In utero exposure to genistein decreased intranasal house dust mite-induced respiratory allergy in middle-aged male B6C3F1 offspring

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Abstract

Despite many hypothesized benefits of dietary isoflavone genistein (GEN) deriving from soy-based products, questions surrounding GEN's developmental effects are increasing. To understand if *in utero* GEN exposure modulated postnatal respiratory allergies in the middle age, we conducted a time course study in the B6C3F1 offspring (PND 240–330) using a common household allergen (house dust mites: HDM; 10 μg/mouse for PND 240 and 290, and 50 μg/mouse for PND 330, a middle age in mice) 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 body weight). Female and male offspring were sensitized with HDM allergens beginning about one month prior to sacrifice followed by challenges with three weekly dosings of HDM extracts, and they were euthanized at day 3 following the final HDM exposure. *In utero* exposure to GEN decreased HDM allergen-induced respiratory allergy in male B6C3F1 offspring at PND 330 as reflected by decreases in airway hyperresponsiveness (e.g., Penh value), HDM-specific IgG$_1$ (a Th2 type Ab) and the activity of eosinophil peroxidase in the lung (an indication of eosinophil recruitment to the lungs). However, *in utero* exposure to GEN had minimal effects on HDM allergen-induced respiratory allergy in the middle-aged female offspring. Changes in serum total IgE, HDM-specific IgE, and lung histopathology scores in both male and female offspring were not biologically significant. Overall,

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in utero GEN exposure exerted a protective effect on respiratory allergy in the middle-aged male, but not female, B6C3F1 offspring following later-life HDM exposures.

Keywords

Genistein; house dust mites; respiratory allergy; *in utero* exposure; IgE

Introduction

Genistein (GEN), a major isoflavone in soy products, can interact with estrogen receptors (Guo, Auttachoat, & Chi, 2005; Martin, Horwitz, Ryan, & McGuire, 1978). Despite the hypothesized beneficial effects of GEN, e.g., higher GEN intake in adults is associated with better lung function (Bime, Wei, Holbrook, Smith, & Wise, 2011; Smith et al., 2004), 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 (Katchy et al., 2014; Patisaul & Jefferson, 2010; Setchell, ZimmerNechemias, Cai, & Heubi, 1997). The possible long-term effects of these relatively high levels of phytoestrogens during infancy are unknown. Phytoestrogens have been detected in amniotic fluid (Doerge, Churchwell, & Delclos, 2000; Jefferson, Patisaul, & Williams, 2012), suggesting that *in utero* exposure indeed occurs.

The prevalence of asthma has doubled in the past decades and continues to rise (Greenwood, 2011; Robinson, Larche, & Durham, 2004). 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). In our previous studies, we have demonstrated that *in utero* exposure to GEN increased IgE production in young B6C3F1 offspring, e.g., postnatal day (PND) 56–120, following adult exposure to a respiratory sensitizer trimellitic anhydride (TMA) (Guo et al., 2005) or HDM (Guo & Meng, 2016). There is evidence that midlife systemic inflammation is associated with frailty in later life (Walker et al., 2019). To further understand how *in utero* GEN exposure modulated respiratory allergy, we hypothesized that exposure to GEN during a sensitive period (e.g., *in utero* exposure) would differentially modulate allergic sensitization in middle-aged male and female offspring to the respiratory allergen HDM. To this end, we have conducted a time course study in the middle-aged offspring at three time points (PND 240, 290, and 330) following *in utero* GEN and later intranasal HDM exposures.

In this study, we have evaluated the effects of *in utero* GEN exposure through dosing dams from gestation day 14 (GD14) to parturition on various allergic responses following HDM stimulation in middle-aged B6C3F1 offspring, including airway hyperresponsiveness (AHR), the total and antigen-specific IgE responses, and eosinophil peroxidase (EPO) activity. 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 (Frawley et al., 2011; Ng, Steinetz, Lasano, & Zelikoff, 2006; Papaconstantinou, Goering, Umbreit, & Brown, 2003). 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 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*. Previous studies reported that a negligible amount of bisphenol A leached from new or used polycarbonate cages maintained at room temperature (Delclos et al., 2014; Johnson et al., 2016; Thigpen et al., 2013). It has been reported that the dietary concentration of isoflavones in the NIH-07 diet is approximately 33 ppm (Brown & Setchell, 2001). In our previous studies (Guo et al., 2005), two different diets, e.g., the 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. Thus, 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 (VH) by gavage (0.1 ml/10 g body) via an 18 G gavage needle from GD 14 to parturition, which produced a dose of 20 mg/kg body. The 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. About one month prior to sacrifice, mice were sensitized with one dose of HDM extract (*Dermatophagoides farinae*, Greer Laboratories, Lenoir, NC) followed by challenges with three dosings of HDM allergens (e.g., four dosings of HDM extract were given once a week except for a two-week interval between dosings 1 and 2; Figure 1). Mice were anesthetized with an intraperitoneal (ip) injection of ketamine (100 mg/kg)/xylazine (10 mg/kg body), followed by intranasal instillation of HDM extract (10 μg/mouse for PND 240 and 290, and

50 μg/mouse for PND 330) in 50 μl (25 μl/nostril) of physiological Hank's balanced salt solution (HBSS). 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, it 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 (Guo & Meng, 2016; Ward, Chung, Copeland, & Doerfler, 2010).

Mice were sacrificed and sera were collected at day 3 following the final HDM exposure. The animals in an additional group were treated with 50 μl 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 for GEN.

Enzyme linked immunosorbent assay (ELISA)

ELISA for total IgE was performed according to the manufacturer's instructions (BD Pharmigen, San Diego, CA). Briefly, 100 μl of diluted capture antibody in the bicarbonate/carbonate coating buffer (100 mM) 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 μl 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 μl 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 H_2SO_4).

Eosinophil peroxidase (EPO) assay

The EPO assay is a colorimetric assay used for detecting eosinophils by measuring the eosinophil peroxidase activity (Davoine et al., 2013; Lintomen et al., 2008; Strath, Warren, & Sanderson, 1985). Mouse spleen and lung were homogenized and then sonicated (30–45 seconds per sample) until the cells were disrupted. The organ homogenates were centrifuged and the resulting supernatants were used for assay. Briefly, Tris-HCl buffer (50 μl) was added to 96-well plates initially except for the wells for undiluted samples (100 μl). 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 H_2O_2 (1.3 μl/ml) in 0.05 M Tris-HCl. The reaction was stopped by adding 2N H_2SO_4, and the optical density measured at 490 nm on an ELISA plate reader (Molecular Devices Thermomax Plate Reader, Rockville, MD).

Determination of airway responsiveness

Measurements of the physiological response to a standard methacholine (MCh) challenge (0 and 5, 20 and 50 mg/ml MCh) were conducted using whole body plethysmographic techniques, similar to those described previously (Rasid, Chirita, Iancu, Stavaru, & Radu, 2012; Whitehead, Walker, Berman, Foster, & Schwartz, 2003). Briefly, the mice were individually placed in a whole-body plethysmograph (Buxco Electronics, Sharon, CT) that contained built-in pneumotachographs at day 2 following the final HDM exposure. The mice were ventilated by 0.8 L/min regulated bias airflow (PLY1040; Buxco Electronics) through the plethysmograph. A differential pressure transducer was used to measure the pressure differential across the pneumotachograph to determine flow. The flow through the pneumotachograph was linear to the differential pressure and integrated. Transducer signals were conditioned using an amplifier (Emka Technologies; France), digitized, and processed in real time. Real-time calculations of frequency and breath waveform [expiratory time (Te), relaxation time (Tr), peak expiratory flow (PEF), peak inspiratory flow (PIF)] were performed and recorded electronically by computer software (Emka Technologies). Estimates of airway responsiveness, expressed as enhanced pause (Penh), were derived from the ventilation and flow-derived parameters. Penh was calculated as $[(Te - Tr/Tr) \times (PEF/PIF)]$. Penh values were averaged every 30 s and recorded for a minimum of 3-min intervals at baseline and after stimulation with each concentration of MCh. The different concentrations of aerosolized MCh were administered for 1 min using an ultrasonic nebulizer and nebulizer control unit (Emka Technologies). The dilution flow through the mixing chamber was 10 L/min. This noninvasive physiological approach offered the advantages of eliminating the effects of anesthesia and surgical trauma and permitting repeated assessment of the same mice while they breathed spontaneously. The noninvasive index of airway hyperresponsiveness, Penh, is an empirically derived, unit-less value based on the pressure waveform in the plethysmograph box (Albertine et al., 2002).

HDM-specific IgE, IgG_1, IgG_{2a} and IgG_{2b} levels in sera

The removal of IgG has been shown to increase the sensitivity of detecting antigen-specific IgE (Fischer et al., 2005). On day 1, dilutions of serum samples (1:8 and 1:16) in blocking solution (PBS + 10% FBS) were first depleted of IgG by overnight incubation at 4°C in protein G-coated 96-well plates (Reacti-Bind plates; Pierce, Rockford, IL). Protein G is a surface protein found on the bacteria *Staphylococcus aureus* that binds Fc portion of IgG. Meanwhile, HDM extract diluted with coating buffer was plated on 96 well (NUNC) plates and incubated overnight at 4°C. On day 2, HDM extract (50 μg/ml) coated plate was washed, then blocked with 10% FBS-PBS for 1 h at room temperature. After additional washings, the IgG depleted sera were transferred to the HDM coated plates and incubated for 2 h at room temperature. After washing, 100 μl of working solution including detector antibody (biotinylated anti-mouse IgE monoclonal) and avidin-HRP reagent were added to each well, then incubated for 1 h at room temperature. After washing, 100 μl 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 H_2SO_4). For the measurement of HDM-specific IgG_1, IgG_{2a} and IgG_{2b} levels, sera without IgG depletion with higher dilutions were used.

Histopathology

Lung tissues were fixed in 10% phosphate-buffered formalin, sectioned and then stained with hematoxylin and eosin, and examined for pathological findings. The sections were also stained with mucicarmine for enumeration of mucin secreting cells in similar peribronchial regions (Cai, Zhou, & Webb, 2012). Mucicarmine is specific to the mucins of epithelial origin, and has been used for the assessment of goblet cells in human specimens (Osho, Wang, Horn, & Adeola, 2017). Grading was performed within the study and a score was assigned based the total lesion severity (4= Marked; 0 = no lesion).

Statistical analysis

Results are presented as mean ± 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. Homogenous data were analyzed using a one-way analysis of variance, and the Dunnett's t test was used to determine differences between the control and experimental groups. Non-homogenous data were analyzed using a nonparametric analysis of variance and the Wilcoxon Rank Test to determine differences between the vehicle control group and exposure groups. A group was considered statistically significant from the control group if p 0.05.

Results

Effect of in utero GEN exposure on the body weight and spleen weight in middle-aged female and male offspring

To determine if *in utero* GEN exposure affected allergic responses in middle-aged mice, a time course study at three different time points (PND 240, 290, and 330) using HDM allergens was conducted in the B6C3F1 offspring (Figure 1). All dams receiving GEN by gavage from GD 14 to parturition at doses of 20 mg/kg body survived the experimental period and showed no overt signs of toxicity as manifested in changes in gait, fur status, drainage from orifices or pregnancy complications. All dams delivered their pups successfully, and total pups per litter, female pups per litter and litter sex (F:M) ratios were not significantly affected by GEN treatment. However, male pups per litter was significantly decreased by GEN (Table 1). In general, there was an age-related increase in body weight in both female and male offspring (Table 2), and no significant differences were observed among the groups in females at any timepoints. Treatment with HDM decreased terminal body weight in male offspring at PND 240, 290 and 330 when compared to naïve mice that received HBSS during intranasal dosing. However, *in utero* GEN treatment in the males did not have a significant effect on body weight when compared with vehicle group.

For absolute spleen weight, there was an increasing trend with time in the female offspring except that the spleen weight leveled off at PND 330. This leveling off effect was seen in both naïve and vehicle groups, but was not observed in GEN-exposed female offspring (Table 2). In addition, at PND 240, there was a significant increase of spleen weight in HDM-treated vehicle female offspring when compared to naïve group, and this increase was abrogated by *in utero* GEN exposure. When the spleen weights were expressed as relative values (%body), GEN treatment significantly increased relative weights of spleen at PND

330 in female offspring when compared to either the NAF group or VHF group. The spleen weight in male offspring also increased with age in all treatment groups (Table 2); however, no significant changes were observed at any time points among the three groups in either absolute or relative spleen weights.

Total IgE levels in middle-aged female and male offspring following in utero GEN exposure

Total serum IgE levels were measured using ELISA. When compared to the vehicle group, *in utero* GEN exposure induced significant increases in IgE levels in female offspring at PND 240, but not at PND 290 and 330 (Figure 2A, top panels); however, there was no difference between NAF and VHF groups at PND 240. When compared to naïve group, significant increases were observed in the vehicle group at PND 330 (right panel) and in the GEN treatment group at all three time points (PND 240, 290 and 330). Taken together, *in utero* GEN exposure had no biologically significant increase of total IgE levels in middle-aged female offspring (Figure 2A).

In male offspring, the HDM treatment (10 μg/mouse) in VHM group stimulated a significant increase in IgE levels when compared to the naïve mice at PND 240 (Figure 2B, left panel), while there was no significant difference between VHM and GEM groups. At PND 290 (Figure 2B, middle panel), there were no significant differences among the three groups. At PND 330, the HDM treatment (50 μg/mouse) stimulated a significant increase in IgE levels when compared to the naïve group (Figure 2B, right panel); however, the vehicle and GEN-treated offspring did not differ significantly in their IgE levels. These data suggest that *in utero* GEN exposure had minimal effects in total IgE production in middle-aged male B6C3F1 offspring.

Antigen-specific IgE in female and male offspring following in utero GEN exposure

Antigen-specific IgE assays were performed using IgG depleted sera from offspring at PND 240 and 290. At both time points tested, there were significant differences between naïve group and mice dosed with HDM (Figure 3 and data not shown), suggesting both male and female offspring developed sufficient respiratory sensitization. At PND 240, *in utero* GEN exposure had no effects on the levels of HDM-specific IgE in either female or male offspring (data not shown). At PND 290, a significant increase of HDM-specific IgE was observed in GEN exposed male offspring when compared to VH group (Figure 3B), but not in the female offspring (Figure 3A). However, the levels of HDM-specific IgE in male offspring were much lower than that in female offspring. Therefore, *in utero* GEN exposure did not induce a biologically meaningful increase of HDM-specific IgE levels in these male offspring.

Antigen-specific IgG$_1$, IgG$_{2a}$ and IgG$_{2b}$ levels in middle-aged female and male offspring

To determine if *in utero* GEN exposure affected the production of other antibodies in the offspring, the antigen-specific IgG$_1$, IgG$_{2a}$ and IgG$_{2b}$ levels in the sera were measured. In females, *in utero* GEN exposure induced significant increases in the levels of antigen-specific IgG$_{2b}$ (a Th1 type Ab) at PND 330 when compared to vehicle group (Table 3). In males, *in utero* GEN exposure induced a significant increase in the levels of antigen-specific IgG$_1$ (a Th2 type Ab) at PND 290 when compared to vehicle group (Table 3). In contrast, a

significant decrease in the level of antigen-specific IgG_1 at PND 330 was observed in male offspring following *in utero* GEN exposure (Table 3).

Eosinophil peroxidase activity in middle-aged female and male offspring following in utero GEN exposure

Eosinophilia in the bronchial region is a sign of bronchial hyperreactivity (Afshar, Vucinic, & Sharm, 2007; Mathias et al., 2013). EPO assays (Figure 4A) were performed on homogenized lungs at PND 330, and *in utero* GEN exposure numerically ($p > 0.05$) increased lung EPO activities in female offspring (Figure 4B). However, in male offspring, *in utero* GEN exposure decreased lung EPO activity at PND 330 (Figure 4C). EPO assays on homogenized spleens were also tested at PND 330, and there were no significant differences between the GEN and vehicle groups in either male or female offspring (data not shown).

Airway hyperresponsiveness in middle-aged female and male offspring

Plethysmography was used to measure changes in lung volume and airway resistance in both female and male offspring at PND 330, and *in utero* GEN exposure did not significantly alter basal Penh values in either female (Figure 5A) or male offspring (Figure 5B). No significant changes in Penh values after MCh challenge were observed in the *in-utero* GEN exposed female offspring (Figure 5A). However, decreases in Penh value were observed in the male offspring with a significant change at MCh dose of 20 mg/kg following *in utero* GEN exposure (Figure 5B).

Lung histopathology in female and male offspring following in utero GEN exposure

Histopathological analysis of the lung samples (Figure 6A) included: peribronchial inflammatory infiltrate (Figure 6B), perivascular inflammatory infiltrate, prominent endothelial lining, subendothelial eosinophils, bronchi-associated lymphoid tissue and mucin producing cells (Figure 6C). At PND 290, the summed pathological score was higher in female offspring than male offspring (Figure 6D); however, there were no significant differences between the vehicle control and *in utero* GEN exposed female or male offspring (Table 4).

Discussion

Increases in asthma prevalence and severity over the last several decades are due at least in part to environmental factors (e.g., diet), and allergic reaction to airborne allergens is an important risk factor for severe asthma in adults (Zureik et al., 2002). In our evaluation of the relationship between soy isoflavone GEN and respiratory allergy, we have demonstrated that *in utero* exposure to GEN increased IgE production in young B6C3F1 offspring following adult exposure to a respiratory sensitizer TMA (Guo et al., 2005) or HDM (Guo & Meng, 2016). However, the effects in aging mice were unknown. Most aging studies focus on two or three specific life phases: mature adult, middle age, and old (Petry, 2002). Middle age refers to a phase of life during which senescent changes can be detected in some, but not all, biomarkers of aging. Using a survival curve that is based on a large cohort of C57BL/6J mice (150 males and 150 females), it has been suggested that mice should be approximately 10–15 months of age (corresponding to 38–47 years old in humans) to be considered the

middle-aged group (Brinton, 2012). The mice in our studies were 8 –11 months old with the last time point (PND 330) being in the range of middle age. The other two time points PND 240 and 290 were between the mature adult (3–6 months of age in a mouse) and middle age.

In our studies with young adult mice (Guo & Meng, 2016), the dose of 10 μg/mouse of HDM was considered optimal because of less variation, and it also permitted further increases following exposures. However, as the mice became aged, the HDM seemed to be less effective in eliciting IgE production when compared to IgE levels in the naïve mice (e.g., PND 240 in male offspring and PND 290 in female offspring in Figure 2). Nonetheless, the PND 240 and 290 offspring did develop respiratory sensitization following HDM challenge because: (1) At both time points tested, there were significant differences in HDM-specific IgE between naïve group and mice dosed with HDM (Figure 3 and data not shown); (2) At both time points tested, there were significant increases in HDM-specific IgG antibodies between naïve group and mice dosed with HDM. For PND 330, the HDM dose was increased to 50 μg/mouse. We performed plethysmographic tests for the B6C3F1 offspring at PND 330, a middle age, and found that *in utero* GEN treatment decreased AHR (e.g., Penh value) at the 20 mg/ml MCh challenge in male offspring. At PND 330, *in utero* GEN treatment in male offspring had no effects on serum total IgE, while decreased the level of HDM-specific IgG_1 (a Th2 type Ab). Furthermore, the EPO activity was decreased following *in utero* GEN exposure in male offspring at PND 330. Thus, in contrast to our findings in young adult offspring, *in utero* exposure to GEN decreased intranasal HDM allergen-induced respiratory allergic reactions in middle-aged male B6C3F1 offspring.

Pulmonary eosinophilia has been considered as one of the major mechanisms for allergen-induced bronchial hyperreactivity (Afshar et al., 2007; Mathias et al., 2013). Using extracts from whole lung homogenates, a significant decrease of EPO activity in the GEN treatment group over the vehicle was observed at PND 330 for male offspring. Consistent with our findings, there is evidence that GEN inhibits eosinophil leukotriene C4 synthesis (a key pathway that may contribute to asthma severity), reduces exhaled nitric oxide and *ex vivo* leukotriene C4 synthesis in a small group of patients with inadequately controlled asthma (Kalhan et al., 2008; Smith et al., 2015). In a male guinea pig model of asthma, GEN (15 mg/kg; i.p.) also reduced ovalbumin-induced eosinophil peroxidase activity and attenuated airway hyperresponsiveness to inhaled Mch (Duan et al., 2003). Similarly, dietary phytoestrogens also significantly attenuated ovalbumin-induced eosinophilia in the lung tissue of guinea pigs (Regal, Fraser, Weeks, & Greenberg, 2000). However, it should be noted that GEN exposure in our study was in early life, e.g., *in utero*, and there was not any dosed GEN left in the body when the HDM respiratory allergic reactions occurred.

As the immune system ages, the IgE response generally decreases (Busse & Mathur, 2010; Hanneuse, Delespesse, Hudson, Dehalleux, & Jacques, 1978; Yagi, Sato, Hayakawa, & Ide, 1997). Our total IgE data also showed a trend towards decline at a later time point. It is possible that GEN modulation of respiratory allergic responses is age-related. A retrospective multiple controlled cohort study has shown that there was an increase in the use of asthma or allergy drugs in young adults with significant changes observed in females 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). This observation was

somewhat confirmed in our animal studies that *in utero* exposure to GEN increased respiratory sensitization in both male and female young B6C3F1 offspring (Guo et al., 2005; Guo & Meng, 2016). In our study with middle-aged female offspring, however, *in utero* GEN treatment did not change AHR, which was consistent with the EPO activity that was not significantly altered following *in utero* GEN exposure in female offspring at PND330. In addition, *in utero* GEN treatment had no effects on serum total IgE in female offspring, while the level of antigen-specific IgG_{2b} (a Th1 type Ab) was increased at PND 330. Thus, *in utero* exposure to GEN had minimal effect on intranasal HDM allergen-induced respiratory allergic responses in middle-aged female B6C3F1 offspring. Interestingly, an increase of spleen weight was observed in the *in utero*-GEN treated female offspring at PND 330. Although the phenotype of splenocytes and their functions in these mice were not examined in this study, developmental GEN exposure decreased $CD5^+$ cells and IL-10, and did not affect $CD4^+CD25^+$ T cells, while increased $CD24^+$ cells in our studies with the middle-aged female NOD mouse offspring (Huang et al., 2018). It was possible that IL-10 producing regulatory B cell subset might control the T cell-dependent inflammatory responses (Vighi, Marcucci, Sensi, Di Cara, & Frati, 2008).

Although the mechanisms underlying a fetal basis of adult effects are currently unclear, the immune-related sex difference, e.g., females tend to have an increased Th1 response in early life compared to males (Bao, Yang, Jun, & Yoon, 2002), and gut microbiota-driven hormone difference (Markle et al., 2013) might be responsible for our observations that male offspring exposed to GEN *in utero* showed a protection of respiratory sensitization. Similar sex-specific phenomena of developmental GEN exposure have been shown in our studies of middle-aged NOD mice in which a protective effect on type 1 diabetes in males while a detrimental effect in females was observed (Huang et al., 2018). It was concluded that the exacerbation of type 1 diabetes in NOD females was associated with gut microbiota-related immunomodulation. Interestingly, S-(−)equol, a biologically active gut microbiota-derived metabolite of the soy isoflavones daidzin/daidzein, is also developmentally regulated and related to early diet composition (Brown et al., 2014). In addition, 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 and postnatal HDM exposures showed a different pattern from similarly treated male offspring. There is evidence that prenatal exposure to phytoestrogen GEN triggers epigenetic changes in immune function genes (Vanhees et al., 2011). Rodents are sexually mature by 3–6 months of age and approach the endocrine equivalent of human perimenopause by 9 months of age (Brinton, 2012; Mobbs, Gee, & Finch, 1984). However, irregularity of cycle length, a reliable indicator of irregular fertility and impending reproductive senescence, starts occurring approximately 8 months of age (Finch, Felicio, Mobbs, & Nelson, 1984). It was possible that the female offspring (8 – 11 months old) were in perimenopause, and thus, the estrogen-dependent imprinting effects could not manifest.

Although it has been reported that higher GEN intake is associated with better lung function (Bime et al., 2011; Smith et al., 2004), this is the first study reporting a protective effect on respiratory allergic reactions in middle-aged male offspring following developmental GEN

exposure. 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 (Katchy et al., 2014; Setchell et al., 1997). The serum level of GEN (1.4–7.5 μM) 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 (Bhandari, Crawford, Huang, & Reenstra, 2003; Djuric, Chen, Doerge, Heilbrun, & Kucuk, 2001; Yellayi et al., 2002), and in infants on soy formula (Cao et al., 2009). As the microbiome play a central role in respiratory allergy, and they are highly influenced by multiple environmental and dietary factors, further studies of the molecular mechanisms by determining how developmental GEN exposure creates a diet− linked, age− related microbiome modulation of the respiratory allergic phenotype are warranted.

Acknowledgments

Abbreviations used:

AHR	airway hyperresponsiveness
ELISA	enzyme linked immunosorbent assay
EPO	eosinophil peroxidase
FBS	fetal bovine serum
GD	gestation day
GEN	genistein
HBSS	Hank's balanced salt solution
HDM	house dust mites
HRP	horseradish peroxidase
MCh	methacholine
PBS	phosphate buffered saline
Penh	enhanced pause
PND	postnatal day
TMA	trimellitic anhydride
TMB	tetramethylbenzidine

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Highlights:

- *In utero* genistein exposure decreased lung EPO activity in middle-aged male offspring

- *In utero* genistein exposure decreased airway hyperresponsiveness in middle-aged males

- *In utero* genistein exposure decreased HDM-specific IgG_1 in middle-aged males
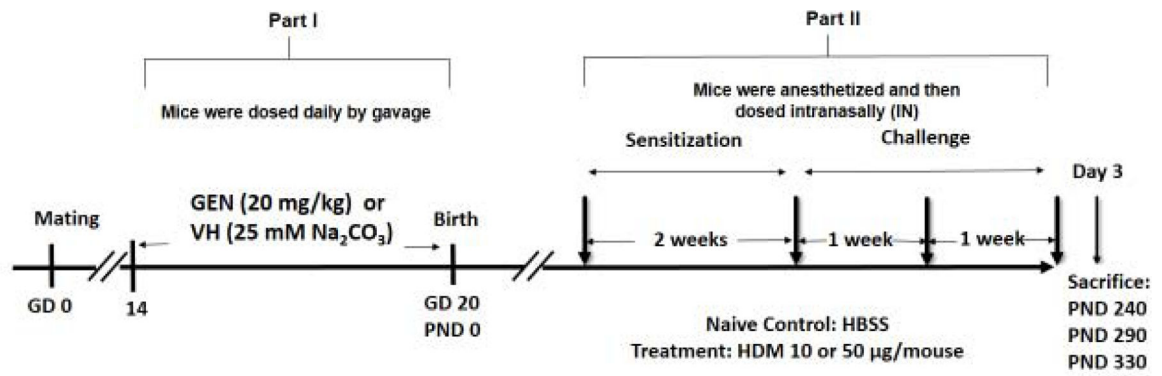
Figure 1.
Experimental design for animal treatments. In our studies with young mice (Guo & Meng, 2016), the dose of 10 μg/mouse of HDM was considered the optimal dose to be used for studies because of less variation, and it also permitted further increases. However, as the mice became aged, the HDM seemed to be less effective in eliciting IgE production when compared to IgE levels in the naïve mice. Thus, for the PND 330 time point, the HDM dose was increased to 50 μg/mouse. PND = postnatal day; gd = gestation day.

Figure 2.
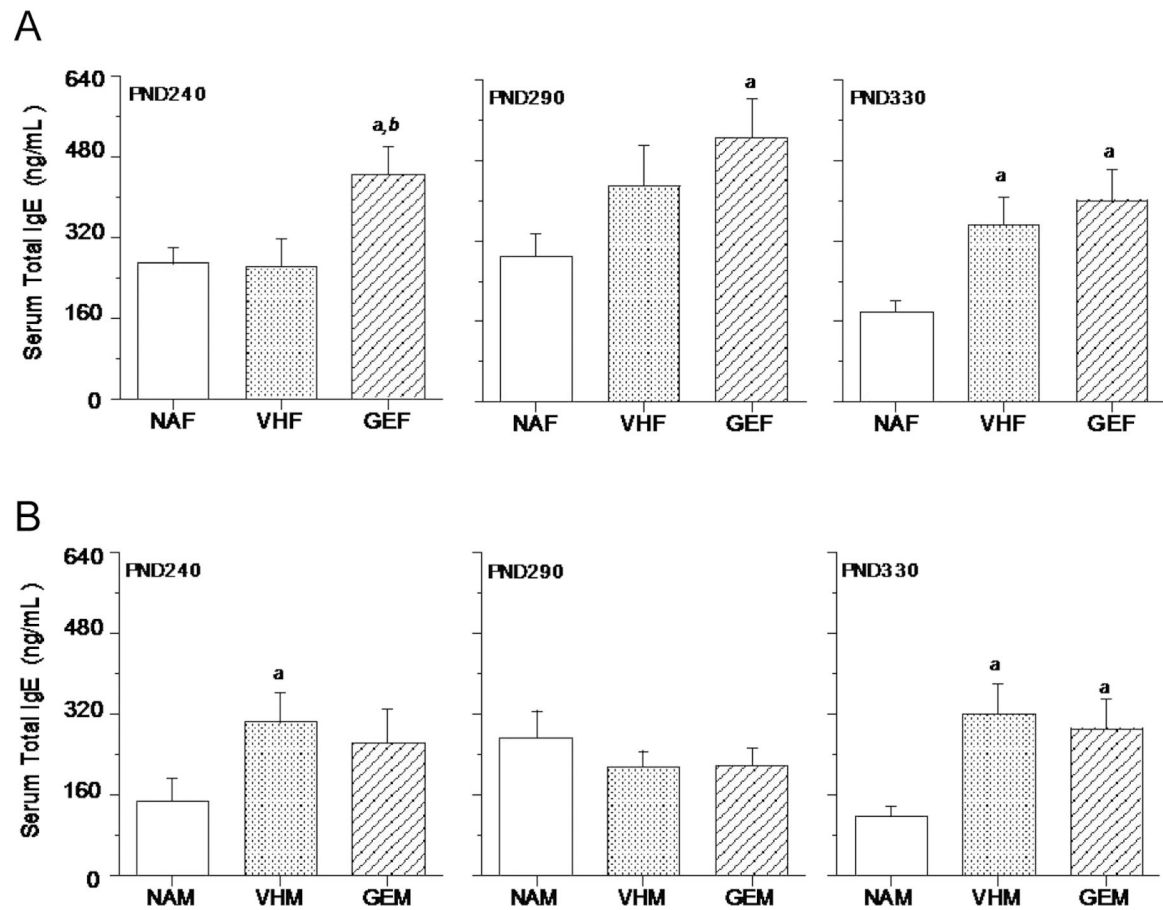Effect of *in utero* GEN exposure on serum total IgE levels in female (A) and male (B) B6C3F1 offspring. Pregnant mice were treated with genistein (GEN) or vehicle (VH) from GD14 to birth by gavage. Female or male offspring were dosed with HDM at intervals as described (Figure 1) and sacrificed 3 days after the last intranasal HDM dosing at three different time points (PND 240, 290, and 330). The serum total IgE levels were measured using ELISA. 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 group; *b*, significantly different from VH group. The number of mice in each group at each time point is shown in Table 2.

Figure 3.

In utero GEN exposure had no effects on antigen-specific IgE in female B6C3F1 offspring (A) but increased antigen-specific IgE in male B6C3F1 offspring (B) at PND 290. Pregnant mice were treated with GEN or VH from GD14 to birth by gavage. Female or male offspring were dosed with HDM or HBSS (NA) at intervals as described in Figure 1 and sacrificed 3 days after the last intranasal dosing. HDM-specific IgE assays using IgG depleted sera were performed 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 group; *b*, significantly different from VH group. The number of mice in each group at each time point is shown in Table 2.
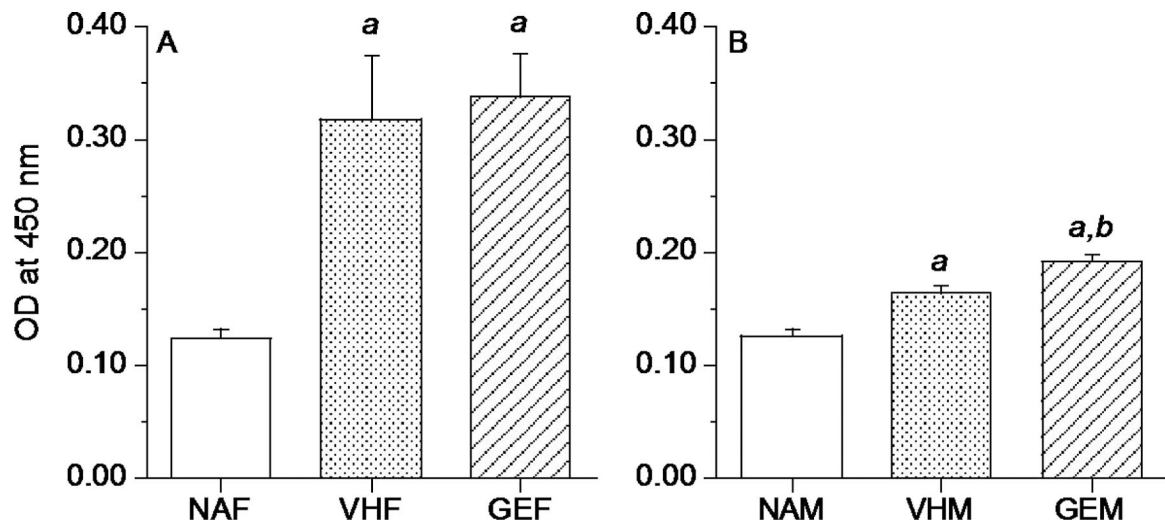
Figure 4.
Eosinophil peroxidase activity in lungs of female and male B6C3F1 offspring at PND 330. (A) Optical density (OD) values decreased with dilution of samples from 1:2 to 1:4096; the linear region of the curve is where proper concentration of the analytes can be compared. In earlier regions where the concentration is in extreme excess (the prozone region), analytes can form large complexes and skew concentration. (B) Female offspring lung samples (both VHF and GEF) from dilutions of 1:32 to 1:512, the linear region of the curve. (C) Male lung samples (both VHM and GEM) from dilutions of 1:32 to 1:512. *, p 0.05 when compared to VH. VHF = VH females dosed with HDM, VHM = VH males dosed with HDM, GEF = GEN females dosed with HDM, GEM = GEN males dosed with HDM. The number of mice in each group at each time point is shown in Table 2.
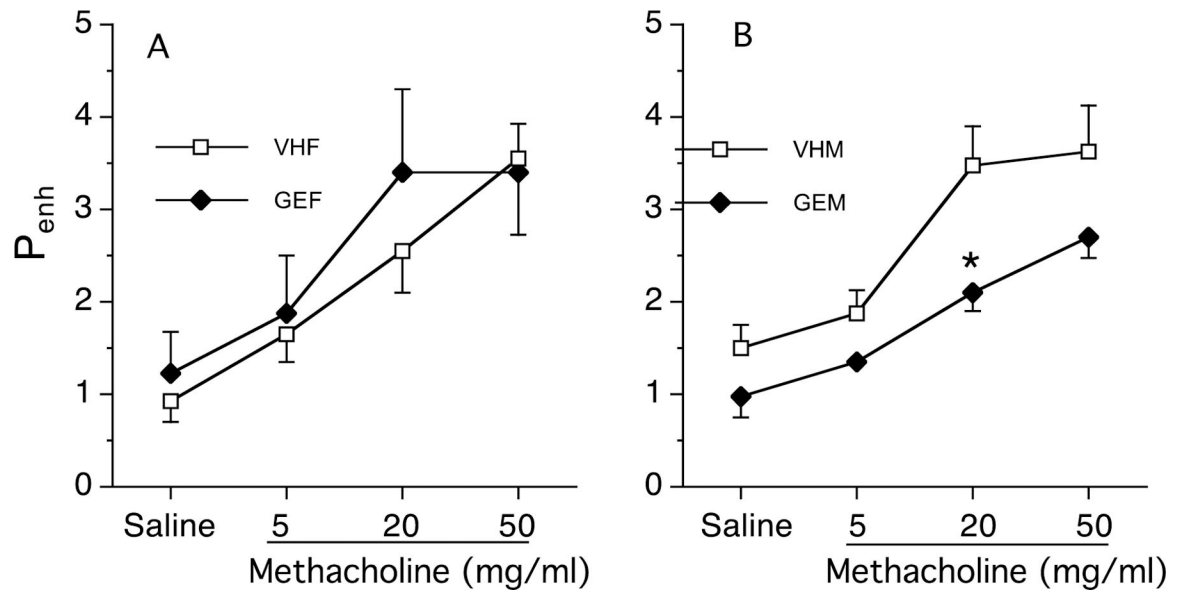
Figure 5.
Effects of *in utero* GEN exposure on airway hyperresponsiveness in female (A) and male (B) B6C3F1 offspring at PND 330. Pregnant mice were treated with GEN or VH from GD14 to birth by gavage. Twenty-four hours after last HDM dosing, female or male offspring were individually placed in a whole-body plethysmograph. Estimates of airway responsiveness, expressed as enhanced pause (Penh), were derived from the ventilation and flow-derived parameters. Penh values were recorded for a minimum of 3-min intervals at baseline and after stimulation with each concentration of MCh (0 and 5, 20 and 50 mg/ml). 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 group; *b*, significantly different from VH group. The number of mice in each group at each time point is shown in Table 2.
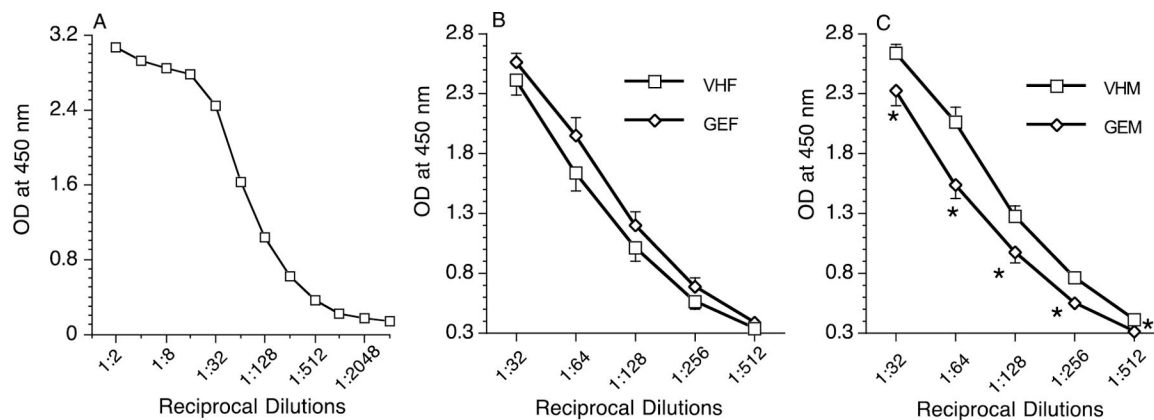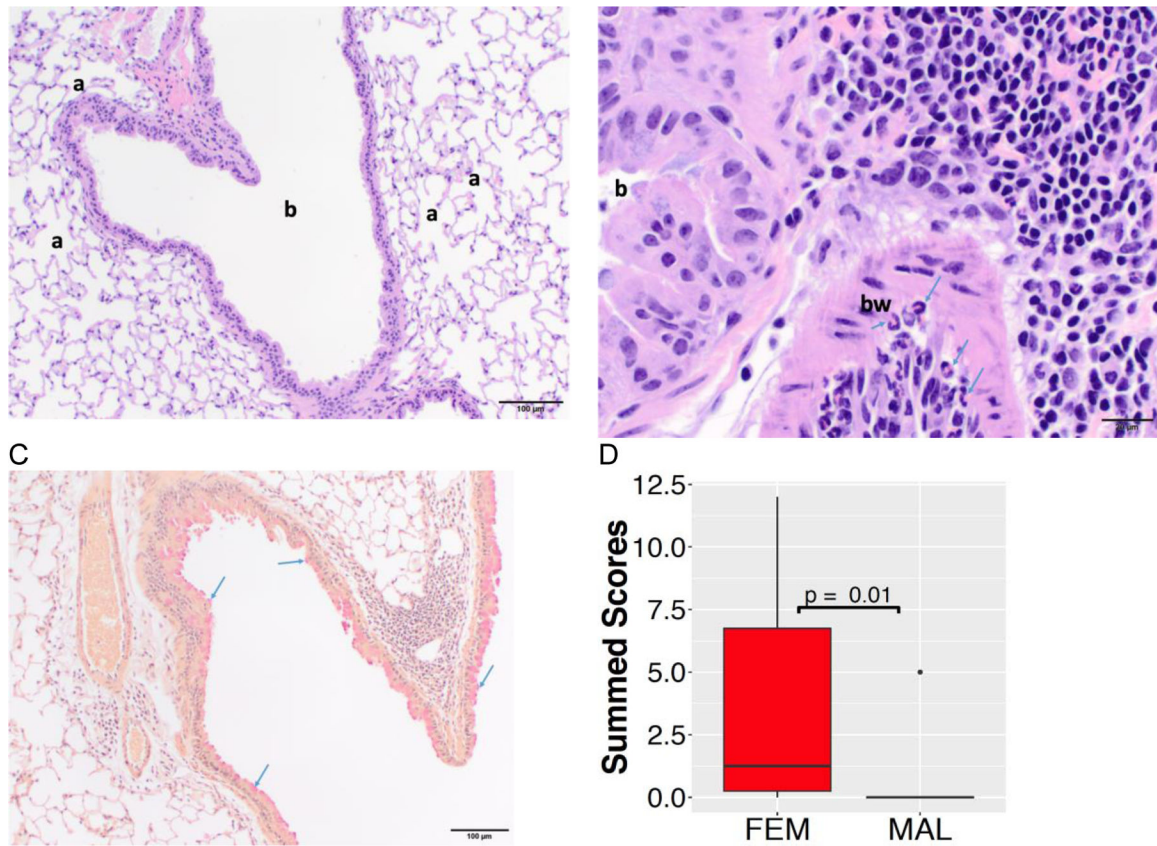
Figure 6.
Lung histopathology in female and male offspring at PND 290. (A) Low magnification photomicrograph depicting a bronchiole (b) and surrounding alveoli (a) from a male mouse. The section is histologically normal. Hematoxylin and eosin staining, 100x original magnification, scale bar = 100μm. (B) High magnification photomicrograph of inflammatory infiltrate (right and top right sections of the image) around a bronchiole (b) and within the bronchiolar wall (bw) from a female mouse. Within the bronchiolar wall, there are numerous eosinophils (arrows). Hematoxylin and eosin staining, 400x original magnification, scale bar = 20μm. (C) Low magnification photomicrograph of mild goblet cell hyperplasia (bright red globules, arrows) in the bronchiolar epithelial lining from a female mouse. Mucicarmine staining, 100x original magnification, scale bar = 100μm. (D) The summed pathological score was higher in female offspring (FEM) than male offspring (MAL).

Table 1.

Effect of GEN exposure during pregnancy on the offspring parameters

Treatment	Litter #	Female #	Male #	Total pups	Sex ratio (F:M)
VH	1	4	4	8	1.00
	2	6	4	10	1.50
	3	4	6	10	0.67
	4	5	5	10	1.00
	5	2	7	9	0.29
	6	3	8	11	0.38
	7	5	6	11	0.83
	8	1	7	8	0.14
	9	2	7	9	0.29
	10	6	3	9	2.00
	11	5	6	11	0.83
	12	7	6	13	1.17
	Average	4.17 ± 0.53	5.75 ± 0.43	9.92 ± 0.42	0.84 ± 0.16
GEN	1	1	7	8	0.14
	2	6	5	11	1.20
	3	6	4	10	1.50
	4	6	5	11	1.20
	5	6	4	10	1.50
	6	0	2	2	0.00
	7	5	5	10	1.00
	8	6	4	10	1.50
	9	5	5	10	1.00
	10	1	2	3	0.50
	11	1	5	6	0.20
	12	4	6	10	0.67
	13	7	4	11	1.75
	14	6	5	11	1.20
	Average	4.28 ± 0.65	4.50 ± 0.36[*]	8.79 ± 0.80	0.95 ± 0.15

Pregnant mice were treated with genistein (GEN) or vehicle (VH) from GD14 to birth by gavage. Female or male pups were counted, and the sex ratio determined.

[*] p 0.05 when compared to VH.

Table 2.

Effect of *in utero* GEN exposure on body weight, absolute and relative spleen weight in middle-aged B6C3F1 offspring

PND		Parameters	Naïve	Vehicle	Genistein
Female	240	Body wt (g)	33.43 ± 1.88	35.12 ± 2.59	33.93 ± 1.74
			(7)	(6)	(7)
		Spleen (mg)	81.25 ± 2.46	105.50 ± 8.79 [a]	87.86 ± 2.76 [b]
		%Body Wt	0.244 ± 0.014	0.307 ± 0.030	0.263 ± 0.018
	290	Body wt (g)	43.49 ± 2.38	41.02 ± 1.59	39.91 ± 1.96
			(8)	(6)	(7)
		Spleen (mg)	106.88 ± 8.82	113.83 ± 5.64	98.14 ± 5.32
		%Body Wt	0.247 ± 0.016	0.279 ± 0.017	0.253 ± 0.025
	330	Body wt (g)	42.16 ± 2.06	40.97 ± 2.74	36.43 ± 2.42
			(8)	(6)	(7)
		Spleen (mg)	105.50 ± 5.27	103.17 ± 2.65	126.29 ± 8.74 [b]
		%Body Wt	0.253 ± 0.016	0.258 ± 0.018	0.356 ± 0.032 [a,b]
Male	240	Body wt (g)	44.48 ± 0.97	41.79 ± 0.30 [a]	42.17 ± 0.72
			(8)	(8)	(8)
		Spleen (mg)	89.63 ± 5.15	80.63 ± 1.59	80.25 ± 2.85
		%Body Wt	0.201 ± 0.009	0.193 ± 0.005	0.190 ± 0.006
	290	Body wt (g)	46.25 ± 1.29	43.98 ± 0.91	43.34 ± 0.46 [a]
			(6)	(8)	(8)
		Spleen (mg)	99.17 ± 5.84	90.25 ± 6.26	95.25 ± 3.00
		%Body Wt	0.214 ± 0.008	0.204 ± 0.011	0.220 ± 0.006
	330	Body wt (g)	47.66 ± 1.18	44.25 ± 0.67 [a]	43.29 ± 0.87 [a]
			(8)	(8)	(8)
		Spleen (mg)	113.13 ± 9.86	110.13 ± 10.25	105.88 ± 5.73
		%Body Wt	0.236 ± 0.016	0.249 ± 0.023	0.244 ± 0.010

Pregnant mice were treated with genistein or vehicle from GD14 to birth by gavage. Female or male offspring were sacrificed at three different time points (PND 240, 290, and 330) following HDM dosing, and their body weight and spleens were weighed. Naive = untreated, non-sensitized naïve animals.

[a,] significantly different from naive;

[b,] significantly different from vehicle. The number in parenthesis indicates the number in each group.

Table 3

Effect of in utero GEN exposure on antigen-specific IgG1, IgG2a and IgG2b levels in middle-aged B6C3F1 offspring.

PND		IgG1		IgG2a		IgG2b	
		VH	GEN	VH	GEN	VH	GEN
Female	240	0.359 ± 0.106	0.236 ± 0.092	0.839 ± 0.3S5	0.457 ± 0.229	0.789 ± 0.247	0.365 ±0.123
	290	0.193 ± 0.051	0.183 ±0.019	0.410 ± 0.229	0.196 ± 0.052	0.393 ± 0.023	0.358 ± 0.014
	330	0.534 ± 0.217	0.535 ± 0.147	0.569 ± 0.207	0.737 ± 0.128	0.409 ± 0.009	0.783 ± 0.176[*]
Male	240	0.162 ± 0.019	0.126 ±0.024	0.246 ± 0.062	0.320 ± 0.136	0.245 ± 0.026	0.415 ±0.182
	290	0.120 ± 0.033	0.172 ±0.018[*]	0.237 ± 0.071	0.263 ± 0.101	0.341 ± 0.014	0.609 ± 0.182
	330	0.413 ±0.039	0.245 ± 0.038[*]	0.698 ± 0.129	0.519 ± 0.131	0.819 ± 0.187	0.634 ±0.118

Pregnant mice were treated with genistein (GEN) or vehicle (VH) from GDI4 to birth by gavage. Female or male offspring were dosed with HDM at intervals as described in methods and sacrificed 3 days after the last intranasal dosing at three different time points (PND 240. 290. and 330). The serum levels of HDM-specific IgG subclasses were measured using ELISA as described at dilutions of 1:5,000 – 1:32.000 for IgG_1, 1:100 – 1:800 for IgG_{2a}, and at 1:100 – 1:200 for lgG_{2b}.

[*]p 0.05 when compared to VH.N = 6 – 8. Optical density (OD) values were shown as mean ± SEM.

Table 4.

Effect of *in utero* GEN exposure on selected lung findings in middle-aged B6C3F1 offspring at PND290

	Treatment	BALT Hyperplasia Score*	Muc Score	PBII Score	PVII Score	PEL Score	SeE Score	Comments:
Female	VH	1	0	0	0	0	0	Normal, Scattered nuetrophil infiltration
	VH	1	0	0	1	0	0	Normal, Perivascular inflammatory foci
	VH	2	1	0	0	0	0	Goblet cell hyperplasia
	VH	0	3	2	2	2	0	Lots of eosinophils
	VH	NA	3	2	3	2	2	Lots of eosinophils,Trickling inflammatory infiltrates shallowly into adjacent alveoli, acute vascular injury
	GEN	NA	2	1	2	1	1	Few eosinophils
	GEN	NA	2	1	1	1	1	Few eosinophils
	GEN	1	0	0	0	0	0	Normal, Scattered nuetrophil infiltration
	GEN	NA	1	0	0.5	0	0	Lots of eosinophils, normal vessels
	GEN	1	0	0	0	0	0	Single PV focus, no eosin
Male	VH	1	0	0	0	0	0	Normal
	VH	1	0	0	0	0	0	Normal
	VH	1	0	0	0	0	0	Normal
	VH	1	0	0	0	0	0	Normal
	VH	0	0	0	0	0	0	Normal, no score
	GEN	0	0	0	0	0	0	Normal, no score
	GEN	0	0	0	0	0	0	Normal, no score
	GEN	0	0	0	0	0	0	Normal, no score
	GEN	NA	1	1	1	1	1	Many Eosinophil
	GEN	0	0	0	0	0	0	Normal, no score

PBII Peribronchial Inflammatory Infiltrate

PVII PerivascularInflammatory Infiltrate

PEL Prominent Endothelial Lining

SeE Subendothelial eosinophils

BALT Bronchi-associated lymphoid tissue

Muc Mucin producing cells

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*Likely to be irrevelant