Maternal ASHMI-therapy prevents airway inflammation and modulates pulmonary innate immune responses in young offspring mice

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Introduction

Allergic asthma is a disease with origins in early life\(^1\), with several studies indicating 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 (OR = 5.0, 95% CI = 1.7 to 14.9), and maternal allergic airway diseases such as allergic rhinitis are also associated with asthma development in children.\(^7-10\) Maternal effect on asthma susceptibility in offspring has been a focus of several animal model studies. These studies have largely explored the question of offspring susceptibility to sensitization using protocols that vary in antigen dose or timing but always include a period of sensitization prior to challenge. Using such methods, Hamada et al\(^{11}\) demonstrated that offspring of mice with OVA-induced “chronic asthma” were more susceptible to asthma after a suboptimal protocol consisting of one intraperitoneal injection of allergen in adjuvant at 4 days of life, followed by 3 consecutive challenges with aerosolized allergen on days 12-14 of life. Likewise, Fedulov et al showed that offspring of OVA-asthmatic mice develop a full asthma phenotype even if they are initially sensitized as late as 6 weeks of age. A similar model using canines has also been developed by Royer et al.\(^{12}\) where offspring from ragweed (RW)-sensitized, but not normal, female dogs developed asthma-like features upon post sensitization exposure to RW. Since specific antibodies to maternally-sensitized antigen are transmitted to offspring and can be detected in absence of direct offspring sensitization, we questioned if offspring could mount airway responses to first-ever exposure with the same antigen. In earlier studies we found that offspring of peanut allergic mice anaphylaxed to first-ever exposure to peanut. Whether offspring of asthmatic mothers develop airway disease in response to first-ever exposure to maternally encountered antigen is yet unknown. In additional experiments we extended our studies to include investigation of
preconception maternal asthma therapy on offspring pulmonary responses to antigen exposure without sensitization. Corticosteroids are the cornerstone of allergic asthma treatment 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 high asthma risk offspring. **Anti-asthma Simplified**

Herbal Medicine Intervention (ASHMI) is a Traditional Chinese Medicine herbal asthma formula consisting of three herbs: Ling-Zhi (*Ganoderma lucidum*), Ku-Shen (*Sophora flavescens*), and Gan-Cao (*Glycyrrhiza uralensis*). In previous work we have reported its ability to decrease airway hyperreactivity (AHR) and eosinophilic inflammation by down-regulating the **SPELL OUT Th2** response in murine models. In clinical trials, ASHMI treatment significantly improved lung function and reduced symptom scores of asthma to a similar extent as standard asthma therapy. Although ASHMI treatment in humans and mice has been shown to be efficacious, ability of maternal ASHMI therapy to alter offspring airway disease has not been explored. In this current report we explored airway responses in offspring (F1 generation) to first-ever exposure of 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

ASHMI is composed of 3 herbs, identified as the fruiting body of *Ganoderma lucidum* (Ling-Zhi), the roots of *Sophora flavescens* Ait (Ku-Shen), and the roots and rhizome of *Glycyrrhiza uralensis* Fischer (Gan-Gao), respectively. Voucher specimens of the raw herbs are archived in the botanical chemistry laboratory, Center for Chinese Herbal Medicine for Allergy and Asthma, Mount Sinai School of Medicine, New York. ASHMI was provided by the Sino-Lion Pharmaceutical Company (Weifang, China) as previously described 18. In brief, herbs were boiled together twice in water. The decoctions were combined, concentrated under reduced pressure and dried to powder. The yield of ASHMI extract was 11.5%.

Antigen sensitization/challenge, ASHMI treatment

A chronic asthma model was developed as depicted in Figure 1A. Standard guidelines for the care and use of animals were followed 19. Briefly, female BALB/c mice (6 weeks old) (Jackson Laboratory, Bar Harbor, ME, USA) were sensitized by 2 weekly intraperitoneally (i.p.) injections with 100 μg OVA (TypeV; Sigma-Aldrich, St Louis, MO, USA) and 2 mg of alum in phosphate buffered saline (PBS) and challenged intratracheally (i.t.) with 100 μg OVA in PBS weekly for 3 weeks. Four weeks after the last i.t. challenge, mice were given 2 consecutive i.t. daily challenges. In a group of mice, 4.5 mg of ASHMI in 0.5 mL of water were administered intragastrically (i.g.) twice daily during 6 weeks beginning one day after the initial i.t. 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 20.
Additional OVA-sensitized/challenged mice received 0.5 mL water, i.g. twice daily for 6 weeks as sham treatment controls (OVA/Sham). Naïve mice served as normal controls. Another set of maternal mice were generated as part of separate ongoing experiments utilizing a chronic asthma model. In this experiment, a similar protocol to those described in Fig 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, final set of intratracheal challenges was given on days 78 and 79 (Fig 1B). For both sets of experiments maternal mice were evaluated for airway hyperreactivity (AHR) using invasive methods 2 days following the 5th i.t. challenge in five 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 naïve BALB/c males until signs of pregnancy became evident.

Following mating and delivery, 12 day old offspring from maternal groups shown in Fig 1A received 3 consecutive intranasal (i.n.) exposures with PBS or OVA (5µg) and 12 day old offspring from maternal groups shown in Fig 1B were given OVA, RW (Greer laboratories, NC) (5µg for both) or PBS. Some offspring from protocol 1B were given high dose OVA exposure (50µg). 48 h later, airway inflammation, goblet cell hyperplasia, serum antibodies and splenocyte’s culture /broncoalveolar fluid (BALF) cytokine levels were determined.

**Measurement of late phase Airway Hyper-responsiveness**

Airway hyper-responsiveness (AHR) to acetylcholine provocation was measured from the mothers 2 days following the 5th challenge by measuring the Airway Pressure Time Index.
(APTI) (Kent Scientific Corporation, Torrington, CT, USA), a classical invasive measure of AHR, as previously described \(^{21,22}\).

**Evaluation of BALF cell differential counts, cytokines and lung histology**

Immediately after sacrifice, lungs were lavaged, and BALF and cytospin slides were prepared as previously described \(^{25}\). Cell differential counts were obtained by counting at least 500 cells per slide by light microscopy after staining with HEMA 3 (Fischer Scientific, Pittsburgh, PA, USA). BALF supernatants were used for analysis of cytokines by ELISA (IL-4, IL-5, IL-13, IL-17, IFN-γ, CXCL-1 and Eotaxin-1 (R&D Systems, Minneapolis, MN for CXCL-1; BDBiosciences, San Jose, CA for all others). Lung samples were fixed in neutral buffered formalin, and embedded in paraffin. Five-micron sections were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) for analysis of inflammatory cells and goblet cells. Goblet cell hyperplasia was expressed as the 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 \(^{26}\).

**Measurement of ovalbumin-specific antibodies**

Blood was collected via the descending vena cava from offspring at sacrifice. Sera was isolated and stored at \(-80^\circ\text{C}\) until OVA-specific IgE, IgG1 and IgG2a levels were determined as previously described \(^{23}\).

**Spleen cell culture and cytokine analysis**
After sacrifice, spleen cell culture was conducted as previous described (28). Briefly, splenocytes were cultured in 24-well plates \((4 \times 10^6/\text{well/mL})\) in the presence or absence of OVA \((100 \, \mu\text{g/mL})\) or Concanavalin A \((\text{ConA}, 2.5 \, \mu\text{g/mL})\). Seventy-two hours later, supernatants were collected and IL-4, IL-5, IL-10, IL-13, IL-17, IFN-\(\gamma\), TNF-\(\alpha\) levels were determined by ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN for IL-13 and IL-17; BDBiosciences, San Jose, CA for all others).

**Statistical analysis**

ANOVA was performed followed by a Bonferroni’s t test for all pairwise comparisons if the data were approximately normal. Differences between groups were analyzed by ANOVA on ranks followed by all pairwise comparisons if the data were not normally distributed. P values < 0.05, based on two-tailed tests, is considered statistical significant. All statistical analyses were performed with Sigma Stat 3.5 (Systat Software Inc, Chicago, IL).
RESULTS

Establishment of allergic airway disease in mothers and effects of ASHMI treatment

We verified establishment of asthma-like phenotype in mothers (F0 generation) and confirmed expected therapeutic effects of ASHMI. AHR was measured 2 days following the last OVA challenge as described in (Figure 1A). As expected OVA/Sham mothers had increased peribronchial and perivascular inflammation as compared with Naive mothers (Repository Figure. 1A panels i and iii). Treatment with ASHMI dramatically reduced peribronchial and perivascular inflammation as showed in Repository Figure 1A (panel ii). PAS staining, which is a marker for mucus hyperproduction was significantly higher in OVA/Sham mothers compared to Naive mothers as shown in Repository Figures 1A (panels iv and vi) and 1B (83.33% ± 6.09 vs 0; P<0.001). This increased staining was markedly reduced when mothers were treated with ASHMI (Repository Figures 1A, panel vi and 2B; 25.0% ± 6.16; P<0.001). Classically, having an increased APTI value indicates AHR to acetylcholine provocation. As shown in Repository Figure 1C, APTI values for sensitized/sham-treated mothers (OVA/Sham) were significantly higher than naïve mothers (532.4 ± 41.29 vs 122.2 ± 16.41; P<0.001), indicating induction of AHR. APTI values from sensitized/ASHMI-treated mothers (OVA/ASHMI) were significantly lower than in OVA/Sham mothers (331.9 ± 63.78; P<0.05), showing the anti-inflammatory effects of ASHMI. Consistent with allergic airway inflammation, a significant number of eosinophils were present in BALF infiltrate of OVA/Sham mice which was absent in Naïve mice and markedly reduced in OVA/ASHMI mice (Repository Fig 1D)

Ovalbumin exposure in offspring from OVA-asthmatic mothers induces airway inflammation that is reduced by maternal ASHMI therapy
F1 Offspring from each of the maternal groups in the first set of experiments were i.n. exposed to 5µg 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 we first evaluated total BALF cell number and differential cells counts immediately after sacrifice. Offspring from OVA/Sham mothers showed a significant increase in total cell number in their BALF when compared with offspring from Naïve (Figure 2A; P<0.001). The total cell number increase offspring of OVA/Sham mothers was reflected by a significant increase in macrophages, eosinophils, neutrophils and lymphocytes numbers (Figure 2B-2E) when compared to offspring of naïve mothers(P<0.001 for macrophages and eosinophils; P<0.01 for the rest). Surprisingly, those offspring from OVA/ASHMI mothers had airway inflammatory responses clearly diminished, having a significantly decreased infiltration of total cells, macrophages, eosinophils and lymphocytes when compared with OVA-exposed offspring of OVA/Sham mothers (Figure 2A-2E; NS for neutrophils, P<0.01 for lymphocytes and P<0.001 for all the rest). The cell concentrations seen in offspring of OVA/ASHMI mothers were similar to naive levels for all cell types with exception of neutrophils.

Maternal therapy impacts 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. 3A, panel i, indicated by inset) as compared with offspring of naïve mothers (Fig. 3A, panel iii). As illustrated in Fig. 4A, panel ii, inflammation was dramatically reduced 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 as compared
with offspring of naive mothers, indicating an increase in mucus production (Fig 3 A, panel iv and Figure 3B; 35.10% ± 11.17 vs 0%; P<0.001). In offspring of ASHMI/OVA mothers, mucus production was markedly reduced (Figure. 3A, panel v and Figure 3B; 8.50% ± 1.24; P<0.001)

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 levels were significantly different from PBS- or OVA exposed offspring of naïve mothers. (P<0.001; Figure 4A. Levels of OVA-IgG2a were measurable and found to be significantly increased in offspring of OVA/ASHMI mothers when compared to offspring of OVA/Sham mothers (Fig.4B, 23.533 ± 3.798 vs 7.821 ± 904.6 ng/mL; P<0.001). Calculations were performed to generate the OVA-IgG1/OVA-IgG2a ratio for offspring of all maternal groups as shown in Figure 4C. Maternal ASHMI treatment significantly shifted the IgG1/IgG2a ratio from 180.9 ± 52.11 in offspring of OVA/Sham mothers compared to 39.89 ± 9.00 in offspring of OVA/ASHMI mothers (P<0.01).

Maternal ASHMI therapy beneficially regulates systemic cytokine profile in offspring mice

Immediately after sacrifice, offspring splenocyte cytokine profiles- including Th1 (IFN-γ), Th2 (IL-4, IL-5 and IL-13), Th3 (IL-10) and Th17 (IL-17) were determined in response to OVA or ConA stimulation. As expected, splenocytes from offspring in all groups did not respond to OVA stimulation (data not shown), thus ConA stimulated cytokines were evaluated. Negligible levels
of IL-4, IL-5 and IL-17 were found in the splenocytes cultures from all the 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 when compared with offspring of OVA/Sham mothers (Table 1; P<0.001). Furthermore, offspring of OVA/ASHMI mothers showed significantly lower levels of IL-13 when compared with OVA/Sham offspring (Table 1; P<0.01). Furthermore, the ratios of IL-10 to IL-13 and IFN-γ to IL-13 were both higher in offspring of OVA/ASHMI mothers as compared to the rest of groups, showing a clear shift from Th2 responses to Th1/Th3 (Table 1).

Airway inflammation in offspring mice exposed to low dose OVA is antigen specific and reduced by maternal therapy with ASHMI or Dexamethasone

To determine antigen specificity of offspring airway inflammation, offspring mice generated in a second set of experiments (Figure 1B) were exposed intranasally to low dose (5µg) OVA or RW, or PBS on three consecutive days without prior sensitization. In addition we sought to compare benefits of maternal ASHMI therapy with effects of maternal treatment with dexamethasone (standard asthma therapy). The effectiveness of DEX treatment on the mother’s airway inflammation (data not shown) were consistent with previously published results. In BALF fluid harvested 48 hours after final exposure, we did not observe significant differences in numbers of macrophages (Figure 5A). We observed significantly higher numbers of lymphocytes (Figure 5B), eosinophils (Figure 5C) and neutrophils (Figure 5D) in offspring of OVA/Sham mothers exposed to OVA but not RW or PBS (P<0.05-0.001). Furthermore, significantly decreased numbers of these cells were observed in BALF of offspring of OVA/ASHMI and OVA/DEX mothers to all exposures (P<0.05-0.001 vs OVA-exposed offspring of OVA/Sham
mothers). These data suggest that low dose OVA-challenge of offspring resulted in mild mixed
eosinophilic/neutrophilic inflammation that was observed only in OVA-exposed but not RW-
exposed offspring of Sham mothers implying specificity of offspring responses to the antigen
used for maternal sensitization. Furthermore, we observed protection from low dose OVA
exposure-induced pulmonary inflammation indicated by fewer BALF inflammatory cells in
offspring of both ASHMI and DEX treated mothers. Absence of inflammation in offspring of
naïve mothers in response to OVA or RW indicates that potential endotoxin contamination was
not a factor in observed responses.

High Dose OVA exposure amplifies offspring airway inflammation which is decreased by
maternal therapy with ASHMI but not Dexamethasone

In light of only mild inflammation observed in response to low dose OVA exposure, offspring
were subjected to 3 intranasal high dose (50µg) OVA challenges without prior sensitization. We
found that BALF macrophage (6A) and lymphocyte (6B) counts were not significantly different
across offspring groups although a modest decline in lymphocyte numbers in offspring of
OVA/ASHMI mothers was observed. BALF eosinophil numbers (6C) were markedly elevated in
OVA-challenged offspring of sham mothers when compared to offspring of ASHMI-treated
mothers (P<0.05). Interestingly, different from observations with low dose OVA challenge (Fig
6C), 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<0.05). A similar profile was observed for neutrophil counts where
significantly higher number of BALF neutrophils were observed in high dose OVA-challenged
offspring of Sham and DEX-treated mothers compared to offspring from ASHMI-treated
mothers (P<0.05 for both, Figure 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 lowers levels of pro-inflammatory chemokines and higher levels of OVA-IgG2a**

To determine factors that could explain differential susceptibility to inflammation in offspring of ASHMI and DEX–treated mothers, we evaluated OVA-specific IgG1 and IgG2a in offspring of ASHMI and DEX-treated mothers (Figure 7A,B). While offspring of ASHMI and DEX treated mice had essentially similar decreases in OVA-IgG1 (P<0.05 for both vs offspring of OVA/Sham), Offspring of OVA/ASHMI mice showed increased levels of OVA-IgG2a when compared to offspring of OVA/DEX mice (P<0.001) as well as offspring of OVA /Sham mice (P<0.01). Elevated levels of OVA-IgG2a in this set of experiments were consistent with data shown for the previous set of offspring in Figure 5B. Further, given the mixed nature of neutrophilic/eosinophilic airway inflammatory responses in offspring groups and absence of classical Th2 cytokines (IL-4, IL-5 and IL-13) or Th1 cytokine IFN-γ in BALF samples following OVA exposure in un-sensitized mice, we measured BALF CXCL1 (murine homologue of IL-8) and eotaxin to understand mechanisms underlying recruitment of inflammatory cells in offspring airways. BALF samples from low dose OVA challenge showed significantly increased CXCL1 in BALF of OVA-challenged but not RW or PBS challenged offspring from Sham mothers (Repository figure 2) whereas levels of BALF CXCL1 in all other offspring groups challenged with low dose OVA, RW or PBS were low and indistinguishable across groups. When we examined BALF chemokine levels in offspring mice after high-dose
OVA challenge, we found that CXCL1 levels in offspring 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 towards reduced BALF eotaxin offspring of ASHMI-treated mice when compared with offspring of Sham mothers (P<0.05) and DEX-treated mothers (P=0.06) as shown in Fig-7D. Together these data suggest that reduced pulmonary inflammation to high dose OVA exposure observed in offspring of ASHMI but not DEX-treated or sham mothers is associated with higher levels of OVA-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 airway inflammation disease. This maternal risk has been confirmed by several animal model studies reviewed in by Lim and Kobzik in which offspring were sensitized to allergens prior to challenge. In the current study we demonstrate, for the first time, that offspring from OVA-sensitized asthmatic mothers develop an asthmatic phenotype in response to first-ever exposure to OVA. We observed mixed eosinophilic/neutrophilic infiltration in BALF fluid and mucus hypersecretion in offspring. These responses were specific to maternally encountered antigen (OVA) and absent in offspring of naïve mothers. Our data suggest that maternally transmitted OVA-specific immunoglobulins were instrumental to offspring airway inflammation as serum OVA-specific immunoglobulins were undetectable in offspring of naïve mothers and airway inflammation was absent when offspring of OVA-asthmatic mothers were challenged with RW, an irrelevant antigen in this study. We assumed that transmission of immunoglobulins in our study occurred via breastmilk since the receptor for IgG (FcRn) is not expressed on the placenta of rodents. Offspring disease directly attributable to maternally inherited IgG antibodies has been demonstrated previously in autoimmune disease but not in the context of allergic asthma.

In this study we also evaluated whether maternal therapeutic treatment of allergic asthma, with either the traditional Chinese medicine herbal formula (ASHMI) or DEX could decrease the risk for disease development in offspring. Both 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 that associated with differential susceptibility to airway
inflammation included lower ratio of OVA-specific serum IgG1:IgG2a and decreased BALF chemokine levels in offspring of ASHMI-mothers. We have demonstrated in a murine model of peanut allergy that maternal peanut-specific IgG1 is associated with anaphylactic reactions in the offspring. In addition to that, IgG2a is considered a protective antibody shown to reduce offspring sensitization. Mechanistically, IgG2a could protect the offspring by clearing the antigen from the circulation prior to the recognition by cells of the adaptive immune system due to the formation of antigen-IgG2a complexes. Therefore, we speculate that transmission of OVA-specific IgG2a likely in breast milk may be partly responsible for the reduced inflammatory response observed in offspring of ASHMI-treated mothers similar to previously published studies. More research needs to be done to elucidate the role of IgG2a in protective effects observed.

The pro-inflammatory innate chemokines CXCL1 and eotaxin appeared to be critical for development of pulmonary inflammation in offspring as other candidate cytokines such as IL-4, IL-5 and IL-13 were absent. CXCL1, the murine homologue of IL-8 is a well-established neutrophil attractant and is produced by multiple cell types including epithelial cells, endothelial cells, macrophages and monocytes. The eosinophil chemoattractant eotaxin-1 is also expressed by many pulmonary cells including airway epithelium, fibroblasts, airway smooth muscle cells and macrophages. CXCL1 and eotaxin-1 were markedly decreased in offspring of ASHMI-mothers. In previously published work we 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 CXCL1 and IL-17. Thus ability of ASHMI to regulate innate chemokines such as CXCL1 may be playing a role in our current study. Further studies are needed to elucidate the specific cellular sources of these
chemokines in our system and mechanisms by which these cells are modulated in offspring by maternal ASHMI therapy. Recent reports have suggested that epigenetic alteration of offspring gene activity may regulate asthma susceptibility\textsuperscript{37}. In ongoing work we have found that ASHMI and DEX treatment affects CpG methylation profiles in promoter regions of IFN-\(\gamma\) and IL-4 (Srivastava et al manuscript in preparation). While epigenetic regulation of CXCL1\textsuperscript{38} has been described, whether such mechanisms contribute to maternal ASHMI effects on offspring in this model are being considered.

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


Table 1: Cytokine levels in spleen cell culture supernatants from the offspring with Con A stimulation

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<th>Offspring Group</th>
<th>IL-10 (pg/mL) Mean±SEM</th>
<th>IFN-γ (pg/mL) Mean±SEM</th>
<th>IL-13 (pg/mL) Mean±SEM</th>
<th>IL10/IL13 (ratio)</th>
<th>IFNγ/IL13 (ratio)</th>
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<td>OVA/Sham</td>
<td>5327±281.7</td>
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<td>754.1±205.9</td>
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<td>1723±82.4***</td>
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<td>1153±39.8</td>
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** P<0.01; ***P<0.001 vs OVA/Sham/OVA
Figure Legends

Figure 1. Experimental protocol. (A) 6 week old BALB/c females were sensitized, challenged and treated with ASHMI as described in the methods section. 48 h after the last challenge mice were sacrificed for analysis. Mice that were not sacrificed were mated with aged-matched naïve BALB/c males until signs of pregnancy became evident. 12 day old offspring from every group of mothers received 3 consecutive i.n. OVA challenges. 48 h after the final i.n. challenge, mice were euthanized and airway inflammation, goblet cell hyperplasia, serum antibodies and splenocyte and BALF cytokines were determined. (B) In a separate set of experiments maternal groups were generated using an extended protocol where ASHMI or DEX treatment was started at Day 29. After final OVA challenge mice were mated and 12 day old offspring were exposed to 3 intranasal OVA or RW exposures. Some offspring received PBS. Other offspring were exposed to high dose OVA (50ug).

Figure 2. Offspring inflammatory cells in BALF. Cell differential counts were obtained by counting at least 500 cells per cytospin slide by light microscopy after staining with HEMA 3. (A) Total cells number, (B) macrophages/mL, (C) eosinophils/mL, (D) neutrophils/mL, (E) lymphocytes/mL and. Values are expressed as means±SEMs (n = 6-9/group). *P<0.05, **P<0.01, ***P<0.001 vs. OVA /Sham.

Figure 3. Offspring lung histological analysis and percentage of PAS positive cells. (A) Lungs from the indicated mice sectioned and stained with Hematoxylin-Eoxin (H&E) or Periodic Acid of Schiff (PAS). Data show representative images at 10x magnification for H&E and 20x
magnification for PAS. (B) percentage of PAS positive cells (number of PAS positive cells/total epithelial cell number in at least 6 bronchioles per animal). Values are expressed as means±SEMs (n = 6-9/group). ***P<0.001 vs. OVA /Sham.

Figure 4. Offspring humoral response. (A) Serum OVA-specific IgG1 (ng/mL). (B) Serum OVA-specific IgG2a (μg/mL) were evaluated by ELISA. (C) OVA-specific IgG1/IgG2a ratio. Data are expressed as means ± SEMs of duplicates for each group (n=6-9 mice/group). **P<0.01, ***P<0.001 vs. OVA/Sham.

Figure 5. Offspring airway inflammation in response to low dose OVA exposure
BALF cells were cytospun onto glass slides and differentially stained with Hema-3 stain. Cell types identified on the basis of cell morphology and differential staining were counted and data for A-Macrophages, B-Lymphocytes, C-Eosinophils and D-Neutrophils were calculated for number of cells/ml of BALF fluid. P-PBS exposure; O-OVA exposure; R-ragweed exposure. Data are shown as Means ±SD. N=3-5 mice/challenge condition. *P<0.05

Figure 6 Offspring airway inflammation in response to high dose OVA exposure BALF cells for high dose OVA-challenged offspring were cytospun onto glass slides and differentially stained with Hema-3 stain. Cell types identified on the basis of cell morphology and differential staining were counted and data for A-Macrophages, B-Lymphocytes, C-Eosinophils and D-Neutrophils were calculated for number of cells/ml of BALF fluid. Data are shown as Means ±SD. N= mice/group. *=P<0.05
Figure 7 Effect of maternal therapy on offspring immunoglobulin and chemokine levels

OVA-specific IgG1 (A) and IgG2a (B) in serum were measured by ELISA. CXCL1 (C) and Eotaxin-1 (D) in BALF supernatant were measured by commercial ELISA kit. Data shown as group means±SD. N=4-5 mice/group. *=P<0.05; **=P<0.01
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E Figure 1 legend **Lung histological analysis, APTI values, and percentage of eosinophils present in BALF fluid in maternal groups.** (A) Lungs from the indicated mice sectioned and stained with Hematoxylin-Eosin (H&E) or Periodic Acid of Schiff (PAS). Data show representative images at 10x magnification for H&E and 20x magnification for PAS. (B) Percentage of PAS positive cells (number of PAS positive cells/total epithelial cell number in at least 6 bronchioles per animal). (C) APTI following Ach provocation was measured 2 days after
the 5\textsuperscript{th} OVA challenge. \textbf{(D)} Percentage of eosinophils present in BALF fluid. Values are expressed as means ± SEMs (n = 4-5/group). *P<0.05, ***P<0.001 vs. OVA/Sham.

\textbf{E Figure 2}

\begin{figure}
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\includegraphics[width=\textwidth]{figure2}
\caption*{\textbf{CXCL1 levels in BALF of offspring after low dose challenge.} CXCL1 in BALF supernatant from offspring mice that were challenged with PBS (P), 5\textmu g OVA (O) or 5\textmu g ragweed were measured by commercial ELISA kit. Data shown as group means±SD. N=3-4 mice/group. ***=P<0.001}
\end{figure}