

Female mice are more susceptible to the development of allergic airway inflammation than male mice

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Summary

Background In humans the prevalence of asthma is higher among females than among males after puberty. The reason for this phenomenon is not clear.

Objective We tested the hypothesis that female mice are more susceptible to the development of allergic asthma than male mice and studied allergic immune responses in the lung.

Methods We compared allergic airway inflammation, i.e. methacholine (MCh) responsiveness, serum IgE, and cytokines, and the number of the different leucocytes in lungs of male and female BALB/c mice, twice-sensitized to ovalbumin (OVA) and subsequently challenged with OVA (OVA-mice) or phosphate-buffered saline (PBS-mice) aerosols on days 24–26, 30, and 31.

Results OVA challenge significantly increased MCh responsiveness, numbers of eosinophils, CD4⁺ T cells, CD4⁺/CD25⁺ T cells, B cells, and levels of Thelper (Th)2 cytokines, total, and OVA-specific IgE. There was, however, also an effect of gender, with female mice responding to OVA challenges with higher numbers of eosinophils, CD4⁺ T cells, B cells, and levels of IL-4, IL-13, IFN- γ , total, and OVA-specific IgE than male mice. In contrast, female PBS-mice had significantly lower percentages of regulatory CD4⁺/CD25⁺ T cells than males (females $4.2 \pm 0.2\%$ vs. males $5.3 \pm 0.1\%$ of CD4⁺ T cells, $P < 0.05$).

Conclusion Female mice develop a more pronounced type of allergic airway inflammation than male mice after OVA challenge. The reduced percentage of regulatory T cells in the lungs of female PBS-mice may indicate that the level of these cells in the lung during the sensitization phase is important for the development and/or progression of an allergic immune response after multiple OVA challenges.

Keywords airway hyper-responsiveness, allergy, asthma, eosinophils, females, gender, IgE, ovalbumin, regulatory T cells, sex hormones

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Introduction

A striking common feature of many autoimmune and inflammatory diseases in humans and experimental animals is that females are more susceptible to specific immunological disorders than males [1, 2]. In general, females have a more pronounced B cell-mediated immunity than age-matched males, and therefore have higher Ig levels and stronger antibody responses to various foreign antigens [3, 4]. Gender differences in T cell-mediated immunity also exist, but these appear to be more complex. Females tend to secrete higher levels of IL-4, IFN- γ , and IL-1 and have higher CD4/CD8 ratios [5, 6].

Many studies have shown a female preponderance for adult asthma [7, 8]. Asthma is a widely prevalent chronic

inflammatory disease of the airways, characterized by a dysregulated type-2 immune response to inhaled antigens. This leads to the production of IgE, infiltration of T cells and eosinophils in the airway wall and lung tissue, airway hyper-responsiveness (AHR), mucus cell hyperplasia, and airway tissue remodelling.

Recently, three studies have suggested that female mice may be more susceptible to some aspects of allergic airway inflammation than male mice [9–11]. Seymour et al. [9] reported higher total and ovalbumin (OVA)-specific IgE levels in female mice sensitized and challenged with OVA as compared with male mice exposed to the same protocol. This was confirmed by Corteling and Trifileff [10], but they did not find gender differences in the number of eosinophils in bronchoalveolar lavage fluid (BALF). Hayashi et al. [11], however, did find increased numbers of eosinophils in the BALF of female mice sensitized and challenged with OVA as compared with similar male mice. These studies, that did not investigate allergic inflammation in the lung tissue compartment, provided somewhat conflicting results. It has been

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demonstrated previously that changes in BALF do not closely mirror the situation in lung tissue [12]. We therefore hypothesized that female mice are more sensitive to the development of allergic inflammation in lung tissue than male mice. To study this, a mouse model of allergic airway inflammation was employed to characterize the local immune response in the lungs of male and female mice. In lung homogenates, we studied the number of B cells, different subsets of T cells, and cytokine concentrations of mice with and without allergic airway inflammation.

Methods

Animals

Male and female BALB/c mice (aged 8–10 weeks) were obtained from Harlan (Zeist, the Netherlands) and were held under specific pathogen-free conditions in groups of six mice per cage. The experiments, approved by the local Committee on Animal Experimentation, were performed under strict governmental and international guidelines on animal experimentation.

Sensitization and provocation procedures

Both male and female mice were sensitized to OVA on days 1 and 14 by intraperitoneal injections of 10 µg OVA (Grade V, Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands), emulsified in 1.5 mg aluminium hydroxide (Aluminject, Pierce Chemical, Etten-Leur, the Netherlands), and diluted to 200 µL with phosphate buffered saline (PBS). They were subsequently challenged with 1% OVA aerosols in PBS (OVA-mice) or PBS aerosols (PBS-mice) for 20 min on days 24, 25, 26, 30, and 31 (Fig. 1) as described previously [13].

Male and female PBS and OVA study groups consisted of 12 mice each, and methacholine (MCh) responsiveness (as a substitute measure of AHR) was assessed on day 31. On day 32, six out of 12 animals of each group were killed to isolate inflammatory cells from lung tissue and collect BALF. The remaining six animals of each group were killed for histological analysis of lung tissue. Total and OVA-specific serum IgE concentrations were assessed on days 1 and 32.

Assessment of methacholine responsiveness

MCh responsiveness was assessed as changes in enhanced pause (Penh) in conscious, spontaneously breathing animals using a whole-body plethysmography system (Buxco Electronics, Petersfield, UK) as described previously [13]. Although there has been discussion about using this method to study

lung mechanics [14], Adler et al. [15] showed that for BALB/c mice exposed to MCh, Penh correlates with resistance as measured with invasive techniques. It should, however, not be used to compare different strains or as a more general measure of airway function. We have therefore restricted the use to comparing MCh responsiveness between OVA and PBS-exposed animals as a substitute measure of AHR.

From the dose–response curves to doubling concentrations of MCh (0–64 mg/mL), the provocative concentration causing a 300% increase in the baseline Penh (PC300) was calculated using GraphPad Prism software (V3.1, GraphPad Software Inc., San Diego, CA, USA). In those cases in which the 300% increase was not reached with the highest concentration of 64 mg/mL MCh, a provocative concentration of 128 mg/mL was assigned.

Measurement of serum immunoglobulin E (total and ovalbumin-specific)

Total and OVA-specific serum IgE were measured by ELISA as described previously [13].

Bronchoalveolar lavage fluid

BALF was collected and processed for cytoplots as described previously [13].

Cytokines

Concentrations of IL-4, IL-5, IL-10, IL-13, IFN-γ, and regulated upon activation, normal T cell expressed and secreted (RANTES) in lung tissue were measured with a multiplex ELISA system (Lincplex Systems, St Charles, MO, USA). Lung tissue was homogenized in 50 mM Tris-HCl buffer, containing 150 mM NaCl and 0.002% Tween-20 (pH 7.5), and centrifuged at 12 000 *g* for 10 min to remove any insoluble material. Supernatants were subsequently stored at –80 °C until further analysis.

Cell preparation and lung digestion

Single-cell suspensions were obtained from lungs for flow cytometric analysis and preparation of cytoplots as described previously [16]. Cells were quantified using a Coulter Counter Z1 (Coulter, Hialeah, FL, USA).

Flow cytometric analysis

Expressions of CD3, CD4, CD25, and B220 cell surface markers on isolated leucocytes were examined with four-colour flow cytometry to determine frequencies of B cells and T cell subsets. Isolated lung tissue leucocytes were stained with a combination of directly conjugated antibodies directed against these cell surface markers as described previously [16]. All antibodies were obtained from Pharmingen and were conjugated with one of the following commonly used fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), and allophycocyanin (APC). Frequencies of B cells and T cell subsets were based on the label combinations: CD3-APC, CD4-PE, B220-PerCP, and CD25-FITC. Cell populations (4×10^4 events) were analysed using an Epics Elite flow

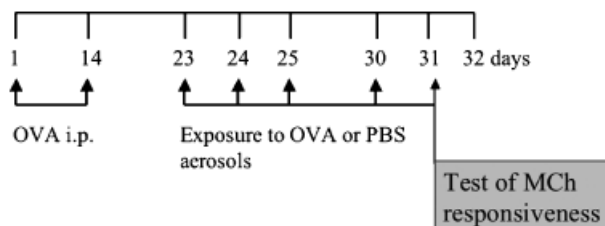


Fig. 1. Experimental design of the study (MCh: methacholine).

cytometer (Coulter Epics, Hialeah, FL, USA) and data analysis was performed using FlowJo software (ThreeStar, San Carlos, CA, USA).

Histology

Formalin-fixed (10%), paraffin-embedded lung sections of 3 µm were stained with haematoxylin and eosin (HE) for morphological evaluation of lung tissue.

Macrophages were identified with anti-MAC3 (BD Biosciences, Alphen a/d Rijn, the Netherlands) on paraffin-embedded lung sections and neutrophils were identified with anti-GR-1 (BD Biosciences) on 4 µm sections of frozen lung tissue. Both antibodies were followed by a biotinylated goat-anti-rat antibody (SBA, Uithoorn, the Netherlands). This complex was subsequently visualized with a streptavidin alkaline phosphatase conjugate (DAKO, Glostrup, Denmark) and Fast Red chromogen (DAKO).

Eosinophils were determined in 4 µm sections of frozen lung tissue and in cytospsots of BALF by staining for cyanide-resistant endogenous peroxidase activity with diaminobenzidine (Sigma). Eosinophils in lung tissue were quantified in a blinded manner by two independent observers by morphometric analysis using Leica QWin image analysis software (Leica Microsystems BV, Rijswijk, the Netherlands). The total area of a lung section stained positive for endogenous peroxidase (excluding vessels and airways) was measured and related to the total area of the section and subsequently expressed as volume percentages. To determine the percentage of eosinophils in cytospsots of BALF, the number of peroxidase-positive cells was counted in a total of 500 cells by two independent observers in a blinded manner.

Statistical analysis

Homogeneity of variances was checked with Levene's test and data were log transformed when the variances were not homogeneously distributed. Two-way analysis of variance (ANOVA) was used to establish the statistical significance of differences in terms of gender and the effect of OVA challenge for each parameter. A Sidak multiple comparison test was used for *post hoc* analysis when specific experimental groups were compared (as indicated in the text). A value of $P < 0.05$ was considered significant. Data analysis was performed with S-Plus software (V6.0, Insightful, Hampshire, UK). Measured values are presented as medians unless stated otherwise.

Results

General histopathology

No obvious differences in histology were found between lung tissue of male and female PBS-mice (data not shown). They had the same numbers of macrophages, neutrophils, and eosinophils in lung tissue and displayed no signs of inflammation. After OVA challenges, both male and female mice demonstrated substantial pulmonary inflammation with infiltrating eosinophils, neutrophils, and macrophages (Figs 2a and b). Female mice showed a more severe type of airway inflammation than male mice with more and bigger

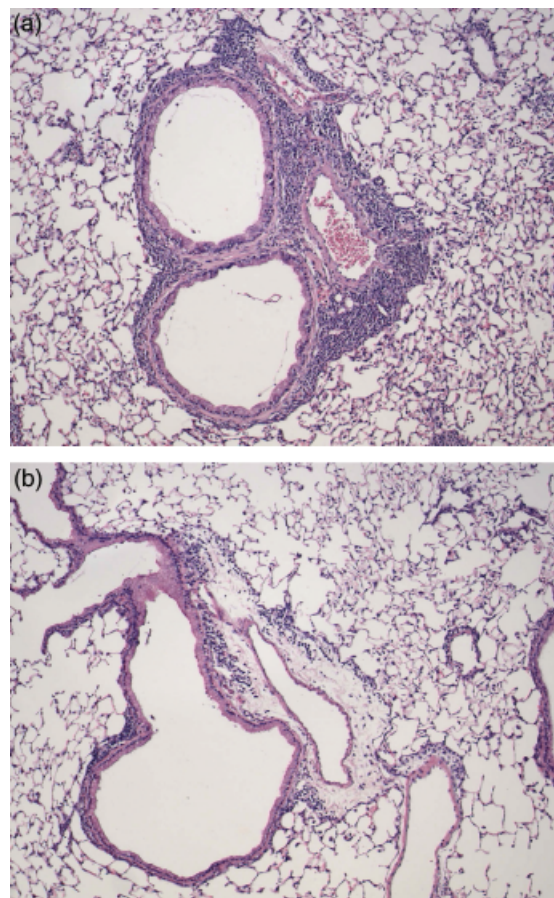


Fig. 2. Female mice (a) respond to ovalbumin (OVA) challenges with a more severe type of airway inflammation than male mice (b). Representative photomicrographs of haematoxylin and eosin-stained lung sections from male and female OVA-mice (magnification $\times 25$).

inflammatory infiltrates and more infiltrating eosinophils, neutrophils, and macrophages.

More eosinophils in bronchoalveolar lavage fluid and lung tissue of female ovalbumin-mice than in male ovalbumin-mice

OVA challenge significantly increased the number of leucocytes in BALF of female mice (6 ± 1 vs. $314 \pm 48 \times 10^4$ cells/mL for PBS-mice vs. OVA-mice, respectively), whereas in male mice such an effect was not seen (5 ± 1 vs. $6 \pm 2 \times 10^4$ cells/mL for PBS-mice vs. OVA-mice, respectively).

In both male and female mice, OVA challenge significantly increased the percentage of eosinophils in BALF cells (Fig. 3a). The significant interaction between the effects of OVA challenge and gender indicates that female mice responded to OVA with a significantly higher increase in eosinophils than male mice did.

For eosinophils in lung tissue the ANOVA showed a more complicated picture (Fig. 3b). The significant effect of OVA and the near-significant interaction between the effects of OVA and gender ($P = 0.08$) are caused by a significant increase of eosinophils after OVA challenge in female mice and the lack of such an effect in male mice. Female mice did have a significantly higher number of eosinophils after OVA challenge than male mice.

Higher total and ovalbumin-specific immunoglobulin E levels in females when compared with males

OVA challenge significantly increased the levels of both total and OVA-specific IgE in male and female mice (Figs 4a and b). Female mice had significantly higher total and OVA-specific IgE levels than male mice after OVA challenge.

Ovalbumin challenge increases the number of B cells in female, but not in male lung tissue

Gender, OVA challenge, and their interaction significantly affected the number of B cells in lung tissue (Fig. 5A). These effects are caused by the significant increase in B cells in

female mice after OVA challenge and the lack of such an effect in male mice.

Female phosphate buffered saline-mice have a lower percentage of CD4⁺/CD25⁺ T cells than male phosphate buffered saline-mice

The number of CD4⁺ T cells in lung tissue was also significantly affected by gender, OVA challenge and their interaction (Fig. 5b). These effects can again be accounted for by the significant increase in CD4⁺ T cells in female mice after OVA challenge and the lack of such an effect of OVA challenge in male mice.

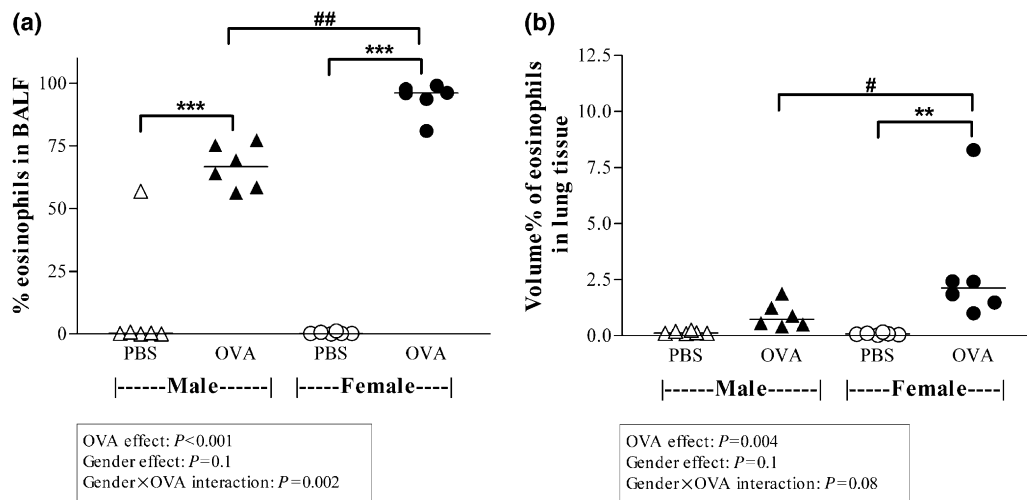


Fig. 3. (a) Ovalbumin (OVA) exposure (closed symbols) induced an increase in percentages of eosinophils in bronchoalveolar lavage fluid (BALF) of both male and female mice as compared to phosphate buffered saline (PBS)-exposed animals (open symbols). Female OVA-mice (closed circles) had higher percentages of eosinophils in BALF than male OVA-mice (closed triangles). ****Post hoc* analysis of PBS vs. OVA $P < 0.001$; ##: *post hoc* analysis of male OVA-mice vs. female OVA-mice $P < 0.01$. (b) OVA exposure induced an increase in the number of eosinophils in lung tissue (expressed as volume percentages in frozen sections of lung tissue) of female mice as compared with PBS-exposed animals. Female OVA-mice had higher numbers of eosinophils in lung tissue than male OVA-mice. ***Post hoc* analysis of PBS vs. OVA $P < 0.01$; #: *post hoc* analysis of male OVA-mice vs. female OVA-mice $P < 0.05$.

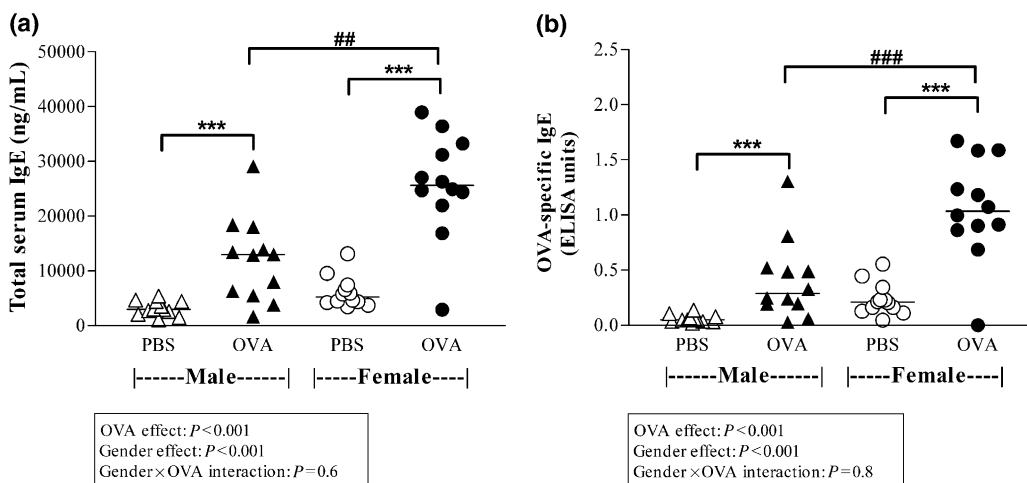


Fig. 4. (a) Ovalbumin (OVA) exposure (closed symbols) induced an increase in total serum IgE in both male and female mice as compared to phosphate buffered saline (PBS)-exposed animals (open symbols). Female OVA-mice (closed circles) had a higher total serum IgE than male OVA-mice (closed triangles). ****Post hoc* analysis of PBS vs. OVA $P < 0.001$; ##: *post hoc* analysis of male OVA-mice vs. female OVA-mice $P < 0.01$. (b) OVA exposure induced an increase in OVA-specific serum IgE in both male and female mice as compared with PBS-exposed animals. Female OVA-mice had a higher OVA-specific serum IgE than male OVA-mice. ****Post hoc* analysis of female PBS vs. OVA $P < 0.001$; **Post hoc* analysis of male PBS vs. OVA $P < 0.001$; ###: *Post hoc* analysis of male OVA-mice vs. female OVA-mice $P < 0.001$.

With respect to $CD4^+/CD25^+$ T cells, OVA challenge significantly increased the percentage of these cells in both male and female mice, and there was no effect of gender (Fig. 5c). However, there was a significant interaction between the effects of OVA challenge and gender. This interaction indicates that female mice respond with a higher increase in $CD4^+/CD25^+$ T cells than male mice after OVA challenge. This may be because

of the fact that female PBS-mice had a significantly lower percentage of $CD4^+/CD25^+$ T cells than male PBS-mice ($P < 0.05$ *post hoc* analysis), but go up to equally high numbers after OVA-challenge.

Ovalbumin challenge increases the levels of T helper2 cytokines in lung tissue of male and female mice

OVA challenge significantly increased the levels of Th2 cytokines IL-4, IL-5, and IL-13 in lung tissue of both male and female mice (Table 1). With respect to IL-4 and IL-13, both gender and the interaction between gender and OVA challenge had significant effects, indicating that female mice responded to an OVA-challenge with higher levels of IL-4 and IL-13. IL-10 levels were only significantly affected by gender, implying that female mice had higher levels of IL-10 as compared with male mice, although *post hoc* analysis did not give a significant difference between these two groups for either PBS or OVA-exposed animals.

Levels of the eosinophil chemokine RANTES were also only affected by gender, but in this case both female PBS and OVA-mice had higher levels of RANTES than their male counterparts.

Furthermore, OVA challenge significantly increased the level of the Th1 cytokine IFN- γ in lung tissue of both male and female mice. Females, again, had higher levels than males after OVA challenge.

Increased methacholine responsiveness after ovalbumin challenge in both male and female mice

In both male and female mice OVA challenge significantly increased the responsiveness to MCh (Figs 6a and b). This can also be seen from the decreased PC300 values after OVA challenge in Fig. 6c. Male PC300 values were significantly lower than female PC300 values after PBS and OVA challenge, suggesting that male mice were intrinsically more sensitive to MCh than female mice.

Discussion

With this study we have shown that female mice developed a more pronounced type of allergic airway inflammation

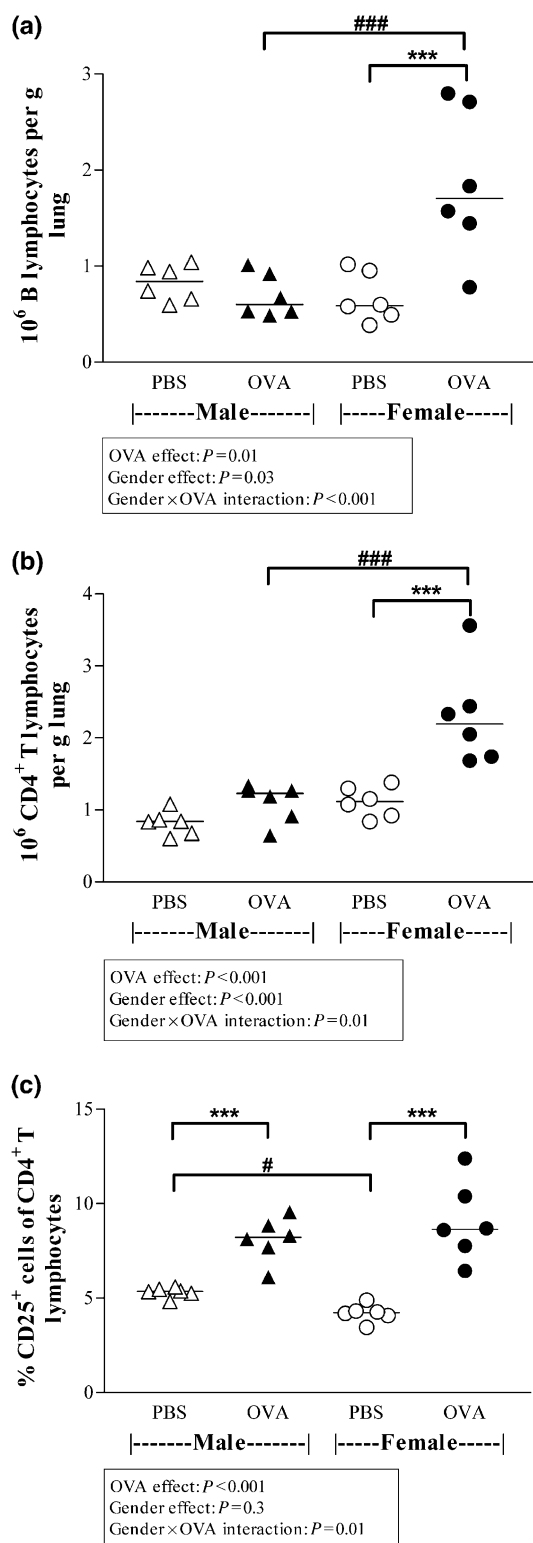


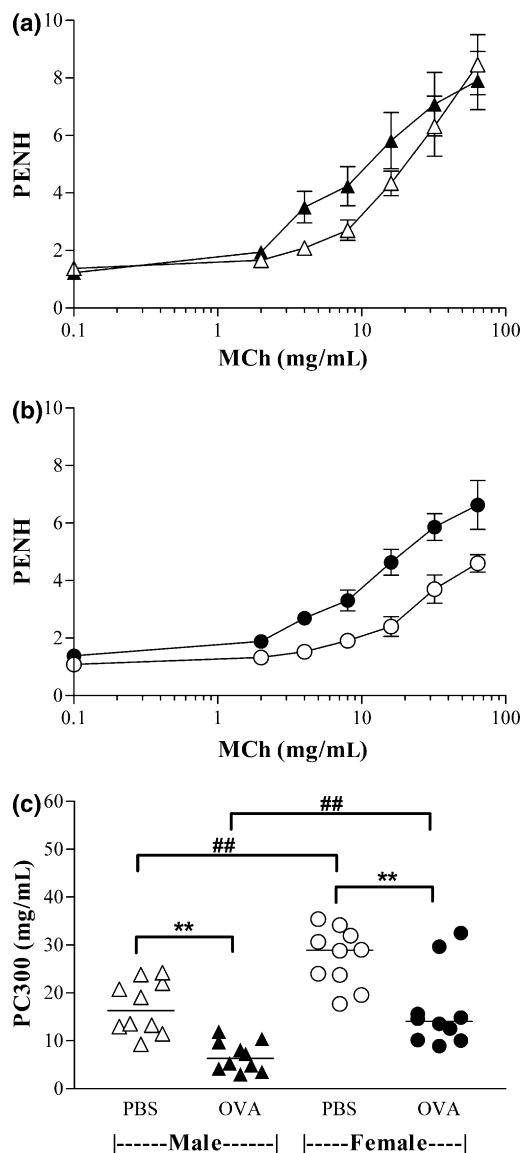
Fig. 5. (a) Ovalbumin (OVA) exposure (closed symbols) induced an increase in B cell numbers (expressed as the number of cells per gram of lung tissue) only in lung tissue of female mice as compared to phosphate buffered saline (PBS)-exposed animals (open symbols). Female OVA-mice (closed circles) also had a higher number of B cells in lung tissue than male OVA-mice (closed triangles). *** *post hoc* analysis of female PBS-mice vs. female OVA-mice $P < 0.001$; ###: *post hoc* analysis of male OVA-mice vs. female OVA-mice $P < 0.001$. (b) OVA-exposure (closed symbols) induced an increase in $CD4^+$ T cell numbers (expressed as the number of cells per gram of lung tissue) only in lung tissue of female mice as compared with PBS-exposed animals (open symbols). Female OVA-mice (closed circles) also had a higher number of $CD4^+$ T cells in lung tissue than male OVA-mice (closed triangles). *** *Post hoc* analysis of female PBS-mice vs. female OVA-mice $P < 0.001$; ###: *post hoc* analysis of male OVA-mice vs. female OVA-mice $P < 0.001$. (c) OVA exposure induced an increase in the percentage of $CD25^+/CD4^+$ T cells in lung tissue (expressed as percentage of $CD4^+$ T cells) of both male and female mice as compared with PBS-exposed animals. Female PBS-mice had a lower percentage of $CD25^+/CD4^+$ T cells in lung tissue than male PBS-mice. *** *Post hoc* analysis of PBS vs. OVA $P < 0.001$; #: *Post hoc* analysis of male PBS-mice vs. female PBS-mice $P < 0.05$.

Table 1. The effect of OVA-challenge on the cytokine levels in lung tissue in male mice and female mice ($n = 6$ per group, mean $\pm$ SEM)

Cytokines (pg/g lung tissue)	Male		Female		P-value		
	PBS	OVA	PBS	OVA	Gender effect	OVA effect	Gender $\times$ OVA interaction
IL-4	2 $\pm$ 0.2	46 $\pm$ 11*	2 $\pm$ 0.3	137 $\pm$ 11*†	<0.001	<0.01	<0.01
IL-5	5 $\pm$ 0.9	582 $\pm$ 138*	9 $\pm$ 4	972 $\pm$ 226*	0.1	<0.001	0.8
IL-10	22 $\pm$ 4	26 $\pm$ 4	41 $\pm$ 20	64 $\pm$ 10	0.02	0.3	0.4
IL-13	12 $\pm$ 1	73 $\pm$ 29*	12 $\pm$ 2	160 $\pm$ 23*‡	0.03	<0.001	0.02
RANTES	60 $\pm$ 5	85 $\pm$ 15	127 $\pm$ 11	166 $\pm$ 29‡	<0.001	0.08	0.7
INF- γ	7 $\pm$ 0.7	12 $\pm$ 2	19 $\pm$ 12§	50 $\pm$ 4¶‡	<0.001	<0.01	0.06

*Post hoc analysis of male PBS vs. male OVA or female PBS vs. female OVA $P < 0.001$. †Post hoc analysis of male OVA vs. female OVA $P < 0.001$. ‡Post hoc analysis of male OVA vs. female OVA $P < 0.01$. §Post hoc analysis of male PBS vs. female PBS $P < 0.05$. ¶Post hoc analysis of male PBS vs. male OVA or female PBS vs. female OVA $P < 0.01$.

OVA, ovalbumin; PBS, phosphate buffered saline; RANTES, regulated upon activation, normal T cell expressed and secreted.



after OVA challenge than male mice, as judged by the elevated numbers of eosinophils, B cells, CD4⁺ T cells, levels of IL-4 and IL-13 in lung tissue, and elevated total and OVA-specific IgE levels. These findings cannot be explained by the fact that female mice were sensitized with a relatively higher dose of OVA intraperitoneally because of their lower body weight (male vs. female mice: 27 $\pm$ 1 vs. 21 $\pm$ 1 g), since Sakai et al. [17] showed that a higher sensitization dose resulted in an attenuation of asthma symptoms in BALB/c mice. Based on the results of this study we may even be underestimating the difference between male and female mice in our protocol.

Our study is in accordance with the study of Hayashi et al. [11], who also found a larger number of eosinophils in BALF of female mice than in male mice. However, a study by Corteling and Trifileff [10] only showed a trend towards elevated eosinophil numbers in BALF of female mice as compared with males. This may have been the result of a difference in protocol, as Corteling et al. challenged their animals through OVA-aerosol inhalation and Hayashi et al. intranasally. Moreover, both studies did not investigate the lung tissue compartment. We studied eosinophils in two compartments of the lung (i.e. BALF and lung tissue) and found significantly higher numbers of eosinophils for females in BALF and lung tissue after OVA challenge. These increased eosinophil numbers in females were accompanied with increased levels of IL-5 and RANTES in the lung, both of which are important in the recruitment of eosinophils to the lung [18, 19]. We think we can therefore safely conclude that female mice respond with higher eosinophil numbers than males to the induction of allergic airway inflammation.

The elevated total and OVA-specific IgE levels in female OVA-mice are in good agreement with the results of both Seymour et al. [11] and Corteling and Trifileff [10], who found elevated levels of both types of IgE in female mice

Fig. 6. (a) Ovalbumin (OVA) exposure (closed triangles) induced an increase in the sensitivity for methacholine (MCh) in male mice as compared to phosphate buffered saline (PBS) exposure (open triangles). Values presented as means $\pm$ SEM. (b) OVA exposure (closed circles) induced an increase in the sensitivity for MCh in female mice as compared to PBS exposure (open circles). Values presented as means $\pm$ SEM. (c) Matching PC300 values as calculated from the MCh dose-response curves. Each group consisted of 12 mice. **: Post hoc analysis of PBS vs. OVA $P < 0.01$; ##: Post hoc analysis of male mice vs. female mice $P < 0.01$.

Table 2. The equation for the calculation of Penh and the measured T_E -values for male and female mice (PBS-challenged) during a 10-min baseline assessment of Penh (values represented as means $\pm$ SEM)

$\text{Penh} = \left(\frac{P_{ip}}{P_{ep}} \right) \times \left(\frac{T_E - T_R}{T_R} \right)$	Male PBS	Female PBS
T_E (ms)	$0.089 \pm 0.004^*$	0.078 ± 0.002

* $P < 0.05$ vs. female PBS as tested with a Mann–Whitney test.

Pip, peak inspiratory pressure; Pep, peak expiratory pressure; T_E , expiratory time; T_R , time interval that encompasses 63% of the integrated expiratory pressure signal; PBS, phosphate buffered saline.

sensitized and challenged with OVA as compared with males treated identically. The amplified levels of total and OVA-specific IgE in females were accompanied by increased levels of both IL-4 and IL-13. Both of these cytokines have been implicated in the production of IgE [20, 21].

Why female mice respond with higher levels of eosinophils, $CD4^+$ T cells, B cells, cytokines, and IgE remains an intriguing question. An interesting observation in this respect is the finding that female mice exposed to PBS have less $CD4^+/CD25^+$ T cells in lung tissue than male PBS-mice. The expression of CD4 and CD25 on T cells in a non-activated environment, such as the lungs of mice exposed to PBS, is a phenotype for naturally occurring regulatory T cells. These cells are thought to suppress immune responses through cell–cell contact rather than cytokine production, in contrast to the adaptive regulatory T cells that work through production of IL-10 and transforming growth factor- β [22, 23]. The lower percentage of regulatory $CD4^+/CD25^+$ T cells that we found in the lungs of female mice exposed to PBS may indicate that healthy female animals are less protected against inflammatory stimuli such as OVA challenges than male mice. Hadeiba and Locksley [24] recently published that lung $CD4^+/CD25^+$ regulatory T cells suppress type 2 immune responses, leading to decreased IgE levels and eosinophil numbers, but not reduced AHR, which correlates well with our study.

We cannot tell whether the lower number of regulatory T cells persists in female mice after OVA challenges. Under inflammatory conditions, such as allergic airway inflammation induced by OVA challenges, the expression of CD4 and CD25 can also represent activated $CD4^+$ T cells. As we did not further specify the regulatory phenotype for the T cells isolated from the OVA-exposed mice, we cannot distinguish between activated $CD4^+$ T cells and regulatory T cells in these animals. The increase in B cells in females after OVA challenge, linked with a corresponding increase in total and OVA-specific IgE levels, does point towards an increase in activated $CD4^+$ T cells. This is confirmed by the corresponding increase in cytokines and chemokines secreted by, among others, activated T cells: IL-4, IL-5, IL-10, IL-13, IFN- γ , and RANTES.

The higher basal levels of IFN- γ , RANTES, and IL-10 in the PBS-challenged females as compared with the males may be caused by a lack of regulatory control of effector T cells and a subsequent basal production of these cytokines from effector T cells.

These results warrant further research into the exact nature of the phenotype of the $CD4^+$ T cells in lungs of male and female mice during allergic inflammation. It would also be interesting to investigate whether the lower number

of regulatory T cells before OVA challenges in females actually influences the progression of the allergic airway inflammation.

There are several possible explanations for immune diamorphism as found in asthma. There seems to be a role for sex hormones in the pathogenesis of asthma. About 30–40% of menstruating female asthmatics experience perimenstrual worsening [25], and asthma incidence declines in females at menopause when compared with males and younger females [7]. Several animal models have suggested that progesterone and oestrogen directly affect the lungs by relaxing bronchial smooth muscle [26, 27]. However, progesterone has also been proven to have an aggravating effect on the phenotype of eosinophilic airway inflammation in male mice [28]. Little has been published, although, on the subject of regulatory T cells and gender. A study from Polanczyk et al. [29] showed that oestrogen drives expansion of the $CD4^+/CD25^+$ regulatory T cell compartment. The amount of estrogen used, however, was similar to a state of pregnancy, which is quite different from normal physiological concentrations of oestrogen. Further studies will be required to determine how sex hormones can interact with the immune system and modify inflammatory responses during the development of asthma.

Another possible explanation for the gender differences found in mice is the effect of stress because of social housing. Stress is a well-known factor influencing the immune system [30, 31]. Palanza et al. [32] have recently shown that social housing of male mice induces a higher level of anxiety than individual housing, whereas in females the opposite was true. The lower number of $CD4^+$ T cells found in male mice could be explained by colony conditions that are more stressful for male mice than for female mice, as was found by Stefanski et al. [33] for subdominant male rats. A similar explanation can be found for the difference in $CD4^+/CD25^+$ T cells between male and female PBS-mice: acute-cold/restraint stress was found to increase $CD4^+/CD25^+$ T cells in BALB/c mice [34].

The effects of stress may also explain why it appears that male mice (with PBS- or OVA challenge) are more sensitive to MCh than female mice. We tested responsiveness to MCh with unrestrained whole body plethysmography and the response to MCh is measured as the enhanced Pause or Penh (Table 2). The absolute Penh differences between males and females are therefore probably caused by an increase in the expiration time (T_E) in male mice because of the stress of social housing. Fokkema et al. [35] found that social stress induces a prolonged expiration in male rats. If we assume that this is also true for mice, then it may mean that the social housing we applied induces stress and prolonged expiration times in males, but not in females. To test this we recorded a 10-min baseline Penh of our male and female PBS-mice and also assessed the mean expiration time over that period (Table 2) and found this assumption to be true. Male mice had significantly longer expiration times than females, and as a result higher Penh values as well.

In conclusion, we have shown that female mice are more sensitive to the development of allergic airway inflammation than male mice, which was accompanied by a reduced percentage of pulmonary $CD4^+/CD25^+$ regulatory T cells in female PBS-mice as compared with male PBS-mice. This

lower percentage of regulatory CD4⁺/CD25⁺ T cells in the lungs of female PBS-mice may indicate that the level of these cells in the lung during the induction of airway inflammation is important for the development and/or progression of the allergic immune response. Further studies in humans will have to show whether these mechanisms also play a role in gender differences in human asthmatics.

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References

- 1 Ansar Ahmed S, Penhale WJ, Talal N. Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. *Am J Pathol* 1985; 121:531–51.
- 2 Grossman C. Possible underlying mechanisms of sexual dimorphism in the immune response, fact and hypothesis. *J Steroid Biochem* 1989; 34:241–51.
- 3 Whitacre CC, Reingold SC, O'Looney PA. A gender gap in autoimmunity. *Science* 1999; 283:1277–8.
- 4 Da Silva JA. Sex hormones and glucocorticoids: interactions with the immune system. *Ann N Y Acad Sci* 1999; 876:102–17; discussion 17–8.
- 5 Amadori A, Zamarchi R, De Silvestro G et al. Genetic control of the CD4/CD8 T-cell ratio in humans. *Nat Med* 1995; 1: 1279–83.
- 6 Grossman CJ. Regulation of the immune system by sex steroids. *Endocr Rev* 1984; 5:435–55.
- 7 Watson L, Boezen HM, Postma DS. Differences between males and females in the natural history of asthma and COPD. In: Wouters EFM, ed. *European respiratory monograph*. Sheffield, UK: European Respiratory Society, 2003; 50–73.
- 8 Venn A, Lewis S, Cooper M, Hill J, Britton J. Questionnaire study of effect of sex and age on the prevalence of wheeze and asthma in adolescence. *BMJ* 1998; 316:1945–6.
- 9 Seymour BW, Friebertshauser KE, Peake JL, Pinkerton KE, Coffman RL, Gershwin LJ. Gender differences in the allergic response of mice neonatally exposed to environmental tobacco smoke. *Dev Immunol* 2002; 9:47–54.
- 10 Corteling R, Trifilieff A. Gender comparison in a murine model of allergen-driven airway inflammation and the response to budesonide treatment. *BMC Pharmacol* 2004; 4:4.
- 11 Hayashi T, Adachi Y, Hasegawa K, Morimoto M. Less sensitivity for late airway inflammation in males than females in BALB/c mice. *Scand J Immunol* 2003; 57:562–7.
- 12 Rutgers SR, Timens W, Kaufmann HF, van der Mark TW, Koeter GH, Postma DS. Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD. *Eur Respir J* 2000; 15:109–15.
- 13 Melgert BN, Postma DS, Geerlings M et al. Short-term smoke exposure attenuates ovalbumin-induced airway inflammation in allergic mice. *Am J Respir Cell Mol Biol* 2004; 30:880–5.
- 14 Bates J, Irvin C, Brusasco V et al. The use and misuse of Penh in animal models of lung disease. *Am J Respir Cell Mol Biol* 2004; 31:373–4.
- 15 Adler A, Cieslewicz G, Irvin CG. Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57Bl/6 mice. *J Appl Physiol* 2004; 97:286–92.
- 16 Hylkema MN, Hoekstra MO, Luinge M, Timens W. The strength of the OVA-induced airway inflammation in rats is strain dependent. *Clin Exp Immunol* 2002; 129:390–6.
- 17 Sakai K, Yokoyama A, Kohno N, Hiwada K. Effect of different sensitizing doses of antigen in a murine model of atopic asthma. *Clin Exp Immunol* 1999; 118:9–15.
- 18 Leckie MJ. Anti-interleukin-5 monoclonal antibodies: preclinical and clinical evidence in asthma models. *Am J Respir Med* 2003; 2:245–59.
- 19 Gonzalo JA, Lloyd CM, Wen D et al. The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J Exp Med* 1998; 188:157–67.
- 20 Wynn TA. IL-13 effector functions. *Annu Rev Immunol* 2003; 21:425–56.
- 21 Renaud JC. New insights into the role of cytokines in asthma. *J Clin Pathol* 2001; 54:577–89.
- 22 Stassen M, Jonuleit H, Muller C et al. Differential regulatory capacity of CD25⁺ T regulatory cells and preactivated CD25⁺ T regulatory cells on development, functional activation, and proliferation of Th2 cells. *J Immunol* 2004; 173:267–74.
- 23 Akbari O, Stock P, DeKruyff RH, Umetsu DT. Role of regulatory T cells in allergy and asthma. *Curr Opin Immunol* 2003; 15:627–33.
- 24 Hadeiba H, Locksley RM. Lung CD25 CD4 regulatory T cells suppress type 2 immune responses but not bronchial hyperreactivity. *J Immunol* 2003; 170:5502–10.
- 25 Vrieze A, Postma DS, Kerstjens HA. Perimenstrual asthma: a syndrome without known cause or cure. *J Allergy Clin Immunol* 2003; 112:271–82.
- 26 Foster PS, Goldie RG, Paterson JW. Effect of steroids on beta-adrenoceptor-mediated relaxation of pig bronchus. *Br J Pharmacol* 1983; 78:441–5.
- 27 Perusquia M, Hernandez R, Montano LM, Villalon CM, Campos MG. Inhibitory effect of sex steroids on guinea-pig airway smooth muscle contractions. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1997; 118:5–10.
- 28 Hellings PW, Vandekerckhove P, Claeys R, Billen J, Kasran A, Ceuppens JL. Progesterone increases airway eosinophilia and hyper-responsiveness in a murine model of allergic asthma. *Clin Exp Allergy* 2003; 33:1457–63.
- 29 Polanczyk MJ, Carson BD, Subramanian S et al. Estrogen drives expansion of the CD4⁺CD25⁺ regulatory T cell compartment. *J Immunol* 2004; 173:2227–30.
- 30 McEwen BS, Seeman T. Protective and damaging effects of mediators of stress. Elaborating and testing the concepts of allostasis and allostatic load. *Ann N Y Acad Sci* 1999; 896:30–47.
- 31 Biondi M, Zannino LG. Psychological stress, neuroimmunomodulation, and susceptibility to infectious diseases in animals and man: a review. *Psychother Psychosom* 1997; 66:3–26.
- 32 Palanza P, Gioiosa L, Parmigiani S. Social stress in mice: gender differences and effects of estrous cycle and social dominance. *Physiol Behav* 2001; 73:411–20.
- 33 Stefanski V, Knopf G, Schulz S. Long-term colony housing in Long Evans rats: immunological, hormonal, and behavioral consequences. *J Neuroimmunol* 2001; 114:122–30.
- 34 Cao L, Hudson CA, Lawrence DA. Immune changes during cold/restraint stress-induced inhibition of host resistance to *Listeria*. *Toxicol Sci* 2003; 74:325–34.
- 35 Fokkema DS, Koolhaas JM, van der Meulen J, Schoemaker R. Social stress induced pressure breathing and consequent blood pressure oscillation. *Life Sci* 1986; 38:569–75.