

ORIGINAL PAPER

Role of sex hormones in allergic inflammation in mice

Y. Roffo-Vasquez*, A. P. Ligeiro de Oliveira†, C. P. Page*, D. Spina* and W. Tavares-de-Lima†

*The Sackler Institute of Pulmonary Pharmacology, King's College London, London, UK and †Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

Clinical and
Experimental
Allergy

Summary

Background A number of clinical studies have documented both a pro- and anti-inflammatory role for sex hormones in the context of lung inflammation and worsening of asthma.

Objective To determine the role of sex hormones in a murine model of allergic inflammation and airway hyper-responsiveness (AHR) induced by ovalbumin (OVA).

Methods Female BALB/c were sensitized to OVA on days 0 and 7 and subsequently challenged on day 14 over a 3-day period. Mice had their ovaries removed either 7 days before or 8 days after the first OVA injection on day 0. Pulmonary eosinophilia and AHR were measured 24 h following the last antigen challenge. In other experiments, ovariectomized mice (Ovx) were pre-treated with oestradiol benzoate. In further studies, the effect of the oestradiol antagonist tamoxifen on allergic inflammation in intact mice was evaluated. Splens from all groups were collected for proliferation assays and measurement of cytokine release.

Results Removal of the ovaries 7 days before sensitization to OVA significantly inhibited lung eosinophilia and IL-5 levels in lung lavage. Furthermore, airway reactivity (maximum response) but not sensitivity (PC100) to methacholine were significantly reduced in these mice. Proliferation of spleen cells and release of IL-5 collected from Ovx mice was significantly attenuated compared with spleen cells obtained from non-Ovx mice. Ovx mice treated with oestradiol benzoate presented partially restored levels of eosinophils and IL-5 in sensitized mice. Moreover, pharmacological antagonism of the effect of endogenous oestrogen with tamoxifen significantly reduced the number of eosinophils in the lung of intact sensitized mice, reproducing the effect of ovariectomy, and suggested a role for oestrogen in the process of antigen sensitization in female mice. In contrast, removal of ovaries 8 days after the first OVA injection failed to alter significantly pulmonary eosinophilia or AHR to methacholine in comparison with non-Ovx mice. Moreover, removal of the ovaries 8 days after the sensitization period induced a significant increase in levels of IL-5 in lung fluid. Spleen cells collected from these mice also had a significantly higher proliferation index and production of IL-5 in response to OVA than non-Ovx mice. Treatment with oestradiol benzoate partially reduced levels of eosinophils present in the lung of Ovx mice, supporting an anti-inflammatory role of sex hormones during the effector phase of the response to inhaled antigen.

Conclusion Sex hormones play a dual role in regulating allergic lung inflammation in mice.

Keywords airway hyper-responsiveness, allergic inflammation, eosinophil migration, oestrogen, mice

Submitted 20 October 2005; revised 23 November 2006; accepted 29 December 2006

Correspondence:

D. Spina, Sackler Institute of Pulmonary Pharmacology, Pharmaceutical Science Research Division, King's College London, School of Biomedical and Health Sciences, 5th floor Hodgkin Building, Guy's Campus, London SE1 1UL, UK.
E-mail: domenico.spina@kcl.ac.uk

Introduction

An estimated 33–52% of asthmatic women reporting worsening of asthma symptom before or during menses [1] have a higher ratio of hospitalization than men, with

50% of these women identified as perimenstrual at the time of hospitalization [2]. In addition, some studies have reported a significant worsening of asthma symptoms and decrease of peak expiratory flow rate from the late follicular stage in asthmatic women, when levels of

oestrogens are higher compared with the luteal phase, the period before menses when levels of oestrogens and progesterone are lower [3–5]. However, despite these clinical observations, the association between sex hormones and asthma deterioration remains controversial, as some authors failed to find any significant correlation between the level of sex hormones and asthma morbidity [1, 6, 7].

It has been reported that oestrogens stimulate, whereas progesterone and androgen suppress the immune system in animal models [8, 9]. In contrast, progesterone worsened allergic airway inflammation in ovalbumin (OVA)-challenged mice, as shown by enhanced bronchial responsiveness to inhaled methacholine and increased pulmonary eosinophilia [10].

In view of these conflicting reports, we have investigated the role of sex hormones in a murine model of pulmonary allergic inflammation. The timing of ovariectomy permitted us to investigate the role of sex hormones in antigen sensitization *per se*, and the effector phase of the inflammatory response following antigen challenge in immunized mice.

Methods

Ovariectomy

Female BALB/c mice (Charles River, Kent, UK) between 5 and 7 weeks old were injected by the intraperitoneal (i.p.) route with mydazolam (2 mg/kg; Roche, Welwyn, UK) and anaesthesia was maintained with isoflurane (Abbot, Kent, UK) throughout the entire procedure. An incision was made in the shaved lower part of the abdomen; the ovaries were identified, held tightly and removed (Ovx). The incision was sutured and the animals received 20 µg/kg of buprenorphine i.m. (Alstoe Animal Health, Melton Mowbray, UK) immediately after and 24 h later. In some animals, the operation was performed 7 days before first sensitization with OVA (protocol 1). In other groups, the ovariectomy was performed 24 h after the second OVA injection on day 8 (protocol 2). In some experiments, all the incisions were performed but the ovaries were not removed (sham-operated). These studies were undertaken under the Animals (Scientific Procedures) Act 1986, UK.

Determination of circulating oestradiol levels

Blood samples were collected 9 days after ovariectomy. The aliquots were centrifuged and the plasma obtained was stored at -70°C . Oestradiol levels were determined using an RIA kit (Cayman Chemicals Co., Ann Arbor, MI, USA) according to the manufacturer's instructions. The detectable range of oestradiol was 7.8–1000 pg/mL.

Antigen-induced allergic inflammation

Female BALB/c mice (Charles River) between 6 and 8 weeks old were immunized i.p. on days 0 and 7 with 10 µg of chicken egg OVA grade V (Sigma Chemical Co., Saint Louis, MO, USA) absorbed to 0.4 mL of a saturated solution of aluminium hydroxide (2.5 mg/mL; Sanofi-Synthelabo, São Paulo, Brazil). On days 14–16 (protocol 1) or days 15–17 (protocol 2), mice were placed in a 12 L plexiglas container and exposed once a day (25 min) to an aerosol solution of OVA (30 mg/mL; Devilbiss Ultra-neb 90, DeVilbiss Healthcare Ltd, Heston, UK). Control mice were injected with the adjuvant only and challenged with OVA on days 14–16. All measurements were performed 24 h after the last aerosol challenge.

Bronchoalveolar lavage

Mice were anaesthetized with urethane (2 g/kg i.p.; Sigma Chemical Co.) and a cannula was inserted into the exposed trachea. The lungs were washed with three 0.5 mL aliquots of sterile saline injected into the cannula. From the bronchoalveolar lavage (BAL) fluid, an aliquot (50 µL) was added to 50 µL of haemolysis solution (0.1% methylene blue in 1% acetic acid). The total number of cells in the lavage was counted with an improved Neubauer haemocytometer (Fisher Scientific, Loughborough, UK). For differential cell counts, cytopsin preparations were prepared from aliquots of BAL fluid (100 µL) centrifuged at 300 g for 1 min using a Shandon Cytospin 2 (Shandon Southern Instruments, Sewickley, PA, USA) at room temperature. Cells were stained with Diffquick (DADE Behring, Marbourg, Switzerland), and a total of 200 cells were counted to determine the proportion of neutrophils, eosinophils and monocytes using standard morphological criteria.

Cytokine and immunoglobulin E levels

Lavage, blood samples and cell culture supernatants were collected, centrifuged and stored at -70°C . Levels of IL-4, IL-5, IFN- γ and IgE were measured by conventional ELISA (BD Pharmingen, Devon, UK and eBioscience, San Diego, CA, USA), following the instructions of the manufacturers.

Respiratory lung mechanics

Mice from all groups were anaesthetized with urethane (2 g/kg i.p.) 24 h after the last aerosol challenge. A tracheostomy was performed and a cannula was inserted into the trachea. After surgery, the mice were placed in a plethysmograph and the tracheostomy tube was connected to a four-way manifold with one port attached to a differential pressure transducer ($\pm 20\text{ cmH}_2\text{O}$; Validyne,

Validyne Engineering Corp., Northridge, CA, USA) for the measurement of mouth pressure (Pao). Two ports were connected to the inspiratory and expiratory ports of a volume-cycled ventilator (SAR-830A, Model 963217, CWE Inc., Ardmore, PA, USA). Mice were ventilated at 150 breaths/min with a tidal volume of 0.15–0.2 mL and positive end expiratory pressure between 3 and 5 cmH₂O. Transpulmonary pressure was estimated as the difference between Pao, and box pressure as the chest wall contributes little to the overall compliance of the respiratory system. Changes in flow were determined with a Fleisch pneumotachograph (size 00000) connected to a side port of the chamber. Flow was measured with a differential pressure transducer (± 2 cmH₂O, Model MP 45-14-871, Validyne Engineering Corp., Northridge, CA, USA). The flow was integrated to give a continuous recording of tidal volume. Breath-by-breath recording of total lung resistance (RL, cmH₂O/L/s) was calculated by an online respiratory analyser on a personal computer (LFR version 6, Mumed Ltd, London, UK). Mice instrumented in this fashion were neuromuscular blocked with pancuronium bromide (0.2 mg/kg; Sigma Chemical Co.) before administration of methacholine (Sigma Chemical Co.).

Measurement of airway responsiveness

Aerosols of methacholine (3.125–50 mg/mL) generated from an ultrasonic nebulizer (Mumed Ltd) were administered directly to the lungs via a port of the plethysmograph. Aerosolized solution was delivered to the mouse with a second ventilator for 8 s, at a rate of 60 breaths/min with a tidal volume of 0.3–0.4 mL. Total airway resistance was measured before, and following administration of saline (0.9%), and then at 2-min intervals thereafter, before and following administration of methacholine. The maximum increase in RL was expressed as a percentage increase of the values of RL obtained following saline challenge. Indices of airway hyper-responsiveness (AHR) included the provocative concentration inducing a 100% increase in post-saline total resistance (PC100) and the peak response (% increase in post-saline RL) and indicate changes in sensitivity and reactivity, respectively.

Treatments

In some experiments, intact mice were treated with the oestradiol receptor antagonist tamoxifen (7 mg/kg i.p. diluted in ethanol and resuspended in a 10% solution of polyethyleneglycol; Tocris, Bristol, UK). In other experiments, ovariectomized mice received the glucocorticosteroid receptor antagonist RU 486 (30 mg/kg i.p. diluted in ethanol and resuspended in a 10% solution of polyethyleneglycol; Sigma Chemical Co.) or synthetic oestradiol benzoate [10 µg/mouse subcutaneous (s.c.); diluted in

ethanol and resuspended in arachis oil, Sigma Chemical Co.]. In experiments where the effect of pharmacological intervention was examined during the sensitization phase (protocol 1), compounds were administered once a day for 3 consecutive days, commencing 24 h before the first OVA injection (–24, –1 and +24 h post-sensitization). In other experiments, oestradiol benzoate and RU 486 were administered once a day for 4 consecutive days, commencing 24 h before the first antigen challenge to investigate the effect of pharmacological treatment during the effector phase (i.e. protocol 2).

Spleen cell cultures

Spleens from control, OVA-immunized and Ovx-immunized mice were collected at day 14 of the sensitization period. The animals were not challenged with OVA aerosol. Spleen cells were obtained by maceration of the tissue and the suspension was passed through a 40 µm cell strainer (BD Falcon, Marsbourg, MA, USA) for removal of debris. Cells were centrifuged at 420 *g* for 7 min and resuspended in RPMI 640 medium supplemented with 10% of fetal calf serum (Sigma Chemical Co.). The number of viable cells was determined using Trypan blue exclusion (Sigma Chemical Co.) and adjusted to 10⁷ cells/mL. Cells were plated in flat-bottom 96-well plates at a volume of 100 µL of cells/well and pulsed for 24 h with 100 µL of OVA (0.1, 1, 10 and 100 µg/mL). The effect of each concentration of stimulus on cell proliferation and cytokine release was performed in quadruplicate. After 24 h, plates were centrifuged at 300 *g* for 4 min and 100 µL of supernatants were collected and frozen at –70 °C for determination of cytokine levels. The remaining cells were resuspended and proliferation was measured using the XTT assay (Sigma Chemical Co.), absorbance measured at 450 nm, following the instructions of the manufacturer.

Statistical analysis

Data are expressed as mean and standard error of the mean (SEM). Unless otherwise specified, treatment effects were compared with analysis of variance (ANOVA) and differences between mean values determined using an appropriate multiple comparison test and were considered to be significant if $P < 0.05$.

Results

Plasma oestradiol levels

The plasma level of oestradiol in non-ovariectomized mice was 496.5 ± 113.1 pg/mL ($n = 5$). Nine days after ovariectomy, only one out of five animals showed detectable levels of circulating oestradiol (83.6 pg/mL).

Ovariectomy performed 7 days before sensitization: allergic lung inflammation

The total number of cells quantified in the BAL obtained from immunized sham-operated mice 24 h following OVA challenge was significantly higher compared with non-sensitized sham-operated mice (OVA/Sham: $90.4 \pm 8.4 \times 10^4$ cells/mL, vs. C/Sham: $25.1 \pm 1.45 \times 10^4$ cells/mL; $n = 10-15$; $P < 0.05$, Fig. 1a). This was reflected by a significantly greater number of eosinophils recruited to the airways in OVA/Sham mice ($42.9 \pm 4.8 \times 10^4$ cells/mL vs. C/Sham: $0.03 \pm 0.03 \times 10^4$ cells/mL; $P < 0.05$, Fig. 1b). The number of neutrophils was $<3\%$ of the total cell counts and not significantly different between groups (data not shown). The intensity of the inflammatory response in the lung was significantly impaired in animals in which the ovaries had been removed 7 days before sensitization (OVA/Ovx). This was reflected by a reduced total number of cells in BAL (OVA/Ovx: $36.8 \pm$

3.1×10^4 cells/mL; $P < 0.05$, Fig. 1a) and number of eosinophils ($6.2 \pm 0.4 \times 10^4$ cells/mL; $n = 15$; $P < 0.05$, Fig. 1b) relative to the OVA/Sham group. These observations were confirmed by histological examination of lung collected from these mice (Fig. 2). There was evidence of peri-bronchial eosinophilia in OVA/Sham-operated mice (Fig. 2b) that was absent in sham-immunized (C/Sham: Fig. 2a) or diminished in allergic mice with their ovaries removed (OVA/Ovx, Fig. 2c).

We confirmed that the surgical procedure alone did not promote a non-specific inflammatory response in the airways. The number of inflammatory cells recovered in lavage fluid from non-sensitized/sham-operated (C/Sham) animals was not significantly different from non-sensitized/non-operated animals (total cells: C/Sham: $24.0 \pm 2.9 \times 10^4$ /mL; $n = 10$ vs. C: $25.1 \pm 1.4 \times 10^4$ cells/mL; $n = 32$). Similarly, the removal of ovaries did not promote a non-specific inflammatory response in the lung in non-sensitized/ovarectomized mice (C/Ovx: $29.2 \pm$

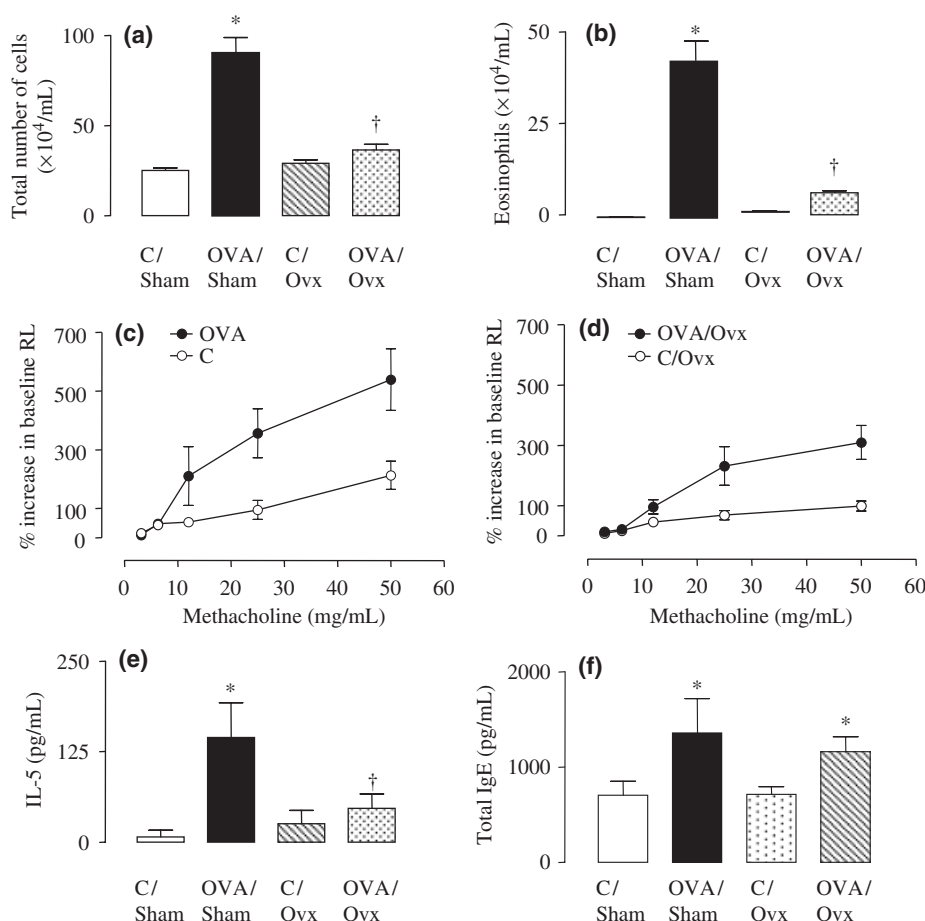


Fig. 1. Effect of sex hormone depletion before the sensitization phase on total number of cells (a), eosinophils (b), increase in baseline resistance (c and d), levels of IL-5 in bronchoalveolar lavage fluid (e) and levels of IgE in serum (f) 24 h post-ovalbumin (OVA) challenge in control-immunized/sham-operated animals (C/Sham), OVA-immunized/sham-operated animals (OVA/Sham), control-immunized/ovarectomized animals (C/Ovx) and OVA-immunized/ovarectomized animals (OVA/Ovx). Vertical lines represent standard error of the mean of 10–30 animals/group. * $P < 0.05$ cf. C/Sham group; † $P < 0.05$ cf. OVA/Sham group.

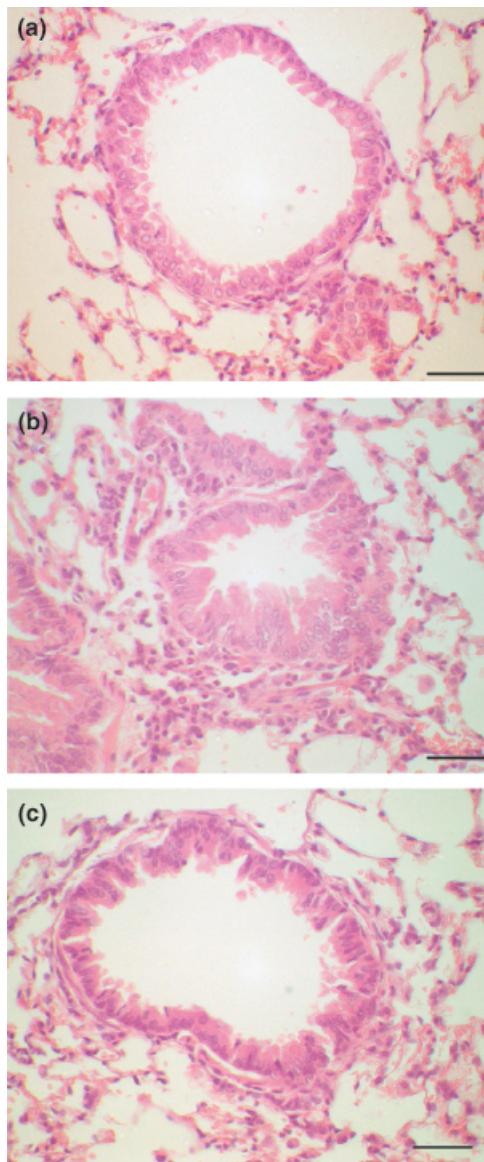


Fig. 2. Photomicrographs of lung tissue collected 24 h post-ovalbumin (OVA) challenge in control-immunized animals (a), OVA-immunized (b) and OVA-immunized/ovariectomized animals (OVA/Ovx) (c). Note the absence of peri-bronchial inflammation in sections from control-immunized and OVA/Ovx animals. The horizontal bar represents 40 μ m.

2.2×10^4 /mL, Figs 1a and b). We also confirmed that the surgical procedure did not interfere with the immunization process *per se* as there was no significant difference in the number of total cells (OVA/intact: 88.2 ± 21.16 ; $n = 26$ vs. OVA/Sham-operated: $91.35 \pm 8.10 \times 10^4$ cells/mL; $n = 10$) and of eosinophils (OVA/intact: $42.9 \pm 4.8 \times 10^4$ vs. OVA/Sham-operated: $38.8 \pm 3.93 \times 10^4$ cells/mL) between sham-operated and non-operated allergic mice. As allergic sensitization was not compromised by surgery *per se*, it was considered unethical to use immu-

nized/sham-operated (OVA/Sham) or non-immunized/ovariectomized mice (C/Ovx) in all our experiments.

Ovariectomy performed 7 days before sensitization: airway hyper-responsiveness to methacholine

Twenty-four hours following the last antigen challenge, there was a significant increase in airway responsiveness (Fig. 1c) as measured by increases in peak response (% increase baseline RL mean $\pm$ SEM; C/Sham: 138 ± 30 vs. OVA/Sham: 444 ± 85 , $n = 11-12$, $P < 0.05$) and sensitivity (Geometric mean and 95% CI; PC100 RL; C/Sham: 36 (24–58) mg/mL vs. OVA/Sham: 8 (5–16) mg/mL; $n = 11-12$; $P < 0.05$). Ovariectomy significantly reduced airway responsiveness to methacholine (Fig. 1d) as reflected in a suppression of the maximum response to methacholine (C/Ovx: 101 ± 11 vs. OVA/Ovx: 238 ± 48 ; $n = 12-13$; $P > 0.05$), but not airway sensitivity to methacholine (C/Ovx: 50 (34–78) mg/mL vs. OVA/Ovx: 14 (8–25) mg/mL; $n = 12-13$; $P < 0.05$).

Ovariectomy performed 7 days before sensitization: cytokine levels in bronchoalveolar lavage and immunoglobulin E levels in serum

The level of IL-4 and IFN- γ was not significantly different between groups 24 h after the last OVA challenge (data not shown). In contrast, the level of IL-5 was significantly increased in allergic mice following OVA challenge (OVA/Sham: 144.6 ± 48 pg/mL vs. C/Sham: 12 ± 7 pg/mL; $P < 0.05$, Fig. 1e). The removal of the ovaries significantly reduced the level of IL-5 in sensitized animals (OVA/Ovx: 46.9 ± 18.2 pg/mL vs. C/Ovx: 25 ± 18 pg/mL) compared with immunized animals with intact ovaries ($P < 0.05$, Fig. 1e).

The amount of total IgE was significantly increased in OVA immunized mice ($P = 0.016$, ANOVA) that were unaffected by removal of the ovaries ($P > 0.05$, Fig. 1f).

Ovariectomy performed 7 days before sensitization: spleen cell proliferation

OVA significantly increased *in vitro* proliferation of spleen cells obtained from the OVA-immunized group in comparison with control-immunized mice (Fig. 3a). However, spleen cells obtained from OVA/Ovx mice were characterized by a significantly reduced proliferative response to OVA *in vitro* when compared with the OVA-immunized group (Fig. 3a).

Spleen cells obtained from OVA/Ovx mice produced significantly lower amounts of IL-5 compared with OVA mice (Fig. 3b). Levels of IL-4 and IFN- γ were not significantly different between OVA and OVA/Ovx mice in these assays (data not shown).

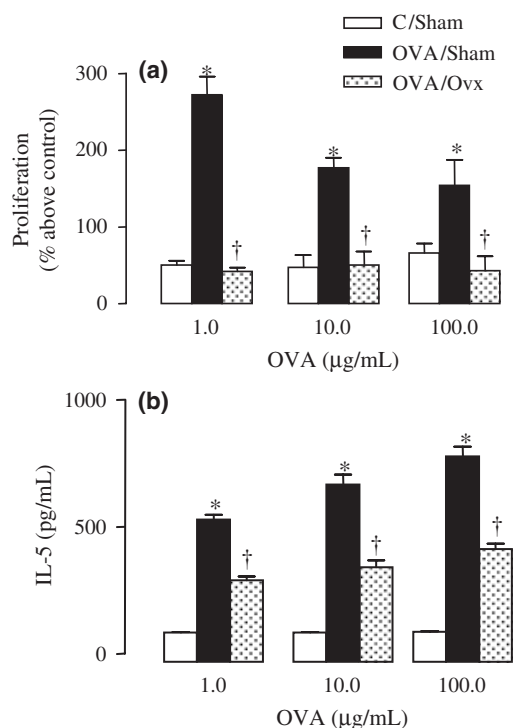


Fig. 3. Bar graph representing the effect of sex hormone depletion before the sensitization phase on spleen cell proliferation (a) and IL-5 levels in culture supernatants (b). Cells were collected from control-immunized animals (C/Sham), ovalbumin (OVA)-immunized (OVA/Sham) and OVA-immunized/ovariectomized animals (OVA/Ovx). Cells were stimulated with OVA for 24 h and tested in quadruplicate. Proliferation is expressed as percentage of increase above non-stimulated cells, and each column represents the mean of three experiments. * $P < 0.05$ cf. C/Sham group; † $P < 0.05$ cf. OVA/Sham group.

Ovariectomy performed 7 days before sensitization: effect of RU 486 on eosinophil migration

In order to investigate a possible mechanism by which the removal of ovaries before immunization reduced the intensity of the inflammatory response in the lung, we investigated the role of endogenous glucocorticosteroids in this model. As with previous experiments, ovariectomy suppressed eosinophilic inflammation in allergic mice (Fig. 4a). Treatment with the glucocorticosteroid receptor antagonist RU 486 during sensitization to OVA failed to reverse the inhibitory effect of removal of the ovaries on this response (OVA/Ovx/RU 486: $0.8 \pm 0.1 \times 10^4$ vs. OVA/Ovx: $1.6 \pm 0.4 \times 10^4$ cells/mL; $P > 0.05$, Fig. 4a).

Ovariectomy performed 7 days before sensitization: the effect of oestradiol on allergic lung inflammation

In order to investigate the role of oestradiol in this model, ovariectomized animals were treated with oestradiol benzoate (10 µg/mouse) before and during the sensitization protocol. The total number of cells quantified in the BAL obtained from oestradiol-treated ovariectomized

mice 24 h following OVA challenge was not significantly different compared with non-treated ovariectomized animals (OVA/Ovx: $65.3 \pm 4.1 \times 10^4$ cells/mL vs. OVA/Ovx/E2: $71.0 \pm 27.9 \times 10^4$ cells/mL; $n = 5$). Oestradiol-treated mice showed a partial recovery of eosinophil recruitment to the airways in ovariectomized mice, although this was not statistically significant in comparison with the OVA/Ovx group (OVA/Ovx: $3.95 \pm 2.3 \times 10^4$ cells/mL vs. OVA/Ovx/E2: $17.94 \pm 2.8 \times 10^4$ cells/mL; $P > 0.05$, Fig. 4b).

In contrast, intact sensitized animals treated with the oestrogen receptor antagonist tamoxifen (7 mg/kg i.p.) before and during the sensitization period were unable to mount an inflammatory response to OVA, mimicking the effect of removal of the ovaries on airway inflammation. Treatment with tamoxifen significantly reduced the total number of cells in the lungs of intact mice after OVA challenge (OVA: $72.2 \pm 12.1 \times 10^4$ cells/mL vs. OVA/tamoxifen: $24.4 \pm 2.5 \times 10^4$ cells/mL; $P < 0.05$). This inhibition was reflected by a significantly lower number of eosinophils in the lung of tamoxifen-treated mice (OVA: $28.9 \pm 2.8 \times 10^4$ cells/mL vs. OVA/tamoxifen: $1.32 \pm 0.3 \times 10^4$ cells/mL; $P < 0.05$, Fig. 4c).

Ovariectomy performed after sensitization: allergic lung inflammation and hyper-responsiveness to methacholine

Using a different experimental approach to investigate the role of sex hormones in the effector phase of the allergic inflammatory response in mice, the ovaries were removed after the sensitization protocol. Removal of the ovaries 24 h after the second OVA injection did not significantly reduce the recruitment of inflammatory cells to the lung following OVA challenge (OVA: $134.6 \pm 16 \times 10^4$ cells/mL vs. OVA/Ovx: $124.4 \pm 15 \times 10^4$ cells/mL; $n = 13-16$; $P > 0.05$, Fig. 5a). This was also reflected by a lack of inhibitory effect on the numbers of eosinophils recruited to the airways in OVA/Ovx mice compared with OVA mice (OVA: $61.4 \pm 14.6 \times 10^4$ cells/mL vs. OVA/Ovx: $72.8 \pm 10 \times 10^4$ cells/mL; $P > 0.05$, Fig. 5b). Similarly, removal of the ovaries after sensitization to OVA did not significantly attenuate AHR induced by antigen challenge in comparison with the OVA group (Figs 5c and d).

Ovariectomy performed after sensitization: cytokine levels in bronchoalveolar lavage and immunoglobulin E levels in serum

Levels of IL-4, IL-5 and IFN- γ were quantified in BAL and total IgE levels quantified in serum collected 24 h after the last OVA challenge. Significantly higher levels of IL-5 were measured in BAL fluid obtained from Ovx mice compared with the OVA group (OVA: 520.01 ± 44 pg/mL vs. OVA/Ovx: 1063.34 ± 44 pg/mL; $P < 0.05$, Fig. 6a). The increased levels of IL-4 and IFN- γ in OVA mice were not significantly different from the levels observed in

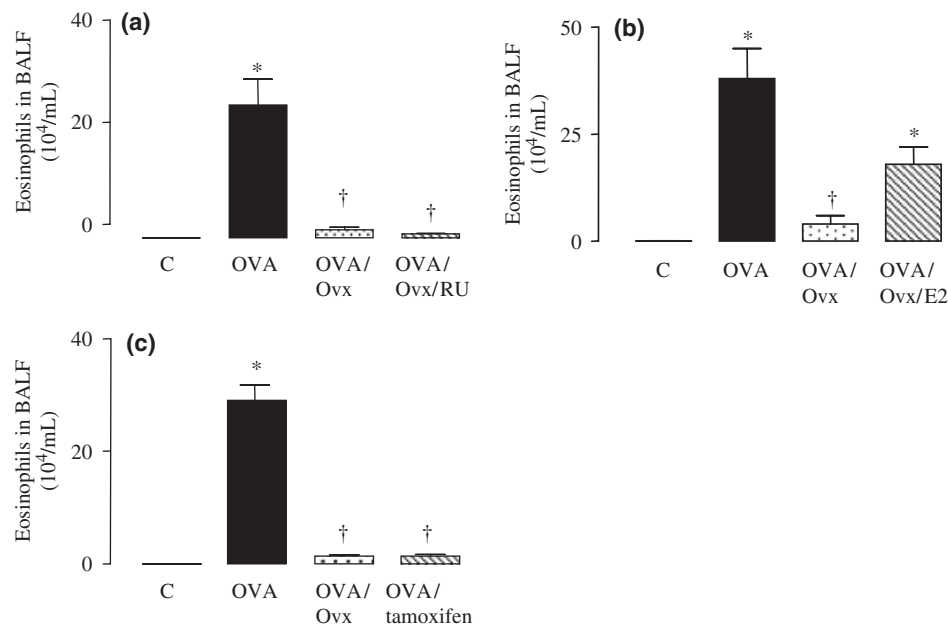


Fig. 4. Bar graph representing the effect of RU 486 (a), oestradiol benzoate (b) and tamoxifen (c) on the number of eosinophils in bronchoalveolar lavage (BAL) fluid collected 24 h post-ovalbumin (OVA) challenge from control-immunized (C), OVA-immunized (OVA), OVA-immunized/ovariectomized (OVA/Ovx), OVA-immunized/ovariectomized animals treated with RU 486 (OVA/Ovx/RU), OVA-immunized animals treated with tamoxifen (OVA/tamoxifen) and OVA-immunized/ovariectomized animals treated with oestradiol benzoate (OVA/Ovx/E2). Vertical lines represent standard error of the mean of 5–12 animals/group. * $P < 0.05$ cf. C; † $P < 0.05$ cf. OVA group.

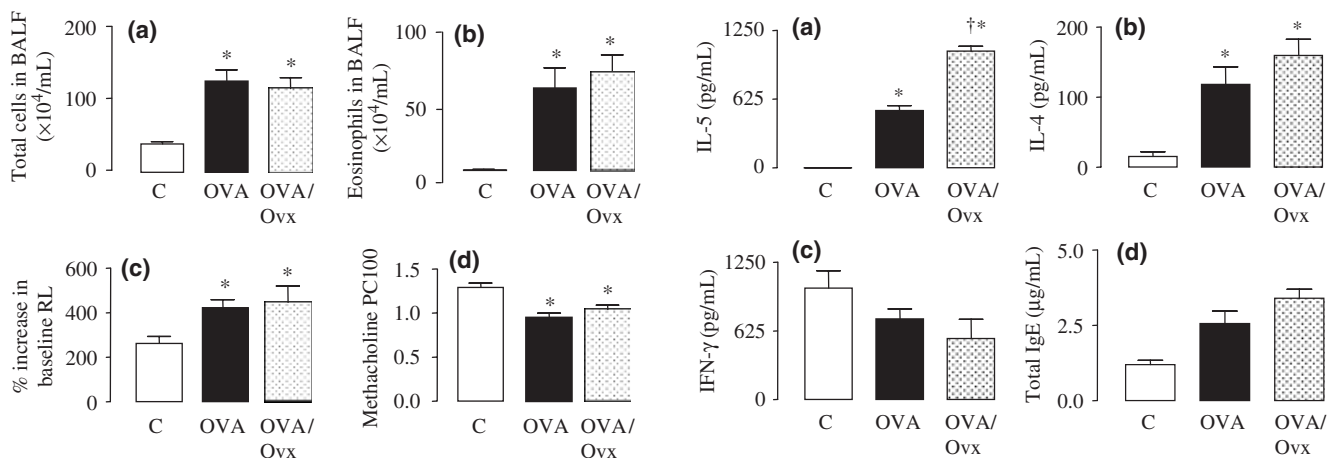


Fig. 5. Bar graph representing the effect of sex hormone depletion after the sensitization phase on the total number of cells (a), eosinophils (b), maximum response (c) and log PC100 to methacholine (d) 24 h post-ovalbumin (OVA) challenge in control-immunized animals (C), OVA-immunized animals (OVA) and OVA-immunized/ovariectomized animals (OVA/Ovx). Vertical lines represent standard error of the mean of 10–16 animals/group. * $P < 0.05$ cf. C group. In the case of (c), data were analysed using the Kruskal–Wallis test, followed by Dunn's test as the sample variances were not homogeneous.

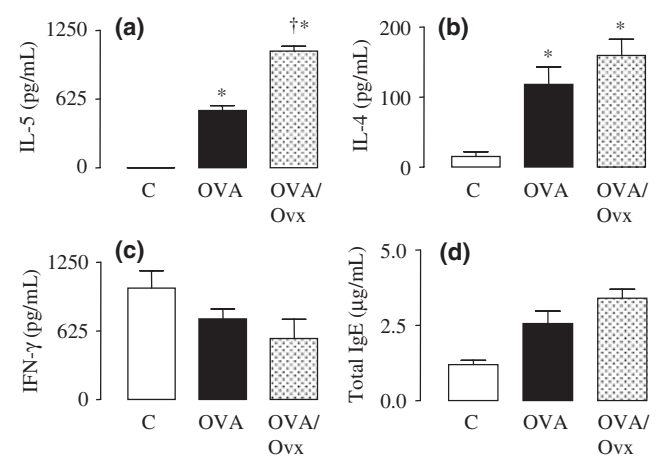


Fig. 6. Bar graph representing the effect of sex hormone depletion after the sensitization phase on IL-5 (a), IL-4 (b) and IFN- γ (c) levels in bronchoalveolar lavage fluid collected 24 h after last ovalbumin (OVA) challenge from control-immunized animals (C), OVA-immunized animals (OVA) and OVA-immunized/ovariectomized animals (OVA/Ovx). Vertical lines represent standard error of the mean of 4–8 animals/group. * $P < 0.05$ cf. C group; † $P < 0.05$ cf. OVA group.

Ovariectomy performed after sensitization: spleen cell cultures

OVA/Ovx mice (Figs 6b and c). The amount of total IgE was increased in OVA-immunized mice and this was unaffected by removal of the ovaries after sensitization ($P > 0.05$, Fig. 6d).

Spleen cells were obtained and processed in the same manner as described earlier. OVA significantly increased *in vitro* proliferation of spleen cells obtained from the OVA-immunized group in comparison with control-

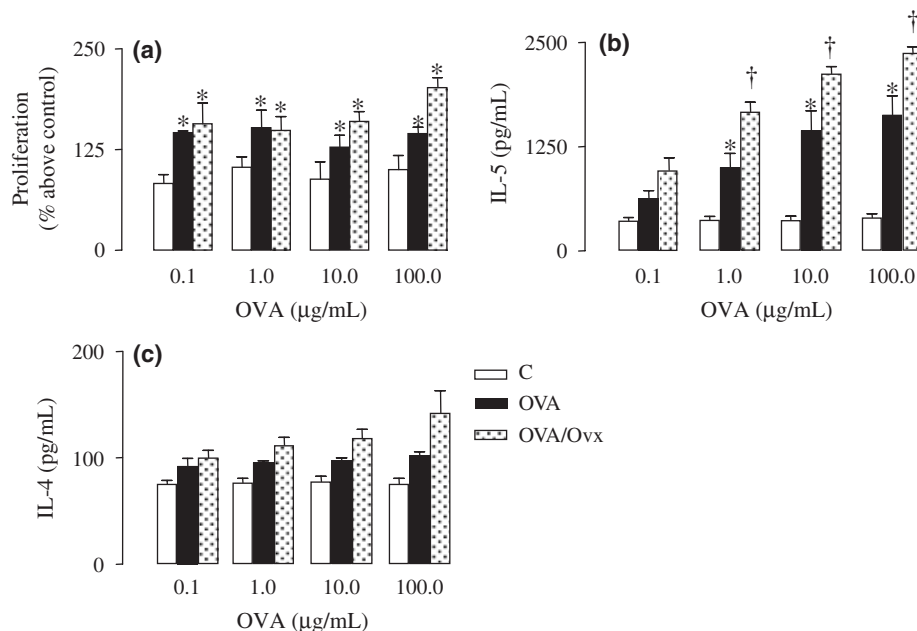


Fig. 7. Bar graph representing the effect of sex hormone depletion before the effector phase on spleen cell proliferation (a), IL-5 (b) and IL-4 (c) levels in the supernatants. Cells were collected from control-immunized animals (C), ovalbumin (OVA)-immunized animals (OVA) and OVA-immunized/ovariectomized animals (OVA/Ovx). Cells were stimulated with OVA for 24 h and tested in quadruplicate. Each column represents the mean of three experiments. Proliferation is expressed as percentage of increase above non-stimulated cells. * $P < 0.05$ cf. C group; † $P < 0.05$ cf. to OVA group.

immunized mice (Fig. 7a). OVA also induced a significant increase in the proliferation of cells obtained from OVA/Ovx mice. However, this increase was not significantly different from the OVA group. OVA significantly increased the levels of IL-5 measured in the supernatants obtained from sensitized spleens compared with control spleens. Spleen cells obtained from ovariectomized mice (OVA/Ovx) produced significantly greater amounts of IL-5 in response to OVA compared with OVA mice (Fig. 7b), confirming *in vivo* results (Fig. 6a). The levels of IL-4 were not significantly different between groups (Fig. 7c).

Ovariectomy performed after sensitization: effect of RU 486 on eosinophil migration

In order to investigate a possible mechanism by which the removal of ovaries after immunization intensified the inflammatory response in the lung, we investigated the role of endogenous glucocorticosteroids in this model. Treatment with RU 486 failed to significantly increase the number of eosinophils recruited to the airways of sensitized ovariectomized mice (Fig. 8a).

Ovariectomy performed after sensitization: effect of oestradiol on allergic lung inflammation

In order to investigate the role of oestradiol in this model, ovariectomized animals were treated with oestradiol benzoate (10 µg/mouse) before and during the OVA chal-

lenge protocol. Removal of the ovaries before the effector phase in immunized mice did not reduce the total numbers of cells recruited to the lung following OVA challenge (OVA: 55 ± 8.0 vs. OVA/Ovx: $82 \pm 10 \times 10^4$ cells/mL; $n = 13-14$; $P < 0.05$). This was also reflected by a lack of an effect in the numbers of eosinophils recruited to the lung of Ovx-treated mice (OVA: $17 \pm 4 \times 10^4$ cells/mL vs. OVA/Ovx: $32 \pm 5 \times 10^4$ cells/mL; $n = 8-14$; $P < 0.05$, Fig. 8b).

The treatment of ovariectomized mice with oestradiol benzoate led to no effect on the total number of cells observed in these animals (OVA/Ovx/E2: $53 \pm 7 \times 10^4$ cells/mL cf. OVA/Ovx; $n = 13$; $P > 0.05$). However, treatment with oestradiol significantly reduced the intensity of the eosinophilia in ovariectomized mice (OVA/Ovx/E2: $13 \pm 3 \times 10^4$ cells/mL vs. OVA/Ovx; $n = 13$; $P < 0.05$, Fig. 8b).

Discussion

The incidence of asthma is lower among pre-pubescent girls in comparison with boys and this status is inverted after the first years of puberty, when sex hormones play a more important role in development [11]. It has also been shown that children with onset of asthma during childhood tend to go into remission after puberty [12]. Other reports show that sex hormone levels during the menstrual cycle in fertile women and hormone replacement in menopausal women are associated with the deterioration of their symptoms [2, 4, 13, 14]. These clinical studies

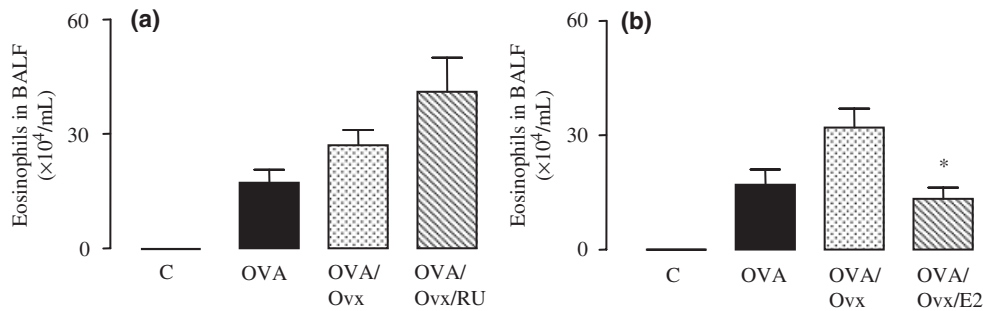


Fig. 8. Bar graph representing the effect of pharmacological intervention before the effector phase. Animals were treated with RU 486 (a) and oestradiol benzoate (b) on the number of eosinophils collected 24 h post-ovalbumin (OVA) challenge from control-immunized (C), OVA-immunized (OVA), OVA-immunized/ovariectomized (OVA/Ovx) and OVA-immunized/ovariectomized animals treated with RU 486 (OVA/Ovx/RU). OVA-immunized/ovariectomized animals treated with oestradiol benzoate (OVA/Ovx/E2). Vertical lines represent standard error of the mean of 3–5 (RU 486) or 8–14 (oestradiol benzoate) animals/group.

suggest that sex hormones may play a role in modulating the asthma phenotype.

Our results show that depletion of sex hormones in mice before sensitization to OVA prevented the development of lung inflammation and AHR to methacholine. Moreover, the ability of the immune system to produce IL-5 is also impaired in the absence of sex hormones before immunization with OVA, as assessed in our *in vivo* experiments and *in vitro* cultured splenocytes, which may explain the reduced influx of eosinophils to the lung observed in these animals. However, we did not observe significant differences in the levels of IL-4 and INF- γ between groups at this time-point. In addition, we have not observed a significant reduction in total IgE levels in ovariectomized mice, suggesting that the inhibition of eosinophil migration in the lung of these animals is not correlated to an impaired Ig production in response to the antigen.

The modulatory effect of oestrogen in the immune system is well documented. It has been shown that oestrogen enhances antigen presentation, dendritic cell differentiation, cytokine release by T cells, T cell-clonal expansion and homing [15–20]. It is possible that the low levels of IL-5 and low proliferation capacity of spleen cells observed in our study reflected the non-availability of oestrogen during the sensitization period, and as a consequence, T cell clonal expansion and homing are compromised when animals are exposed to the antigen during the challenge protocol. Nonetheless, there is clearly sufficient T cell activity to allow normal Ig production by B cells. The importance of oestradiol in regulating airway inflammation in our model was confirmed using two different approaches. Treatment of intact mice with the oestradiol antagonist tamoxifen before and during immunization with OVA inhibited the migration of eosinophils to the lungs of sensitized animals, recapitulating the effects observed with removal of the ovaries. In addition, treatment with oestradiol benzoate before and during the immunization process partially re-established the inflam-

matory response to OVA in ovariectomized mice, although the values did not reach statistical significance. Together, these support a role for oestradiol in the sensitization process in female mice.

Depletion of sex hormones could affect the negative feedback mechanism of corticosterone on the hypothalamic–pituitary axis by increasing the levels of type II glucocorticosteroid receptors in the pituitary gland [21]. In addition, sex hormones could also interfere with the magnitude of the response to corticosterone as oestradiol increases plasma levels of corticosterone-binding protein [22]. We treated ovariectomized mice with the glucocorticosteroid antagonist RU 486 before and during sensitization to OVA in order to investigate whether oestradiol depletion was inhibiting cellular migration to the lung secondary to increased glucocorticosteroid activity *in vivo*. In these experiments, we did not consider the effect of RU 486 as a progesterone antagonist to be relevant because the animals had been depleted of this hormone by the removal of the ovaries. Treatment of ovariectomized mice with RU 486 during the protocol of sensitization failed to reverse the inhibition of the inflammatory response observed in these animals, suggesting that the anti-inflammatory effect induced by sex hormone depletion observed in this model is not glucocorticosteroid dependent.

We performed ovariectomy after mice had been immunized to OVA in order to investigate the role of sex hormones in the effector phase of the inflammatory response. Lung inflammation and AHR was not inhibited in ovariectomized immunized mice. There was a trend for a greater recruitment of eosinophils to the lung in ovariectomized mice, although this did not achieve statistical significance. Interestingly, there was a significant increase in IL-5 levels in BAL fluid collected 24 h after the last antigen challenge in ovariectomized mice, suggesting an intensification of the inflammatory process. We also observed an increase in IL-4 and a decrease of INF- γ levels in BAL fluid. Although these data did not achieve

statistical significance at this time-point, they follow the trend observed for eosinophil migration. We cannot rule out the possibility that differences in IL-4 and IFN- γ may be significant at other time-points as has been reported elsewhere [23]. Moreover, spleen cells from mice ovariectomized after sensitization proliferated to a greater extent to the highest concentrations of OVA in comparison with intact sensitized mice, recapitulating results obtained *in vivo*. Moreover, spleen cells obtained from mice ovariectomized after immunization also released significantly higher levels of IL-5 when stimulated with OVA, suggesting that sex hormones can modulate the release of this cytokine *in vivo* and *in vitro*.

The role of oestrogen as an anti-inflammatory stimulus during the effector phase was demonstrated by treatment of ovariectomized animals with oestradiol benzoate. Ovariectomized animals supplemented with oestrogen during antigen challenge had reduced numbers of eosinophils in the lung in comparison with ovariectomized mice not supplemented with oestrogen. When oestradiol is present during the sensitization period, cells from the immune system retain their capacity of proliferation and expansion and a normal response to the antigen is observed, i.e., high numbers of eosinophils in the lung. However, if oestradiol is removed during the antigen challenge period, a greater number of eosinophils are observed in the lung. This is also observed when levels of IL-5 are measured *in vivo* and *in vitro*, suggesting that oestradiol is important in modulating the intensity of the inflammatory response to the antigen in the effector phase and moreover, this appears to be independent of glucocorticosteroids as RU 486 did not significantly inhibit this response. The anti-inflammatory effects of oestradiol are well documented in a number of studies. Oestradiol can down-regulate neutrophil trafficking and inflammatory mediator release in non-allergic models of inflammation and haemorrhagic shock, where oestradiol seems to modulate leucocyte adhesion and diapedesis [24–28]. It has also been shown that a combination of exogenous oestrogens significantly reduced leucocyte migration, platelet activation, mast cell activation and endothelial wall disruption in a rat model of amyloid- β -induced mesenteric inflammation [29]. The transcription factor p65/relA, a member of the nuclear factor κ B family, plays a major role in inflammation and drives the expression of pro-inflammatory mediators. Using a time course experiment and the microtubule-disrupting agent nocodazole, it has been shown that oestradiol inhibits p65 intracellular transport to the nucleus of macrophages via an oestrogen receptor α -mediated mechanism, down-regulating the production of pro-inflammatory cytokines [30]. In addition, we have previously shown that a significant increase in the numbers of circulating eosinophils in peripheral blood and total numbers of cells in the bone marrow of rats is observed in the absence of

oestrogen that was partially reversed when the animals were treated with this hormone [31]. Similarly, other authors have shown that oestrogen significantly suppressed bone marrow production of leucocytes and affected the distribution of polymorphonuclear cells in peripheral blood and down-regulated the traffic of these cells to tissue [32]. It is possible that in our model, the increased numbers of cells and significantly higher levels of IL-5 observed *in vivo* and *in vitro* are a reflection of this lack of negative control exerted by oestrogen on leucocyte generation by the bone marrow and subsequent migration to the tissue.

Together, our results suggest that sex hormones can regulate allergic lung inflammation by two different pathways. Oestradiol has a pro-inflammatory effect regulating the response to allergen, possibly modulating T cell function, clonal expansion and/or T cell homing [18]. On the other hand, oestradiol has an anti-inflammatory effect, down-regulating the traffic of eosinophils to the lung, possibly suppressing bone marrow activity, pro-inflammatory mediators release and/or adhesion molecule expression. A modulatory role for sex hormones has been suggested for other inflammatory diseases like arthritis [33, 34], diabetes [35] and glomerulonephritis [36] where oestrogen seems to modulate the immune and inflammatory responses by different pathways.

Sex hormones could be one of the factors influencing the low incidence of asthma observed in girls before puberty in comparison with boys, when sex hormone levels are very low [37–39]. It could also be involved in the reported remission of the disease observed in asthmatic girls once they reach puberty, when levels of oestradiol increase [11, 39, 40]. Similarly, the correlation observed between the level of sex hormones and asthma symptoms observed in fertile, menopausal and pregnant asthmatic women could also be better understood in light of these dual effects of sex hormones [1, 5, 13, 14, 41–43]. This dual effect of sex hormone on the allergic inflammatory response in the lung may explain the conflicting clinical data reported in the literature.

To our knowledge, our study is the first to dissociate the effect of sex hormone on the sensitization period from the effector period in terms of sex hormone depletion in an allergic response. The exact mechanisms by which oestrogen regulates the allergic response in the airways remain to be established and is the subject of future studies.

References

- 1 Ensom MH, Chong E, Carter D. Premenstrual symptoms in women with premenstrual asthma. *Pharmacotherapy* 1999; 19:374–82.
- 2 Skobeloff EM, Spivey WH, Silverman R, Eskin BA, Harchelroad F, Alessi TV. The effect of the menstrual cycle on asthma

- presentations in the emergency department. *Arch Intern Med* 1996; 156:1837–40.
- 3 Chandler MH, Schuldheisz S, Phillips BA, Muse KN. Premenstrual asthma: the effect of estrogen on symptoms, pulmonary function, and beta 2-receptors. *Pharmacotherapy* 1997; 17:224–34.
 - 4 Pauli BD, Reid RL, Munt PW, Wigle RD, Forkert L. Influence of the menstrual cycle on airway function in asthmatic and normal subjects. *Am Rev Respir Dis* 1989; 140:358–62.
 - 5 Hanley SP. Asthma variation with menstruation. *Br J Dis Chest* 1981; 75:306–8.
 - 6 Weinmann GG, Zacur H, Fish JE. Absence of changes in airway responsiveness during the menstrual cycle. *J Allergy Clin Immunol* 1987; 79:634–8.
 - 7 Dorhofer DM, Sigmon ST. Physiological and psychological reactivity in women with asthma: the effects of anxiety and menstrual cycle phase. *Behav Res Ther* 2002; 40:3–17.
 - 8 Shoenberger ZM, Chiorazzi N, Lahita RG. Regulation of the immune response by sex hormones. I. In vitro effects of estradiol and testosterone on pokeweed mitogen-induced human B cell differentiation. *J Immunol* 1988; 141:91–8.
 - 9 Grossman CJ. Interactions between the gonadal steroids and the immune system. *Science* 1985; 227:257–61.
 - 10 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.
 - 11 Debley JS, Redding GJ, Critchlow CW. Impact of adolescence and gender on asthma hospitalization: a population-based birth cohort study. *Pediatr Pulmonol* 2004; 38:443–50.
 - 12 Guerra S, Wright AL, Morgan WJ, Sherrill DL, Holberg CJ, Martinez FD. Persistence of asthma symptoms during adolescence: role of obesity and age at the onset of puberty. *Am J Respir Crit Care Med* 2004; 170:78–85.
 - 13 Carlson CL, Cushman M, Enright PL, Cauley JA, Newman AB. Hormone replacement therapy is associated with higher FEV1 in elderly women. *Am J Respir Crit Care Med* 2001; 163:423–8.
 - 14 Troisi RJ, Speizer FE, Willett WC, Trichopoulos D, Rosner B. Menopause, postmenopausal estrogen preparations, and the risk of adult-onset asthma. A prospective cohort study. *Am J Respir Crit Care Med* 1995; 152:1183–8.
 - 15 Karpuzoglu-Sahin E, Zhi-Jun Y, Lengi A, Sriranganathan N, Ansar AS. Effects of long-term estrogen treatment on IFN-gamma, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice. *Cytokine* 2001; 14:208–17.
 - 16 Komi J, Lassila O. Nonsteroidal anti-estrogens inhibit the functional differentiation of human monocyte-derived dendritic cells. *Blood* 2000; 95:2875–82.
 - 17 Maret A, Coudert JD, Garidou L *et al.* Estradiol enhances primary antigen-specific CD4 T cell responses and Th1 development *in vivo*. Essential role of estrogen receptor alpha expression in hematopoietic cells. *Eur J Immunol* 2003; 33:512–21.
 - 18 Murphy HS, Sun Q, Murphy BA *et al.* Tissue-specific effect of estradiol on endothelial cell-dependent lymphocyte recruitment. *Microvasc Res* 2004; 68:273–85.
 - 19 Salem ML. Estrogen, a double-edged sword: modulation of TH1- and TH2-mediated inflammations by differential regulation of TH1/TH2 cytokine production. *Curr Drug Targets Inflamm Allergy* 2004; 3:97–104.
 - 20 Offner H, Adlard K, Zamora A, Vandenbark AA. Estrogen potentiates treatment with T-cell receptor protein of female mice with experimental encephalomyelitis. *J Clin Invest* 2000; 105:1465–72.
 - 21 Coyne MD, Kitay JL. Effect of ovariectomy on pituitary secretion of ACTH. *Endocrinology* 1969; 85:1097–102.
 - 22 Turner BB. Sex difference in glucocorticoid binding in rat pituitary is estrogen dependent. *Life Sci* 1990; 46:1399–406.
 - 23 Riffo-Vasquez Y, Spina D, Page C *et al.* Effect of Mycobacterium tuberculosis chaperonins on bronchial eosinophilia and hyper-responsiveness in a murine model of allergic inflammation. *Clin Exp Allergy* 2004; 34:712–9.
 - 24 Cuzzocrea S, Santagati S, Sautebin L *et al.* 17beta-estradiol antiinflammatory activity in carrageenan-induced pleurisy. *Endocrinology* 2000; 141:1455–63.
 - 25 Speyer CL, Rancilio NJ, McClintock SD *et al.* Regulatory effects of estrogen on acute lung inflammation in mice. *Am J Physiol Cell Physiol* 2005; 288:C881–90.
 - 26 Xing D, Miller A, Novak L, Rocha R, Chen YF, Oparil S. Estradiol and progestins differentially modulate leukocyte infiltration after vascular injury. *Circulation* 2004; 109:234–41.
 - 27 Vegeto E, Bonincontro C, Pollio G *et al.* Estrogen prevents the lipopolysaccharide-induced inflammatory response in microglia. *J Neurosci* 2001; 21:1809–18.
 - 28 Miller AP, Feng W, Xing D *et al.* Estrogen modulates inflammatory mediator expression and neutrophil chemotaxis in injured arteries. *Circulation* 2004; 110:1664–9.
 - 29 Thomas TN, Rhodin JA, Clark L, Garces A, Bryant M. A comparison of the anti-inflammatory activities of conjugated estrogens and 17-beta estradiol. *Inflamm Res* 2003; 52:452–60.
 - 30 Ghisletti S, Meda C, Maggi A, Vegeto E. 17{beta}-estradiol inhibits inflammatory gene expression by controlling NF- κ B intracellular localization. *Mol Cell Biol* 2005; 25:2957–68.
 - 31 Ligeiro de Oliveira AP, Oliveira-Filho RM, da Silva ZL, Borelli P, Tavares de LW. Regulation of allergic lung inflammation in rats: interaction between estradiol and corticosterone. *Neuroimmunomodulation* 2004; 11:20–7.
 - 32 Josefsson E, Tarkowski A, Carlsten H. Anti-inflammatory properties of estrogen. I. In vivo suppression of leukocyte production in bone marrow and redistribution of peripheral blood neutrophils. *Cell Immunol* 1992; 142:67–78.
 - 33 Cutolo M, Capellino S, Montagna P *et al.* New roles for estrogens in rheumatoid arthritis. *Clin Exp Rheumatol* 2003; 21:687–90.
 - 34 Rovinsky J, Radikova Z, Imrich R, Greguska O, Vigas M, Macho L. Gonadal and adrenal steroid hormones in plasma and synovial fluid of patients with rheumatoid arthritis. *Endocr Regul* 2004; 38:143–9.
 - 35 Mittendorfer B. Insulin resistance: sex matters. *Curr Opin Clin Nutr Metab Care* 2005; 8:367–72.
 - 36 Carlsten H, Nilsson N, Jonsson R, Backman K, Holmdahl R, Tarkowski A. Estrogen accelerates immune complex glomerulonephritis but ameliorates T cell-mediated vasculitis and sialadenitis in autoimmune MRL lpr/lpr mice. *Cell Immunol* 1992; 144:190–202.

- 37 Hermann C, De Fine ON, Host A, Begtrup K, Hollnagel H. Prevalence, severity and determinants of asthma in Danish five-year-olds. *Acta Paediatr* 2006; **95**:1182–90.
- 38 Hallberg J, Anderson M, Wickman M, Svartengren M. Sex influences on lung function and medication in childhood asthma. *Acta Paediatr* 2006; **95**:1191–6.
- 39 Schatz M, Clark S, Camargo CA Jr. Sex differences in the presentation and course of asthma hospitalizations. *Chest* 2006; **129**:50–5.
- 40 Tollefsen E, Langhammer A, Romundstad P, Bjermer L, Johnsen R, Holmen TL. Female gender is associated with higher incidence and more stable respiratory symptoms during adolescence. *Respir Med* 2006, (in press).
- 41 Gibbs CJ, Coutts II, Lock R, Finnegan OC, White RJ. Premenstrual exacerbation of asthma. *Thorax* 1984; **39**:833–6.
- 42 Haggerty CL, Ness RB, Kelsey S, Waterer GW. The impact of estrogen and progesterone on asthma. *Ann Allergy Asthma Immunol* 2003; **90**:284–91.
- 43 Eliasson O, Scherzer HH, DeGraff AC Jr. Morbidity in asthma in relation to the menstrual cycle. *J Allergy Clin Immunol* 1986; **77**:87–94.