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Sex-related splenocyte function in a murine model of allergic asthma

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Clinical and
Experimental
Allergy

Summary

Background The prevalence and severity of asthma are higher among boys than girls, but the ratios are reversed after puberty. These observations strongly suggest that sex hormones have a role in the pathogenesis of the disease. However, the mechanisms underlying the gender differences in asthma are not fully understood.

Objective The aim of this study was to investigate sex differences in allergic inflammation in terms of immune function.

Methods Male and female C57BL/6 mice were sensitized and challenged with ovalbumin (OVA). OVA-specific IgE in serum and airway inflammation were compared between sexes. Splenocytes from OVA-sensitized male or female donor mice were transferred to male or female naïve recipient mice. Subsequently, the recipient mice were challenged, followed by the evaluation of OVA-specific IgE and airway inflammation. Cytokines secreted from splenocytes of the sensitized mice were measured.

Results The levels of OVA-specific IgE and the allergen-induced airway inflammation were higher in female than in the male mice. The contents of T-helper type 2 (Th2) cytokines, IL-4, IL-5 and IL-13, in the bronchoalveolar lavage fluid from female mice were higher than those from male mice. The airway inflammation in female recipients transferred with splenocytes from female donors was more severe than that in any other combination of recipients and donors. Splenocytes from the sensitized female mice produced more of the Th2 cytokine, IL-5, than those from the sensitized male mice upon stimulation with OVA.

Conclusion Our findings suggest that the sex difference in allergic airway inflammation may be attributable to the sex difference in not only the hormonal environment but also in the immune cells themselves.

Keywords airway inflammation, asthma, sex difference, splenocyte, Th2 cytokine

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Introduction

Bronchial asthma is a disease characterized by chronic airway inflammation associated with the accumulation and activation of inflammatory cells, such as eosinophils and mast cells, in the bronchial wall and airway lumen. The behaviour of the inflammatory cells in asthmatic airways is tightly regulated by a network of T-helper type 2 (Th2) cytokines, such as IL-4, IL-5 and IL-13, which are produced mainly by Th2 cells. Inflammatory mediators released from these cells lead to airway narrowing and airway hyperresponsiveness, which are the pathophysiological features observed in asthmatics [1–3].

Clinical observations and epidemiologic studies indicate that in children, asthma is more prevalent in boys

than in girls, but that the ratio reverses after puberty [4]. Similarly, the admission rate for asthma in patients 2–17 years of age is higher in boys than in girls, but in patients 18–54 years of age the rate is approximately three times higher in women than in men [5]. The reversal of the male/female prevalence of asthma at puberty strongly suggests that sex hormones have a role in the pathogenesis of the disease [6]. The female sex hormones progesterone and oestradiol have been shown to have effects on several cell functions that favour the exacerbation of asthmatic airway inflammation, such as the enhancement of the differentiation of naïve Th lymphocytes towards the Th2 lineage [7], and the antigen-specific production of IL-4 and IL-5 [8, 9]. In contrast, the male sex hormone testosterone inhibited eosinophilic inflammation [10] and

mast cell degranulation [11] *in vitro*. Consistent with these findings *in vitro*, recent studies using murine models of allergic asthma have demonstrated more susceptibility to allergic airway inflammation in female mice [12–14], an enhancement of the inflammation in castrated and progesterone-administered male mice [12, 15] and a reduction of the inflammation in androgen-administered and ovariectomized female mice [16, 17]. However, the underlying mechanisms of the gender differences in the prevalence and severity of asthma are not fully understood. On the other hand, there is evidence from a human study suggesting that a genetic factor related to gender, not an environmental factor such as the presence of sex hormones, could affect the Th1/Th2 balance. Giron-Gonzalez *et al.* reported that the IFN- γ : IL-4 ratio was significantly higher in the supernatants of phytohaemagglutinin-stimulated T cell cultures from men compared with those from women, and furthermore, that the supplementation of the T cell cultures with oestradiol, progesterone or testosterone did not modify the secretion of these cytokines [18].

Therefore, we hypothesized that the gender difference in asthma symptoms may be attributable to differences not only in the hormonal environment but also in the immune cells themselves. To address this hypothesis, we examined the effects of a combination of changes in the sex hormonal environment and immune cells in each sex on antigen-induced airway inflammation, using adoptive transfer of splenocytes in a murine model of allergic asthma.

Materials and methods

Sensitization and antigen challenge protocols

Specific pathogen-free male and female C57BL/6 (B6) mice, 6–8 weeks old, were purchased from Japan SLC

(Hamamatsu, Japan). The sensitization, antigen challenge and sample collection were performed as described previously [19]. Briefly, mice were sensitized by intraperitoneal (i.p.) injections of ovalbumin (OVA) (Sigma, St Louis, MO, USA) (8 μ g/mouse) adsorbed with aluminium hydroxide (Wako Pure Chemical Industries, Osaka, Japan) on days 0 and 5. On day 17, mice were challenged with aerosolized OVA (5% in saline) or saline as a control for 1 h on two occasions 4 h apart. At 3 and 5 days after the challenge, peripheral blood, bronchoalveolar lavage (BAL) fluids and lung tissues were collected (Fig. 1a). Serum was obtained by centrifugation of peripheral blood after clotting at room temperature. Total cell numbers in the BAL fluids were counted with a haemocytometer. Smears of BAL cells prepared with a Cytospin IV (Shandon, Runcorn, UK) were stained with Diff-Quik solution (International Reagents Corp., Kobe, Japan) for differential cell counting. The percentages of cell differentials were determined by counting at least 200 cells under light microscopy. After centrifugation of the BAL fluid, supernatants were stored at -80°C for cytokine measurements by ELISA. Lung tissues were fixed with 10% buffered formaldehyde and embedded in paraffin. Four-micrometre-thick sections were stained with haematoxylin and eosin for the counting of tissue eosinophils. Eosinophils in the peribronchial area were counted in a $5.4 \times 10^{-3} \text{ mm}^2$ square, defined by a squared eyepiece, along the epithelial basement membrane [20]. At least five squares in each bronchus and five bronchi for each mouse were examined, and the average of the data was used as the result of each mouse. Counts of cells in BAL fluids and lung tissues were performed by a person unaware of the experiment. All experiments were approved by and conformed to the guidelines of the Committee of Animal Experiments at Tohoku Pharmaceutical University.

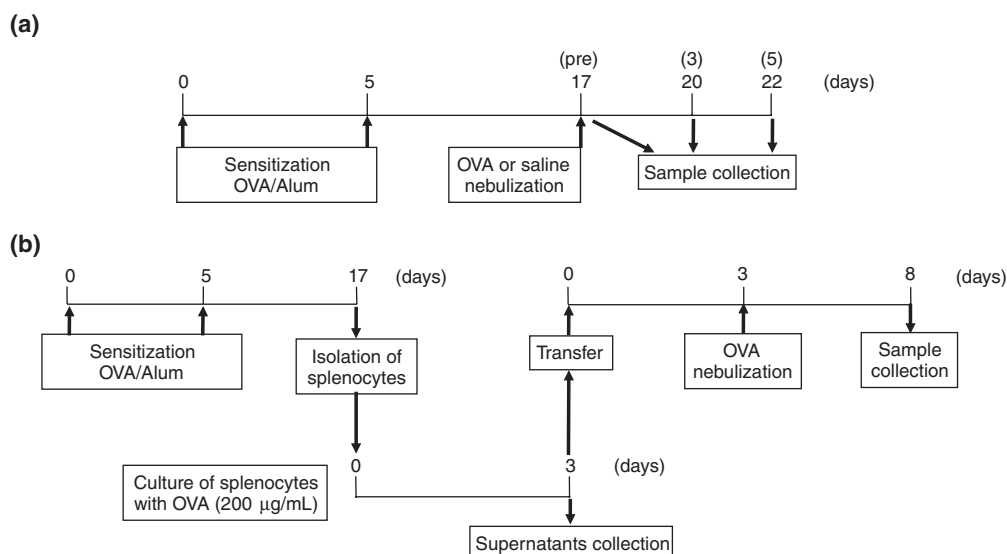


Fig. 1. Diagrammatic representation of the protocols for sensitization, antigen challenge and splenocyte transfer.

Ovalbumin-specific immunoglobulin E measurement

Serum levels of OVA-specific IgE were measured with sandwich ELISA according to the method described previously [20]. Briefly, 96-well microtitre plates (Nunc, Roskilde, Denmark) were coated with 100 µL/well of 10 µg/mL OVA in 0.05 M bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. After washing with 0.5% Tween 20/PBS and blocking with 1% bovine serum albumin (Sigma)/PBS, samples (five times diluted), pooled serum of naïve BALB/c female mice as a background (100 times diluted) and pooled serum of sensitized BALB/c female mice [19] as a standard (serially twofold diluted) were added to the wells and incubated for 2 h at 37 °C. Subsequently, plates were washed and 1 : 10 000 diluted horseradish peroxidase-conjugated goat anti-mouse IgE antibody (Bethyl Laboratories Inc., Montgomery, TX, USA) was added and incubated for 1.5 h. After a further wash, all plates were developed with tetramethylbenzidine substrate solution (BioFX Laboratories, Owing Mills, MD, USA), stopped with 1 N HCl and read at 450 nm using a Bio-Rad microplate reader (Bio-Rad, Munich, Germany). OVA-specific IgE levels in samples were related to an internal standard obtained from the pooled serum of the sensitized mice that was arbitrarily assigned as 1000 experimental units (EU)/mL.

Cytokine assays

The IL-4, IL-5, IL-13 and IFN- γ contents in the BAL fluid and culture supernatants of the splenocytes were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The sensitivity of detection was 2 pg/mL for IL-4 and IFN- γ , 7 pg/mL for IL-5 and 1.5 pg/mL for IL-13.

Preparation of splenocytes for adoptive transfer and cytokine assay

The preparation, culture and adoptive transfer of splenocytes were performed as described previously [21]. Briefly, on day 17, spleens were excised from sensitized mice, and splenocytes were disaggregated. The washed splenocytes were resuspended at 5×10^6 cells/mL in complete medium consisting of RPMI-1640 (Gibco BRL, Rockville, MD, USA) with 10% heat-inactivated fetal bovine serum (ICN Bio-medicals Inc., Aurora, OH, USA), 55 µM 2-mercaptoethanol (Wako Pure Chemical Industries), 2 mM L-glutamin (Wako Pure Chemical Industries), 50 U/mL penicillin G (Meiji Seika, Tokyo, Japan) and 50 µg/mL streptomycin sulphate (Meiji Seika). These were then cultured at 37 °C in the presence or absence of 200 µg/mL OVA. Three days after the culture, the cultured splenocytes were harvested and washed three times and resuspended at 2×10^7 cells/mL in sterile saline. Unless specified, 500 µL (1×10^7 cells)

of this suspension was injected i.p. into recipient naïve mice. Three days after the injection, the recipient mice were aerosol challenged, and 5 days after the challenge, peripheral blood, BAL fluids and lung tissues were collected and processed as mentioned above (Fig. 1b). In the preliminary experiments, freshly isolated splenocytes from OVA-sensitized mice were unable to prime naïve recipient mice to respond to a subsequent OVA inhalation, as reported previously [21]. For the cytokine assay, the culture supernatants of the splenocytes were collected 3 days after the culture. The contents of cytokines in the supernatants were measured by ELISA as mentioned above.

Data analysis

Data are presented as mean $\pm$ SD. Significant differences between two groups were determined using the non-parametric Mann-Whitney *U*-test. These analyses were performed using Prism4 (GraphPad Software, San Diego, CA, USA). A *P*-value of < 0.05 was considered to be significant.

Results

Sex differences in allergen-induced airway inflammation

In both male and female mice, the numbers of total cells, eosinophils and lymphocytes in the BAL fluid were significantly increased at 5 days after OVA inhalation compared with those before the inhalation while saline inhalation did not induce a significant increase in any type of cells (data not shown). However, the degree of increments was different between males and females. The numbers of total cells, eosinophils and lymphocytes in female mice were significantly higher than those in male mice (Fig. 2). Peribronchial infiltration of inflammatory cells, particularly eosinophils and mononuclear cells, was observed in lung tissues from OVA-challenged mice (Fig. 3a). Female mice showed a more severe type of airway inflammation than male mice, with more infiltrating eosinophils. Cell counting revealed that the number of eosinophils in female tissues was significantly greater than that in male tissues at 5 days after OVA challenge (283.6 ± 148.3 vs. 98.4 ± 78.0 cells/mm², $P < 0.05$) (Fig. 3b).

Ovalbumin-specific immunoglobulin E in serum

The levels of OVA-specific IgE in serum from OVA-sensitized mice before OVA challenge were not significantly different between males (2.5 ± 1.8 EU/mL, $n = 5$) and females (5.0 ± 4.7 EU/mL, $n = 6$). However, 5 days after OVA challenge, the levels in females were significantly higher than those in males (20.6 ± 27.0 EU/mL, $n = 12$ vs. 3.1 ± 1.1 EU/mL, $n = 9$, $P < 0.01$) (Fig. 4).

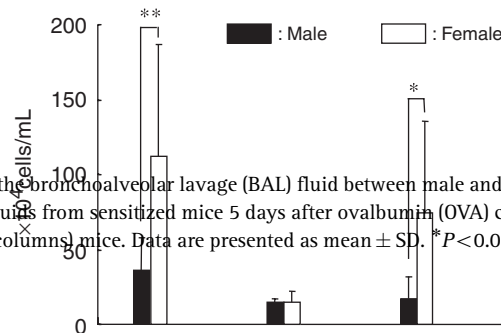
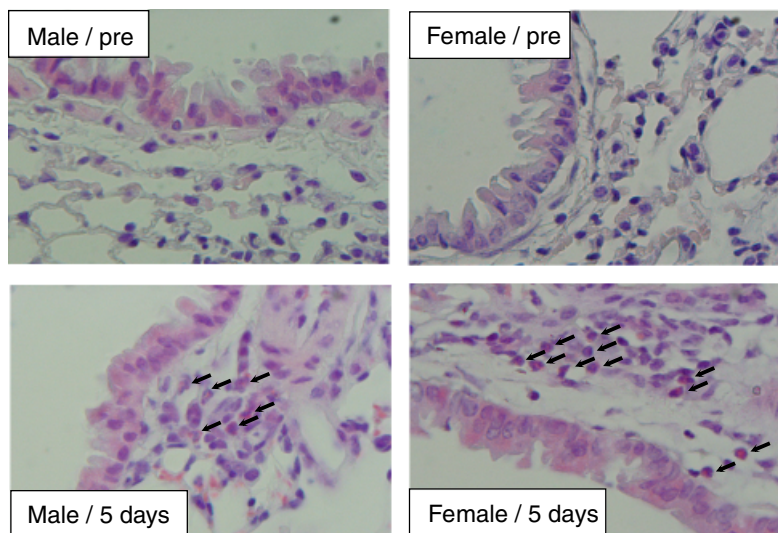


Fig. 2. Comparison of cell numbers in the bronchoalveolar lavage (BAL) fluid between male and female mice. The numbers of total cells, macrophages, eosinophils and lymphocytes in BAL fluids from sensitized mice 5 days after ovalbumin (OVA) challenge were compared between male ($n = 12$) (closed columns) and female ($n = 12$) (opened columns) mice. Data are presented as mean $\pm$ SD. * $P < 0.05$ and *** $P < 0.01$.

(a)



(b)

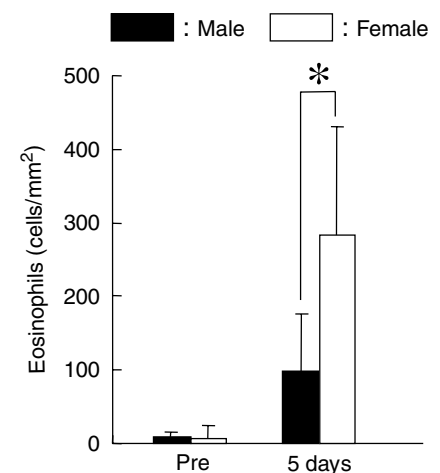


Fig. 3. Eosinophil infiltration to peribronchial area in male and female mice. (a) Pictures show lung tissues obtained from sensitized male or female mice before (pre) and 5 days after ovalbumin (OVA) challenge. $\times 1000$, haematoxylin and eosin staining. Arrows indicate eosinophils. (b) The number of eosinophils in lung tissues obtained from sensitized male (closed columns) or female (opened columns) mice before (pre) and 5 days after OVA challenge was counted as described in 'Materials and methods'. Data are presented as mean $\pm$ SD ($n = 5-10$). * $P < 0.05$.

Cytokine contents in bronchoalveolar lavage fluids after ovalbumin inhalation

A sex difference was also observed in terms of the contents of Th2 cytokines in BAL fluids from mice after OVA inhalation. The contents of IL-4, IL-5 and IL-13 in BAL fluids of female mice were significantly higher than those of male mice (IL-4: 23.5 ± 7.7 vs. 3.9 ± 2.0 pg/mL, $P < 0.01$; IL-5: 51.8 ± 18.8 vs. 13.3 ± 13.6 pg/mL, $P < 0.05$; IL-13: 79.0 ± 25.6 vs. 16.6 ± 17.2 pg/mL, $P < 0.05$) at 3 days after OVA inhalation (Fig. 5). In contrast to Th2 cytokines, the IFN- γ content in BAL fluids from OVA-challenged mice was below the sensitivity of detection in both sexes (data not shown).

Sex-mismatched adoptive transfer of splenocytes from sensitized mice

Splenocytes from OVA-sensitized female donor mice were transferred to male or female naïve recipient mice. The numbers of total cells and eosinophils in the BAL fluid from the female recipients were significantly higher than those from the male recipients at 5 days after OVA inhalation (left side in Fig. 6). On the other hand, in the case of the transfer from male donor mice to male or female naïve recipient mice, the numbers of inflammatory cells in the BAL fluid 5 days after OVA inhalation were not significantly different in any type of cells between the male and female recipients (on the right side in Fig. 6). On

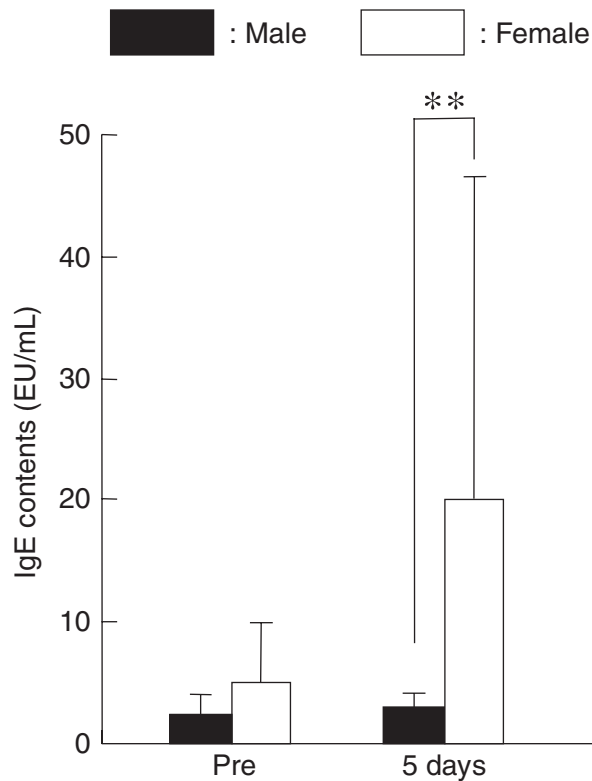


Fig. 4. Ovalbumin (OVA)-specific IgE levels in serum from male and female mice. OVA-specific IgE contents in serum obtained from sensitized male (closed columns) or female (opened columns) mice before (pre) and 5 days after OVA challenge were measured as described in 'Materials and methods'. Data are presented as mean $\pm$ SD ($n = 5-12$). ** $P < 0.01$.

the other hand, in the comparison between the donors, splenocytes from female donors caused more severe inflammation, in terms of the numbers of total cells, eosinophils and lymphocytes, than splenocytes from male donors in female recipients. However, when the recipient mice were male, there were no significant differences in any type of cells between the donors (Fig. 6). Conversely, female recipients transferred with female splenocytes exhibited the most intense allergen-induced inflammation. Saline inhalation did not significantly increase the numbers of inflammatory cells in any recipient mice compared with before the inhalation (data not shown). The numbers of eosinophils in the peribronchial area were not significantly different between the groups (Fig. 7). The levels of OVA-specific IgE were significantly different only between female donors/male recipients and male donors/male recipients (Fig. 8).

Sex differences in cytokine production by splenocytes

The content of IL-5 in the culture supernatants of female splenocytes stimulated with OVA was significantly higher than that of male splenocytes (746.1 ± 556.7 vs. 242.8 ± 262.8 pg/mL, $P < 0.01$) (Table 1). Sex differences

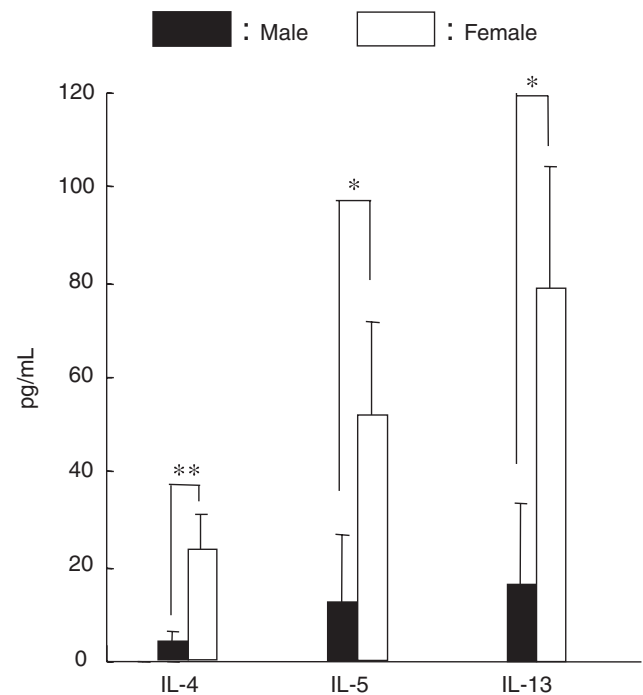


Fig. 5. Cytokine contents in bronchoalveolar lavage (BAL) fluids of sensitized and challenged mice. BAL fluids were obtained from sensitized male (closed columns) or female (opened columns) mice 3 days after ovalbumin (OVA) challenge. The contents of IL-4, IL-5 and IL-13 in the BAL fluid were measured by ELISA as described in 'Materials and methods'. Data are presented as mean $\pm$ SD ($n = 5-8$). * $P < 0.05$ and ** $P < 0.01$.

were not significant in the contents of other Th2 cytokines, IL-4 and IL-13, and the Th1 cytokine, IFN- γ . The contents of all cytokines from either male or female splenocytes cultured in the absence of OVA were much lower than those in the presence of OVA, and there was no difference in the cytokine contents between sexes (data not shown).

Discussion

In the present study, we demonstrated for the first time that the sex difference in allergic airway inflammation is attributable, at least in part, to a sex-related difference in immune function, by investigating the effects of sex-mismatched adoptive transfer of splenocytes from sensitized male and female mice and by comparing the cytokine production by female mice-derived splenocytes with male mice-derived splenocytes. In murine models of allergic asthma, a higher sensitivity for the development of allergen-induced airway inflammation in adult female mice compared with adult male mice has been demonstrated using the BALB/c strain [12-14]. We observed more severe inflammation in female mice not only in the BALB/c strain (data not shown) but also in the B6 strain

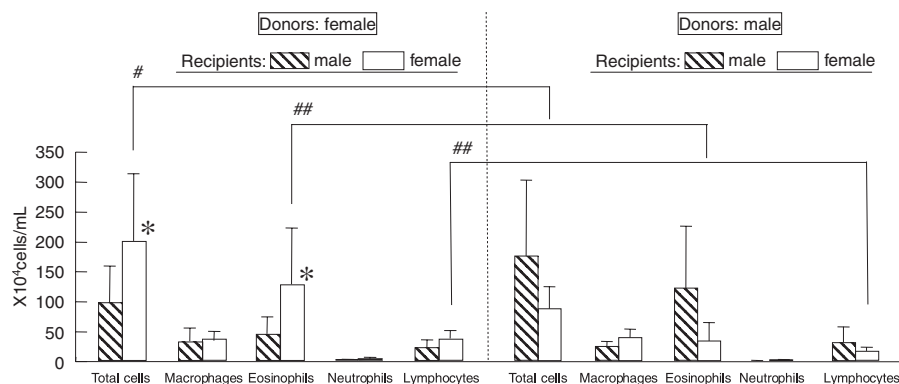


Fig. 6. Cell numbers in bronchoalveolar lavage (BAL) fluids of mice adoptively transferred with splenocytes. Splenocytes from sensitized female or male mice (donors) were transferred to naïve male (hatched columns) and female (opened columns) mice (recipients). BAL fluid was obtained from the recipient mice 5 days after ovalbumin challenge. The numbers of total cells, macrophages, eosinophils, neutrophils and lymphocytes in BAL fluids were counted as described in 'Materials and methods'. Data are presented as mean $\pm$ SD ($n = 7-9$). *, $P < 0.05$ and ** $P < 0.01$.

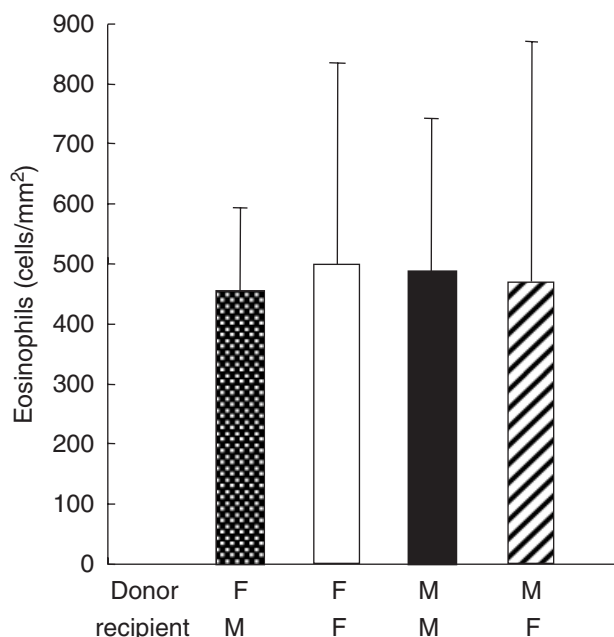


Fig. 7. Eosinophil infiltration to peribronchial area in mice adoptively transferred with splenocytes. Splenocytes were transferred as described in Fig. 6. Lung tissues were obtained from the recipient mice 5 days after ovalbumin challenge. The number of eosinophils in lung tissues was counted as described in 'Materials and methods'. Data are presented as mean $\pm$ SD ($n = 10-11$).

using the same protocol. These findings from BALB/c and B6 strains indicated that the more severe airway inflammation in female mice is not dependent on the strain.

Our results from the adoptive transfer experiments suggested that a sex-related difference in allergen-specific immune function was involved in the worsening of the airway inflammation in females. The allergen-induced airway inflammation in female mice transferred with female splenocytes (F $\rightarrow$ F) was more severe than in male mice transferred with female splenocytes (F $\rightarrow$ M) (on the

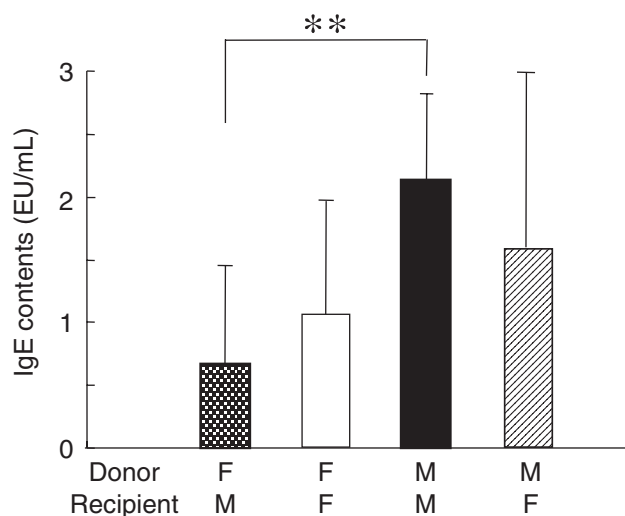


Fig. 8. Ovalbumin (OVA)-specific IgE levels in serum from mice adoptively transferred with splenocytes. Splenocytes were transferred as described in Fig. 6. OVA-specific IgE contents in serum obtained from the recipient mice 5 days after OVA challenge were measured as described in 'Materials and methods'. Data are presented as mean $\pm$ SD ($n = 6-11$). ** $P < 0.01$.

left side in Fig. 6). However, when male splenocytes were transferred, the airway inflammation was not significantly different between the female (M $\rightarrow$ F) and male (M $\rightarrow$ M) recipients (on the right side in Fig. 6). These results suggest that the hormonal environment in females is necessary but not sufficient to account for the sex difference in allergen-induced airway inflammation. On the other hand, when the effect of splenocyte transfer on the airway inflammation was compared in female recipients, female splenocyte transfer (F $\rightarrow$ F) showed more intense inflammation than male splenocyte transfer (M $\rightarrow$ F). However, in the case of male recipients (F $\rightarrow$ M and M $\rightarrow$ M), no significant difference in the airway inflammation was observed. These results suggest that

Table 1. Cytokine production by splenocytes from male and female mice

	Male	Female
IL-4 (pg/mL)	24.3 ± 10.3	18.0 ± 8.9
IL-5 (pg/mL)	242.8 ± 262.8	746.1 ± 556.7**
IL-13 (pg/mL)	3691.3 ± 2516.2	4050.0 ± 458.2
IFN- γ (pg/mL)	2475.0 ± 652.6	2533.5 ± 1082.5

** $P < 0.01$.

Mean ± SD ($n = 7-12$).

allergen-specific responses by female immune cells are necessary but not sufficient to account for the sex difference in allergen-induced airway inflammation. Together with these findings, it could be proposed that both the sex-related hormonal environment and the allergen-specific immune function are required for the development of the sex difference in allergic airway inflammation. In the current study, male mice adoptively transferred with male splenocytes had more allergen-induced airway inflammation than male mice conventionally sensitized and challenged with allergen, while there was no significant difference between transfer and conventional females. In a study using male mice, in contrast to our results, eosinophilia in BAL fluids in transfer-mice was significantly lower than that in conventional mice [22]. They transferred antigen-specific Th2 cells without the culture. The effect of adoptive transfer on allergic airway inflammation might be different between sexes and strains of mice, and types and preparation procedures of transferred cells. The reason why the differences in the numbers of peribronchial eosinophils between the groups in the adoptive transfer experiments were not significant remains elusive at present. The levels of OVA-specific IgE in any recipient were much lower than those in sensitized and challenged mice, while the levels were significantly different only between $F \rightarrow M$ and $M \rightarrow M$ mice. These results of OVA-specific IgE were consistent with previous reports in which naïve mice transferred with antigen-specific T cells were devoid of the increase of antigen-specific IgE even after antigen challenge, in contrast to actively sensitized mice [22, 23]. Therefore, in our adoptive transfer experiments, the contribution of the antigen/IgE interaction to antigen-induced airway inflammation could be excluded.

To elucidate the sex-related differences in allergen-specific immune function, we compared the antigen-stimulated Th1/Th2 cytokine synthesis by splenocytes of female and male sensitized mice. We found that the secretion of IL-5, one of the Th2 cytokines, from female splenocytes was higher than that from male splenocytes upon antigen stimulation, while the secretion of IFN- γ , one of the Th1 cytokines, was not significantly different. Progesterone (0.114 ± 0.007 nM, $n = 4$) and testosterone (0.034 ± 0.076 nM, $n = 4$) were detected in the culture medium containing 10% fetal bovine serum, and phenol

red (13.3 mM), which is known to have sex hormone-like activity [24], was present in the medium. Sex hormones and the hormone-like activity present in the medium could affect the antigen-specific immune responses [7-9]. Nevertheless, the IL-5 production by splenocytes in response to specific antigen was different between sexes. Therefore, our findings suggest that there is a sex difference in the potential of splenocytes to produce IL-5 even if the sex hormones in the culture had effects on the IL-5 production. Our findings are supported by those of Giron-Gonzalez et al, who reported that the Th1:Th2 ratio was significantly higher in supernatants of phytohaemagglutinin-stimulated T cell cultures from men compared with those from women in a culture medium containing 10% fetal bovine serum and that even the presence of supplementary sex hormones did not modify the production of these cytokines [18]. Together with these findings, we assume that the increased synthesis of Th2 cytokines in female lymphocytes could contribute to the development of gender differences in allergic asthma, in cooperation with differences in the hormonal environment. The secretions of Th2 cytokines other than IL-5, such as IL-4 and IL-13, were not significantly different between female and male splenocyte cultures. However, the contents of IL-4 and IL-13 in the BAL fluid of female mice after OVA challenge were significantly higher than those of male mice. Depending on the localization of the immune cells, antigen-induced IL-4/IL-13 production might be different. It will be necessary to investigate sex differences in the cytokine production using lymphocytes in bronchus-associated lymphoid tissues where antigen-specific immune responses occur after antigen challenge.

We used all kinds of cells in the spleen, without the isolation/purification of T cells and antigen-presenting cells such as dendritic cells required for allergen-specific immune responses, for the adoptive transfer and *ex vivo* stimulation experiments. Therefore, it remains to be determined which cells in splenocytes are responsible for the sex difference in allergen-specific immune function. It will be necessary to perform transfer and culture experiments using isolated T cells and dendritic cells.

In conclusion, we demonstrated that the sex difference in allergic airway inflammation is attributable not only to the hormonal environment but also to immune cells. The mechanisms by which the hormonal environment interacts with the immune system still remain unclear. However, investigation of the genetic and hormonal regulation of the immune system in each sex would be helpful for understanding the pathogenesis of diseases associated with gender differences such as asthma and collagen diseases.

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