston et al., 2009). This response suggests that insufficient maternal exposure is unlikely to be the cause of the lack of demonstrable maternal treatment effect in this study, particularly in light of our previous work demonstrating poor tolerance of higher doses of MACA per IA exposure (Ward et al., unpublished data). Nonetheless, the capability to induce an allergic response should not be taken to imply that the spectrum of responses to a given allergen (such as MACA) may be expected to be equivalent to the response to any other allergen. Although no maternal treatment effect on subsequent pup responses was seen in this study, it remains possible that maternal exposure to a different allergen (such as HDM) might produce different results.

Alternately, the lack of maternal treatment effect observed in this study may have been a function of the method of dam sensitization. In this study, all mice were sensitized and

challenged strictly via the respiratory tract. This method closely mimics the natural exposure scenario, in which allergens (including aerosolized fungal fragments) are typically inhaled (Gorny et al., 2002; Green et al., 2006). In contrast, in the studies described above, dam sensitization was achieved by intraperitoneal (IP) and/or subcutaneous (SC) injection with adjuvant (Herz et al., 2001; Hamada et al., 2003; Uthoff et al., 2003; Victor et al., 2003; Fedulov et al., 2007; Fusaro et al., 2007). Dams were either bred soon thereafter, or a high level of sensitization was maintained by frequent aerosol or intranasal allergen exposures until breeding. These exposure methods would be expected to produce more intense sensitization than would be seen in spontaneous disease. It is possible that the quantity of maternally-derived enhancing and/or inhibitory factors may have been increased to supra-physiologic levels in these studies, producing alterations in pup responses beyond those that would be expected following natural exposure.

Comparison of these results to available human data has proven difficult. It can be difficult to distinguish the impact of maternal sensitization and/or gestational exposure to a specific aeroallergen from the effects of neonatal allergen exposure, as controlled exposure or avoidance of that allergen may be impossible. Several studies have demonstrated increased cord blood IgE or allergen-stimulated proliferation of cord blood T-cells, and these results have been attributed to *in utero* sensitization (Jones et al., 1996; Miller et al., 2001). The relevance of these phenomena has been questioned as they may be transient in nature and do not necessarily correlate with later sensitization (Smillie et al., 2001; Bønnelykke et al., 2008).

In summary, the present study demonstrates that IA administration of either MACA or HDM extract to young adult BALB/c mice induces the development of an airway inflammatory response similar to human allergic asthma. However, in contrast to previous reports, neither maternal sensitization nor gestational allergen exposure could be demonstrated to have a clear effect on pup sensitization to homologous or heterologous allergen in this study. The reasons for these discrepancies are unclear, but may reflect differences in pup age at initiation of sensitization as well as differences in dam sensitization and exposure protocols. The respiratory sensitization and exposure protocol used in this study mimics natural allergen exposure more closely than do parenteral routes of exposure, suggesting that the results of this study may be more reflective of spontaneous disease.

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