



Contents lists available at ScienceDirect



Maternal Antiasthma Simplified Herbal Medicine Intervention therapy prevents airway inflammation and modulates pulmonary innate immune responses in young offspring mice

Iván López-Expósito, PhD^{*,†}; Kamal D. Srivastava, PhD^{*}; Neil Birmingham, PhD^{*}; Alexandra Castillo, BS^{*}; Rachel L. Miller, MD[‡]; and Xiu-Min Li, MD, MS^{*}

^{*} Department of Pediatrics, Mount Sinai School of Medicine, New York, New York

[†] Department of Bioactivity and Food Analysis, Institute in Food Science Research (CIAL), CSIC-UAM, Madrid, Spain

[‡] Division of Pulmonary, Allergy, Critical Care Medicine, Department of Medicine; Division of Allergy, Immunology, and Rheumatology, Department of Pediatrics, Columbia University, New York, New York

ARTICLE INFO

Article history:

Received for publication August 29, 2014.

Received in revised form October 8, 2014.

Accepted for publication October 14, 2014.

ABSTRACT

Background: Maternal asthma is a risk factor for asthma in offspring; however, transmission of the risk for allergic asthma without direct offspring sensitization has not been explored.

Objective: To determine whether offspring from mothers with ovalbumin (OVA)-sensitized asthma would develop airway disease at first-ever exposure to OVA and whether preconception maternal treatment with the Antiasthma Simplified Herbal Medicine Intervention (ASHMI) or dexamethasone (DEX) could modify this risk in offspring.

Methods: Female BALB/c mice (F0) with OVA-induced asthma were generated using established protocols. Mice with asthma were treated with ASHMI, DEX, or water for 6 to 7 weeks. Naive mice served as controls. Subsequently, mice were mated. Twelve-day-old F1 offspring received 3 consecutive intranasal low- or high-dose OVA exposures without sensitization. Forty-eight hours later, airway inflammation, mucus hypersecretion, serum antibodies, and cytokines were evaluated.

Results: Offspring from OVA-sensitized mothers, but not naive mothers, showed eosinophilic and neutrophilic airway inflammation, and mucus hyperplasia after OVA exposure and the presence of OVA-specific IgG1 and IgG2a. Offspring of ASHMI- and DEX-treated mothers showed decreased airway inflammation and mucus hypersecretion after low-dose OVA ($P < .05$ – $.001$ for the 2 comparisons vs offspring of OVA/Sham mothers). Offspring of ASHMI-treated, but not DEX-treated, mothers were protected after the high-dose OVA challenge ($P < .05$ – $.01$ vs offspring OVA/Sham). Maternal ASHMI therapy was associated with increased IgG2a ($P < .01$ vs offspring of OVA/Sham mothers) and decreased bronchoalveolar lavage fluid CXCL-1 and eotaxin-1 levels ($P < .01$ and $P < .05$, respectively, vs offspring of OVA/Sham mothers).

Conclusion: Offspring of mothers with OVA-induced asthma developed airway inflammation and mucus to first-ever OVA exposure without prior sensitization. Maternal therapy with ASHMI was superior to DEX in decreasing offspring susceptibility to airway disease and could be a strategy to lower asthma prevalence.

© 2014 American College of Allergy, Asthma & Immunology. Published by Elsevier Inc. All rights reserved.

Introduction

Allergic asthma is a disease with origins in early life,¹ with several studies indicating that allergic sensitization can occur during prenatal development.^{2–4} Having a parental history of asthma,⁵ specifically maternal asthma,⁶ increases the risk that a child will

develop asthma (odds ratio 5.0, 95% confidence interval 1.7–14.9), and maternal allergic airway diseases, such as allergic rhinitis, also are associated with asthma development in children.^{7–10} The maternal effect on asthma susceptibility in offspring has been a focus of several animal model studies. These studies have largely

Dr López-Expósito and Dr Srivastava contributed equally to this study.

Reprints: Kamal D. Srivastava, PhD, Pediatric Allergy and Immunology, The Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029-6574; E-mail: kamal.srivastava@mssm.edu.

Disclosure: Dr López-Expósito acknowledges financial support from the Consejo Superior de Investigaciones Científicas through a JAE-Doc grant. Dr Srivastava was supported by a KL2 Faculty Scholar Award (KL2TR000069) from the Mount Sinai Clinical and Translational Science Award. Dr Li received grants from the National

Institutes of Health, the FAI, the Chris Burch Fund, the Sean Parker Foundation, and Winston Wolkoff Integrative Medicine for Allergies and Wellness. Dr. Li holds US Patent PCT/US05/08600 for ASHMI™ and is shareholder of Herb Springs LLC.

Funding: This study was supported from the National Center for Complementary and Alternative Medicine, National Institutes of Health (grant 1P01 AT002644725-01); the Center for Chinese Herbal Therapy for Asthma; the Sean Parker Foundation (Antiasthma Simplified Herbal Medicine Intervention active compounds for asthma therapy); and Winston Wolkoff Integrative Medicine for Allergies and Wellness.

<http://dx.doi.org/10.1016/j.anai.2014.10.018>

1081-1206/© 2014 American College of Allergy, Asthma & Immunology. Published by Elsevier Inc. All rights reserved.

explored the question of offspring susceptibility to sensitization using protocols that vary in antigen dose or timing but always include a period of sensitization before challenge. Using such methods, Hamada et al¹¹ demonstrated that offspring of mice with ovalbumin (OVA)-induced “chronic asthma” were more susceptible to asthma after a suboptimal protocol consisting of 1 intraperitoneal injection of allergen in adjuvant at 4 days of life, followed by 3 consecutive challenges with aerosolized allergen on days 12 to 14 of life. Likewise, Fedulov et al showed that offspring of mice with OVA-induced asthma develop a full asthma phenotype even if they are initially sensitized as late as 6 weeks of age.¹² A similar model using canines was developed by Royer et al¹³ in which offspring from ragweed (RW)-sensitized, but not normal, female dogs developed asthma-like features after sensitization exposure to RW. Because specific antibodies to maternally sensitized antigen are transmitted to offspring and can be detected in the absence of direct offspring sensitization, the authors questioned whether offspring could mount airway responses to first-ever exposure with the same antigen. In previous studies, the authors found that offspring of mice allergic to peanut developed anaphylaxis at first-ever exposure to peanut. Whether offspring of mothers with asthma develop airway disease in response to first-ever exposure to maternally encountered antigen is unknown. In additional experiments, the authors extended their studies to include the investigation of preconception maternal asthma therapy on offspring pulmonary responses to antigen exposure without sensitization. Corticosteroids are the cornerstone of allergic asthma treatment,¹⁴ and their impact on offspring asthma risk has received attention. Multiple studies have shown that their use during pregnancy does not increase offspring asthma risk, but no information is currently available regarding their potential to protect offspring against a high risk of asthma. The Antiasthma Simplified Herbal Medicine Intervention (ASHMI) is a traditional Chinese medicine herbal asthma formula consisting of 3 herbs: *ling-zhi* (*Ganoderma lucidum*), *ku-shen* (*Sophora flavescens*), and *gan-cao* (*Glycyrrhiza uralensis*). In previous work, the authors reported its ability to decrease airway hyperreactivity (AHR) and eosinophilic inflammation by downregulating the T-helper type (T_H) 2 response in murine models.^{15–17} In clinical trials, ASHMI treatment significantly improved lung function and lowered symptom scores of asthma to a similar extent as standard asthma therapy.¹⁸ Although ASHMI treatment in humans and mice has been shown to be efficacious, the ability of maternal ASHMI therapy to alter offspring airway disease has not been explored. In the present study, the authors explored airway responses in offspring (F1 generation) to first-ever exposure to maternally encountered and irrelevant antigens without prior sensitization and further investigated their modification by preconception maternal (F0) treatment with ASHMI or dexamethasone (DEX).

Methods

ASHMI Preparation

The ASHMI preparation is composed of 3 herbs, identified as the fruiting body of *G lucidum*, the roots of *S flavescens* Ait, and the roots and rhizome of *G uralensis* Fischer. Voucher specimens of the raw herbs are archived in the Botanical Chemistry Laboratory at the Center for Chinese Herbal Medicine for Allergy and Asthma, Mount Sinai School of Medicine (New York, New York). ASHMI was provided by the Sino-Lion Pharmaceutical Company (Weifang, China) as previously described.¹⁹ In brief, herbs were boiled together twice in water. The decoctions were combined, concentrated under decreased pressure, and dried to powder. The yield of ASHMI extract was 11.5%.

Antigen Sensitization and Challenge and ASHMI Treatment

A chronic asthma model was developed as depicted in Figure 1A. Standard guidelines for the care and use of animals were

followed.²⁰ Briefly, female BALB/c mice (6 weeks old; Jackson Laboratory, Bar Harbor, Maine) were sensitized by 2 weekly intraperitoneal injections with 100 µg of OVA (Type V; Sigma-Aldrich, St Louis, Missouri) and 2 mg of alum in phosphate buffered saline (PBS) and challenged intratracheally with 100 µg of OVA in PBS weekly for 3 weeks. Four weeks after the last intratracheal challenge, mice were given 2 consecutive intratracheal daily challenges. In a group of mice, 4.5 mg of ASHMI in 0.5 mL of water was administered intragastrically twice daily during 6 weeks beginning 1 day after the initial intratracheal challenge. This group was denoted OVA/ASHMI. The dose was determined by a conversion table of equivalent human-to-animal dose ratios based on body surface area.²¹

Additional OVA-sensitized and challenged mice received 0.5 mL of water intragastrically twice daily for 6 weeks as sham treatment controls (OVA/Sham). Naive mice served as normal controls. Another set of maternal mice was generated as part of separate ongoing experiments using a chronic asthma model. In this experiment, a similar protocol to that shown in Figure 1A was used with the exception that maternal treatment was started on day 29, after the third intratracheal sensitization, and a DEX-treated group was added (OVA/DEX) as a control, representing steroid therapy. After therapy concluded on day 77, a final set of intratracheal challenges was given on days 78 and 79 (Fig 1B). For the 2 sets of experiments, maternal mice were evaluated for AHR using invasive methods 2 days after the fifth intratracheal challenge (5 mice per group). In addition, AHR studies and goblet cell hyperplasia were analyzed. Mice that were not sacrificed for AHR studies were mated with aged-matched naive male BALB/c mice until signs of pregnancy became evident.

After mating and delivery, 12-day-old offspring from the maternal groups shown in Figure 1A received 3 consecutive intranasal exposures with PBS or OVA (5 µg) and 12-day-old offspring from the maternal groups shown in Figure 1B were given OVA (5 µg), RW (5 µg; Greer Laboratories, Lenoir, North Carolina) or PBS. Some offspring from the protocol shown in Figure 1B were given high-dose OVA exposure (50 µg). Forty-eight hours later, airway inflammation, goblet cell hyperplasia, serum antibodies, and cytokine levels in splenocyte cultures and bronchoalveolar lavage fluid (BALF) were determined.

Measurement of Late-Phase AHR

Airway hyperresponsiveness to acetylcholine provocation was measured in mothers 2 days after the fifth challenge by measuring the Airway Pressure Time Index (APTI; Kent Scientific Corporation, Torrington, Connecticut), a classic invasive measurement of AHR, as previously described.^{22–25}

Evaluation of BALF Cell Differential Counts, Cytokines, and Lung Histology

Immediately after sacrifice, the lungs were lavaged, and BALF and cytospin slides were prepared as previously described.²⁶ Cell differential counts were obtained by counting at least 500 cells per slide by light microscopy after staining with HEMA 3 (Fischer Scientific, Pittsburgh, Pennsylvania). BALF supernatants were used for the analysis of cytokines by enzyme-linked immunosorbent assay (interleukin [IL]-4, IL-5, IL-13, IL-17, interferon-γ [IFN-γ], CXCL-1 [R&D Systems, Minneapolis, Minnesota], and eotaxin-1; BD Biosciences, San Jose, California for all others). Lung samples were fixed in neutral buffered formalin and embedded in paraffin. Five-micron sections were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) for analysis of inflammatory cells and goblet cells, respectively. Light microscopy and image acquisition were performed at the Microscopy Shared Resource Facility at Mount Sinai. Goblet cell hyperplasia was expressed as the

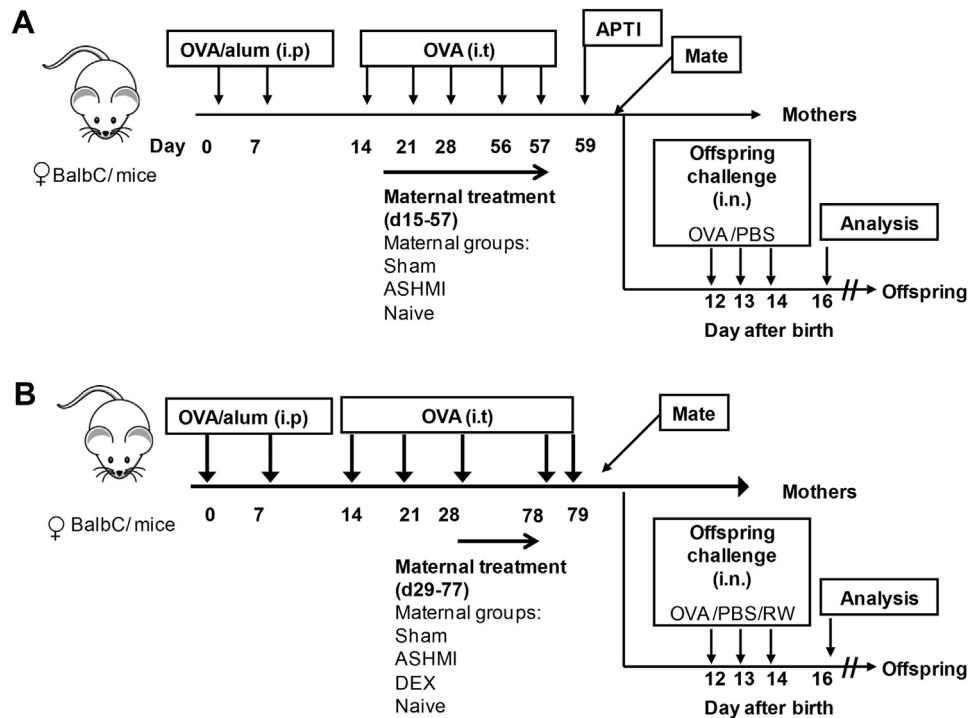


Figure 1. Experimental protocol. (A) Six-week-old female BALB/c mice were sensitized, challenged, and treated with the Antiasthma Simplified Herbal Medicine Intervention (ASHMI) as described in the Methods section. Forty-eight hours after the last challenge, mice were sacrificed for analysis. Mice that were not sacrificed were mated with matched by age to naive male BALB/c mice until signs of pregnancy became evident. Twelve-day-old offspring from every group of mothers received 3 consecutive intranasal ovalbumin (OVA) challenges. Forty-eight hours after the final intranasal challenge, mice were euthanized, and airway inflammation, goblet cell hyperplasia, serum antibodies, and cytokines in splenocyte cultures and bronchoalveolar lavage fluid were determined. (B) In a separate set of experiments, maternal groups were generated using an extended protocol in which ASHMI or dexamethasone (DEX) treatment was started at day 29. After the final OVA challenge, mice were mated and 12-day-old offspring were exposed to 3 intranasal OVA or ragweed (RW) exposures. Some offspring received phosphate buffered saline (PBS). Other offspring were exposed to high-dose OVA (50 µg). APTI, Airway Pressure Time Index.

percentage of PAS-positive epithelial cells (number of PAS-positive cells divided by the total epithelial cell number) in at least 6 randomly selected bronchioles per animal.²⁷

Measurement of OVA-Specific Antibodies

Blood was collected from the descending vena cava from offspring at sacrifice. Sera were isolated and stored at -80°C until OVA-specific IgE, IgG1, and IgG2a levels were determined, as previously described.²⁴

Spleen Cell Culture and Cytokine Analysis

After sacrifice, spleen cell culture was conducted as previous described.^{28,29} Briefly, splenocytes were cultured in 24-well plates (4×10^6 /well/mL) in the presence or absence of OVA (100 µg/mL) or concanavalin A (2.5 µg/mL). Seventy-two hours later, supernatants were collected and IL-4, IL-5, IL-10, IL-13, IL-17, IFN- γ , and tumor necrosis factor- α levels were determined by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems for IL-13 and IL-17; BD Biosciences for all others).

Statistical Analysis

Analysis of variance was performed followed by the Bonferroni *t* test for all pairwise comparisons if the data were approximately normal. Differences between groups were analyzed by analysis of variance on ranks followed by all pairwise comparisons if the data were not normally distributed. A *P* value less than .05, based on 2-tailed tests, was considered statistically significant. All statistical

analyses were performed with SigmaStat 3.5 (Systat Software, Inc, Chicago, Illinois).

Results

Establishment of Allergic Airway Disease in Mothers and Effects of ASHMI Treatment

The authors verified the establishment of the asthma-like phenotype in mothers (F0 generation) and confirmed the expected therapeutic effects of ASHMI based on their previous studies.¹⁶ AHR was measured 2 days after the last OVA challenge (Fig 1A). As expected, OVA/Sham mothers had increased peribronchial and perivascular inflammation compared with naive mothers (eFig 1Ai, iii). Treatment with ASHMI dramatically decreased peribronchial and perivascular inflammation, as shown in eFigure 1Aii. PAS staining, a marker of mucus hyperproduction, was significantly higher in OVA/Sham mothers compared with naive mothers ($83.33 \pm 6.09\%$ vs 0% , $P < .001$; eFig 1Aiv and vi and 1B). This increased staining was markedly decreased when mothers were treated with ASHMI ($25.0 \pm 6.16\%$, $P < .001$; eFigs 1Avi and 2B). Classically, having an increased APTI value indicates AHR to acetylcholine provocation. As shown in eFigure 1C, APTI values for OVA/Sham mothers were significantly higher than for naive mothers (532.4 ± 41.29 vs 122.2 ± 16.41 , $P < .001$), indicating induction of AHR. APTI values from OVA/ASHMI mothers were significantly lower than in OVA/Sham mothers (331.9 ± 63.78 , $P < .05$), showing the anti-inflammatory effects of ASHMI. Consistent with allergic airway inflammation, a significant number of eosinophils was present in the BALF infiltrate of OVA/Sham mice that was absent in naive mice and markedly decreased in OVA/ASHMI mice (eFig 1D).

OVA Exposure in Offspring from Mothers with OVA-Induced Asthma Induces Airway Inflammation That Is Decreased by Maternal ASHMI Therapy

The F1 offspring from each maternal group in the first set of experiments were intranasally exposed to 5 μ g of OVA during 3 consecutive days starting at 12 days of age and sacrificed for analysis at day 16, as depicted in Figure 1A. As a measurement of airway inflammation, total BALF cell number and differential cells counts were evaluated immediately after sacrifice. Offspring from OVA/Sham mothers showed a significant increase in total cell number in their BALF compared with offspring from naive mothers ($P < .001$; Fig 2A). The total cell number increase in offspring of OVA/Sham mothers was reflected by a significant increase in macrophages, eosinophils, neutrophils, and lymphocytes (Fig 2B–E) compared with offspring of naive mothers ($P < .001$ for macrophages and eosinophils; $P < .01$ for the rest). Surprisingly, offspring from OVA/ASHMI mothers had clearly diminished airway inflammatory responses, with significantly decreased infiltration of total cells, macrophages, eosinophils, and lymphocytes compared with OVA-exposed offspring of OVA/Sham mothers (not significant for neutrophils, $P < .01$ for lymphocytes, $P < .001$ for the rest; Fig 2A–E). The cell concentrations seen in the offspring of OVA/ASHMI mothers were similar to naive levels for all cell types with the exception of neutrophils.

Maternal Therapy Affects Lung Pathology in Offspring Exposed to OVA

Consistent with the BALF data, offspring of OVA/Sham mothers exhibited pronounced peribronchial and perivascular inflammation containing numerous eosinophils (Fig 3Ai, inset) compared with offspring of naive mothers (Fig 3Aii). As illustrated in Figure 3Aii, inflammation was dramatically decreased in the lungs from OVA-exposed offspring of OVA/ASHMI mothers. OVA exposure in offspring of OVA/Sham mothers also showed an increase of

positively staining PAS epithelial cells in bronchioles compared with offspring of naive mothers, indicating an increase in mucus production ($35.10 \pm 11.17\%$ vs 0% , $P < .001$; Fig 3Aiv and B). In offspring of ASHMI/OVA mothers, mucus production was markedly decreased ($8.50 \pm 1.24\%$, $P < .001$; Fig 3Av and B).

Levels of Maternally Transmitted OVA-Specific Antibodies in Offspring Are Differentially Altered by Maternal ASHMI Therapy

A hallmark of systemic allergic inflammation is the presence of allergen-specific IgE. Serum OVA-specific IgE was undetectable in offspring from all maternal groups (data not shown). Measurement of another known anaphylactic antibody, OVA-specific IgG1, yielded detectable and similar levels in offspring of OVA/Sham and OVA/ASHMI mothers and these levels were significantly different from PBS- or OVA-exposed offspring of naive mothers ($P < .001$; Fig 4A). Levels of OVA-specific IgG2a were measurable and found to be significantly increased in offspring of OVA/ASHMI mothers compared with offspring of OVA/Sham mothers (23.533 ± 3.798 vs 7.821 ± 904.6 ng/mL, $P < .001$; Fig 4B). Calculations were performed to generate the OVA-specific IgG1/OVA-specific IgG2a ratio for offspring of all maternal groups, as shown in Figure 4C. Maternal ASHMI treatment significantly shifted the IgG1/IgG2a ratio. A ratio of 180.9 ± 52.11 was observed for offspring of OVA/Sham mothers compared with 39.89 ± 9.00 in offspring of OVA/ASHMI mothers ($P < .01$).

Maternal ASHMI Therapy Beneficially Regulates Systemic Cytokine Profile in Offspring Mice

Immediately after sacrifice, offspring splenocyte cytokine profiles, including T_H1 (IFN- γ), T_H2 (IL-4, IL-5, and IL-13), T_H3 (IL-10), and T_H17 (IL-17), were determined in response to OVA or concanavalin A stimulation. As expected, splenocytes from offspring in all groups did not respond to OVA stimulation (data not shown); thus, concanavalin A-stimulated cytokines were evaluated. Negligible levels of IL-4, IL-5, and IL-17 were found in

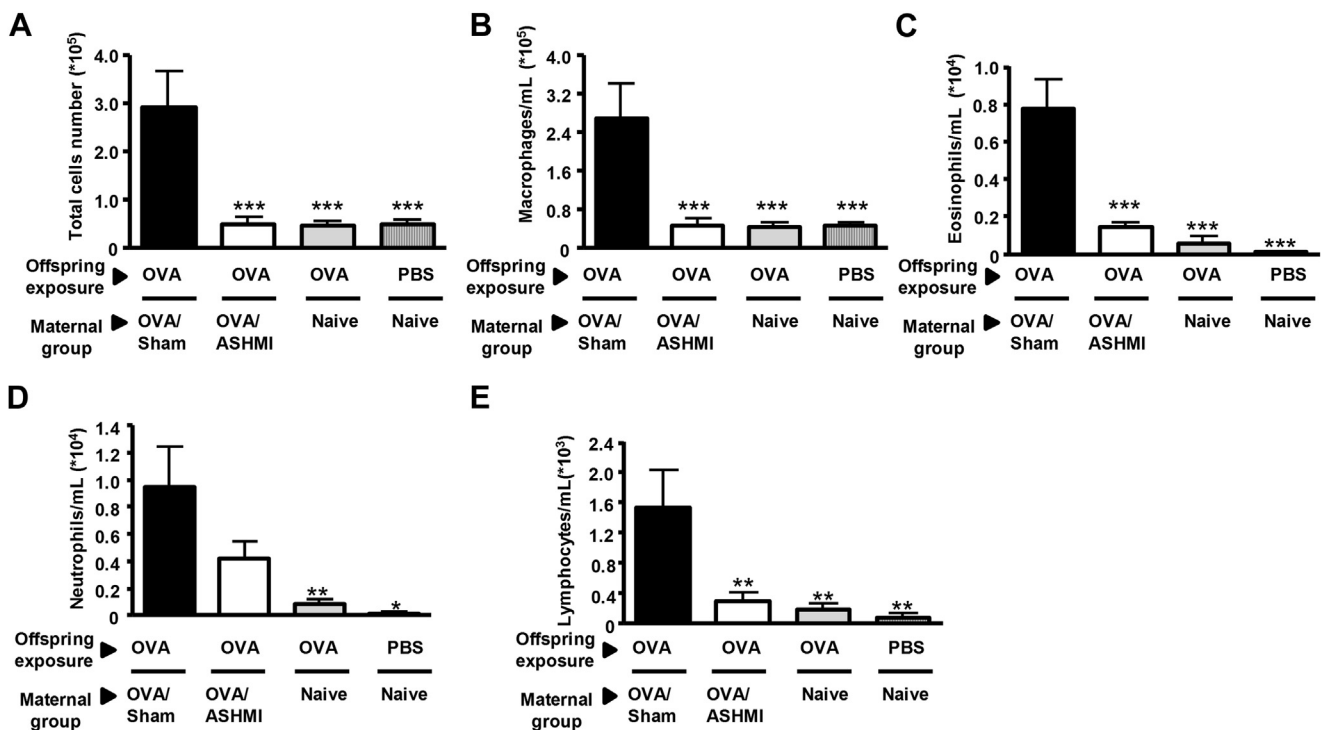


Figure 2. Offspring inflammatory cells in bronchoalveolar lavage fluid. Cell differential counts were obtained by counting at least 500 cells per cytospin slide by light microscopy after differential staining with HEMA 3: (A) total cell numbers, (B) macrophages per milliliter, (C) eosinophils per milliliter, (D) neutrophils per milliliter, (E) lymphocytes per milliliter. Values are expressed as mean $\pm$ SEM ($n = 6-9$ /group). * $P < .05$, ** $P < .01$, *** $P < .001$ vs OVA/Sham. ASHMI, Antiasthma Simplified Herbal Medicine Intervention; DEX, dexamethasone; OVA, ovalbumin; PBS, phosphate buffered saline; RW, ragweed.

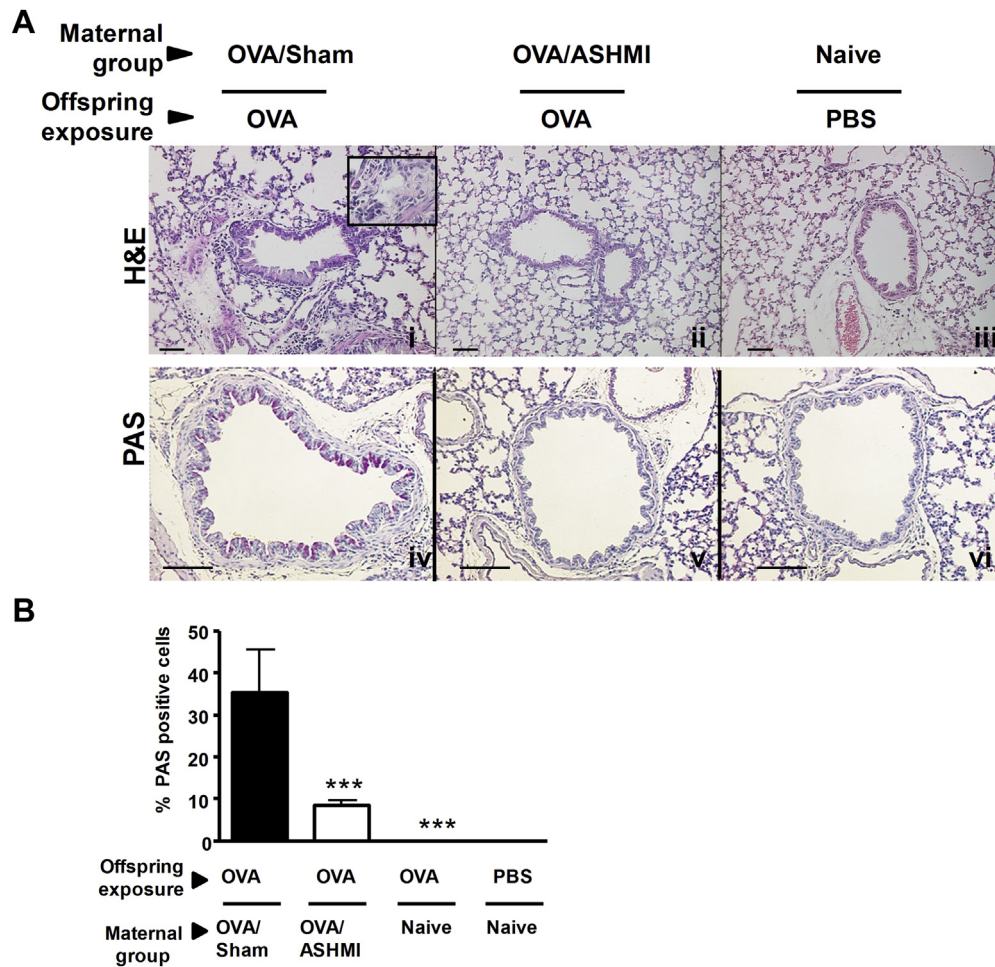


Figure 3. Offspring lung histologic analysis and percentage of cells positive for periodic acid-Schiff (PAS). (A) Lungs from the indicated mice were sectioned and stained with hematoxylin and eosin (H&E) or PAS. Panels show representative images at 10× magnification for H&E and 20× magnification for PAS. (B) Percentage of PAS-positive cells (number of PAS-positive cells divided by the total epithelial cell number in ≥6 bronchioles per animal). Values are expressed as mean ± SEM (n = 6–9/group). ****P* < .001 vs OVA/Sham. ASHMI, Antiasthma Simplified Herbal Medicine Intervention; OVA, ovalbumin; PBS, phosphate buffered saline.

splenocyte cultures from all offspring groups included in the study (data not shown); however, a significantly elevated production of IL-10 and IFN- γ was found in offspring of OVA/ASHMI mothers compared with offspring of OVA/Sham mothers (*P* < .001; Table 1). Furthermore, offspring of OVA/ASHMI mothers showed significantly lower levels of IL-13 compared with OVA/Sham offspring (*P* < .01; Table 1). Moreover, the ratios of IL-10 to IL-13 and IFN- γ to IL-13 were higher in offspring of OVA/ASHMI mothers compared with the other groups, showing a clear shift from T_H2 responses to T_H1 or T_H3 (Table 1). For reasons that need to be explored further, IL-13 levels in cultures from offspring of naive mice were higher than those observed from the other groups; however, overall T_H2 bias was lower in offspring from naive mothers compared with offspring from Sham mothers.

Airway Inflammation in Offspring Mice Exposed to Low-Dose OVA Is Antigen Specific and Decreased by Maternal Therapy with ASHMI or DEX

To determine antigen specificity of offspring airway inflammation, offspring mice generated in a second set of experiments (Fig 1B) were exposed intranasally to low-dose (5 μ g) OVA or RW or PBS on 3 consecutive days without prior sensitization. In addition, the benefits of maternal ASHMI therapy were compared

with the effects of maternal treatment with DEX (standard asthma therapy). The effectiveness of DEX treatment on the mother's airway inflammation (data not shown) was consistent with previously published results.²⁵ In BALF harvested 48 hours after final exposure, no significant differences in the numbers of macrophages were observed (Fig 5A). Significantly larger numbers of lymphocytes (Fig 5B), eosinophils (Fig 5C), and neutrophils (Fig 5D) were observed in offspring of OVA/Sham mothers exposed to OVA but not to RW or PBS (*P* < .05–.001). Furthermore, significantly decreased numbers of these cells were observed in BALF of offspring of OVA/ASHMI and OVA/DEX mothers to all exposures (*P* < .05–.001 vs OVA-exposed offspring of OVA/Sham mothers). These data suggest that the low-dose OVA challenge of offspring resulted in mild mixed eosinophilic and neutrophilic inflammation that was observed only in OVA-exposed, but not in RW-exposed, offspring of Sham mothers, implying specificity of offspring responses to the antigen used for maternal sensitization. Furthermore, protection from low-dose OVA exposure-induced pulmonary inflammation was observed as indicated by fewer BALF inflammatory cells in the offspring of ASHMI- and DEX-treated mothers. Absence of inflammation in offspring of naive mothers in response to OVA or RW indicates that potential endotoxin contamination was not a factor in observed responses.

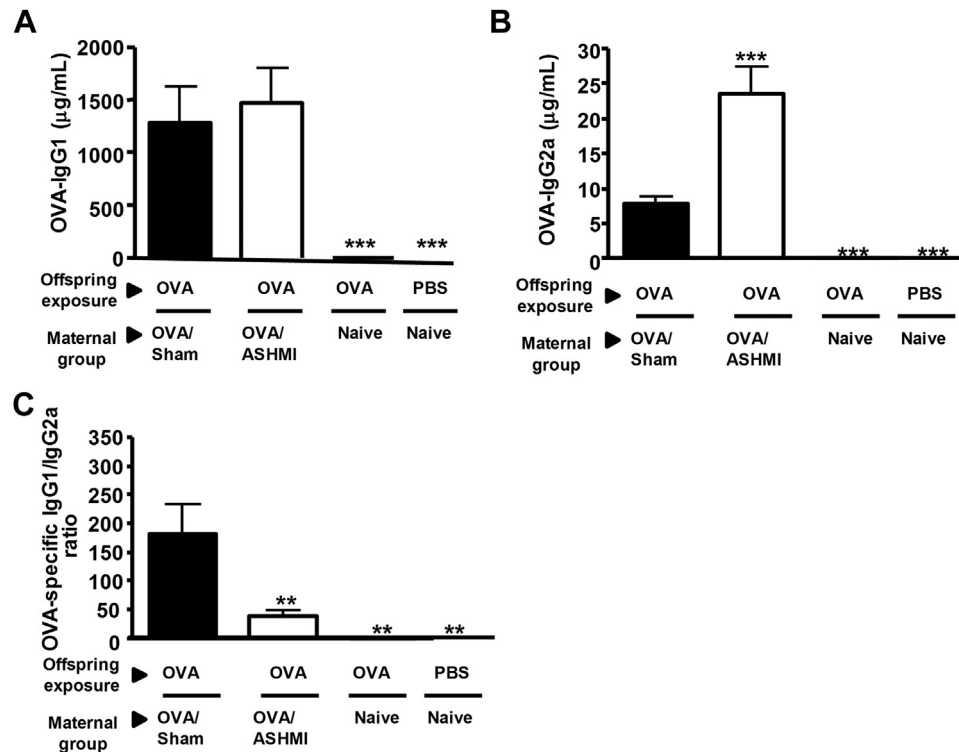


Figure 4. Offspring humoral response. (A) Serum OVA-specific IgG1 (nanograms per milliliter). (B) Serum OVA-specific IgG2a (micrograms per milliliter) were evaluated by enzyme-linked immunosorbent assay. (C) OVA-specific IgG1/IgG2a ratio. Data are expressed as mean $\pm$ SEM of duplicates for each group ($n = 6-9$ mice/group). ** $P < .01$, *** $P < .001$ vs OVA/Sham. ASHMI, Antiasthma Simplified Herbal Medicine Intervention; OVA, ovalbumin; PBS, phosphate buffered saline.

High-Dose OVA Exposure Amplifies Offspring Airway Inflammation That Is Decreased by Maternal Therapy with ASHMI but not with DEX

In light of the only mild inflammation observed in response to low-dose OVA exposure, offspring were subjected to 3 intranasal high-dose (50 μ g) OVA challenges without prior sensitization. BALF macrophage (Fig 6A) and lymphocyte (Fig 6B) counts were not significantly different across offspring groups, although a modest decrease in lymphocyte numbers in offspring of OVA/ASHMI mothers was observed. BALF eosinophil numbers (Fig 6C) were markedly elevated in OVA-challenged offspring of Sham mothers compared with offspring of ASHMI-treated mothers ($P < .05$). Interestingly, different from observations with the low-dose OVA challenge (Fig 5C), BALF eosinophil numbers in high-dose OVA-challenged offspring from DEX-treated mothers also showed markedly elevated counts that were significantly higher than those in ASHMI offspring ($P < .05$). A similar profile was observed for neutrophil counts in which a significantly larger number of BALF neutrophils was observed in high-dose OVA-challenged offspring of Sham and DEX-treated mothers compared with offspring from ASHMI-treated mothers ($P < .05$ for the 2 comparisons; Fig 6D). Taken together these findings suggest that

preconception maternal ASHMI, but not DEX, therapy protects offspring from pulmonary inflammation in response to high-dose OVA exposure without prior sensitization.

Protection in OVA/ASHMI Offspring but not OVA/DEX Offspring to High-Dose OVA Exposure Is Linked to Lower Levels of Proinflammatory Chemokines and Higher Levels of OVA-Specific IgG2a

To determine factors that could explain the differential susceptibility to inflammation in offspring of ASHMI- and DEX-treated mothers, OVA-specific IgG1 and IgG2a were evaluated in offspring of ASHMI- and DEX-treated mothers (Fig 7A, B). Although offspring of ASHMI- and DEX-treated mice had essentially similar decreases in OVA-specific IgG1 ($P < .05$ for the 2 comparisons vs offspring of OVA/Sham mothers), offspring of OVA/ASHMI mice showed increased levels of OVA-specific IgG2a compared with offspring of OVA/DEX mice ($P < .001$) and offspring of OVA/Sham mice ($P < .01$). Elevated levels of OVA-specific IgG2a in this set of experiments were consistent with data shown for the previous set of offspring shown in Figure 5B. Further, given the mixed nature of neutrophilic and eosinophilic airway inflammatory responses in offspring

Table 1
Cytokine levels in spleen cell culture supernatants from offspring with concanavalin A stimulation

Offspring group	IL-10 (pg/mL), mean $\pm$ SEM	IFN- γ (pg/mL), mean $\pm$ SEM	IL-13 (pg/mL), mean $\pm$ SEM	IL-10/IL-13 ratio	IFN- γ /IL-13 ratio
OVA/Sham	5,327 $\pm$ 281.7	3,495 $\pm$ 274.8	754.1 $\pm$ 205.9	7.5	4.6
OVA/ASHMI	8,629 $\pm$ 260.4 ^b	16,046 $\pm$ 396.2 ^b	75.36 $\pm$ 75.3 ^a	114	212.9
OVA/naive	4,433 $\pm$ 174.0 ^b	19,290 $\pm$ 1038.0 ^b	1,723 $\pm$ 82.4 ^b	2.5	11.2
PBS/naive	2,159 $\pm$ 72.4 ^b	15,027 $\pm$ 1253.0 ^b	1,153 $\pm$ 39.8	1.9	13.0

Abbreviations: ASHMI, Antiasthma Simplified Herbal Medicine Intervention; IFN- γ , interferon- γ ; IL, interleukin; OVA, ovalbumin; PBS, phosphate buffered saline.

^a $P < .01$ vs OVA/Sham.

^b $P < .001$ vs OVA/Sham.

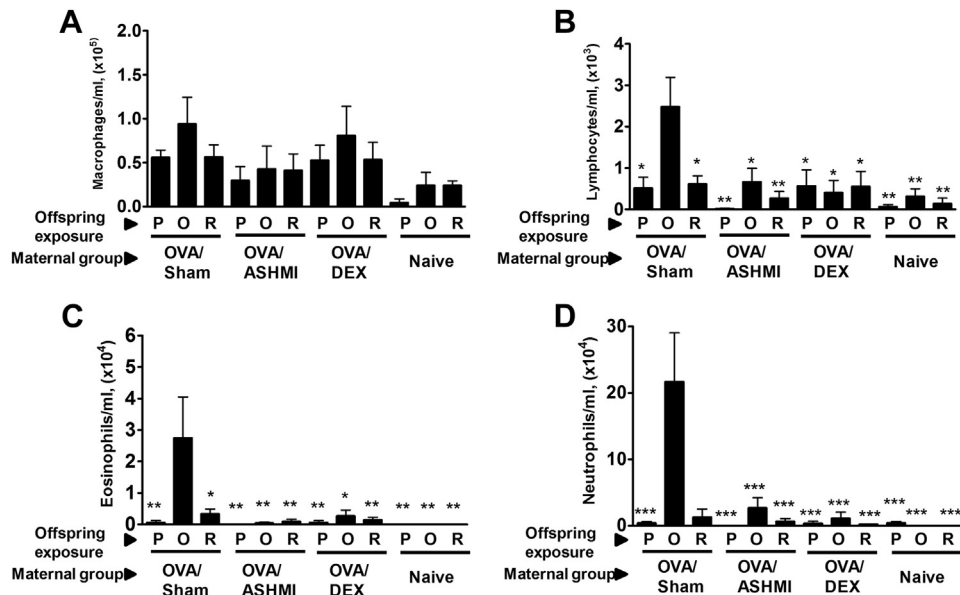


Figure 5. Offspring airway inflammation in response to low-dose OVA exposure. Bronchoalveolar lavage fluid cells were placed on cytospin glass slides and differentially stained with HEMA 3 stain. Cell types identified based on cell morphology and differential staining were counted, and data for (A) macrophages, (B) lymphocytes, (C) eosinophils, and (D) neutrophils were calculated for the number of cells per milliliter of bronchoalveolar lavage fluid. Data are shown as mean $\pm$ SEM ($n = 3-5$ mice/challenge condition). * $P < .05$, ** $P < 0.01$, *** $P < 0.001$. ASHMI, Antiasthma Simplified Herbal Medicine Intervention; DEX, dexamethasone; O, ovalbumin exposure; OVA, ovalbumin; P, phosphate buffered saline exposure; PBS, phosphate buffered saline; R, ragweed exposure; RW, ragweed.

groups and the absence of classic T_H2 cytokines (IL-4, IL-5, and IL-13) or the T_H1 cytokine IFN- γ in BALF samples after OVA exposure in unsensitized mice, BALF CXCL-1 (murine homolog of IL-8) and eotaxin were measured to understand the mechanisms underlying the recruitment of inflammatory cells in offspring airways. BALF samples from the low-dose OVA challenge showed significantly

increased CXCL-1 in the BALF of OVA-challenged but not RW- or PBS-challenged offspring from Sham mothers (eFig 2), whereas levels of BALF CXCL-1 in all other offspring groups challenged with low-dose OVA, RW, or PBS were low and indistinguishable across groups. When BALF chemokine levels were examined in offspring mice after the high-dose OVA challenge, CXCL-1 levels in offspring

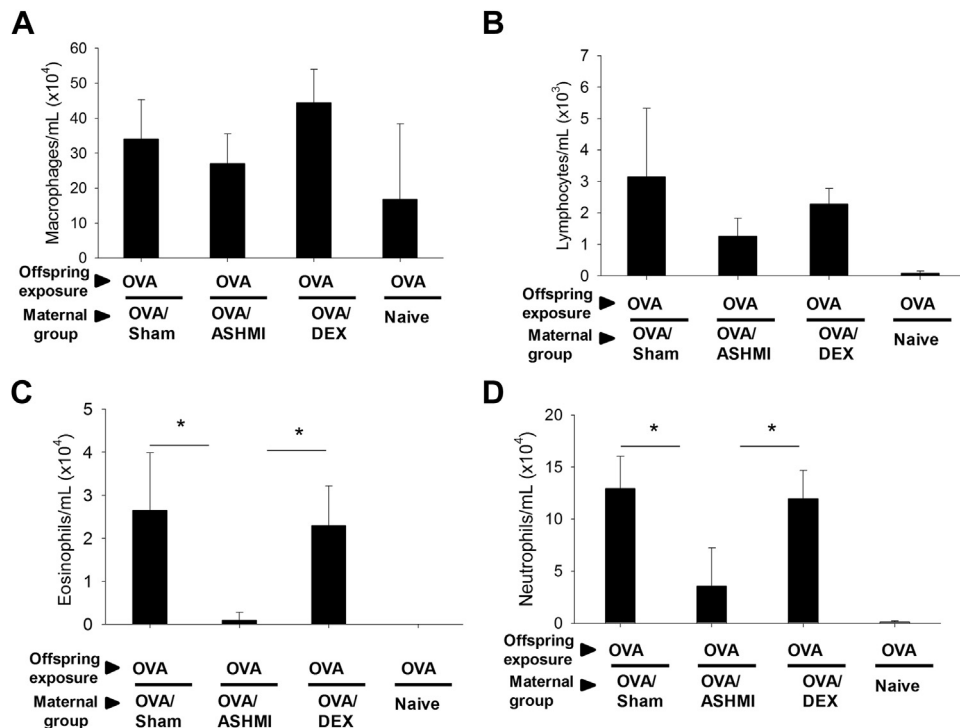


Figure 6. Offspring airway inflammation in response to high-dose OVA exposure. Bronchoalveolar lavage fluid cells from high-dose OVA-challenged offspring were placed on cytospin glass slides and differentially stained with HEMA 3 stain. Cell types identified based on cell morphology and differential staining were counted, and data for (A) macrophages, (B) lymphocytes, (C) eosinophils, and (D) neutrophils were calculated for the number of cells per milliliter of bronchoalveolar lavage fluid. Data are shown as mean $\pm$ SEM ($n =$ mice/group). * $P < .05$. ASHMI, Antiasthma Simplified Herbal Medicine Intervention; DEX, dexamethasone; OVA, ovalbumin.

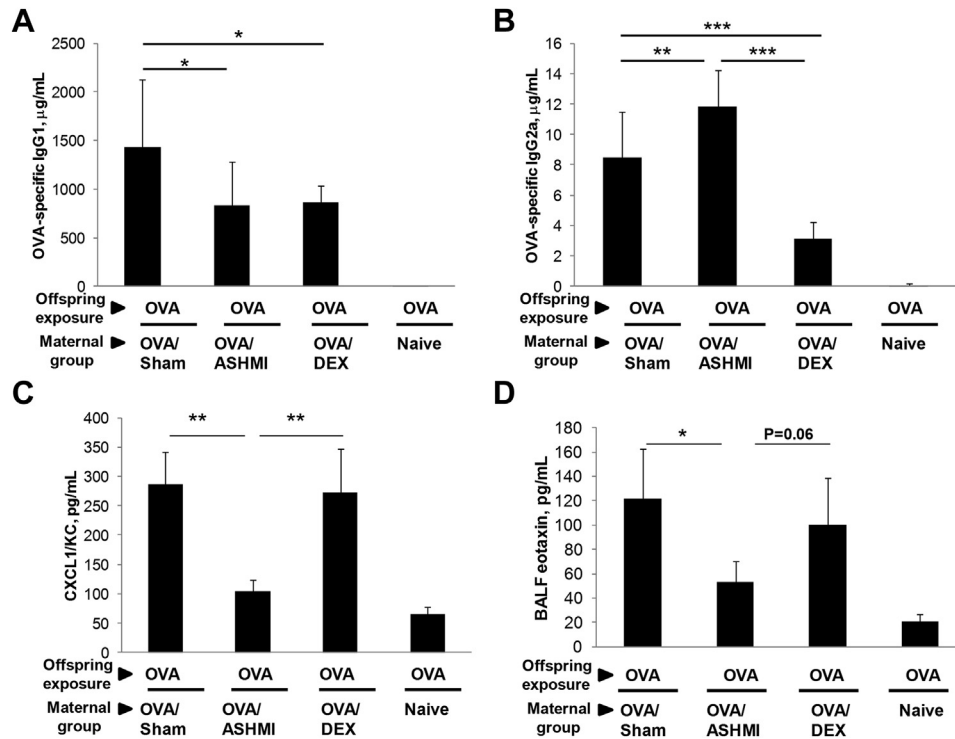


Figure 7. Effect of maternal therapy on offspring immunoglobulin and chemokine levels. OVA-specific (A) IgG1 and (B) IgG2a in serum were measured by enzyme-linked immunosorbent assay. (C) CXCL-1 and (D) eotaxin-1 in bronchoalveolar lavage fluid supernatant were measured by a commercial enzyme-linked immunosorbent assay kit. Data shown as group mean $\pm$ SEM ($n = 4$ –5 mice/group). * $P < .05$, ** $P < .01$. ASHMI, Antiasthma Simplified Herbal Medicine Intervention; DEX, dexamethasone; OVA, ovalbumin.

of Sham and DEX-treated mothers were significantly higher than those found in samples from offspring of ASHMI-treated mothers (Fig 7C). Similarly, there was a trend toward lower BALF eotaxin in offspring of ASHMI-treated mice compared with offspring of Sham mothers ($P < .05$) and DEX-treated mothers ($P = .06$; Fig 7D). Together these data suggest that decreased pulmonary inflammation to high-dose OVA exposure observed in offspring of ASHMI-but not of DEX-treated or Sham mothers is associated with higher levels of OVA-specific IgG2a and lower BALF chemokine levels.

Discussion

Children of parents with allergic asthma, specifically maternal asthma, have been shown to have a significantly higher risk to develop allergic inflammatory airway disease.²⁶ This maternal risk has been confirmed by several animal model studies reviewed by Lim and Kobzik²⁷ in which offspring were sensitized to allergens before challenge. In the present study, the authors demonstrate, for the first time, that offspring from mothers with OVA-sensitized asthma develop an asthmatic phenotype in response to first-ever exposure to OVA. The authors observed mixed eosinophilic and neutrophilic infiltration in BALF and mucus hypersecretion in offspring. These responses were specific to a maternally encountered antigen (OVA) and were absent in offspring of naive mothers. These data suggest that maternally transmitted OVA-specific immunoglobulins were instrumental to offspring airway inflammation because serum OVA-specific immunoglobulins were undetectable in offspring of naive mothers and airway inflammation was absent when offspring of mothers with OVA-induced asthma were challenged with RW, an irrelevant antigen in this study. The authors presume that transmission of immunoglobulins in this study occurred by breast milk because the receptor for IgG (FcRn) is not expressed on the placenta of rodents.²⁸ Offspring disease directly attributable to maternally inherited IgG antibodies has been

demonstrated previously in autoimmune disease^{29,30} but not in the context of allergic asthma.

In this study, the authors also evaluated whether maternal therapeutic treatment of allergic asthma with the traditional Chinese medicine herbal formula (ASHMI) or DEX could decrease the risk for disease development in offspring. The 2 treatments protected offspring from pulmonary inflammation to low-dose OVA exposure, but only offspring of ASHMI-treated mothers remained protected when given higher doses of OVA. Differences between offspring of ASHMI-treated and DEX-treated mothers associated with differential susceptibility to airway inflammation included a lower ratio of OVA-specific serum IgG1 to IgG2a and decreased BALF chemokine levels in offspring of ASHMI-treated mothers. The authors previously reported in a murine model of peanut allergy that maternal peanut-specific IgG1 is associated with anaphylactic reactions in the offspring.³¹ In addition, IgG2a is considered a protective antibody shown to decrease offspring sensitization.³² Mechanistically, IgG2a could protect the offspring by clearing the antigen from the circulation before the recognition by cells of the adaptive immune system owing to the formation of antigen-IgG2a complexes.³³ Therefore, the authors speculate that transmission of OVA-specific IgG2a likely through breast milk could be responsible in part for the decreased inflammatory responses observed in offspring of ASHMI-treated mothers, similar to previously published studies.³⁴ More research needs to be done to elucidate the role of IgG2a in the protective effects observed.

The proinflammatory innate chemokines CXCL-1 and eotaxin appeared to be critical for the development of pulmonary inflammation in offspring because other candidate cytokines, such as IL-4, IL-5, and IL-13, were absent in the BALF. CXCL-1, the murine homolog of IL-8, is a well-established neutrophil attractant and is produced by multiple cell types, including epithelial cells, endothelial cells, macrophages, and monocytes.³⁵ The eosinophil chemoattractant eotaxin-1 also is expressed by many pulmonary cells,

including airway epithelium, fibroblasts, airway smooth muscle cells, and macrophages.³⁶ CXCL-1 and eotaxin-1 were markedly decreased in offspring of ASHMI-treated mothers. In previously published work, the authors found that in a RW model of asthma characterized by mixed granulocytic inflammation, ASHMI, but not DEX, treatment protected mice from airway inflammation that was linked to decreases in BALF CXCL-1 and IL-17.³⁷ Thus, the ability of ASHMI to regulate innate chemokines such as CXCL-1 might have played a role in the present study. Further studies are needed to elucidate the specific cellular sources of these chemokines in the system and the mechanisms by which these cells are modulated in offspring by maternal ASHMI therapy. Recent reports have suggested that epigenetic alteration of offspring gene activity might regulate asthma susceptibility.³⁸ In ongoing work, the authors have found that ASHMI and DEX treatment affects CpG methylation profiles in promoter regions of IFN- γ and IL-4 (Srivastava et al, unpublished manuscript). Although epigenetic regulation of CXCL-1 has been described,³⁹ whether such mechanisms contribute to maternal ASHMI effects on offspring in this model are being considered.

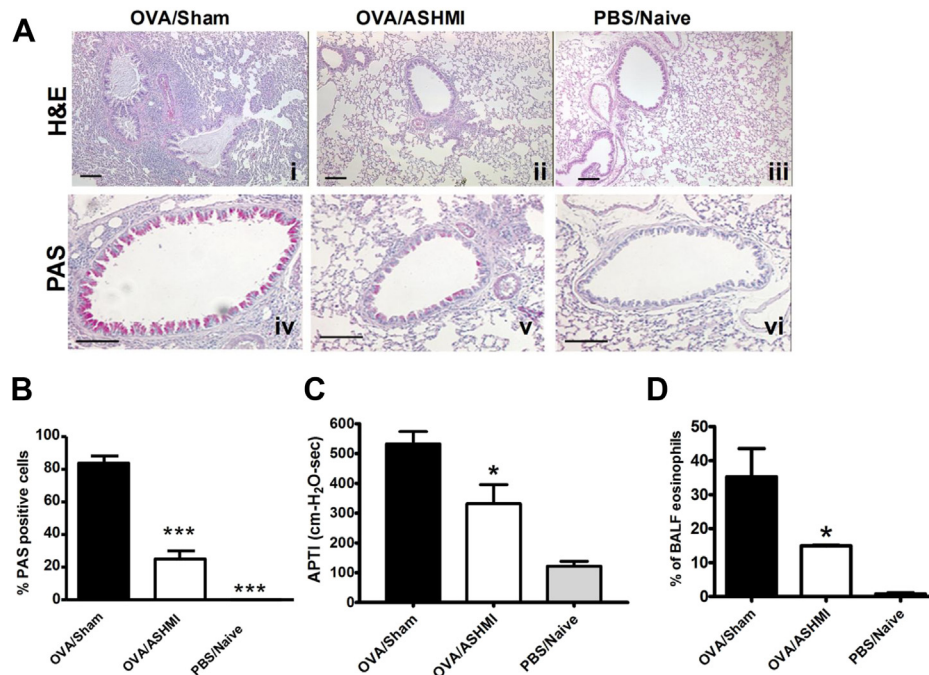
In summary, offspring from mothers with OVA-sensitized asthma develop airway inflammation and mucus hypersecretion to first-ever OVA exposure, which is absent in offspring of naive mothers. This susceptibility to airway disease was greatly decreased when mothers were given preconception ASHMI therapy and to a limited extent with maternal DEX therapy. Thus, asthma therapy with ASHMI in the current generation might be of value as preventive therapy for decreasing asthma risk in the future generation.

Supplementary Data

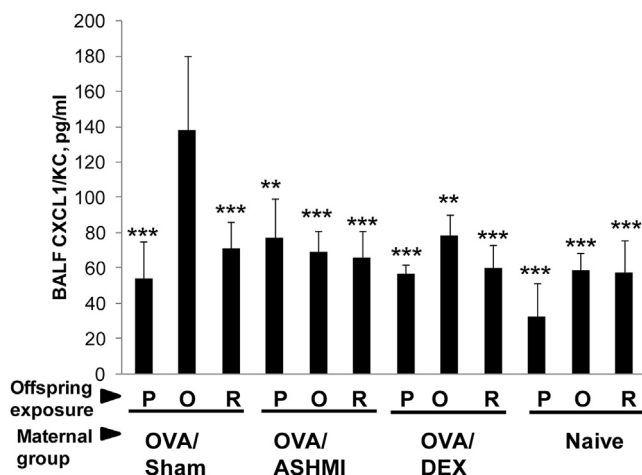
Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2014.10.018>.

References

- [1] Gern JE, Lemanske RF Jr, Busse WW. Early life origins of asthma. *J Clin Invest*. 1999;104:837–843.
- [2] Uthoff H, Spenner A, Reckelkamm W, et al. Critical role of preconceptional immunization for protective and nonpathological specific immunity in murine neonates. *J Immunol*. 2003;171:3485–3492.
- [3] Devereux G, Barker RN, Seaton A. Antenatal determinants of neonatal immune responses to allergens. *Clin Exp Allergy*. 2002;32:43–50.
- [4] Blumer N, Herz U, Wegmann M, Renz H. Prenatal lipopolysaccharide-exposure prevents allergic sensitization and airway inflammation, but not airway responsiveness in a murine model of experimental asthma. *Clin Exp Allergy*. 2005;35:397–402.
- [5] Sears MR, Holdaway MD, Flannery EM, Herbison GP, Silva PA. Parental and neonatal risk factors for atopy, airway hyper-responsiveness, and asthma. *Arch Dis Child*. 1996;75:392–398.
- [6] Litonjua AA, Carey VJ, Burge HA, Weiss ST, Gold DR. Parental history and the risk for childhood asthma. Does mother confer more risk than father? *Am J Respir Crit Care Med*. 1998;158:176–181.
- [7] Latzin P, Frey U, Roiha HL, et al. Prospectively assessed incidence, severity, and determinants of respiratory symptoms in the first year of life. *Pediatr Pulmonol*. 2007;42:41–50.
- [8] Celedon JC, Litonjua AA, Ryan L, et al. Exposure to cat allergen, maternal history of asthma, and wheezing in first 5 years of life. *Lancet*. 2002;360:781–782.
- [9] Kurukulaaratchy RJ, Waterhouse L, Matthews SM, Arshad SH. Are influences during pregnancy associated with wheezing phenotypes during the first decade of life? *Acta Paediatr*. 2005;94:553–558.
- [10] Kurukulaaratchy RJ, Matthews S, Waterhouse L, Arshad SH. Factors influencing symptom expression in children with bronchial hyperresponsiveness at 10 years of age. *J Allergy Clin Immunol*. 2003;112:311–316.
- [11] Hamada K, Suzuki Y, Goldman A, et al. Allergen-independent maternal transmission of asthma susceptibility. *J Immunol*. 2003;170:1683–1689.
- [12] Fedulov AV, Leme AS, Kobzik L. Duration of allergic susceptibility in maternal transmission of asthma risk. *Am J Reprod Immunol*. 2007;58:120–128.
- [13] Royer CM, Rudolph K, Barrett EG. The neonatal susceptibility window for inhalant allergen sensitization in the atopically predisposed canine asthma model. *Immunology*. 2013;138:361–369.
- [14] Expert Panel Report 3 [EPR-3]: guidelines for the diagnosis and management of asthma—summary report 2007. *J Allergy Clin Immunol*. 2007;120:S94–S138.
- [15] Li XM. Traditional Chinese herbal remedies for asthma and food allergy [current perspectives]. *J Allergy Clin Immunol*. 2007;120:25–31.
- [16] Zhang T, Srivastava K, Wen MC, et al. Pharmacology and immunological actions of a herbal medicine ASHMI on allergic asthma. *Phytother Res*. 2010;24:1047–1055.
- [17] Wen MC, Wei CH, Hu ZQ, et al. Efficacy and tolerability of anti-asthma herbal medicine intervention in adult patients with moderate-severe allergic asthma. *J Allergy Clin Immunol*. 2005;116:517–524.
- [18] Kelly-Pieper K, Patil SP, Busse P, et al. Safety and tolerability of an antiasthma herbal formula [ASHMI] in adult subjects with asthma: a randomized, double-blinded, placebo-controlled, dose-escalation phase I study. *J Altern Complement Med*. 2009;15:735–743.
- [19] Kelly-Pieper K, Patil SP, Busse P, et al. Safety and tolerability of an antiasthma herbal formula [ASHMITM] in adult asthmatics: a randomized, double-blinded, placebo-controlled, dose escalation phase I study. *J Altern Complement Med*. 2008.
- [20] Institute of Laboratory Animal Resources Commission of Life Sciences NRC. *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academy Press; 1996.
- [21] Xiu SY. *The Experimental Method of Pharmacology*. Beijing: The People's Public Health Publisher; 1986.
- [22] Busse PJ, Zhang TF, Srivastava K, Schofield B, Li XM. Effect of ageing on pulmonary inflammation, airway hyperresponsiveness and T and B cell responses in antigen-sensitized and -challenged mice. *Clin Exp Allergy*. 2007;37:1392–1403.
- [23] Levitt RC, Mitzner W. Expression of airway hyperreactivity to acetylcholine as a simple autosomal recessive trait in mice. *FASEB J*. 1988;2:2605–2608.
- [24] Li XM, Schofield BH, Wang QF, Kim KH, Huang SK. Induction of pulmonary allergic responses by antigen-specific Th2 cells. *J Immunol*. 1998;160:1378–1384.
- [25] Kumar RK, Herbert C, Thomas PS, et al. Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma. *J Pharmacol Exp Ther*. 2003;307:349–355.
- [26] Martinez FD, Wright AL, Taussig LM, et al. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *N Engl J Med*. 1995;332:133–138.
- [27] Lim RH, Kobzik L. Maternal transmission of asthma risk. *Am J Reprod Immunol*. 2009;61:1–10.
- [28] Roopenian DC, Christianson GJ, Sproule TJ. Human FcRn transgenic mice for pharmacokinetic evaluation of therapeutic antibodies. *Methods Mol Biol*. 2010;602:93–104.
- [29] Tincani A, Nuzzo M, Motta M, et al. Autoimmunity and pregnancy: autoantibodies and pregnancy in rheumatic diseases. *Ann N Y Acad Sci*. 2006;1069:346–352.
- [30] Setiady YY, Samy ET, Tung KS. Maternal autoantibody triggers de novo T cell-mediated neonatal autoimmune disease. *J Immunol*. 2003;170:4656–4664.
- [31] Lopez-Expósito I, Song Y, Jarvinen KM, Srivastava K, Li XM. Maternal peanut exposure during pregnancy and lactation reduces peanut allergy risk in offspring. *J Allergy Clin Immunol*. 2009;124:1039–1046.
- [32] Melkild I, Groeng EC, Leikvold RB, Granum B, Lovik M. Maternal allergen immunization during pregnancy in a mouse model reduces adult allergy-related antibody responses in the offspring. *Clin Exp Allergy*. 2002;32:1370–1376.
- [33] Matson AP, Thrall RS, Rafti E, Lingenheld EG, Puddington L. IgG transmitted from allergic mothers decreases allergic sensitization in breastfed offspring. *Clin Mol Allergy*. 2010;8:9.
- [34] Mosconi E, Rekima A, Seitz-Polski B, et al. Breast milk immune complexes are potent inducers of oral tolerance in neonates and prevent asthma development. *Mucosal Immunol*. 2010;3:461–474.
- [35] Kobayashi Y. The role of chemokines in neutrophil biology. *Front Biosci*. 2008;13:2400–2407.
- [36] Adar T, Shteingart S, Ben Ya'acov A, et al. From airway inflammation to inflammatory bowel disease: eotaxin-1, a key regulator of intestinal inflammation. *Clin Immunol*. 2014;153:199–208.
- [37] Srivastava KD, Dunkin D, Liu C, et al. Effect of antiasthma simplified herbal medicine intervention on neutrophil predominant airway inflammation in a ragweed sensitized murine asthma model. *Ann Allergy Asthma Immunol*. 2014;112:339–347.
- [38] Yang IV, Schwartz DA. Epigenetic mechanisms and the development of asthma. *J Allergy Clin Immunol*. 2012;130:1243–1255.
- [39] Wen X, Wu GD. Evidence for epigenetic mechanisms that silence both basal and immune-stimulated transcription of the IL-8 gene. *J Immunol*. 2001;166:7290–7299.



eFigure 1. Lung histologic analysis, Airway Pressure Time Index (APTI) values, and percentage of eosinophils present in bronchoalveolar lavage fluid in maternal groups. (A) Lungs from the indicated mice were sectioned and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Data show representative images at 10 $\times$ magnification for H&E and 20 $\times$ magnification for PAS. (B) Percentage of PAS-positive cells (number of PAS-positive cells divided by the total epithelial cell number in ≥ 6 bronchioles per animal). (C) APTI after acetylcholine provocation was measured 2 days after the fifth OVA challenge. (D) Percentage of eosinophils present in bronchoalveolar lavage fluid. Values are expressed as mean $\pm$ SEM ($n = 4-5$ /group). * $P < .05$, *** $P < .001$ vs OVA/Sham. ASHMI, Antiasthma Simplified Herbal Medicine Intervention; DEX, dexamethasone; OVA, ovalbumin; PBS, phosphate buffered saline.



eFigure 2. CXCL-1/Keratinocyte Cytokine levels in bronchoalveolar lavage fluid (BALF) of offspring after low-dose challenge. CXCL-1 in bronchoalveolar lavage fluid supernatant from offspring mice that were challenged with phosphate buffered saline (P), 5 μ g of OVA (O), or 5 μ g of ragweed (R) were measured by a commercial enzyme-linked immunosorbent assay. Data are shown as group mean $\pm$ SD ($n = 3-4$ mice/group). ** $P < .01$, *** $P < .001$. ASHMI, Antiasthma Simplified Herbal Medicine Intervention; DEX, dexamethasone; OVA, ovalbumin.