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***In Utero* Exposure to Genistein Enhanced Intranasal House Dust Mite Allergen-Induced Respiratory Sensitization in Young Adult B6C3F1 Mice**

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Abstract

Despite many hypothesized benefits of dietary isoflavone genistein (GEN) deriving from soy-based products, questions surrounding GEN's developmental immunotoxic effects are increasing. To understand how *in utero* GEN exposure may modulate postnatal respiratory sensitization, we conducted a time course study using a common household allergen (house dust mites: HDM; 10 µg/mouse) following intranasal instillation, a physiological route of allergen exposure. GEN was administered to dams by gavage from gestational day 14 to parturition at a physiologically relevant dose (20 mg/kg bw). Female and male offspring were sensitized with HDM allergens beginning about one month prior to sacrifice followed by challenges with three weekly doses of HDM extracts, and they were euthanized at day 3 following the final HDM exposure at four different time points (postnatal day (PND) 80, 120, 160, and 200). *In utero* GEN combined with postnatal HDM exposures (GEN+HDM) increased total IgE production in both young female and male B6C3F1 offspring (e.g., PND 80 in females and PND 120 in males). Increased antigen-specific IgG₁, IgG_{2a} and IgG_{2b} levels were also observed at various time points in both female and male offspring. In addition, increases in macrophage number in bronchoalveolar lavage fluid of both female and male GEN+HDM offspring at PND 80 and PND 120, respectively, were observed when compared to the vehicle group. For T cells, an increase over the vehicle in female GEN+HDM offspring was observed at PND 80. Due to similar patterns of increases, it seems likely that GEN+HDM-induced increases in total IgE and macrophages are related. Overall, *in utero* GEN plus later-life HDM exposures exert increases in total IgE and HDM-specific IgG production as well as macrophage recruitments to the lung in young adult mice.

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Keywords

Genistein; house dust mites; asthma; *in utero* exposure; IgE

Introduction

Genistein (GEN), a major isoflavone in most soy products, can interact with estrogen receptors (Martin et al., 1978). Despite the hypothesized beneficial effects of GEN (e.g., decreased incidences of some hormone-related cancers), there are concerns about the potential long-term effects of this compound on human health, especially that of infants and young children. Infants fed soy milk formulas have plasma isoflavone levels that are orders of magnitude higher than those of infants fed human or cow's milk (Setchell et al., 1997; Patisaul and Jefferson, 2010; Katchy et al., 2014). The possible long-term effects of these relatively high levels of phytoestrogens during infancy are unknown. A retrospective multiple controlled cohort study has indicated that there was an increase in the use of asthma or allergy drugs in young adults who had been fed soy formula during infancy as compared to those who were fed cow milk formula from the age of less than 9 days old (Strom et al., 2001). Additionally, phytoestrogens have been detected in amniotic fluid (Doerge et al., 2001; Jefferson et al., 2012), suggesting that *in utero* exposure also occurs.

The prevalence of asthma has doubled in the past decades and continues to rise (Robinson et al., 2004; Greenwood, 2011). High titers of IgE antibody to common house allergens such as house dust mite (HDM) significantly increased the risk for acute wheezing provoked by infection (e.g., rhinovirus) among asthmatic children (Soto-Quiros et al., 2012). The total serum IgE levels in asthmatics are approximately four times higher than those in nonasthmatic individuals (Siroux et al., 2004; Tanaka et al., 2014). In our previous studies using trimellitic anhydride (TMA) as a respiratory sensitizer, we have demonstrated that *in utero* exposure to GEN at a physiologically relevant concentration (20 mg/kg) increased IgE production at postnatal day (PND) 84 in B6C3F1 mice (Guo et al., 2005). Sensitization to airborne allergens is a powerful risk factor for severe asthma in adults (Zureik et al., 2002). To further understand how *in utero* GEN exposure modulates respiratory sensitization, we conducted a time course study with four time points (PND 80, 120, 160, and 200) using a physiologically relevant route of allergen exposure (intranasal) and a common household allergen HDM.

Abnormal activation of macrophages and T cells has been reported to play a key role in allergic inflammation and in asthma (Madore et al., 2010; Kim et al., 2012; Balhara and Gounni, 2012; Soroosh et al., 2013). It was hypothesized that exposure to GEN during a sensitive period (e.g., *in utero* exposure) would enhance allergic sensitization in adults to have an exaggerated response to the respiratory allergen HDM (e.g., an increase in the IgE response and aberrant activation of T cells and macrophages). In this study, besides other sensitization endpoints, we have evaluated the effects of *in utero* GEN exposure through dosing dams from gestation day 14 (GD14) to parturition on total serum IgE production in response to HDM stimulation in B6C3F1 offspring. The period of GD14 until birth is the period of colonization and establishment of the bone marrow and thymus in mice (Landreth,

2002). The B6C3F1 mouse, a hybrid of male C3H/HeN and female C57BL/6J mice, was selected over randomly bred mice to decrease the variation between individual responses and reduce the number of animals for each experiment, and yet have the vigor associated with the heterozygosity. This model has been widely used for studies of estrogenic effects (Papaconstantinou et al., 2003; Ng et al., 2006; Frawley et al., 2011). Furthermore, our studies on several strains of mice including B6C3F1, C57BL/6, BDF1 and BALB/c have suggested that the B6C3F1 mice was the best responder (e.g., the highest production of IgE and IL-4) following respiratory allergen exposure, and has the potential to detect respiratory sensitization by various treatments (Guo et al., 2002).

Materials and Methods

Animals and animal exposure

Both female C57BL/6 and male C3H mice (8–12 weeks old) were obtained from Charles River Breeding Laboratories (Portage, MI). Timed pregnant primiparous C57BL/6 mice were generated through housing two female C57BL/6 mice and one male C3H mouse in one cage (plug date = gestational day 0). Pregnant mice were housed individually in polycarbonate cages (Lab Products, Inc., Seaford, DE) with hardwood chip bedding, and the animal room was maintained at 21–24°C and the relative humidity between 40 and 70%. The mice consumed Harlan Teklad Laboratory Diets (NIH 07; Madison, WI) and tap water from water bottles *ad libitum*. A recent study reported that a negligible amount of bisphenol A leaches from new or used polycarbonate cages maintained at room temperature (Thigpen et al., 2013). Brown and Setchell (2001) reported the dietary concentration of isoflavones in the NIH-07 diet to be approximately 33 ppm. In our previous studies (Guo et al., 2005), two different diets, phytoestrogen free 5K96 diet and NIH 07 rodent diet, were compared, and it was found that *in utero* GEN exposure by gavage increased serum total IgE in both cases with more enhancement observed in NIH 07 diet-fed female mice. To compare the effects of different allergens (TMA vs. HDM), the NIH 07 rodent diet was used in this study.

GEN solutions were prepared fresh daily in 25 mM Na₂CO₃ at a concentration of 2 mg/ml (Guo et al., 2005). Mice were administered the GEN solution or the vehicle by gavage (0.1 ml/10 g body) via an 18 G gavage needle from GD 14 to parturition. The B6C3F1 offspring were weaned at PND 22, and at this time the offspring were housed up to four same-sex littermates per cage. All animal procedures were conducted under an animal protocol approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Dosing of house dust mite allergen

At each time point, each mouse in a group was randomly collected from different litters to control for bias due to litter effect, and there were no significant differences in the initial body weights between the groups that were assigned to different time points. For each experiment time point, the samples were collected in one day. Mice were anesthetized with an intraperitoneal (ip) injection of ketamine (100 mg/kg)/xylazine (10 mg/kg), followed by intranasal instillation of various amounts (5–40 µg/mouse) of HDM extract (*Dermatophagoides farinae*, Bi-Level extraction at 1:10 and 1:5 w/v in 0.125 M ammonium

bicarbonate followed by dialysis against distilled water; Greer Laboratories, Lenoir, NC) in 50 μ l (25 μ l/nostril) of physiological Hank's balanced salt solution (HBSS). About one month prior to sacrifice, mice were sensitized with one dose HDM allergens followed by challenges with three doses of HDM extracts (e.g., four doses of HDM extract were given once a week except for a two-week interval between doses 1 and 2; **Figure 1**). The rationale for the exposure protocol was based on the immunology paradigm that the primary immune response (e.g., the allergy sensitization phase) was approximately 7–14 days following the initial exposure (doses 1 to 2). The second exposure was 14 days after the first and thus was on the boundary between sensitization and challenge. The last two exposures were challenge exposures. This dosing regimen has been used extensively for various allergens including HDM (Ward et al., 2010).

Mice were sacrificed and sera were collected at day 3 following the final HDM exposure at four different time points: PND 80, 120, 160, and 200. The naïve animals were treated with 50 μ l of HBSS. These HBSS-only mice are herein referred to as “naïve group” to differentiate them from the group of mice that received 25 mM Na₂CO₃ *in utero*, the vehicle (VH) for GEN. In our previous studies (Guo et al., 2002b; Guo et al., 2005; Guo et al., 2005b; Guo et al., 2006), developmental GEN exposure has been shown to enhance the immune response (e.g., increases in T cells and macrophages) in rodents in the absence of allergic sensitization.

Enzyme linked immunosorbent assay (ELISA) for IgE

ELISA for total serum IgE was performed according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). Briefly, 100 μ l of diluted capture antibody were added to each well in a 96-well plate (NUNC MaxiSorp flat-bottom), and allowed to adhere overnight at 4°C. Plates were washed, then blocked with 10% fetal bovine serum–phosphate buffered saline (FBS-PBS) for 1 h at room temperature. After washing, serial dilutions of the standard and samples (from 1:16 to 1:128) were prepared in the plates, and then allowed to adhere for 2 h at room temperature. After washing, 100 μ l of working solution including detector antibody and avidin-horseradish peroxidase (HRP) reagent were added to each well, then incubated for 1 h at room temperature. The detector antibody was biotinylated anti-mouse IgE monoclonal antibody. After washing, 100 μ l of tetramethylbenzidine (TMB) substrate solution were added to each well. After the incubation in the dark for 30 min at room temperature the absorbance was read at 450 nm within 30 min of adding stop solution (2N H₂SO₄).

HDM-specific IgG₁, IgG_{2a} and IgG_{2b} levels in serum

For the measurement of HDM-specific IgG₁, IgG_{2a} and IgG_{2b} levels, HDM extract (50 μ g/ml) diluted with coating buffer was plated on 96 well (NUNC) plates and incubated overnight at 4°C. On day 2, HDM extract coated plate was washed, then blocked with 10% FBS-PBS for 1 h at room temperature. After additional washings, the serially diluted sera were transferred to the HDM coated plates and incubated for 2 h at room temperature. After washing, 100 μ l of working solution including detector antibodies (biotinylated anti-mouse IgG₁, IgG_{2a} or IgG_{2b} monoclonal Abs) and avidin-HRP reagent were added to each well, then incubated for 1 h at room temperature. After washing, 100 μ l of TMB substrate solution

were added to each well. After the incubation in the dark for 30 min at room temperature the absorbance was read at 450 nm within 30 min of adding stop solution (2N H₂SO₄) as described previously for IgE ELISA.

Analysis of cells in the bronchoalveolar lavage fluid (BALF)

Mice were anesthetized with CO₂, and bronchoalveolar lavage performed. Although there have been reports on differential effects of isoflurane and CO₂ inhalation on plasma levels of inflammatory markers (Lawrance et al., 2009), all mice in the present study were anesthetized the same way and were not expected to differentially affect the treatment groups. The lungs were lavaged with 1.0 ml of physiological saline via the tracheal cannula while gently massaging the thorax. After centrifugation, the supernatants were saved and stored in a -80°C freezer for the determination of cytokines and eosinophil peroxidase activity. To ensure full recovery of lung leukocytes, an additional wash using 1.0 ml of physiological saline was performed, and the numbers of differential leukocytes in the BALF were determined via flow cytometry in conjunction with cell counting using a Coulter Counter ZII, with the red blood cells lysed with a ZAP-OGLOBIN II lytic reagent (Coulter Corporation, Miami, Florida). The respective cell types were labeled with an appropriate monoclonal antibody (mAb), conjugated with a fluorescent molecule for visualization. All the antibodies (PerCP-conjugated anti-mouse CD3e mAb, FITC conjugated mAb for Gr-1 and PE conjugated anti-Mac-3) were obtained from BD PharMingen. Isotype-matched irrelevant antibodies were used as controls. Following the addition of the reagents, the cells were incubated at 4°C in the dark for at least 30 min. After incubation, the cells were washed 2x, and enumeration was performed on a Becton Dickinson FACScan Flow Cytometer. For each sample, 5,000 events were counted.

Eosinophil peroxidase (EPO) assay (Lintomen et al., 2008)

The EPO assay is a colorimetric assay used for detecting eosinophils by measuring the eosinophil peroxidase activity (Strath et al., 1985; Davoine et al., 2013). To measure eosinophil peroxidase activity in the undiluted BALF (100 µl), Tris-HCl buffer (50 µl) was added to 96-well plates initially. The samples were serially diluted from 1:2 to 1:4096 in the plates. The diluted samples were incubated for 30 min with 150 µl of substrate solution containing 1.5 mM o-phenylenediamine (2 mg/ml, Sigma, St Louis, MO) and 6.6 mM H₂O₂ (1.3 µl/ml) in 0.05 M Tris-HCl. The reaction was stopped by adding 2N H₂SO₄, and the optical density measured at 490 nm on an ELISA plate reader (Molecular Devices Thermomax Plate Reader, Rockville, MD). For spleen and whole lung EPO determination, mouse spleen and lung were homogenized and then sonicated (30-45 seconds per sample) until the cells were disrupted. The organ homogenates were spun down and the resulting supernatants were serially diluted and incubated with the substrate solution for 30 min. The reaction was stopped and the optical density measured as described.

Levels of cytokines in the BALF

The cell-free supernatant was recovered by centrifuging BALF samples at 150 × g at 4 °C for 10 min. The concentrations of IL-2 and IL-4 in the supernatant were determined using ELISA as described according to the manufacturer's instruction (BD Pharmingen, San Diego, CA).

Statistical Analysis

Results are presented as mean $\pm$ SEM. To determine the type of analysis to be used, the Bartlett's Test for homogeneity was conducted. The software used was JMP Pro 10. All data were homogenous, and the data were analyzed using a one-way analysis of variance. The Dunnett's t test was used to determine differences between the control and experimental groups. A group was considered statistically significant from the control group if $p < 0.05$.

Results

HDM dose response and age-related change of serum IgE levels in naïve mice

A HDM dose response study was conducted initially to determine the amount of HDM that would stimulate the highest IgE levels. Female B6C3F1 mice (12 wks old; approximately PND 84) were given various doses (5-40 μ g/mouse) of HDM at intervals as described in our experimental design (**Figure 1**). As shown in **Figure 2A**, a bell-shaped dose response was produced. When the antigen-specific IgG₁ was measured, a similar pattern was observed (**Figure 2B**). The bell-shaped responses were likely due to tolerance induction at high doses (Haugaard et al., 1993). Thus, the dose of 10 μ g/mouse of HDM was considered the optimal dose for further studies. Additionally, there were less variation for IgE levels at the 10 μ g/mouse group when compared to the 5 μ g/mouse group, and it permitted detection of IgE and IgG changes (either increases or decreases) following GEN treatment.

To determine how age affected serum IgE levels, a time course study at four different postnatal time points (PND 80, 120, 160, 200) was conducted in naïve B6C3F1 mice without HDM dosing. As shown in **Figure 2C**, there was an age-related change in IgE levels for both female and male mice. For the naïve female mice, the IgE level peaked at around PND 160 and reached a plateau. For the naïve male mice, the IgE levels were higher at PND 200.

Increased total IgE levels in both adult female and male offspring following in utero GEN exposure—To determine if *in utero* GEN exposure affected respiratory sensitization, a time course study (PND 80, 120, 160, 200) following treatment with HDM (10 μ g/mouse) allergens was conducted in both female and male offspring. In female vehicle offspring, HDM treatment induced a bell-shaped response with age, which peaked at PND 120 and came down at PND 200 (**Figure 3A**). *In utero* GEN exposure induced significant increases in serum total IgE levels in young (e.g., at PND 80) and old female adult offspring (e.g., at PND 200) over the vehicle control when the total serum IgE levels were low (**Figure 3A**). When compared to naïve group, significantly increased serum total IgE levels were observed at PND 80 and 120 in female vehicle offspring, and at PND 80, 120 and 200 in female GEN-exposed offspring.

In male offspring, HDM treatment significantly increased serum total IgE levels in vehicle control group at PND 80, 120 and 160 when compared to naïve group, which peaked at PND 160 and came down at PND 200 (**Figure 3B**). *In utero* GEN exposure induced significant increases in serum total IgE levels over the vehicle control at PND 120, a time point prior to the peak vehicle response. When compared to naïve group, significantly increased serum total IgE levels were observed at PND 80, 120, and 160 in male GEN-exposed offspring.

Taken together, *in utero* GEN exposure increased total IgE levels in both adult female and male offspring depending on age.

Antigen-specific IgG₁, IgG_{2a} and IgG_{2b} levels in both adult female and male offspring following *in utero* GEN exposure—To determine if *in utero* GEN exposure affected the production of IgG antibodies in the offspring, the antigen (HDM)-specific IgG₁, IgG_{2a} and IgG_{2b} levels in the sera were measured. In female offspring, *in utero* GEN exposure induced significant increases in antigen-specific IgG₁ levels at PND 120 and 160 (**Figure 4A**), in antigen-specific IgG_{2a} levels at PND 120 and 160 (**Figure 5A**) and in antigen-specific IgG_{2b} levels at PND 80 (**Figure 6A**) when compared to vehicle group. In male offspring, *in utero* GEN exposure induced significant increases in antigen-specific IgG₁ levels at PND 120 and 160 (**Figure 4B**), in antigen-specific IgG_{2a} levels at PND 160 (**Figure 5B**) and in antigen-specific IgG_{2b} levels at PND 160 and 200 (**Figure 6B**) when compared to vehicle group.

Numbers of BALF macrophages, T cells and neutrophils in both adult female and male offspring following *in utero* GEN exposure—When the BALF macrophages in the female offspring were examined, significant increases of macrophage numbers in GEN treated female offspring over the vehicle and naïve mice were observed at PND 80 and 160 (**Figure 7A**). At PND 120, HDM treatment significantly increased the number of macrophages in BALF when compared to naïve females, and GEN treatment attenuated this increase (**Figure 7A**). At PND 200, there were no significant differences among these three test groups. In male offspring, there was a significant increase of BALF macrophages in GEN-treated males over both the naïve and vehicle groups at PND 120. At PND 80 and 160, there were increases of macrophages in both the vehicle and GEN treated offspring when compared to naïve mice; however, there were no significant differences between the vehicle and GEN-treated mice at these three time points (**Figure 7B**). At PND 200, there were no significant differences among these three test groups.

In female offspring, HDM treatment elicited a significant increase in T cell numbers over the naïve mice in all the four time points (PND 80, 120, 160 and 200) tested (**Figure 8A**). When compared to the vehicle mice, *in utero* GEN exposure increased the T cell number at PND 80, but decreased T cell number at PND 120. Similar to females, HDM treatment groups showed increases in T cells for the four time points tested in male offspring when compared to the naïve mice, but there were no significant differences between the vehicle and GEN treatment groups (**Figure 8B**).

The number of neutrophils (Gr1⁺) in the BALF were also measured in both males and females at all the four time points, and there were significant increases of Gr-1⁺ neutrophils after HDM treatment when compared to naïve mice; however, no significant differences in the number of neutrophils between vehicle and GEN-treated offspring in all tested time points (data not shown).

Cytokines in BALF and lung eosinophil peroxidase activity in adult female and male offspring following *in utero* GEN exposure—The levels of cytokines IL-2 and IL-4 in BALF at time point 1 (PND 80) were measured using ELISA, and no significant

differences between vehicle and GEN treated groups were found in either female or male offspring (data not shown). EPO assays on homogenized spleens were also tested at time points 1 (PND 80) and 2 (PND 120) when enhanced IgE production was observed, and there were no significant differences between the GEN and vehicle groups in either male or female offspring (data not shown). In addition, when the EPO activity was measured in the BALF from offspring sacrificed at PND 80 (Time point 1), there were again no significant differences between the GEN and the vehicle treated groups in either males or females (data not shown).

Effect of *in utero* GEN exposure on the body weight and spleen weight in adult female and male offspring

—In general, there was an age-related increase in body weight in both female and male offspring (**Table 1**). *In utero* GEN treatment decreased the body weight at PND 200 in the female offspring when compared to the VH group (**Table 1**). For absolute spleen weight, there were no significant differences among the three groups in the female offspring. When the spleen weights were expressed as relative values (%body weight), there was a decreasing trend with age in female offspring. HDM treatment significantly increased %body weight at PND 120 in the females, and this enhancing effect was attenuated by *in utero* GEN exposure (**Table 1**). At PND 200, GEN treatment increased relative weights of spleen when compared to the NA group.

Treatment with HDM decreased terminal body weight in male offspring at PND 120 when compared to naïve mice that received HBSS during intranasal dosing (**Table 1**). *In utero* GEN treatment did not have a significant effect on body weight in the male offspring when compared with vehicle group. The spleen weight in male offspring did not show much differences among the groups except for PND 160 when HDM treatment increased the weight of spleen in male offspring as demonstrated by a significant change observed in vehicle group when compared to the naïve group, and this enhancing effect was attenuated by *in utero* GEN exposure (**Table 1**). In male offspring, HDM treatment significantly increased relative spleen weight at three time points (PND 120, 160, and 200), and this enhancing effect showed trend toward attenuation by *in utero* GEN exposure at PND 160 (**Table 1**), although it did not reach the levels of statistical significance.

Discussion

Several reports suggest that exposure to high levels of soy and isoflavones (e.g., GEN) during sensitive windows of immune system development may increase likelihood of allergic sensitization. A retrospective study has indicated that there was an increase in the use of asthma or allergy drugs in young adults who had been fed soy formula during infancy as compared to those who were fed cow milk formula (Strom et al., 2001). Our previous studies have also shown that *in utero* exposure to GEN increased IgE production in young adult B6C3F1 mice (PND 84) but not at PND 42, a mid-adolescent stage, following treatment with a respiratory sensitizer TMA (Guo et al., 2005). Appropriate animal studies that may be indicative of the potential of a substance (e.g., HDM) to cause sensitization by inhalation in humans may include measurements of IgE and other specific immunological parameters (Basketter and Kimber, 2011). In this study, a house dust mite model was employed to determine if *in utero* exposure to GEN increased IgE production and

exacerbated other immunological parameters. The measurement of total serum IgE levels is recognized to be one of the best methodologies for identifying respiratory sensitizers (Cookson, 1999; Lanier et al., 2003; Tanaka et al., 2014). Belessis et al. (2004) have reported that one of the risk factors for an intensive care unit admission of acute severe asthma is a 1.6-fold increase in serum total IgE levels. Based on these reports, we followed serum total IgE levels as a prime indicator of respiratory sensitization in our study.

It was noteworthy that *in utero* GEN combined with postnatal HDM exposures increased total IgE production in female offspring at PND 80 and male offspring at PND 120, suggesting that *in utero* GEN exposure might exacerbate HDM-induced respiratory sensitization in young adults. This is consistent with the reports that there was an increase in the use of asthma or allergy drugs in young adults who had been fed soy formula during infancy (Strom et al., 2001) and our previous study with TMA that *in utero* exposure to GEN increased IgE production at PND 84 but not at PND 42 (Guo et al., 2005). Although the exact mechanisms underlying a fetal basis (e.g., GEN *in utero* exposure) of the adult disease (e.g., respiratory sensitization) are currently unclear, the age-related epigenetic changes (e.g., methylation increases of estrogen receptor α at an average of 1% every 3 years) of the immune system (Issa, 2003; Liang et al., 2015) and differential sex maturation (e.g., earlier puberty in females) in female and male offspring might partially be responsible for our observations that female offspring following *in utero* GEN + postnatal HDM exposures showed a different pattern of increases (PND 80) in total IgE levels from similarly treated male offspring (PND 120). To this end, prenatal exposure to phytoestrogen GEN has been shown to trigger epigenetic changes in immune function genes (Vanhees et al., 2011).

In our studies, we found that there were age-related changes in serum total IgE levels in the absence of HDM treatment. For the female offspring, the level of serum total IgE peaked at PND 160, and for male offspring it was highest at PND 200. This increase in serum IgE levels with age in the naïve mice might be responsible for a lack of significant differences in the serum IgE levels between mice treated with HBSS (NA) and the mice treated with HDM (VH group) at several time points (PND 160 and 200 for females, and PND 200 for males). It is currently unclear why increased levels of serum IgE were not observed at all the time points. As discussed earlier, it is possible that GEN-mediated increases in IgE production are age-related. Another explanation might be related to the fact that both IgE and IgG contribute to respiratory sensitization, and they can be independently regulated (Finkelman et al., 1991; Hoeger et al., 1994; Williams et al., 2012). We observed increases in HDM-specific IgG₁ and IgG_{2a} at PND 120 and 160 in both female and male offspring.

In female offspring, it was unclear why the total serum IgE from the GEN group at PND 160 was lower than that of PND 120 and PND 200. However, we believe this was not an artifact from a bad experimental design. Although no actual randomization techniques were applied, there were no significant differences in the initial body weights between the groups that were assigned to different time points. Secondly, the fact that serum IgE levels in HDM-treated mice were similar to NAF group was not due to a possible HDM dosing error because HDM-specific IgG₁ and IgG_{2a} were clearly detected at PND 160. In addition, no animals were excluded during analysis. At PND 200, *in utero* GEN combined with postnatal HDM exposures significantly increased total IgE production in female offspring, and a

numerical increase of HDM-specific IgG_{2b} was also observed. Interestingly, *in utero* GEN combined with postnatal HDM exposures significantly increased HDM-specific IgG_{2b} but not total IgE in male offspring at PND 160 and 200. Further examination of *in utero* GEN on respiratory sensitization in older adults is warranted.

Sunyer et al. (1996) reported that asthma-associated serum total IgE is independent of specific IgE levels. Therefore, an additional reason for lacking of effects on total serum IgE at certain time points could be related to antigen-specific IgE. Unfortunately, there was no commercially available HDM-specific ELISA kit for mouse, and we found that the IgG depletion technique to increase the sensitivity for HDM-specific IgE to bind to HDM coated plates was not reliable. In future studies, it would be useful to employ a rat basophilic leukemia cell β -hexosaminidase release assay as an indirect measure of antigen-specific IgE in serum (Ward et al., 2010).

In the analysis of T cells, at every time point tested (PND 80, 120, 160 and 200), HDM treatment resulted in a significant increase of T cells in both female and male offspring. These increases are consistent with the pivotal roles of T cells in acquired immunity, which recognize allergen associated with MHC II on antigen-presenting cells, and respond to them. Part of this response is to facilitate the recruitment and activation of macrophages (Nishimura et al., 2009). HDM treatment significantly increased the number of macrophages in the BALF over the VH controls at PND 80 and 160 in female offspring and at PND120 in male offspring. Importantly, GEN-mediated increase in serum IgE were also observed in female offspring at PND 80 and in male offspring at PND 120 when compared to the vehicle mice. Thus, the number of macrophages in the BALF could be related to the levels of serum total IgE (Wu and Zarrin, 2014).

The dose of 20 mg/kg body weight is physiologically relevant. For a 4-month-old infant who consumes soy formula as directed by the manufacturers, approximately 6-11 mg/kg body weight of isoflavones can be obtained (Setchell et al., 1997; Katchy et al., 2014). The serum level of GEN (1.4-7.5 μ M) in mice that have been fed 1000 ppm GEN-containing diet (~80 mg/kg body weight, which is higher than our 20 mg/kg dose) was equivalent to that in men who received 100 mg GEN/day (Djuric et al., 2001; Yellayi et al., 2002; Bhandari et al., 2003), and in infants on soy formula (Cao et al., 2009).

In summary, this is the first study to investigate the modulation of HDM-induced respiratory sensitization by GEN following *in utero* exposure. The magnitude of IgE enhancement after GEN exposure in our study is potentially significant as one of the risk factors for an intensive care unit admission of acute severe asthma is a 1.6-fold increase in serum total IgE levels (Belessis et al., 2004). Although multiple mechanisms seemed to be involved, modulation of macrophage by *in utero* GEN exposure may contribute to the exacerbating potentials of this compound on respiratory sensitization. Estrogen has been reported to promote macrophage activation and proinflammatory mediator production (Calippe et al., 2010; Campbell et al., 2010). Various ERs including ER α , ER β , non-classical ER and G-protein-coupled receptor 30 have been identified (Molero et al., 2002; Filardo et al., 2007; Holladay et al., 2010), and GEN interacts with them with different affinity. Additionally, GEN can affect the activities of various enzymes (Wiegand et al., 2009). Further study of the

molecular mechanisms underlying the modulatory effect of GEN on asthma are needed to understand contributions of estrogenic and enzyme inhibitory activities (e.g., tyrosine kinase inhibition) to enhanced IgE production, and the safety issues associated with ingestion of this compound.

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Abbreviations used

BALF	bronchoalveolar lavage fluid
BPA	bisphenol A
ELISA	enzyme linked immunosorbent assay
EPO	eosinophil peroxidase
ER	estrogen receptor
FBS	fetal bovine serum
GD	gestation day
GEN	genistein
HBSS	Hank's balanced salt solution
HDM	house dust mites
HRP	horseradish peroxidase
PBS	phosphate buffered saline
PND	postnatal day
TMA	trimellitic anhydride
TMB	tetramethylbenzidine

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Highlights

1. *In utero* genistein exposure increased IgE production in young adult offspring
2. *In utero* genistein exposure increased antigen-specific IgG subclasses
3. *In utero* genistein exposure increased lung macrophage number

Figure 1.
Experimental design for animal treatments; IN = intranasal dosing

Figure 2.

HDM dose response curve for serum total IgE levels (A) and HDM-specific IgG₁ (B). For HDM dose response study, female B6C3F1 mice (12 wks; N = 5) were given 4 doses of HDM ranging from 5 μg/mouse to 40 μg/mouse at intervals as described and were sacrificed 3 days after the last dose. Serum was collected and ELISA performed to obtain serum total IgE level. *, p < 0.05 when compared to VH. (C) Serum total IgE levels in naïve (NA) mice with time. Animals that only received HBSS (50 μl per mouse) during intranasal dosing were sacrificed at four different time points (PND 80, 120, 160, 200) and their serum total IgE levels measured as described. The number of mice in each group at each time point is shown in Table 1. *, p < 0.05 when compared to PND 80 mice.

Figure 3.

Effect of *in utero* GEN exposure on serum total IgE levels in female (A) and male (B) B6C3F1 offspring. Pregnant mice were treated with genistein (GEN) or vehicle (VH) from GD14 to birth by gavage. Female or male offspring were dosed with HDM at intervals as described and sacrificed 3 days after the last intranasal HDM dosing at four different time points (PND 80, 120, 160, 200). The serum total IgE levels were measured using ELISA as described. NAF = untreated, non-sensitized naïve females, NAM = untreated, non-sensitized naïve males, VHF = VH females dosed with HDM, VHM = VH males dosed with HDM, GEF = GEN females dosed with HDM, GEM = GEN males dosed with HDM. *a*, significantly different from naïve mice; *b*, significantly different from VH mice. The number of mice in each group at each time point is shown in Table 1.

Figure 4. Effect of *in utero* GEN exposure on antigen-specific IgG₁ levels in female (A) and male (B) B6C3F1 offspring. Pregnant mice were treated with genistein (GEN) or vehicle (VH) from GD14 to birth by gavage. Female or male offspring were dosed with HDM at intervals as described and sacrificed 3 days after the last intranasal HDM dosing. HDM-specific IgG₁ levels in the sera at dilutions of 1:5,000 – 1:32,000 were measured as described. VHF = vehicle females dosed with HDM, VHM = vehicle males dosed with HDM, GEF = GEN females dosed with HDM, GEM = GEN males dosed with HDM. HDM-specific IgG₁ levels in untreated, non-sensitized naïve animals were undetectable, and therefore no data could be shown. *, significantly different from VH. The number of mice in each group at each time point is shown in Table 1.

Figure 5. Effect of *in utero* GEN exposure on antigen-specific IgG_{2a} levels in female (A) and male (B) B6C3F1 offspring. Pregnant mice were treated with genistein (GEN) or vehicle (VH) from GD14 to birth by gavage. Female or male offspring were dosed with HDM at intervals as described and sacrificed 3 days after the last intranasal HDM dosing. HDM-specific IgG_{2a} levels in the sera at dilutions of 1:100 - 1:800 were measured as described. VHF = vehicle females dosed with HDM, VHM = vehicle males dosed with HDM, GEF = GEN females dosed with HDM, GEM = GEN males dosed with HDM. HDM-specific IgG_{2a} levels in untreated, non-sensitized naïve animals were undetectable, and therefore no data could be shown. *, significantly different from VH. The number of mice in each group at each time point is shown in Table 1.

Figure 6.

Effect of *in utero* GEN exposure on antigen-specific IgG_{2b} levels in female (A) and male (B) B6C3F1 offspring. Pregnant mice were treated with genistein (GEN) or vehicle (VH) from GD14 to birth by gavage. Female or male offspring were dosed with HDM at intervals as described and sacrificed 3 days after the last intranasal HDM dosing. HDM-specific IgG_{2b} levels in the sera at dilutions of 1:100 - 1:200 were measured as described. VHF = vehicle females dosed with HDM, VHM = vehicle males dosed with HDM, GEF = GEN females dosed with HDM, GEM = GEN males dosed with HDM. HDM-specific IgG_{2b} levels in untreated, non-sensitized naïve animals were undetectable, and therefore no data could be shown. *, significantly different from VH. The number of mice in each group at each time point is shown in Table 1.

Figure 7.

In utero GEN exposure affected the number of macrophages in BALF in adult female (A) and male B6C3F1 offspring (B). Pregnant mice were treated with genistein (GEN) or vehicle (VH) from GD14 to birth by gavage. Female or male offspring were dosed with HDM at intervals as described and sacrificed 3 days after the last intranasal HDM dosing at four different time points (PND 80, 120, 160, 200) and their BALF was collected. Macrophage count was conducted using flow cytometry. NAF = untreated, non-sensitized naïve females, NAM = untreated, non-sensitized naïve males, VHF = vehicle females dosed with HDM, VHM = vehicle males dosed with HDM, GEF = GEN females dosed with HDM, GEM = GEN males dosed with HDM. *a*, significantly different from naïve mice; *b*, significantly different from VH mice. The number of mice in each group at each time point is shown in Table 1.

Figure 8.

In utero GEN exposure affected the number of T cells in BALF in adult female (A) and male B6C3F1 offspring (B). Pregnant mice were treated with genistein (GEN) or vehicle (VH) from GD14 to birth by gavage. Female or male offspring were dosed with HDM at intervals as described and sacrificed 3 days after the last intranasal HDM dosing at four different time points (PND 80, 120, 160, 200) and their BALF was collected. T-cell count was conducted using flow cytometry. NAF = untreated, non-sensitized naïve females, NAM = untreated, non-sensitized naïve males, VHF = vehicle females dosed with HDM, VHM = vehicle males dosed with HDM, GEF = GEN females dosed with HDM, GEM = GEN males dosed with HDM. *a*, significantly different from naïve mice; *b*, significantly different from VH mice. The number of mice in each group at each time point is shown in Table 1.

Table 1Effect of *in utero* GEN exposure on body weight, absolute and relative spleen weight in B6C3F1 mice.

	PND	Parameters	Naïve	Vehicle	Genistein
Female	80	Body wt (g)	21.73 ± 0.44	22.03 ± 0.51	21.51 ± 0.49
		Spleen (mg)	(7)	(7)	(8)
		%Body Wt	85.00 ± 4.74	93.71 ± 7.37	92.25 ± 4.40
	120		0.397 ± 0.022	0.423 ± 0.026	0.428 ± 0.015
		Body wt (g)	26.09 ± 0.94	23.90 ± 1.00	23.66 ± 0.99
		Spleen (mg)	(8)	(7)	(7)
		%Body Wt	84.50 ± 2.38	94.29 ± 5.91	81.86 ± 5.37
	160		0.325 ± 0.01	0.382 ± 0.011 ^a	0.329 ± 0.011 ^b
		Body wt (g)	26.44 ± 1.05	26.32 ± 0.99	25.95 ± 0.72
		Spleen (mg)	(7)	(6)	(8)
		%Body Wt	85.86 ± 3.21	90.83 ± 5.74	91.88 ± 2.91
	200		0.326 ± 0.01	0.345 ± 0.018	0.355 ± 0.009
Male	80	Body wt (g)	27.70 ± 1.12	25.75 ± 0.43	25.63 ± 0.52
		Spleen (mg)	(7)	(8)	(8)
		%Body Wt	85.43 ± 9.66	77.38 ± 2.27	73.00 ± 3.71
	120		0.304 ± 0.021	0.301 ± 0.009	0.284 ± 0.011
		Body wt (g)	33.41 ± 0.92	30.93 ± 0.56 ^a	30.46 ± 0.75 ^a
		Spleen (mg)	(7)	(8)	(8)
		%Body Wt	76.29 ± 4.67	78.38 ± 3.47	73.25 ± 1.33
	160		0.215 ± 0.007	0.253 ± 0.009 ^a	0.242 ± 0.007 ^a
		Body wt (g)	34.70 ± 1.01	35.26 ± 1.27	35.59 ± 1.59
		Spleen (mg)	(7)	(8)	(8)
		%Body Wt	75.86 ± 2.15	91.88 ± 3.84 ^a	84.75 ± 5.85
	200		0.220 ± 0.010	0.261 ± 0.009 ^a	0.238 ± 0.009
		Body wt (g)	42.40 ± 1.18	40.18 ± 0.94	39.27 ± 0.71 ^a
		Spleen (mg)	(8)	(8)	(8)
		%Body Wt	79.25 ± 3.18	82.50 ± 2.41	81.13 ± 2.15
			0.187 ± 0.006	0.205 ± 0.003 ^a	0.207 ± 0.005 ^a

Pregnant mice were treated with genistein or vehicle (VH) from GD14 to birth by gavage. Female or male offspring were sacrificed at four different time points (PND 80, 120, 160, 200) following HDM dosing, and their body weight and spleens were weighed. NA = untreated, non-sensitized naïve animals.

^a, significantly different from NA;

^b, significantly different from VH. The number in parenthesis indicates the number in each group.