

Estrogen Stimulates Th2 Cytokine Production and Regulates the Compartmentalisation of Eosinophils during Allergen Challenge in a Mouse Model of Asthma

Yeping Cai Jiansheng Zhou Dianne C. Webb

Immunology Department, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia

Key Words

Asthma • Eosinophils • Estrogen • IL-13 • IL-5 • Mucus

Abstract

Background: The observation that asthma becomes more prevalent following puberty in females suggests estrogen potentiates the development of this disease. However, most studies examining the role of estrogen in rodent models of asthma are complicated by their reliance on ovariectomised mice in which hormones other than estrogen are also attenuated. **Methods:** We aimed to understand the influence of estrogen on allergic airway disease by using type I (tamoxifen) or type II (ICI 182,780) antagonists in female mice or delivering estradiol to male mice during aeroallergen challenge. **Results:** The antagonists showed that estrogen promoted both the mobilisation of bone marrow eosinophils and egression of eosinophils to the airway lumen. These findings were corroborated in male mice treated with estradiol, which increased eosinophil numbers in both blood and airways. Estrogen stimulated goblet cell hyperplasia and baseline lung resistance, but had little effect on the number of eosinophils in the bronchial submucosa or methacholine-induced airway hyperreactivity. Estrogen receptor α was expressed by CD4⁺ T cells from allergic mice, and estrogen pro-

moted the production of IL-5 and IL-13, and suppressed the production of the eicosanoid 12-HETE by mediastinal lymph node cells. **Conclusions:** These data show that during aeroallergen challenge, estrogen stimulates Th2 cytokine production, which may be linked to its ability to suppress 12-HETE. Lung resistance at baseline, goblet cell hyperplasia and the compartmentalisation of eosinophils was also influenced by estrogen. However, estrogen does not play a major role in stimulating enhanced sensitivity to methacholine-induced lung resistance.

Copyright © 2012 S. Karger AG, Basel

Introduction

Coincident with the onset of puberty and increasing levels of circulating estrogen, asthma becomes significantly more common in women than in men, particularly during the reproductive years and pregnancy [reviewed in ref. 1–3]. This suggests a role for estrogen in promoting the development of asthma in women. The influence of estrogen on allergic airway disease has been investigated in rodent models, which show that ovariectomy (OVX) prior to allergen sensitisation attenuates the migration of inflammatory cells to the lungs [4, 5], sug-

gesting estrogen is pro-inflammatory during allergen sensitisation. Using mice who have undergone OVX after sensitisation, Roffo-Vasquez et al. [5] suggest a biphasic scenario in which estrogen enhances T cell activation during allergen sensitisation and yet suppresses eosinophil trafficking to the lung during allergen challenge. However, the role of estrogen in allergic inflammation is controversial with studies such as those by Dimitropoulou et al. [6] showing that implantation of estradiol pellets in OVX mice before sensitisation reduced airway inflammation. The use of models employing OVX to study the role of estrogen in allergic inflammation is complicated because ovarian products other than estrogen may influence the observed effects [7]. Indeed, a recent study suggested an important consideration when analysing the role of sex hormones in allergic disease is not the levels of circulating estrogen per se, but the estrogen/progesterone ratio [7]. In congruence with this concept, estradiol supplementation of OVX mice does not fully compensate the anti-inflammatory effects of OVX in allergic airway disease [5]. However, treatment of allergic rats with the estrogen antagonist ICI 182,780 does not affect the levels of circulating progesterone, which are higher than in allergic OVX rats despite similar levels of broncho-alveolar lavage fluid (BALF) eosinophilia [7], suggesting estrogen rather than the estrogen/progesterone ratio may be more important in regulating allergic inflammation. Although the use of estrogen antagonists assists in understanding the effects of estrogen in allergic disease, antagonists such as the type I estrogen antagonist tamoxifen is not without its complexities. Tamoxifen exhibits mixed agonist/antagonist activities that are dose, tissue and species specific. For example, tamoxifen used throughout sensitisation and challenge convincingly inhibited eosinophilia in the airways of allergic mice [5], but its suppressive effects were not as significant in rats [4]. The interaction of tamoxifen with the estrogen receptor (ER) combines high affinity occupancy of the primary estrogen-binding site and activation of the ER in addition to weaker occupancy of a secondary site to antagonise the receptor [8]. Collectively, the likelihood of the predominance of either event is dependent on the dose of tamoxifen used. Higher doses of tamoxifen can thus overcome its agonistic properties by increasing the occupancy and thus dominance of the inhibitory site. In contrast, type II anti-estrogens, such as ICI 182,780, are devoid of such agonist activity [9]. However, to date, ICI 182,780 has had limited use in investigating the function of estrogen in allergic disease in experimental models of asthma. An additional consideration in the

analysis of the role of estrogen in the development of allergic disease is dissection of both temporal and spatial mechanisms of disease progression. Thus, early estrogen-sensitive events that regulate T cell activation and expansion dictate the subsequent development of allergic inflammation. Considering the levels of circulating estrogen in females are cyclical in nature, the influence of estrogen on the consequences of allergen exposure may vary depending on the stage during the menstrual cycle or pregnancy at which an allergen is encountered. It is also likely that allergen sensitisation occurs during childhood with subsequent episodes of allergen challenge experienced through into puberty when estrogen levels are increased.

The experimental approach in the current study is designed to assess the role of estrogen in asthma models. We will employ two strategies to antagonise estrogen in intact female mice: (1) a high dose of tamoxifen thought sufficient to overcome its agonistic properties, or (2) the type II pure estrogen antagonist ICI 182,780. To dissect out the cyclical effects of estrogen on the dynamics of immune cell recruitment to the allergic lung, we will employ a model in which estrogen is neutralised after sensitisation. We will complement the studies in female mice by determining the effects of supplemental estradiol during allergen challenge of male mice.

Methods

Induction of Allergic Airway Disease

Female BALB/c mice were sensitised by intraperitoneal injection with 50 µg of ovalbumin (OVA, Fraction V; Sigma, St. Louis, Mo., USA), or the equivalent of saline for nonallergic mice, mixed with 20 µl of Rehydralgel (Al₂O₃-based adjuvant) made up to 200 µl with 0.9% sterile saline. On days 12, 14, 16 and 19, all mice were challenged with an aerosol generated from 10 mg/ml OVA in saline for 3 × 30 min per day with 30-min breaks between aerosols [10]. Tamoxifen (Sigma) was dissolved at 100 mg/ml in ethanol, and then diluted in corn oil (Sigma) to 10 mg/ml. ICI 182,780 (Tocris Bioscience, Bristol, UK) was dissolved at 25 mg/ml in ethanol then diluted to 2.5 mg/ml in corn oil. Mice were injected intraperitoneally with 1 mg of tamoxifen, 250 µg of ICI 182,780 or the equivalent of ethanol in corn oil 4 h before OVA aerosols. Estradiol was dissolved at 20 mg/ml in DMSO then diluted to 50 µg/ml in sterile saline. Male mice received 400 µg/kg estradiol [5] or the equivalent of DMSO by intraperitoneal injection. Mice were sacrificed on day 20 and BALF was obtained by flushing the airways with 2 ml of PBS. Numbers of cells in BALF were determined by total cell counts and differential counts of May-Grünwald-Giemsa-stained cytopins. Lungs were dissected from the thoracic cavity for recovery of mediastinal lymph nodes (MLN). All mice were treated according to Australian National University Animal Welfare guidelines (JMB32/07 and JMB55/10).

Quantification of Eosinophils and Goblet Cells

Lung sections were stained with Carbol's chromotrope-haematoxylin or Alcian blue-periodic acid-Schiff for enumeration of peribronchial eosinophils and goblet cells, respectively [10].

Analysis of Lung Function

Lung resistance was determined in anaesthetised mice attached to an electronically controlled mouse ventilator. First saline, then increasing doses of methacholine were nebulised and delivered via the endotracheal tube, and lung resistance was calculated from tracheal pressure measurements with BioSystem XA software (Buxco Electronics, Wilmington, N.C., USA) as described in detail elsewhere [11].

Purification of Dendritic Cells and CD4⁺ T Cells for RT-PCR

Dendritic cells (DCs) and CD4⁺ T cells were enriched from MLN cells or lungs from allergic mice using the MinimaCS CD11c or CD4 magnetic bead system (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously [10].

cDNA Preparation and RT-PCR

RNA was purified with TRIzol reagent (Life Technologies, Rockville, Md., USA) and reverse transcribed using oligo dT and superscript III RT (Invitrogen, Carlsbad, Calif., USA). PCR for ER α was performed using 5'-GCAGTAACGAGAAAGGAAAC/5'-CGAGACCAATCATCAGAATC [12] and for GAPDH with 5'-CATGGAGAAGGCCGGGGC/5'-CGGACACATTGGG-GGTAGG (GenBank NM_008084).

Cytokine Production

MLN cells were stimulated with 1 mg/ml OVA in complete MLC medium for 68 h at 37°C, 5% CO₂ in 96-well plates with 2×10^6 cells per well. IL-5 and IL-13 were measured by ELISA as described elsewhere [10] using capture and detection IL-5 (Pharmingen, San Diego, Calif., USA) and IL-13 antibodies (R&D Systems, Minneapolis, Minn., USA). Limits of detection were 6 and 12 pg/ml, respectively. In some experiments, MLN cells from allergic female mice were incubated for 40 h with estradiol in MLC with 1 mg/ml OVA and charcoal/dextran-treated FBS (HyClone/Thermo Scientific, Logan, Utah, USA).

C18 Extraction of Culture Supernatants and Measurement of 12-HETE

Hydrophobic material was extracted from MLN cell culture supernatants by reverse phase chromatography as described in more detail previously [13]. Samples were assayed using a correlate enzyme immunoassay 12-HETE (12-hydroxytetraenoic acid) kit (Assay Designs, Ann Arbor, Mich., USA). The limit of detection was 50 pg/ml.

Isolation of Bone Marrow Cells

Bone marrow was collected from femurs by flushing with 8 ml of cold PBS. Eosinophils and eosinophilic myelocytes in cytopins stained with May-Grünwald-Giemsa were determined by morphological criteria [14].

Quantitation of Estradiol

Estradiol was measured in culture supernatants using an EIA kit (Enzo Life Sciences, Plymouth, Pa., USA) with a limit of detection of 30 pg/ml.

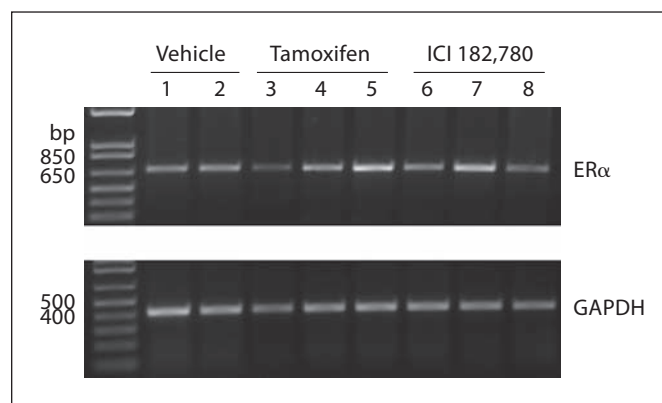


Fig. 1. ER expression in the lungs of allergic female mice. RT-PCR for ER α and GAPDH in the lungs from individual allergic female mice treated with vehicle (lanes 1–2), tamoxifen (lanes 3–5) or ICI 182,780 (lanes 6–8) during aeroallergen challenge.

Statistical Analysis

Significance of differences between experimental groups was analysed using Student's unpaired t test or ANOVA with post hoc Tukey-Kramer multiple comparison test. Values are the mean $\pm$ SEM. Differences in means were considered significant if $p < 0.05$.

Results

ER α Expression in the Allergic Lung

To help understand the role of estrogen in allergic airway disease, we evaluated expression of ER α in the allergic lung. Because some studies suggest that ICI 182,780 inhibits the growth of breast cancer cells by down-regulating expression of ER α [15], we also assessed whether ER α expression was affected by tamoxifen or ICI 182,780. Notably, a higher dose of tamoxifen was used than in previous studies [5] to block its agonistic properties. All vehicle- and antagonist-treated mice expressed ER α (fig. 1), suggesting the allergic lung has the capacity to respond to estrogen and does not down-regulate ER α in response to either tamoxifen or ICI 182,780.

Comparison of Inflammation and Goblet Cell Hyperplasia in Allergic Mice Treated with Tamoxifen or ICI 182,780

To investigate the ability of estrogen to influence allergic inflammation during allergen challenge, we assessed eosinophilia in allergic mice treated with tamoxifen or ICI 182,780. Both estrogen antagonists significantly inhibited the percentage of eosinophils in the blood (fig. 2a, d). In contrast, neither tamoxifen nor ICI 182,780

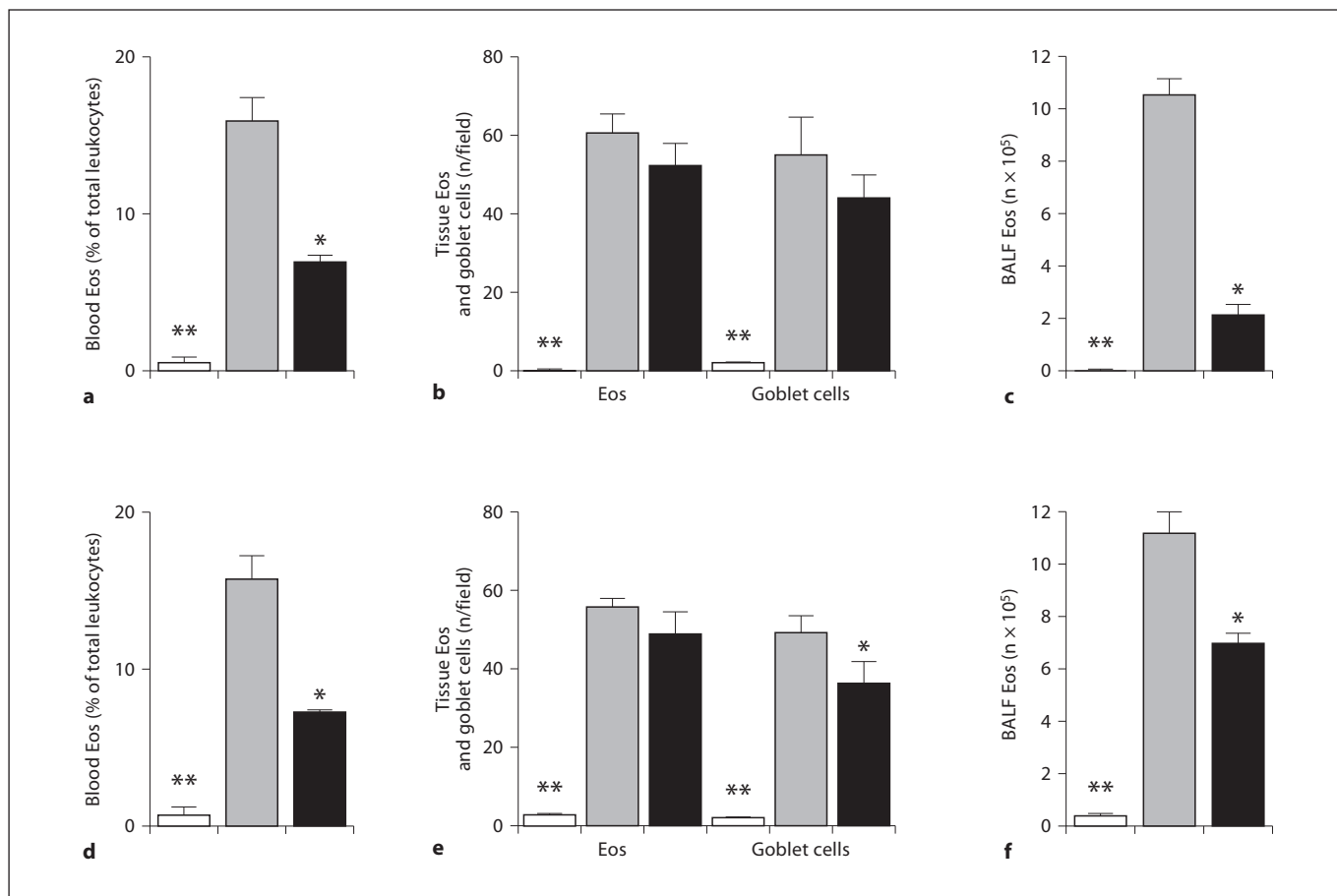


Fig. 2. Influence of estrogen on blood and lung eosinophilia. **a–c** Tamoxifen. **d–f** ICI 182,780. **a, d** Percentages of blood eosinophils were determined by morphological criteria in May-Grünwald-Giemsa-stained smears. **b, e** Lung sections were either stained with Carbol's chromotrope or Alcian blue-periodic acid-Schiff, and the numbers of submucosal eosinophils and goblet cells were counted in the peribronchial region of at least 10 similar fields (magnification $\times 1,000$) per mouse. **c, f** Numbers of

airway eosinophils were quantified by correlating differential counts of May-Grünwald-Giemsa-stained cytopins with total cell counts in the BALF. Samples were from either nonallergic mice (clear bars), allergic mice treated with vehicle (grey bars) or antagonist (black bars). Eos = Eosinophils. Values are the mean $\pm$ SEM of at least 4 mice per group. * $p < 0.05$ vs. vehicle-treated allergic mice, ** $p < 0.05$ vs. all allergic mice.

influenced their numbers in the bronchial submucosa (fig. 2b, e). Both antagonists also reduced the migration of eosinophils from the submucosa of the lung tissue to the airway lumen (BALF: fig. 2c, f). At the concentrations used, tamoxifen was more efficient than ICI 182,780 at inhibiting eosinophilia in BALF. There was a trend towards reduced numbers of goblet cells lining the airways of tamoxifen- and ICI 182,780-treated mice that reached significance with the latter (fig. 2b, e). Collectively, these data show that estrogen influences the compartmentalisation of eosinophils and stimulates goblet cell hyperplasia during aeroallergen challenge.

Estrogen and Allergen-Induced Th2 Cytokine Production

As our data showed that estrogen influenced the compartmentalisation of eosinophils, we investigated whether estrogen also influenced the production of the eosinophil-responsive cytokines IL-5 and IL-13. MLN cells from allergic female mice treated with the estrogen antagonists showed attenuated IL-5 and IL-13 production when cultured *ex vivo* (fig. 3a, b). At the concentrations used, ICI 182,780 was more effective in attenuating both IL-5 and IL-13, which were on average 2.0 and 1.2 ng/ml lower than in mice treated with tamoxifen. ER α was ex-

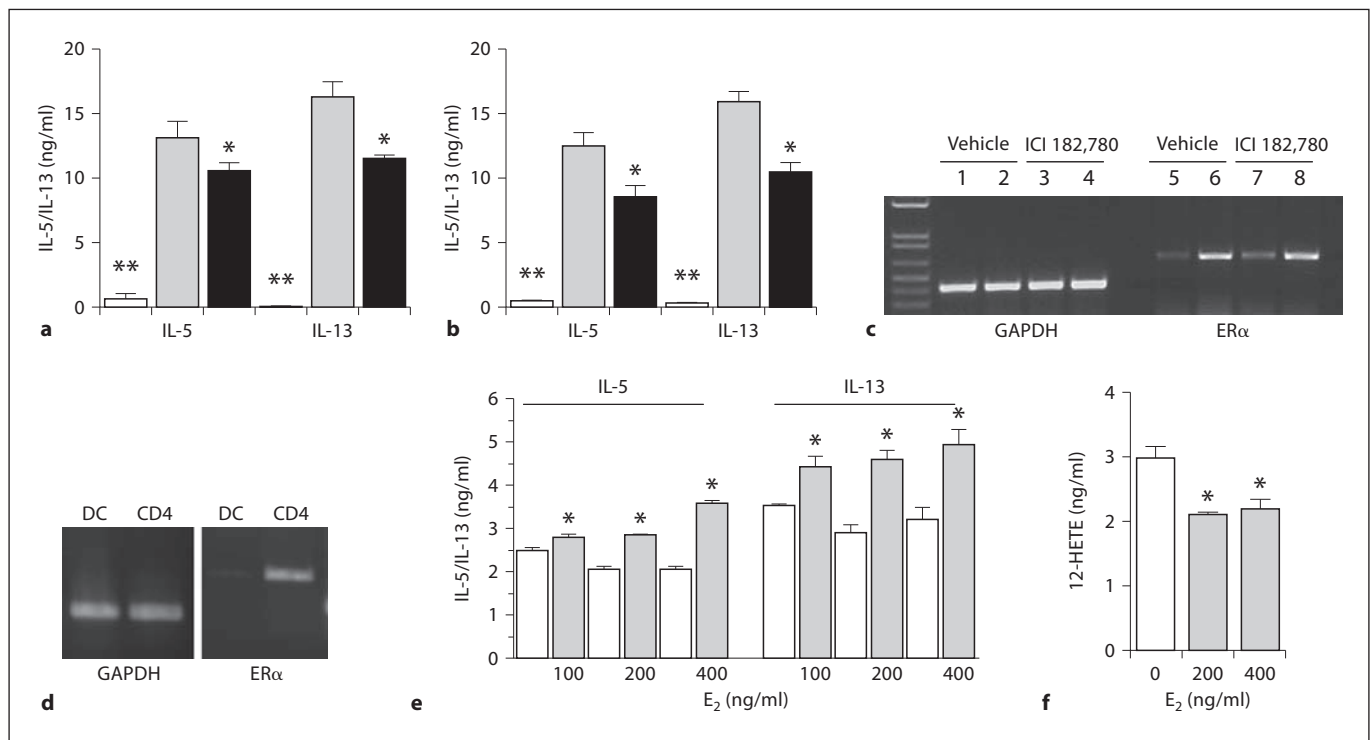


Fig. 3. Effect of estrogen on Th2 cytokine production. **a, b** IL-5 and IL-13 produced by MLN from allergic female mice treated with either tamoxifen (**a**) or ICI 182,780 (**b**) and then cultured with OVA ex vivo were assayed in triplicate by ELISA. Samples were from nonallergic mice (clear bars), allergic mice treated with vehicle (grey bars) or antagonist (black bars). **c** RT-PCR for ER α and GAPDH expressed by MLN from individual allergic female mice treated with vehicle (lanes 1, 2, 5, 6) or ICI 182,780 (lanes 3, 4, 7, 8) during aeroallergen challenge. **d** RT-PCR for ER α ex-

pressed by CD4⁺ T cells and DCs purified from the MLN of allergic mice. IL-5, IL-13 (**e**) and 12-HETE (**f**) produced by MLN from allergic female mice treated with vehicle (white bars) or varying concentrations of estradiol (E₂; grey bars) in vitro were measured by ELISA. All results are representative of duplicate experiments and values are the mean $\pm$ SEM of at least 4 mice per group. * $p < 0.05$ vs. vehicle-treated allergic mice or vehicle-treated MLN, ** $p < 0.05$ vs. both vehicle- and antagonist-treated allergic mice.

pressed by MLN cells from allergic female mice, suggesting these cells may be directly responsive to estrogen (fig. 3c). We did not examine ER α expression in MLN from tamoxifen-treated mice as both antagonists showed a similar pattern of Th2 cytokine production. ER α expression was detected in CD4⁺ T cells, but was barely detectable in DCs from MLN (fig. 3d) and not detected in DCs enriched from the allergic lung (data not shown), suggesting it more likely that estrogen directly influences the capacity of CD4⁺ Th2 cells to produce IL-5 and IL-13 rather than to stimulate antigen presentation by DCs during aeroallergen challenge. MLN cells incubated with estradiol in vitro showed enhanced production of both IL-5 and IL-13 (fig. 3e). Because a study by Stygar et al. [16] suggested estrogen-suppressed expression of 12-lipoxygenase in blood leucocytes, we also investigated the effects of estrogen on the production of 12-HETE (a 12-li-

poxygenase product) by MLN cells from allergic female mice. Compared to vehicle, treatment of MLN cells with estradiol significantly attenuated the production of 12-HETE (fig. 3f). These data show that estrogen influences the production of Th2 cytokines and 12-HETE by CD4⁺ T cells.

Estrogen Promotes Egression of Eosinophils from the Bone Marrow

The observation that estrogen enhanced blood eosinophilia (fig. 2) suggested estrogen might promote mobilisation of eosinophils in the bone marrow. Therefore, we assessed the numbers of mature eosinophils and eosinophilic myelocytes in the bone marrow of allergic female mice treated with vehicle or ICI 182,780. The tamoxifen group was not examined because similar numbers of blood and tissue eosinophils were seen in both antagonist

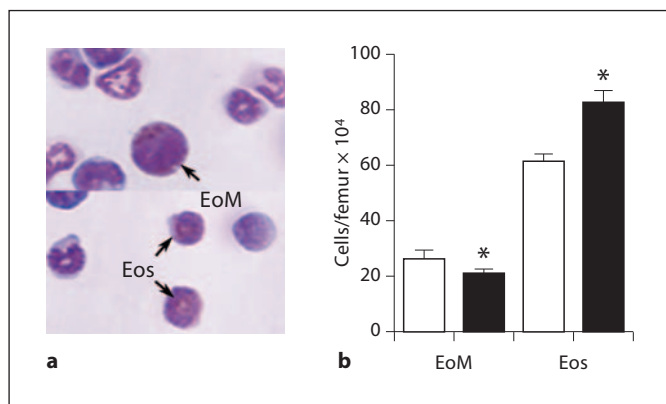


Fig. 4. The influence of estrogen on bone marrow eosinophils. **a** Eosinophil myelocytes (EoM) and mature eosinophils (Eos) were determined in bone marrow cytopspins by morphological criteria. **b** Numbers of eosinophils and precursors were quantified in mice treated with vehicle or ICI 182,780 by correlating the total number of cells in the bone marrow of 5 mice per group with May-Grünwald-Giemsa-stained cytopspins. Values are the mean $\pm$ SEM, * $p < 0.05$ of ICI 182,780-treated (black bars) compared to vehicle-treated (clear bars) mice.

treatment groups. Eosinophils and eosinophil myelocytes in bone marrow cytopspins (fig. 4a) displayed typical morphology [14]. However, the number of mature eosinophils in the bone marrow was significantly elevated in mice treated with ICI 182,780 (fig. 4b). Considering these mice displayed attenuated blood eosinophilia, it is likely that estrogen enhances mobilisation of bone marrow eosinophils, and when estrogen is inhibited, fewer eosinophils egress into the blood, causing their accumulation within the bone marrow. We also noted that the number of eosinophil myelocytes was marginally lower in the ICI 182,780 group, suggesting estrogen also plays a minor role in promoting eosinophilopoiesis during allergen challenge.

Comparison of Airway Function in Allergic Mice Treated with Tamoxifen and ICI 182,780

We next investigated the effects of estrogen on lung mechanics. Baseline lung resistance (lung resistance measured during challenge with saline) was significantly lower in allergic female mice treated with either tamoxifen or ICI 182,780 than in those treated with vehicle during allergen challenge (fig. 5). However, while the difference between vehicle- and tamoxifen- or ICI 182,780-treated mice in lung resistance induced by methacholine challenge reached significance at some concentrations of methacholine (fig. 5a, b), overall this appeared to reflect

inherent differences in baseline lung resistance rather than increased sensitivity to methacholine. Collectively, these data show that during the challenge phase of antigen exposure, estrogen potentiates baseline lung resistance but has minimal effect in enhancing the sensitivity to methacholine-induced bronchoconstriction.

Estrogen Enhances Blood and Airway Eosinophilia in Allergic Male Mice

Previous studies showed that estrogen can suppress airway hyperresponsiveness in male mice [17] suggesting ERs are expressed in the lungs of male mice. Therefore, we tested whether estradiol could influence blood and airway eosinophilia (in BALF) in allergic male mice. Mice treated with estradiol during allergen challenge displayed significantly elevated blood eosinophilia (fig. 6a) and higher numbers of eosinophils in the airways compared to vehicle-treated mice (fig. 6b). These observations confirm those in female mice showing estrogen influences the mobilisation of eosinophils from the bone marrow to the blood and their migration to the airway lumen.

Discussion

The current study demonstrated that estrogen plays a significant role in the development of allergic airway disease by regulating the mobilisation of bone marrow eosinophils and the compartmentalisation of eosinophils, stimulating Th2 cytokine production and goblet cell hyperplasia, and enhancing lung resistance at baseline. ER α expression was detected in the allergic lung, MLN and CD4+ T cells, suggesting these tissues and cells are directly responsive to estrogen. Indeed, MLN cells from allergic female mice treated with the estrogen antagonists exhibited a reduced propensity for production of the Th2 cytokines, IL-5 and IL-13, and MLN cells from allergic mice produced enhanced levels of IL-5 and IL-13 when exposed to estradiol in vitro. While ER α was highly expressed by CD4+ T cells from the MLN, the observation that it was only weakly expressed in MLN DCs and undetectable in lung DCs was somewhat surprising considering that studies have shown that estrogen promotes the differentiation of a subset of DCs from bone marrow precursors in vitro [18]. However, estrogen does not increase the proliferation of differentiated DCs [18], and since a large percentage of DCs in the allergic lung and MLN express an activated phenotype [10], it is not unreasonable to suggest that, as similarly occurs with the nuclear recep-

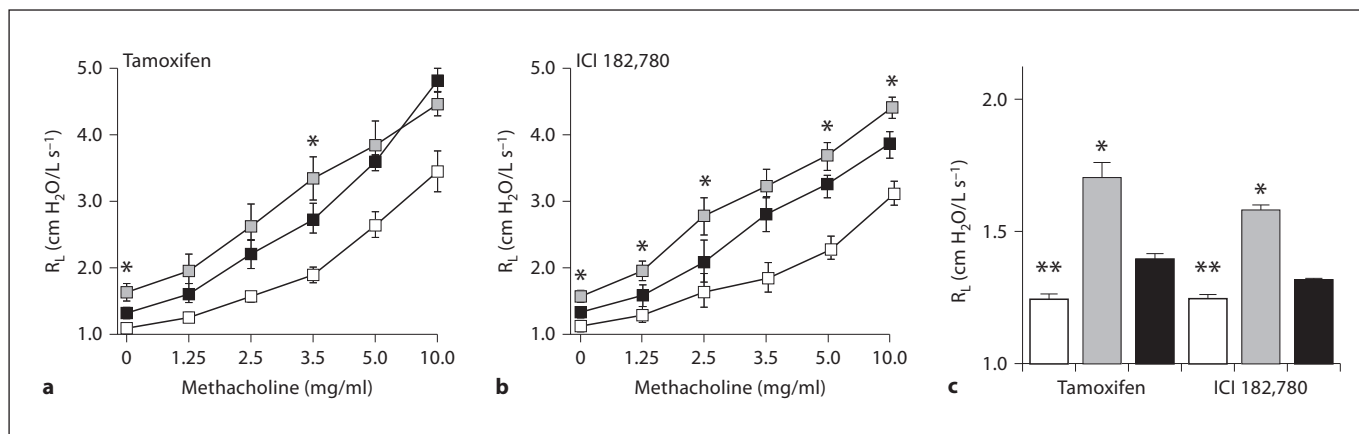


Fig. 5. The effect of estrogen on lung function. **a, b** Anaesthetised mice were intubated and resistance of the lung (R_L) was measured in response to saline and then increasing doses of methacholine in nonallergic mice (clear bars) and allergic mice treated with vehicle (grey bars), tamoxifen (**a**; black bars) or ICI 182,780 (**b**; black bars). All allergic mice exhibited significantly higher resistance than

nonallergic mice. **c** Baseline lung resistance (measured during saline challenge) in nonallergic mice (clear bars), or allergic mice treated with vehicle (grey bars) or tamoxifen or ICI 182,780 (black bars). Values are the mean $\pm$ SEM of at least 4 mice per group. * $p < 0.05$ for vehicle- compared to antagonist-treated allergic mice, ** $p < 0.05$ for nonallergic compared to all allergic mice.

tor, PPAR γ [19], ER α expression is down-regulated following differentiation. In contrast, the strong expression of ER α by CD4 $^+$ T cells makes it likely that estrogen directly influences the function of CD4 $^+$ T cells during aeroallergen challenge in allergic mice. This concept is supported by previous studies suggesting estrogen can increase Th2 responses [reviewed in ref. 20] and potentiate IL-4 and GATA3 transcripts in splenocyte CD4 $^+$ T cells stimulated in vitro [21]. Our observation that estrogen suppresses 12-HETE produced by MLN cells suggests a possible mechanism linking estrogen to Th2 responses. Our recent studies showed that 12-HETE plays a significant role in the cross-talk between DCs and CD4 $^+$ T cells in the MLN from allergic mice [13]. These CD4 $^+$ T cells expressed 12-lipoxygenase and supplementation of DC/CD4 T cells co-cultured with 12-HETE significantly attenuated production of IL-5 and IL-13 [13]. Estrogen is known to down-regulate 12-lipoxygenase in peripheral blood leucocytes [16] and 12-HETE may modulate immune responses by activating the nuclear receptor PPAR γ [22, 23]. Therefore, considering the proven suppressive effects of this eicosanoid on Th2 cytokine production [13], it is likely that estrogen stimulates Th2 cytokine production by suppressing the catalysis of 12-HETE by CD4 $^+$ T cells. Of the Th2 cytokines, IL-5 is long recognised for its ability to promote the differentiation and proliferation of eosinophil precursors [24, 25] and to stimulate mobilisation of eosinophils from the bone marrow compart-

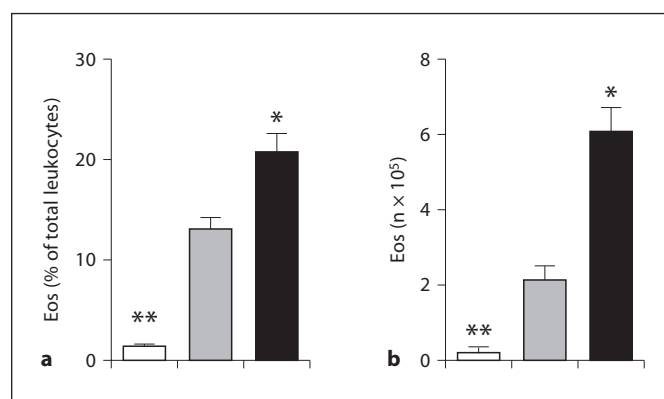


Fig. 6. The influence of estradiol on eosinophilia in allergic male mice. Peripheral blood (**a**) and airway eosinophilia (**b**; BALF) were determined as described in the legend to figure 2 in nonallergic mice (clear bars), or allergic mice treated with either vehicle (grey bars) or estradiol (black bars) during aeroallergen challenge. Values are the mean $\pm$ SEM of 5 mice per group. * $p < 0.05$ vs. vehicle-treated allergic mice, ** $p < 0.05$ vs. allergic mice. Eos = Eosinophils.

ment [26]. It is thus unsurprising that the ability of estrogen to enhance IL-5 production correlated with our observation that estrogen promoted eosinophil mobilisation from the bone marrow. Localised IL-5 also plays a role in the migration of eosinophils from the bronchial submucosa to the airway lumen, as demonstrated in studies

showing mice treated intranasally with anti-IL-5 antibody exhibit attenuated numbers of eosinophils in the airway lumen [25]. However, the role of IL-5 appears more important in the recruitment of eosinophils to the airways than their persistence in the lung tissue. Flood-Page et al. [27] showed that, despite a dramatic reduction in blood and airway eosinophilia, significant levels of eosinophils persisted in the bronchial submucosa following anti-IL-5 treatment of asthmatics. Our observation that estrogen promoted blood and airway eosinophilia, without affecting the numbers of eosinophils in the bronchial submucosa, suggests the ability of estrogen to stimulate production of IL-5 may contribute to the observed compartmentalisation of eosinophils. Transepithelial egression of leucocytes is proposed as a mechanism by which the diseased airway tissue is cleared of eosinophils [28, 29]. Therefore, it is also likely that the increase in availability of eosinophils for lung entry from the blood that is stimulated by estrogen is balanced by the effects of estrogen in enhancing the 'exit' of eosinophils from the bronchial submucosa to the airway lumen. The net effect of these processes seems to be homeostasis in the numbers of eosinophils in the bronchial submucosa. Our data also showed that estrogen potentiated IL-13 production by MLN cells from allergic mice during allergen challenge. IL-13R α is expressed by bronchial epithelial cells and is known to influence critical allergy-related processes including the hypersecretion of mucus [30, 31]. At the concentrations used, ICI 182,780 was more effective than tamoxifen at inhibiting the production of IL-13 by MLN cells, and consistent with the cytokine data, a trend towards reduced numbers of goblet cells was observed in both groups of antagonist-treated mice that reached significance in the ICI 182,780 group. Thus estrogen enhances goblet cell hyperplasia in the allergic lung and this is likely to be mediated through the ability of this hormone to potentiate IL-13 production. Baseline lung resistance in allergic female mice treated with the estrogen antagonists was lower than in the vehicle control allergic groups, suggesting estrogen also contributes to deteriora-

tion in lung function during the effector phase of allergen exposure. When challenged with methacholine, lung resistance was lower in the antagonist-treated group at some concentrations of methacholine, especially with ICI 182,780. However, overall, the ability of estrogen to potentiate methacholine-induced airway hyperresponsiveness in allergic mice seems moderate, with differences in the treatment groups more reflective of inherent differences in baseline resistance, which are likely due to structural changes to the airways, rather than increased sensitivity to methacholine-induced bronchospasm. Previous studies using mice that have been ovariectomised after sensitisation have shown no effect on lung function [5], which may be reflective of the different approaches used to modify the function of oestrogen. In summary, our data show that during the effector phase of allergen exposure, estrogen potentiated IL-5 and IL-13 production by a mechanism that is likely related to its ability to attenuate production of 12-HETE, a known inhibitor of Th2 cytokine production by CD4 $^{+}$ T cells [13]. The enhanced production of IL-5 and IL-13 correlated with elevated blood and airway eosinophilia, goblet cell hyperplasia and increased lung resistance at baseline. Collectively, these findings show that estrogen contributes to the severity of allergic disease during the effector phase of allergen exposure and provides evidence to support the concept that estrogen contributes to the increase in asthma severity in females following the onset of puberty.

Acknowledgements

We thank Anne Prinns, John Curtin School of Medical Research, Australian National University, for preparing the lung sections. The authors gratefully acknowledge financial support from a New South Wales Asthma Foundation Innovation Grant (D.C.W.) and a National Health and Medical Research Project Grant (D.C.W.). None of the authors have a financial relationship with companies that have an interest in the subject matter of this paper.

References

- 1 Almqvist C, Worm M, Leynaert B: Impact of gender on asthma in childhood and adolescence: a GA2LEN review. *Allergy* 2008;63: 47–57.
- 2 McCallister JW, Mastronarde JG: Sex differences in asthma. *J Asthma* 2008;45:853–861.
- 3 Jensen-Jarolim E, Untersmayr E: Gender-medicine aspects in allergology. *Allergy* 2008;63:610–615.
- 4 Ligeiro de Oliveira AP, Oliveira-Filho RM, da Silva ZL, Borelli P, Tavares de Lima W: Regulation of allergic lung inflammation in rats: Interaction between estradiol and corticosterone. *Neuroimmunomodulation* 2004;11:20–27.
- 5 Rizzo-Vasquez Y, Ligeiro de Oliveira AP, Page CP, Spina D, Tavares-de-Lima W: Role of sex hormones in allergic inflammation in mice. *Clin Exp Allergy* 2007;37:459–470.

- 6 Dimitropoulou C, Drakopanagiotakis F, Chatterjee A, Snead C, Catravas JD: Estrogen replacement therapy prevents airway dysfunction in a murine model of allergen-induced asthma. *Lung* 2009;187:116–127.
- 7 Ligeiro de Oliveira AP, Domingos HV, Cavriani G, Damazo AS, Dos Santos Franco AL, Oliani SM, Oliveira-Filho RM, Vargaftig BB, de Lima WT: Cellular recruitment and cytokine generation in a rat model of allergic lung inflammation are differentially modulated by progesterone and estradiol. *Am J Physiol Cell Physiol* 2007;293:C1120–C1128.
- 8 Jensen EV, Khan SA: A two-site model for antiestrogen action. *Mech Ageing Dev* 2004;125:679–682.
- 9 Robertson JF: Faslodex (ICI 182, 780), a novel estrogen receptor downregulator – future possibilities in breast cancer. *J Steroid Biochem Mol Biol* 2001;79:209–212.
- 10 Webb DC, Cai Y, Matthaie KI, Foster PS: Comparative roles of IL-4, IL-13, and IL4R α in dendritic cell maturation and CD4+ Th2 cell function. *J Immunol* 2007;178:219–227.
- 11 Siegle JS, Hansbro N, Herbert C, Yang M, Foster PS, Kumar RK: Airway hyperreactivity in exacerbation of chronic asthma is independent of eosinophilic inflammation. *Am J Respir Cell Mol Biol* 2006;35:565–570.
- 12 Lu B, Dotzlaw H, Leygue E, Murphy LJ, Watson PH, Murphy LC: Estrogen receptor α mRNA variants in murine and human tissues. *Mol Cell Endocrinol* 1999;158:153–161.
- 13 Cai Y, Kumar RK, Zhou J, Foster PS, Webb DC: Ym1/2 promotes Th2 cytokine expression by inhibiting 12/15(S)-lipoxygenase: identification of a novel pathway for regulating allergic inflammation. *J Immunol* 2009;182:5393–5399.
- 14 Travlos GS: Normal structure, function, and histology of the bone marrow. *Toxicol Pathol* 2006;34:548–565.
- 15 Martin LA, Pancholi S, Chan CM, Farmer I, Kimberley C, Dowsett M, Johnston SR: The anti-oestrogen ICI 182,780, but not tamoxifen, inhibits the growth of MCF-7 breast cancer cells refractory to long-term oestrogen deprivation through down-regulation of oestrogen receptor and IGF signalling. *Endocr Relat Cancer* 2005;12:1017–1036.
- 16 Stygar D, Masironi B, Eriksson H, Sahlin L: Studies on estrogen receptor (ER) α and β responses on gene regulation in peripheral blood leukocytes in vivo using selective ER agonists. *J Endocrinol* 2007;194:101–119.
- 17 Matsubara S, Swasey CH, Loader JE, Dakhamma A, Joetham A, Ohnishi H, Balhorn A, Miyahara N, Takeda K, Gelfand EW: Estrogen determines sex differences in airway responsiveness after allergen exposure. *Am J Respir Cell Mol Biol* 2008;38:501–508.
- 18 Paharkova-Vatchkova V, Maldonado R, Kovats S: Estrogen preferentially promotes the differentiation of CD11c+ CD11b (intermediate) dendritic cells from bone marrow precursors. *J Immunol* 2004;172:1426–1436.
- 19 Varga T, Nagy L: Nuclear receptors, transcription factors linking lipid metabolism and immunity: the case of peroxisome proliferator-activated receptor gamma. *Eur J Clin Invest* 2008;38:695–707.
- 20 Pernis AB: Estrogen and CD4+ T cells. *Curr Opin Rheumatol* 2007;19:414–420.
- 21 Lambert KC, Curran EM, Judy BM, Milligan GN, Lubahn DB, Estes DM: Estrogen receptor α (ER α) deficiency in macrophages results in increased stimulation of CD4+ T cells while 17 β -estradiol acts through ER α to increase IL-4 and GATA-3 expression in CD4+ T cells independent of antigen presentation. *J Immunol* 2005;175:5716–5723.
- 22 Huang JT, Welch JS, Ricote M, Binder CJ, Willson TM, Kelly C, Witztum JL, Funk CD, Conrad D, Glass CK: Interleukin-4-dependent production of PPAR-gamma ligands in macrophages by 12/15-lipoxygenase. *Nature* 1999;400:378–382.
- 23 Li Q, Cheon YP, Kannan A, Shanker S, Bagchi IC, Bagchi MK: A novel pathway involving progesterone receptor, 12/15-lipoxygenase-derived eicosanoids, and peroxisome proliferator-activated receptor gamma regulates implantation in mice. *J Biol Chem* 2004;279:11570–11581.
- 24 Yamaguchi Y, Suda T, Suda J, Eguchi M, Miura Y, Harada N, Tominaga A, Takatsu K: Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J Exp Med* 1988;167:43–56.
- 25 Tomaki M, Zhao L-L, Lundahl J, Sjöstrand M, Jordana M, Lindén A, O'Byrne PM, Lötvall J: Eosinophilopoiesis in a murine model of allergic airway eosinophilia: involvement of bone marrow IL-5 and IL-5 receptor α_1 . *J Immunol* 2000;165:4040–4050.
- 26 Palframan RT, Collins PD, Severs NJ, Rothery S, Williams TJ, Rankin SM: Mechanisms of acute eosinophil mobilization from the bone marrow stimulated by interleukin 5: the role of specific adhesion molecules and phosphatidylinositol 3-kinase. *J Exp Med* 1998;188:1621–1632.
- 27 Flood-Page PT, Menzies-Gow AN, Kay AB, Robinson DS: Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am J Respir Crit Care Med* 2003;167:199–204.
- 28 Erjefält JS, Uller L, Malm-Erjefält M, Persson CG: Rapid and efficient clearance of airway tissue granulocytes through transepithelial migration. *Thorax* 2004;59:136–143.
- 29 Persson C, Uller L: Transepithelial exit of leucocytes: inflicting, reflecting or resolving airway inflammation? *Thorax* 2011;65:1111–1115.
- 30 Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA: Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999;103:779–788.
- 31 Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Shepard D, Erle DJ: Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 2002;8:885–889.