Steady-state estradiol triggers a unique innate immune response to allergen resulting in increased airway hyper-reactivity yet decreased eosinophils and ILC2.

Kristi Warren (kristi.warren@hsc.utah.edu)
George E Wahlen Department of Veterans Affairs Medical Center: VA Salt Lake City Health Care System
https://orcid.org/0000-0003-0680-4037

Cassandra Deering-Rice
The University of Utah College of Pharmacy

Tom Huecksteadt
George E Wahlen Department of Veterans Affairs Medical Center: VA Salt Lake City Health Care System

Shubhanshi Trivedi
University of Utah Medical Center: University of Utah Hospital

Alessandro Venossa
The University of Utah College of Pharmacy

Christopher Reilly
The University of Utah College of Pharmacy

Karl Sanders
University of Utah Medical Center: University of Utah Hospital

Frederic Clayton
University of Utah Medical Center: University of Utah Hospital

Todd A. Wyatt
Omaha VA Medical Center: VA Nebraska-Western Iowa Health Care System

Jill A Poole
University of Nebraska Medical Center

Nicola M. Heller
Johns Hopkins University School of Medicine

Daniel Leung
University of Utah Medical Center: University of Utah Hospital

Robert Paine III
University of Utah Medical Center: University of Utah Hospital

Research Article

Keywords:

Posted Date: April 18th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1551796/v1

Version of Record: A version of this preprint was published at Biology of Sex Differences on January 6th, 2023. See the published version at https://doi.org/10.1186/s13293-022-00483-7.

Abstract

Rationale: Asthma is a chronic airway condition that occurs more often in women than men during reproductive years. In males, testosterone attenuates allergic inflammation while estrogens are thought to exacerbate asthma through the estrogen receptors on airway epithelial cells. Population studies have looked at all types of asthma, and collectively shown that long-term use of oral contraceptives decreased the onset of asthma in women of reproductive age. In the current study, we hypothesized that steady-state levels of estrogen would reduce airway inflammation and airway hyperresponsiveness to methacholine challenge.

Methods: Ovariectomized BALB/c mice (Ovx) were implanted with subcutaneous hormone pellets (estrogen, OVX-E2; or placebo, OVX-Pl) that deliver consistent levels of estrogen [49 ± pg/mL] followed by ovalbumin sensitization and challenge. In conjunction with methacholine challenge, immune phenotyping was performed to correlate inflammatory proteins and immune populations with better or worse pulmonary outcomes measured by invasive pulmonary mechanics techniques.

Results: Histologic analysis showed an increase in total cell infiltration around the airways and vasculature leading to an increased inflammatory score in ovarectomized (OVX) animals with steady-state estrogen pellets (OVX-E2-OVA) as compared to other groups including female-sham operated (F-Sham-OVA) and OVX implanted with a placebo pellet (OVX-Pl-OVA). Airway resistance (Rrs) and lung elastance were increased in OVX-E2-OVA in comparison to F-Sham-OVA following aerosolized intratracheal methacholine challenges. Immune phenotyping revealed that steady-state estrogen reduced CD3+ T cells, CD19+ B cells, ILC2 and eosinophils across all experiments. Inflammatory cytokines (IL-5 and IL-13) were also decreased in OVX-E2-OVA treated animals in comparison to Female-Sham-OVA mice. ILC2 that were sorted and stimulated with exogenous IL-33 had less cytokine and chemokine expression when they were isolated from OVX-E2-OVA animals indicating that estrogen suppresses IL-33 mediated activation of ILC2.

Conclusions: Therapeutically targeting estrogen receptors may have a limiting effect on eosinophils, ILC2 and potentially other immune populations that may improve asthma symptoms in those females that experience perimenstrual worsening of asthma, with the caveat, that long-term use of estrogens or hormone receptor modulators may be detrimental to the lung microenvironment over time.

Introduction

Asthma is a chronic respiratory disease that afflicts approximately 350 million people worldwide. Many of these individuals present in clinics and emergency rooms with a range of symptoms that include chronic coughing, frequent night awakenings, and excess mucus production(1, 2). More severe disease is associated with airway constriction, shortness of breath and ultimately reduced FEV_1 and FVC readings(1, 3). While genetics, ethnicity, co-morbidities (e.g., diabetes and obesity)(4–6), and

environmental exposures play a role in the development of asthma(7), biological female sex is correlated with severe asthma in adulthood(8–11).

The overarching theme in sex-biased asthma is that ovarian hormones play a role in the development of severe asthma in women of reproductive age. During menstruation-ovulation cycling (day 0–14) estrogen serum concentrations vary between 20–500 pg/mL among women. Progesterone and estrogen serum concentrations increase simultaneously in females leading up to menstruation [P4; 7–15 ng/mL and E2; 100–300 pg/mL] (i.e. day 15 – day 28), then drop rapidly to baseline levels [P4; 1.5 ng/mL E2; <20 pg/mL] (12, 13). While peak levels of the ovarian hormones are thought to exacerbate asthma symptoms in 30–40% of women around menstruation(13), it has also been postulated that the sudden drop in circulating progesterone and estrogen may explain increased asthma symptomology during that time(13). In longitudinal studies estrogen-based oral contraceptives are linked to reduced onset of asthma in women of reproductive age(14–16). Along this line of reasoning estrogen-based oral contraceptives yield serum concentrations of estrogen equivalent to that of the mid-follicular phase [5–80 pg/mL](17–19), which represents a concentration that is moderately-low in comparison to the levels reached closer to ovulation or menstruation. Short-term and long-term hormone treatments need detailed investigation in asthmatic women as modulating hormones may be a safe strategy for regulating exacerbations in those subsets of asthmatic women who are sensitive to those hormone fluctuations.

Animal modeling is useful to understand hormone mechanisms that govern biological sex differences in male versus female asthmatics (8–11, 20–24). Foundational studies show that aspects of inflammatory signaling pathways, microRNA expression, and precursor myeloid and lymphoid cell progenitors in the bone marrow are influenced by hormone receptor ligation that ultimately influences the lung response to allergen (21, 25–27). In the present studies we examined steady-state estrogen of a pre-ovulatory range in the OVA-induced allergic inflammation model. To our surprise, estrogen had a profound effect on animals that underwent pulmonary function testing by making those animals more responsive to methacholine and thereby increasing airway hyper-reactivity readings. Based on these findings, we hypothesized that type 2 immunity would increase with estrogen treatment, however, this was not the case. In fact, estrogen was associated with decreased type 2 cells and allergic inflammatory cytokines and chemokines. The study highlights distinct roles of estrogens on lung cells and immune cells in female-biased allergic responses.

Materials And Methods

Mice, Ovariectomies & Subcutaneous Hormone Pellets

BALB/c mice were ovarectomized at 3 weeks of age at The Jackson Laboratory (Bar Harbor, ME). After two weeks of post-operation recovery, animals were transported to the animal facility where they acclimated for one week prior to beginning the experimental protocol. Deep anesthesia with achieved using ketamine [100 mg kg^{-1}] and xylazine [16 mg kg^{-1}] and implantation of a subcutaneous, 60-day, slow-release pellet containing estrogen (E2-17β; 0.1 mg) or a placebo pellets purchased from Innovative

Research of America (Sarasota, FL, USA). Male- and/or female-sham operated animals were included as controls. Post-surgery animals were given two doses of buprenorphine 12 hours apart for pain management according to standard protocols. Two weeks after surgery animals were sensitized with ovalbumin and aluminum hydroxide (Sigma, St. Louis, MO) at 3-week intervals followed by 5–10 daily challenges with intranasal ovalbumin (28, 29). All protocols were approved by the IACUC or Research Advisory Committees at the University of Nebraska Medical Center (Omaha, NE) or the Salt Lake City VA Medical Center (Salt Lake City, UT).

Bronchoalveolar Lavage fluid Collection & Analysis

Euthanized animals were placed in the prone position and small incision was made to expose the trachea, followed by a 1–3 mM width incision made to the trachea for cannulation. A 1 mL syringe was filled and fitted onto the cannula and the lungs were slowly flushed three times with 1 mL of Dulbecco's PBS. The first wash was collected into a separate tube and centrifuged at 300 x g for 10 minutes at 4ºC. After centrifugation the supernatants were separated from cell pellets for cytokine and chemokine analysis by ELISA. The second and third washes were collected and centrifuged 300 x g to separate cellular content from the supernatants; the cellular pellets from the three washes were combined and total cells were counted using the TC-20 automated counter (Biorad) with trypan blue exclusion. Approximately 20–40,000 cells were applied to cytospins slides and stained with Giemsa for cell differential determination; Giemsa staining procedure, previously described(30).

Lung Histology & Scoring

Chest cavity was exposed following deep anesthesia and lungs were cleared of blood by cardiac perfusion with saline solution. Whole lungs were fixed by tracheal instillation of 10% neutral buffered formalin at a constant pressure (25 cm H_2O). Following paraffin embedding, 6 µm sections were cut and stained with Hematoxylin & Eosin (H&E) by the Associated Regional and University Pathologists Inc., at the University of Utah. Image acquisition occurred the following day to allow the mounting reagent to fully dry. Images (100x and 200x magnification) were acquired using a Zeiss Axioscope 7 (Carl Zeiss Meditec, Inc., Dublin, CA) and processed using Adobe Photoshop (San Jose, CA)(30). Inflammation scores were calculated as follows: (% of bronchial / bronchiolar epithelium with infiltrate x measured number of cellular depth of peribronchial infiltrate) + (% of pulmonary veins with infiltrate x measured number of cellular depth of perivenous infiltrate). This score was calculated on 2 slides per animal and 2–3 animals per group. Representative images are shown in Fig. 4 at 100x and 200x magnification.

ELISA

Cell culture supernatants BAL fluids, and serum were centrifuged at 400 X g at room temperature for 10 minutes to clear cellular debris prior to testing. IL-5, IL-13, CCL3, CCL11, CCL17, CCL22 and KC Duo-set ELISA (R&D Systems, Minneapolis, MN) were performed according to the manufacturer's instructions. Luminescence was measured on the SpectraMax M3 (Molecular Devices LLC, San Jose, CA) at 450 nm with 570 nm wavelength correction. ILC2 culture supernatants were diluted at least 1:10 with reagent

diluent provided with the Ancillary Reagents Kit II (R&D Systems) that accompanies the ELISA Duo-set kits. Estrogen was measured by ELISA according to the manufacturer's instruction after a 50-fold dilution in reagent buffer provided in each kit (Abnova, Taipei, Taiwan). OVA-specific IgE was measured in serum according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, Michigan)

Pulmonary mechanics

BALB/c mice (n = 4–7/group) were used to assess pulmonary function using a FlexiVent FX -1 small animal ventilator (Flexivent FX1; SCIREQ Inc., Montreal, Qc, Canada). We measured airway resistance (Rrs), compliance (Crs), and elastance (Ers) in anesthetized (ketamine + xylazine 100 + 20 mg/kg), paralyzed (0.1 mg/kg vecuronium bromide), and tracheostomized mice as described previously (31, 32). Baseline measurements were collected using broadband low-frequency forced oscillations for each mouse. This was followed by assessment of bronchial reactivity/contractility elicited by delivery of aerosolized methacholine at 12.5 mg/ml using the Flexivent Aeroneb fine particle nebulizer in saline for 10 seconds per dose at 4–5 min intervals. The maximum response for measured criteria was determined at baseline, after saline and after each methacholine nebulization. Data are represented as methacholine responses (12.5 mg/mL) after baseline subtraction. Baseline PV-loops were generated in seven equal steps between 3 and 30 cm H20 and normalized to the individual mouse weight.

ILC2 Isolation and Cell Culture Conditions

Lungs were perfused with 2 mL of 1X Dulbecco's PBS, excised and digested in Hank's Balanced Salt Solution containing Collagenase, Type I [10 µg/mL] (Worthington Biochemical, Lakewood, NJ) and DNase I [0.02 mg/mL] (Sigma-Aldrich) for 30 minutes at 37C. Single-cell suspensions were passed through 40-micron nylon filters and washed one time with PBS containing 2% FBS. ILC2 were enriched from single cell suspensions using the mouse ILC2 enrichment kit (StemCell Technologies, Vancouver, Canada) followed by labeling with anti-mouse antibodies against CD45, CD3, B220, CD11c, CD11b, F4/80, Ter-119, Ly6c, Ly6g, CD127, CD25, KLRG-1 and ICOS. ILC2 purification was completed by FACS sorting on the ARIA II (BD Biosciences, Franklin Lakes, NJ). We acquired approximately 35,000–50,000 purified ILC2 from 4–5 ovalbumin treated control animals, or approximately 10,000–15,000 purified ILC2 from 6–8 naïve male and female mice. Purified ILC2 were split equally across 4–6 wells of round-bottom 96-well plate (Corning, Corning, NY), followed by the addition of IL-2 and IL-7 with and without IL-33 for 72 hours in culture. Viability was assessed by trypan blue exclusion on the TC-20 cell counter (Biorad, Hercules, CA). Cell culture supernatants were collected and stored at -80C until analysis by ELISA could be completed.

Results

Figure 1. **Airway and vascular cell infiltrates are increased in the airways when estrogen is given at steady-state levels.**

Adult females are more likely to have increased allergic inflammation in comparison to men, yet steady-state levels of estrogen in the form of oral contraceptives improve asthma severity in adult women(14, 15, 33). With these data in mind, we hypothesized that estrogen may improve allergic inflammation in

allergen-challenged animals. Ovarectomized animals (OVX) were implanted with subcutaneous estrogen (OVX-E2) or placebo pellets (OVX-Pl) and allergic inflammation was experimentally generated using chicken egg ovalbumin (OVA) sensitization and airway challenge protocols (Fig. 1A). High circulating levels of estrogen were appropriately detected in ovarectomized animals implanted with an estrogen pellet, while negligible levels of estrogen were detected when animals were implanted with a placebo pellet (Fig. 1B). Histological inflammatory scores were determined by a blinded pathologist (Fig. 1C). Cellular immune infiltration was determined by quantifying bronchial and vascular inflammation and cellular infiltrates (Fig. 1D-G). No inflammation was found in the F-Sham-saline treated animals even after methacholine challenge (Fig. 1C and 1D). F-Sham-OVA had more inflammation than Female-Sham-Saline mice indicating a significant induction of allergic inflammation with OVA sensitization and airway challenges (Fig. 1C,1D,1E; $P < 0.05$). OVX-E2-OVA had more inflammatory cell infiltrate than F-sham-OVA treated when comparing scoring results (Fig. 1C, 1D, 1E). Although not quantified basement membranes were thickened in all OVA treated animals and we noted airway contractility in the OVX-E2-OVA mice indicative of airway contractility (Indicated by black arrows in Fig. 1F, 1I and 1J). In summary these studies showed that estrogen significantly increased the allergic inflammatory response to airway challenge.

Airway resistance is increased in ovarectomized animals treated with estradiol in comparison to female-sham treated animals

In the next studies respiratory system mechanics were measured by determining resistance and elastance following methacholine challenge in each of the treatment groups: Female-Sham-Saline, Female-Sham-OVA, OVX-E2-OVA and OVX-Placebo-OVA. Airway resistance (Rrs) is an indicator of airway flow and airway obstruction, while elastance (Ers) determines the stiffness of the lung tissue. Rrs was higher in OVX-E2-OVA compared to Female-Sham-OVA (Fig. 2A; $p = 0.0185$). Differences in resistance between OVX-placebo-OVA compared to F-Sham-OVA did not reach statistical significance. Elastance (Ers) readings trended upwards in OVX-placebo-OVA animals compared to F-Sham-OVA ($p = 0.0845$), but only reached statistical significance in OVX-E2-OVA mice compared to F-Sham-OVA mice (Fig. 2B; $P < 0.0001$). Pressure volume (PV) loops were also determined over the course of pulmonary function testing (Fig. 2C). We observed changes, or flattening, of the pressure-volume loops which fits with the increased Ers in OVX-E2-OVA mice compared to F-Sham-OVA. No statistical differences were observed between OVX-placebo-OVA mice and F-Sham-OVA. Altogether the pulmonary function testing indicates that estrogen treatment stiffens the lung tissues in combination with increasing airway resistance in ovarectomized mice treated with ovalbumin.

Total immune cell infiltrate was reduced in BAL fluid from ovarectomized, estrogen-treated animals compared to female, sham-operated mice following allergen.

We compared the composition of immune airway infiltrates in each treatment group following OVA treatments and methacholine challenge (Fig. 3). OVA treated mice all had significant increases in neutrophils and eosinophils in comparison to F-Sham-Saline treated mice (Fig. 3A). OVX-E2-OVA treated

animals had a higher percentage of macrophages represented in the BAL cells collected as compared to F-Sham-OVA, while OVX-placebo-OVA had a lower percentage of macrophages in comparison to F-Sham following OVA challenge. In addition, OVX-E2-OVA mice had a lower percentage of total lymphocytes as compared to F-Sham-OVA. Counts for each population were determined using the volume of BAL recovered multiplied by the percentage of cell type counted per field (Fig. 3B-3F). First, total BAL cells were reduced in OVX-E2-OVA treated animals compared to F-Sham-OVA; OVX-placebo-OVA treated mice maintained a comparable number of total cells to F-Sham-OVA in their BAL (Fig. 3B). The counts of macrophages were reduced in OVX-Placebo-OVA compared to F-Sham-OVA, but macrophages were comparable in OVX-E2-OVA and F-Sham-OVA (Fig. 3C). Most interestingly, OVX-E2-OVA had lower counts of neutrophils and eosinophils in comparison to F-Sham-OVA (Fig. 3D, 3E), while OVX-Placebo-OVA mice had increased numbers of neutrophils in comparison to F-Sham-OVA mice. Finally, less lymphocytes were recovered from the BAL fluid of OVX-E2-OVA treated animals compared to F-Sham-OVA controls (Fig. 2F). Together these data suggest that hormones or the lack of hormones significantly alter the immune cell compartment that contributes to pulmonary mechanics outcomes.

Allergic inflammatory cytokines are released at different rates in OVX-E2-OVA treated animals compared to intact female-sham mice treated with airway allergen. In the next studies we wanted to confirm that differences in BAL cell populations were not a byproduct of methacholine challenges, but present because of airway stress and inflammation induced by OVA treatment in conjunction with estrogen treatment. In the next studies we collected BAL following five consecutive daily intranasal OVA challenges. Sex differences are previously reported using the OVA model, and in our previous report, female BALB/c mice specifically had higher ILC2 responses to the allergy-associated type 2-inducing cytokine, IL-33, compared to males. In those studies, IL-5 and IL-13 responses were higher for females in comparison to males. In the remaining studies we include male animals to make an appropriate assessment on ‘sex differences’ in comparison to our ovariectomized-estrogen treated animals. IL-13 and CCL3 (MIP1a) were the cytokine and chemokine that were statistically higher in F-Sham-OVA treated animals compared to Male-Sham-OVA (Fig. 4B, 4G). IL-5 and IL-13 were decreased in OVX-E2-OVA animals compared to Female-Sham-OVA (Fig. 4A, 4B), and CCL11 trended downwards in these OVX-E2-OVA animals in comparison to F-Sham-OVA (Fig. 4F). BAL concentrations of CCL22, CC12, CCL11, CCL3 and IL-33 were equivalent in F-Sham-OVA and OVX-E2-OVA. Notably, CCL22, CCL12, and CCL11 were all increased in OVX-placebo-OVA treated animals compared to F-Sham-OVA, indicating that these allergic inflammatory proteins are regulated to some extent by ovary-derived hormones.

Circulating cytokines and chemokines are selectively altered with steady-state estrogen treatment. Serum was collected from OVA treated animals for detection of allergic-inflammatory cytokines and chemokines. Serum concentration of IL-13, CCL22, and CCL3 were increased in F-Sham-OVA mice compared to Male-Sham-OVA mice (Fig. 5B, 5C, 5D). In contrast to the findings in the BAL fluid, serum concentrations of CCL22 and CCL3 were increased in OVX-E2-OVA mice as compared with F-Sham-OVA mice (Fig. 5C, 5D). Although we saw increases in neutrophil numbers in OVX-Placebo-OVA mice compared to F-Sham-OVA in the previous studies (Fig. 3D), the neutrophil chemoattractant protein, KC, was not different between any of the groups.

Estrogen treatment reduces eosinophils in BAL of OVA sensitized and challenged ovarectomized mice.
Eosinophils are regularly detected in circulation and in sputum of asthmatic patients (34, 35), as such eosinophils are thought of clinically as a determinant of asthma diagnosis and severity of disease (Fig. 6). In these studies, we prepared mice as in Fig. 1A, however these animals did not undergo methacholine challenges prior to characterizing eosinophil levels by flow cytometry. As expected, increased numbers of eosinophils were detected following OVA-allergen challenge in bronchoalveolar lavage fluids in comparison to saline treated (data not shown) animals. Eosinophils were determined at higher numbers in females compared to males in other studies (36, 37), however in the present studies statistical analysis showed no differences in eosinophil percentages or numbers in BAL between M-Sham-OVA and F-Sham OVA animals by flow cytometry. As with the previous data (Fig. 3), we again detected reduced numbers of eosinophils in OVX-E2-OVA treated animals in comparison to F-Sham-OVA. Together this indicates a suppressive effect on eosinophils, again, a well-excepted biomarker of asthma, with pharmacologically-delivered estrogen in the traditional OVA model.

Steady-state estrogen reduces airway T cells and B cells in BAL fluid collected from ovarectomized animals compared to intact female controls.

Total CD3 + T cells (**7A, 7B**) and CD19 + B cells (**7C-7D**) were assessed in the same treatment groups as before by flow cytometry. F-Sham-OVA did have higher numbers of CD3 + T cells detected in the BAL in comparison to M-Sham-OVA, however total CD3 + T cells were significantly reduced in OVA-E2-OVA mice compared to F-Sham-OVA mice. Again, this indicated that E2 is driving a suppressive program that is reducing the allergic inflammatory response to OVA. Similarly, CD19 + B cells (Fig. 7C, 7D) were reduced along with OVA-specific IgE (Fig. 7E) in the OVX-E2-OVA animals as compared with F-Sham-OVA mice. IgE levels are determinant of degree of allergic responses in the clinic and typically determined as another prototypical biomarker of asthmatic disease. These data revealed differential effects of female sex hormones whereby lung immune responses are decreased by estrogen.

Accumulation and IL-33-induced activation of lung ILC2 is reduced in OVX-E2-OVA mice compared to F-Sham-OVA mice.

ILC2 are important for allergic airway inflammation associated with asthma (38) and are increased in peripheral blood of asthmatic patients (39). ILC2 are predominantly responsible for IL-5 and IL-13 production following in vitro and in vivo stimulation of mouse tissues or cells with IL-33 (40). Importantly these cells have been shown to interact with type 2 helper T cells and to directly support eosinophil responses through their production of IL-5 (41–43). First, we identified ILC2 as LIN- CD127 + KLRG-1 + cells in the BAL and lungs following OVA challenge (Fig. 8A, 8B and 8C). Others have reported sex differences in the numbers of ILC2 in allergen challenge models, and we have intermittently determined ILC2 count and frequency differences in males versus females depending on the markers used for ILC2 specific detection. In these studies, we detected sex differences in counts and frequencies of ILC2, not only in BAL or airway-localized ILC2, but also in remaining lung tissue in M-Sham-OVA versus F-Sham-OVA animals (Fig. 8A, 8B). We assessed viability of the ILC2 during flow analysis using a fixable viability

dye and confirmed that estrogen was not associated with lower viability in the BAL or lung ILC2 (Fig. 8D). Next lung ILC2 were sorted from each treatment group for ex vivo culture with and without IL-33. As in past studies we would expect higher allergic cytokine production in F-Sham-OVA mice as compared to M-Sham-OVA mice. As in our previous report, ILC2 were stimulated with IL-33 [10 ng/mL] for 3 days in culture to detect IL-5 and IL-13 production by ILC2. We add to that report by showing that ILC2 from F-Sham-OVA mice produced more CCL22 and CCL3 in response to IL-33 when compared to M-Sham-OVA. While the amount of IL-5, IL-13, CCL22, and CCL3 are higher in females compared to males, the excessive production of these cytokines in females was not due to estrogen stimulation, as we found reduced IL-5 and IL-13 production, and reductions in pro-inflammatory chemokines, CCL22 and CCL3, by lung ILC2 obtained 17β-E2 treated OVX mice (OVX-E2-OVA) compared to Female-Sham-OVA treated animals (Fig. 8E-8H). IL-5, IL-13, CCL22 and CCL3 were all comparable in OVX-PI-OVA and F-Sham-OVA treated animals.

Discussion

In the present study we examined the effect of steady-state estrogen in the well-defined OVA-induced allergic inflammation model. Substantial allergic inflammation was generated with five or ten consecutive days of intranasal (i.n.) OVA administration followed by an assessment of pulmonary mechanics, innate immune populations in whole lung tissue and BAL, and type 2 inflammatory cytokine (IL-5 and IL-13) and chemokines (CCL17 and CCL22) detected in BAL and circulation. We leveraged this well-studied model to show how pulmonary mechanics changes occur in conjunction with E2 levels that correlated with the mid-follicular phase of ovulation [17β-E2 @ 49+/-5 pg/mL]. We documented a significant reduction in ILC2 and eosinophils with this estrogen treatment and showed that ILC2 were reduced and became non-responsive to IL-33 stimulation when we isolated those cells from estrogen-treated animals. However, these reductions did not correlate with reduced airway resistance, as airway resistance remained higher in OVX-E2-OVA mice compared to F-Sham-OVA treated controls. Together, this indicates that estrogen is enhancing the response to allergic inflammatory events directly in lung cells, while reducing the allergic responses in type 2 innate immune cells.

Steady-state 17β-estradiol increased airway resistance and overall inflammatory scores following OVA challenge and PV loops were flattened in estrogen treated animals which indicates stiffening of lung tissues. There are clear airway structural changes occurring with estrogen treatment that resemble results from other studies (44). Our interpretation of the data are that alveolar, airway epithelial and airway smooth muscle cells are directly stimulated by exogenous estrogen through estrogen receptors (44–48), with those studies in mind it is likely that the estrogen receptor alpha (ER-α) controls the airway smooth muscle responses to allergen. Estrogen receptor α knockout animals display spontaneous airway reactivity in early experiments that explored sex differences in asthma(49). More recent work has shown that estrogen receptor α and estrogen receptor β activation have distinct consequences in animal modeling of allergen responses (50, 51). Using an estrogen receptor β agonist precisely activates ER-β, and this report showed reduces airway resistance (Rrs) following allergen challenge. In that same study an ER-α agonist did not significantly alter Rrs in female or ovarectomized mice following allergen

challenge(46). In our studies steady-state estrogen significantly increased Rrs generated in ovalbumin-challenged mice treated with 12.5 mg/mL of MCh to measure airway hyperreactivity. Comparatively, our study only challenged animals with a moderately-low dose of methacholine, whereas Ambhore et al (2019) found the greatest effects with 50 mg/mL of methacholine(46). An additional comparison was the reduction in total BAL cells in our study and the reductions seen in a study that used the estrogen receptor β agonist(51). Those animals given the ER-β agonist had reduced eosinophils, lymphocytes, and macrophages in response to mixed-allergen challenge. Our study similarly showed significant reductions in eosinophils, neutrophils, CD3 + T cells, CD19 + B cells, and ILC2 along the airways. While our model is likely activating both ER-α and ER-β in several cell types, it does suggest that ER-β may be responsible for the reduction of the immune populations. There are very few studies that examine ER-β responses, but data generated in non-allergic animal models do note a regulatory phenotype, or FOXP3 + T regulatory cell induction occurring via ER-β activation(52–55). Perhaps regulatory T cells are decreasing inflammatory responses through direct (co-inhibitory molecules such as PD-1) or indirect immune inhibition (TGF-β, IL-10 production)(56, 57). Of course, examining T regulatory cells was beyond the scope of the current study, but it is likely they are generated since OVA-induced allergic inflammation relies heavily of OVA-specific T cells.

One additional study documented a role for ER-α in the release of IL-33 from EpCAM + airway epithelial cells(50), which indeed is an important factor in type 2 allergic responses. IL-33 induces substantial IL-5 and IL-13 responses from ILC2(28). Therefore, one would think that steady-state estrogen would increase the IL-33 response in the airways, however, we saw no changes in the amount of IL-33 in BAL or circulation with estrogen treatment; we furthermore, did not see a significant effect of biological sex on IL-33 production as previously report(36). Although these conflicting results are likely due to various factors related to experimental design and method of IL-33 detection.

We did confirm a strong inhibitory effect of estrogen on the IL-33 responsiveness of ILC2 and showed that this decline in IL-5, IL-13 and chemokines was not because of reduced cell viability. It will be important to establish whether estrogen is inducing a different phenotype, perhaps the ILC3-like or an ILC regulatory phenotype, in future studies(58, 59). Future studies will also investigate estrogen receptor expression specifically in ILC2. It has yet to be determined whether the estrogen receptors are expressed at the protein level in ILC2, although it was shown that lung ILC2 were not regulated by ER-α(50), and sequencing data showed low read counts for Esr1 (ER-α)and Esr2 (ER-β) transcripts in BM ILC2(60). Contrary to these reports, Bartemes et al, showed that uterine ILC2 were responsive to IL-33 and depleted in ER-α and ER-β knockout animals (61). This suggests that tissue localization is important for estrogen responses involving inflammatory stimuli. Certainly, the uterus is directly exposed to fluctuations in ovarian hormones, and this may influence the expression of various hormone receptors over time. This is a critical gap to fill in allergy and asthma studies as many studies don't verify protein expression of hormone receptors, nor do they examine changes in receptor expression during various activating stimulation (i.e. following IL-33 activation)(62). To date, it is not yet established whether human ILC2 express the estrogen receptors at the protein level, but a recent clinical report defined increased circulating ILC2 in pregnant women compared to non-pregnant women. These data were correlated within each

subject with higher levels of progesterone and estrogen in circulation of early (first trimester) and late pregnant (third trimester) women(63). Together this indicates that the ovarian hormones are strongly associated with expansion of ILC2 in females.

Perspectives And Significance

The current study highlighted a striking reduction in immune-mediated allergic inflammation in the ovalbumin sensitization and challenge model with estrogen. This response was what we hypothesized based on the clinical studies that showed improvements in asthma severity with oral contraceptives. However, what was unexpected was the increase in airway hyperreactivity and inflammation noted in multiple areas in the lung, beyond just the airway cells, that indicated lung airway smooth muscle cells and potentially alveolar cells become hyper-reactive because of estrogen. This again highlights the complicated nature of hormonal responses and their role in amplifying allergic responses in the lung. Future studies will continue to tease apart these interactions, between the various immune cells and lung cells in the context of hormonal fluctuations, to work towards identifying precision medicine strategies for male and female asthmatics.

Abbreviations

AHR: Airway Hyperreactivity

BAL: Bronchoalveolar Lavage

Ers: Respiratory system elastance

FEV_1: Forced Expiratory Volume for the first second of exhalation

FVC: Forced Vital Capacity

ILC2: Group 2 innate lymphoid cells

IL-5: Interleukin 5

IL-13: Interleukin 13

MCh: Methacholine

OVA: Chicken Egg Ovalbumin

OVX: Ovariectomized

Rrs: Respiratory system resistance

Th2: Type 2 CD4 + Helper T cells

Th17: IL-17 + CD4 + Helper T cells

Declarations

Ethics approval and consent to participate: All protocols were approved by the appropriate IACUC and research advisory committees.

Consent for publication: All the authors agree with the content of this study and consent for publication.

Availability of data and material: All data are present in the main text of the manuscript, and saline control data for Figure 4 and 5 are available at request; the values from the various protein assay for the saline treated animals were not detectable and removed to concisely show the relevant results in the paper.

Competing interests: The authors declare that there are no competing interests.

Funding Sources: NIEHS R01ES032553 (AV); Skaggs Foundation for Research Initiatives Grant (AV); NIEHS R01ES017431 and R01ES027015 (CR); NCI 5P30CA042014-24 (Flow Cytometry Core). VA Merit (I01 BX005413) to TAW. TAW is the recipient of a Research Career Scientist Award (IK6 BX003781) and KJW is the recipient of a Career Development Award (IK2 BX004219) from the Department of Veterans Affairs.

Authors' contributions: KJW: conceptualized the experiments, collected the data, wrote the manuscript, and is the corresponding author for the paper. CDR: Conceptualized and executed the pulmonary mechanics studies, analyzed the experimental data, and prepared the figures and wrote the manuscript. TH: Conceptualized and executed the experiments and edited the manuscript. ST: Collected data, ran statistical analysis, and edited the manuscript. AV: Executed the experiments, interpreted, and edited the manuscript. CR: Conceptualized the experiments and edited the manuscript. KS: Conceptualized experiments, interpreted the pulmonary mechanics data and edited the manuscript. FC: Generated the data for the histology studies and edited the manuscript. TW: Conceptualized the experiments, wrote, and edited the final manuscript. JAP: Conceptualized experiments and edited the final manuscript. NMH: Conceptualized experiments and edited the final manuscript. DL: Conceptualized the experiments, interpreted the data throughout preparation of the manuscript. RP: Evaluated the data, interpreted the pulmonary mechanics studies and edited the manuscript.

Acknowledgements: This research was supported by the University of Utah Flow Cytometry Core and ARUP Laboratories. The authors acknowledge these core facilities, the Directors and staff for their essential contributions to this project.

References

1. Padem N, Saltoun C. Classification of asthma. Allergy Asthma Proc. 2019;40(6):385-8.

2. Aaron SD, Boulet LP, Reddel HK, Gershon AS. Underdiagnosis and Overdiagnosis of Asthma. Am J Respir Crit Care Med. 2018;198(8):1012–20.
3. Ruppel GL, Enright PL. Pulmonary function testing. Respir Care. 2012;57(1):165–75.
4. Lang JE, Bunnell HT, Lima JJ, Hossain MJ, Wysocki T, Bacharier L, et al. Effects of age, sex, race/ethnicity, and allergy status in obesity-related pediatric asthma. Pediatr Pulmonol. 2019;54(11):1684–93.
5. Lang JE. Obesity and childhood asthma. Curr Opin Pulm Med. 2019;25(1):34–43.
6. Lang JE. The impact of exercise on asthma. Curr Opin Allergy Clin Immunol. 2019;19(2):118–25.
7. Lang JE, Tang M. Smoking: it's still a big problem in children with asthma. J Pediatr (Rio J). 2019;95(5):506–8.
8. Keselman A, Heller N. Estrogen Signaling Modulates Allergic Inflammation and Contributes to Sex Differences in Asthma. Front Immunol. 2015;6:568.
9. Chowdhury NU, Guntur VP, Newcomb DC, Wechsler ME. Sex and gender in asthma. Eur Respir Rev. 2021;30(162).
10. Shah R, Newcomb DC. Sex Bias in Asthma Prevalence and Pathogenesis. Front Immunol. 2018;9:2997.
11. Yung JA, Fuseini H, Newcomb DC. Hormones, sex, and asthma. Ann Allergy Asthma Immunol. 2018;120(5):488–94.
12. Yen SSC, Strauss JF, Barbieri RL. Yen & Jaffe's reproductive endocrinology: physiology, pathophysiology, and clinical management. Seventh edition. ed. Philadelphia, PA: Elsevier/Saunders; 2014. xvi, 942 pages p.
13. Haggerty CL, Ness RB, Kelsey S, Waterer GW. The impact of estrogen and progesterone on asthma. Ann Allergy Asthma Immunol. 2003;90(3):284–91. quiz 91 – 3, 347.
14. Nwaru BI, Simpson CR, Soyiri IN, Pillinger R, Appiagyei F, Ryan D, et al. Exogenous sex steroid hormones and asthma in females: protocol for a population-based retrospective cohort study using a UK primary care database. BMJ Open. 2018;8(6):e020075.
15. Nwaru BI, Pillinger R, Tibble H, Shah SA, Ryan D, Critchley H, et al. Hormonal contraceptives and onset of asthma in reproductive-age women: Population-based cohort study. J Allergy Clin Immunol. 2020;146(2):438–46.
16. Nwaru BI, Tibble H, Shah SA, Pillinger R, McLean S, Ryan DP, et al. Hormonal contraception and the risk of severe asthma exacerbation: 17-year population-based cohort study. Thorax. 2021;76(2):109–15.
17. Brenner PF, Mishell DR, Stanczyk FZ, Goebelsmann U. Serum levels of d-norgestrel, luteinizing hormone, follicle-stimulating hormone, estradiol, and progesterone in women during and following ingestion of combination oral contraceptives containing dl-norgestrel. Am J Obstet Gynecol. 1977;129(2):133–40.

18. Mishell DR, Thorneycroft IH, Nakamura RM, Nagata Y, Stone SC. Serum estradiol in women ingesting combination oral contraceptive steroids. Am J Obstet Gynecol. 1972;114(7):923–8.
19. Tourgeman DE, Gentzchein E, Stanczyk FZ, Paulson RJ. Serum and tissue hormone levels of vaginally and orally administered estradiol. Am J Obstet Gynecol. 1999;180(6 Pt 1):1480–3.
20. Keselman A, Fang X, White PB, Heller NM. Estrogen Signaling Contributes to Sex Differences in Macrophage Polarization during Asthma. J Immunol. 2017;199(5):1573–83.
21. Kovats S. Estrogen receptors regulate innate immune cells and signaling pathways. Cell Immunol. 2015;294(2):63–9.
22. Laffont S, Guéry JC. Deconstructing the sex bias in allergy and autoimmunity: From sex hormones and beyond. Adv Immunol. 2019;142:35–64.
23. Ambhore NS, Kalidhindi RSR, Sathish V. Sex-Steroid Signaling in Lung Diseases and Inflammation. Adv Exp Med Biol. 2021;1303:243–73.
24. Carey MA, Card JW, Voltz JW, Germolec DR, Korach KS, Zeldin DC. The impact of sex and sex hormones on lung physiology and disease: lessons from animal studies. Am J Physiol Lung Cell Mol Physiol. 2007;293(2):L272-8.
25. Laffont S, Seillet C, Guery JC. Estrogen Receptor-Dependent Regulation of Dendritic Cell Development and Function. Front Immunol. 2017;8:108.
26. Fuseini H, Cephus JY, Wu P, Davis JB, Contreras DC, Gandhi VD, et al. ERα Signaling Increased IL-17A Production in Th17 Cells by Upregulating IL-23R Expression, Mitochondrial Respiration, and Proliferation. Front Immunol. 2019;10:2740.
27. Newcomb DC, Cephus JY, Boswell MG, Fahrenholz JM, Langley EW, Feldman AS, et al. Estrogen and progesterone decrease let-7f microRNA expression and increase IL-23/IL-23 receptor signaling and IL-17A production in patients with severe asthma. J Allergy Clin Immunol. 2015;136(4):1025–34.e11.
28. Warren KJ, Sweeter JM, Pavlik JS, Nelson AJ, Devasure JM, Dickinson JD, Sisson JH, Wyatt TA, Poole JA. Sex differences in activation of lung-related type-2 innate lymphoid cells in experimental asthma. Ann Allergy Asthma Immunol. 2016.
29. Warren KJ, Dickinson JD, Nelson AJ, Wyatt TA, Romberger DJ, Poole JA. Ovalbumin-sensitized mice have altered airway inflammation to agriculture organic dust. Respir Res. 2019;20(1):51.
30. Venosa A, Smith LC, Murray A, Banota T, Gow AJ, Laskin JD, et al. Regulation of Macrophage Foam Cell Formation During Nitrogen Mustard (NM)-Induced Pulmonary Fibrosis by Lung Lipids. Toxicol Sci. 2019;172(2):344–58.
31. Bevans T, Deering-Rice C, Stockmann C, Light A, Reilly C, Sakata DJ. Inhaled Remifentanil in Rodents. Anesth Analg. 2016;122(6):1831–8.
32. Deering-Rice CE, Nguyen N, Lu Z, Cox JE, Shapiro D, Romero EG, et al. Activation of TRPV3 by Wood Smoke Particles and Roles in Pneumotoxicity. Chem Res Toxicol. 2018;31(5):291–301.
33. Nwaru BI, Ekerljung L, Rådinger M, Bjerg A, Mincheva R, Malmhäll C, et al. Cohort profile: the West Sweden Asthma Study (WSAS): a multidisciplinary population-based longitudinal study of asthma,

allergy and respiratory conditions in adults. BMJ Open. 2019;9(6):e027808.

34. Dunican EM, Elicker BM, Gierada DS, Nagle SK, Schiebler ML, Newell JD, et al. Mucus plugs in patients with asthma linked to eosinophilia and airflow obstruction. J Clin Invest. 2018;128(3):997–1009.
35. Yancey SW, Keene ON, Albers FC, Ortega H, Bates S, Bleecker ER, et al. Biomarkers for severe eosinophilic asthma. J Allergy Clin Immunol. 2017;140(6):1509–18.
36. Cephus JY, Stier MT, Fuseini H, Yung JA, Toki S, Bloodworth MH, et al. Testosterone Attenuates Group 2 Innate Lymphoid Cell-Mediated Airway Inflammation. Cell Rep. 2017;21(9):2487–99.
37. Laffont S, Blanquart E, Savignac M, Cenac C, Laverny G, Metzger D, et al. Androgen signaling negatively controls group 2 innate lymphoid cells. J Exp Med. 2017;214(6):1581–92.
38. Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. Immunity. 2012;36(3):451–63.
39. Bartemes KR, Kephart GM, Fox SJ, Kita H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. J Allergy Clin Immunol. 2014;134(3):671–8.e4.
40. Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H. IL-33-responsive lineage- CD25 + CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. J Immunol. 2012;188(3):1503–13.
41. Molofsky AB, Nussbaum JC, Liang HE, Van Dyken SJ, Cheng LE, Mohapatra A, et al. Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. J Exp Med. 2013;210(3):535–49.
42. Nussbaum JC, Van Dyken SJ, von Moltke J, Cheng LE, Mohapatra A, Molofsky AB, et al. Type 2 innate lymphoid cells control eosinophil homeostasis. Nature. 2013;502(7470):245–8.
43. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate Lymphoid Cells: 10 Years On. Cell. 2018;174(5):1054–66.
44. Ambhore NS, Kalidhindi RSR, Pabelick CM, Hawse JR, Prakash YS, Sathish V. Differential estrogen-receptor activation regulates extracellular matrix deposition in human airway smooth muscle remodeling. FASEB J. 2019;33(12):13935–50.
45. Ambhore NS, Katragadda R, Raju Kalidhindi RS, Thompson MA, Pabelick CM, Prakash YS, et al. Estrogen receptor beta signaling inhibits PDGF induced human airway smooth muscle proliferation. Mol Cell Endocrinol. 2018;476:37–47.
46. Ambhore NS, Kalidhindi RSR, Loganathan J, Sathish V. Role of Differential Estrogen Receptor Activation in Airway Hyperreactivity and Remodeling in a Murine Model of Asthma. Am J Respir Cell Mol Biol. 2019;61(4):469–80.
47. Sathish V, Freeman MR, Long E, Thompson MA, Pabelick CM, Prakash YS. Cigarette Smoke and Estrogen Signaling in Human Airway Smooth Muscle. Cell Physiol Biochem. 2015;36(3):1101–15.
48. Bhallamudi S, Connell J, Pabelick CM, Prakash YS, Sathish V. Estrogen receptors differentially regulate intracellular calcium handling in human nonasthmatic and asthmatic airway smooth

muscle cells. Am J Physiol Lung Cell Mol Physiol. 2020;318(1):L112-L24.

49. Carey MA, Card JW, Bradbury JA, Moorman MP, Haykal-Coates N, Gavett SH, et al. Spontaneous airway hyperresponsiveness in estrogen receptor-alpha-deficient mice. Am J Respir Crit Care Med. 2007;175(2):126–35.
50. Cephus JY, Gandhi VD, Shah R, Brooke Davis J, Fuseini H, Yung JA, et al. Estrogen receptor-α signaling increases allergen-induced IL-33 release and airway inflammation. Allergy. 2021;76(1):255–68.
51. Kalidhindi RSR, Ambhore NS, Bhallamudi S, Loganathan J, Sathish V. Role of Estrogen Receptors α and β in a Murine Model of Asthma: Exacerbated Airway Hyperresponsiveness and Remodeling in ERβ Knockout Mice. Front Pharmacol. 2019;10:1499.
52. Aggelakopoulou M, Kourepini E, Paschalidis N, Panoutsakopoulou V. ERβ in CD4 + T Cells Is Crucial for Ligand-Mediated Suppression of Central Nervous System Autoimmunity. J Immunol. 2016;196(12):4947–56.
53. Aggelakopoulou M, Kourepini E, Paschalidis N, Simoes DC, Kalavrizioti D, Dimisianos N, et al. ERβ-Dependent Direct Suppression of Human and Murine Th17 Cells and Treatment of Established Central Nervous System Autoimmunity by a Neurosteroid. J Immunol. 2016;197(7):2598–609.
54. Guo D, Liu X, Zeng C, Cheng L, Song G, Hou X, et al. Estrogen receptor β activation ameliorates DSS-induced chronic colitis by inhibiting inflammation and promoting Treg differentiation. Int Immunopharmacol. 2019;77:105971.
55. Xiong Y, Zhong Q, Palmer T, Benner A, Wang L, Suresh K, et al. Estradiol resolves pneumonia via ERβ in regulatory T cells. JCI Insight. 2021;6(3).
56. Polanczyk MJ, Hopke C, Vandenbark AA, Offner H. Treg suppressive activity involves estrogen-dependent expression of programmed death-1 (PD-1). Int Immunol. 2007;19(3):337–43.
57. Tai P, Wang J, Jin H, Song X, Yan J, Kang Y, et al. Induction of regulatory T cells by physiological level estrogen. J Cell Physiol. 2008;214(2):456–64.
58. Maggi L, Mazzoni A, Capone M, Liotta F, Annunziato F, Cosmi L. The dual function of ILC2: From host protection to pathogenic players in type 2 asthma. Mol Aspects Med. 2021;80:100981.
59. Bernink JH, Ohne Y, Teunissen MBM, Wang J, Wu J, Krabbendam L, et al. c-Kit-positive ILC2s exhibit an ILC3-like signature that may contribute to IL-17-mediated pathologies. Nat Immunol. 2019;20(8):992–1003.
60. Blanquart E, Laffont S, Guéry JC. Sex hormone regulation of innate lymphoid cells. Biomed J. 2021;44(2):144–56.
61. Bartemes K, Chen CC, Iijima K, Drake L, Kita H. IL-33-Responsive Group 2 Innate Lymphoid Cells Are Regulated by Female Sex Hormones in the Uterus. J Immunol. 2018;200(1):229–36.
62. Sathish V, Martin YN, Prakash YS. Sex steroid signaling: implications for lung diseases. Pharmacol Ther. 2015;150:94–108.

63. Zhao Y, Zhu Y, Chen X, Lin H, Qin N, Zhou Z, et al. Circulating Innate Lymphoid Cells Exhibit Distinctive Distribution During Normal Pregnancy. Reprod Sci. 2022;29(4):1124–35.

Figures

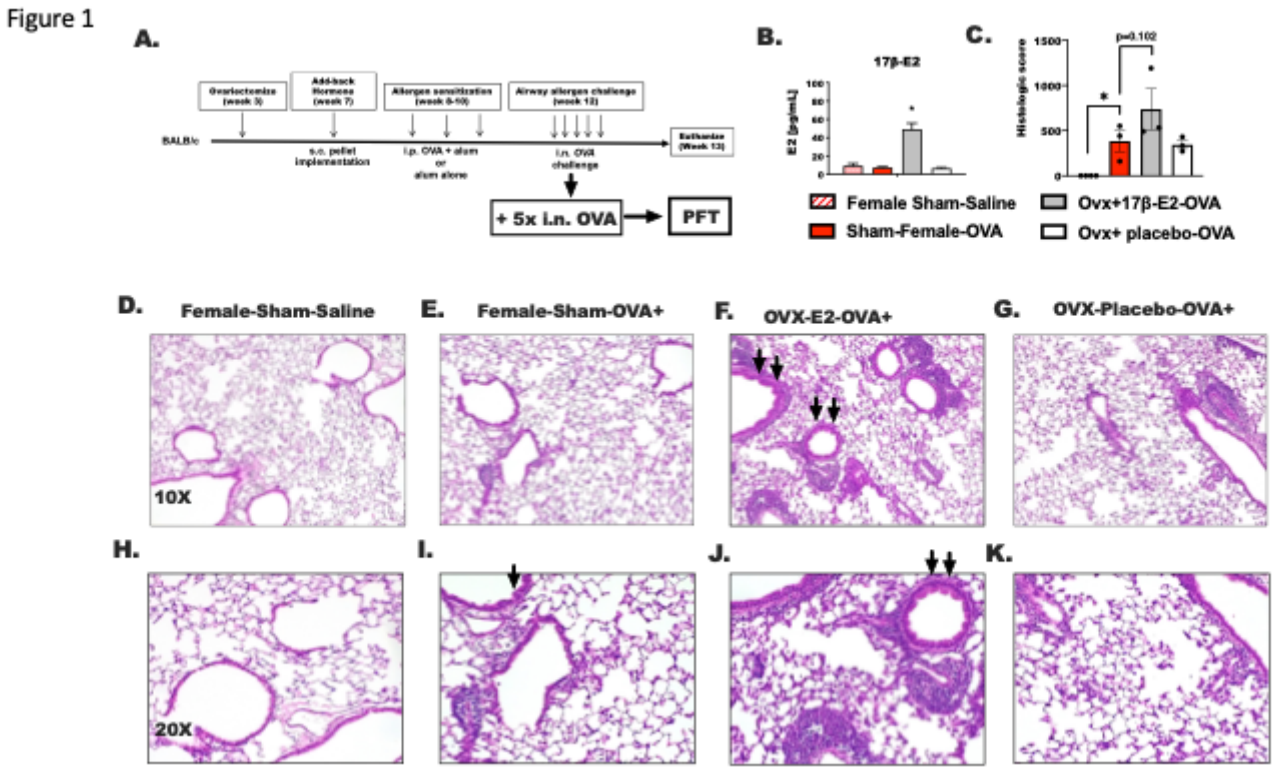

Figure 1

Airway and vascular cell infiltrates are increased in the airways when estrogen is given at steady state levels. A. An overview of experimental protocol is showed where female BALB/c mice were ovarectomized at 3 weeks of age (OVX) and implanted with subcutaneous estrogen (17β-E2, 0.1 mg) or placebo pellets 3 weeks later. Sham-operated female mice were included as controls. **B.** Circulating levels of estrogen were determined by ELISA. **C.** After ten consecutive daily challenges with i.n. ovalbumin, lungs were inflated then excised followed by paraffin embedding and H&E staining. **C-H.** Histological scoring was performed by a blinded pathologist on two sections per animal, 3-4 animals per group. The data shown as mean ± SEM. Results represent 2 independent experiments. One-Way ANOVA were used to determine statistical differences followed by Sidak's post-test to determine between groups statistical effects.

Figure 2

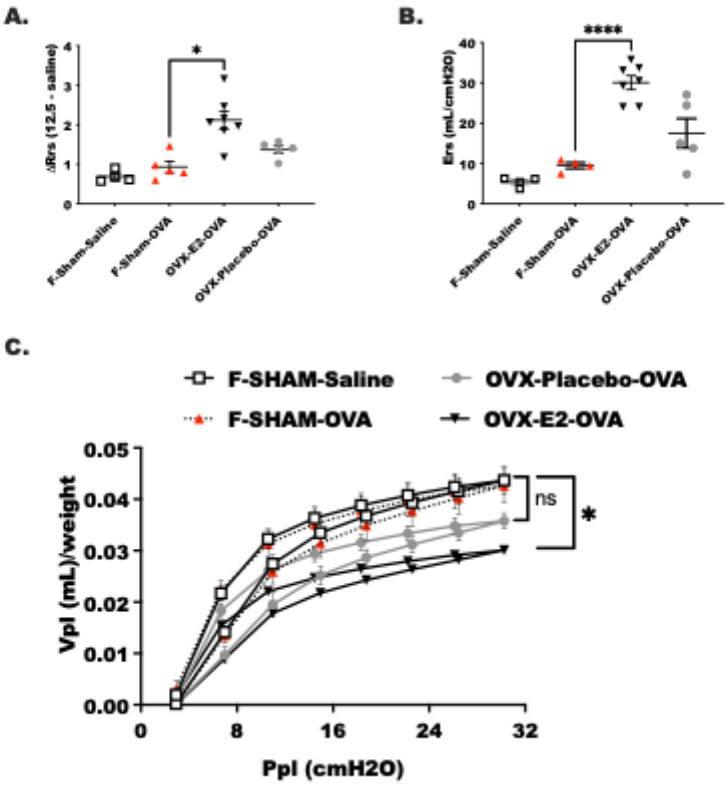

Figure 2

Airway resistance is increased in ovarectomized animals treated with estradiol in comparison to female sham treated animals. After completion of the experimental protocol outline in Figure 1 animals were subjected to methacholine challenge (12.5 mg /mL) using the flexivent system (Scireq); "PFT" = pulmonary function testing . **A.** Resistance (Rrs, baseline subtracted) and **B** elastance (Ers; baseline subtracted) were measured following methacholine challenge. **C**. Pressure Volume loops were determined and normalized to body weights. The data are shown as means ± SEM. Results represent 3 independent experiments, N= 4=7/group. Mixed-ANOVAs were used to determine statistical differences using multiple variate selection followed by Sidak's post-test.

Figure 3

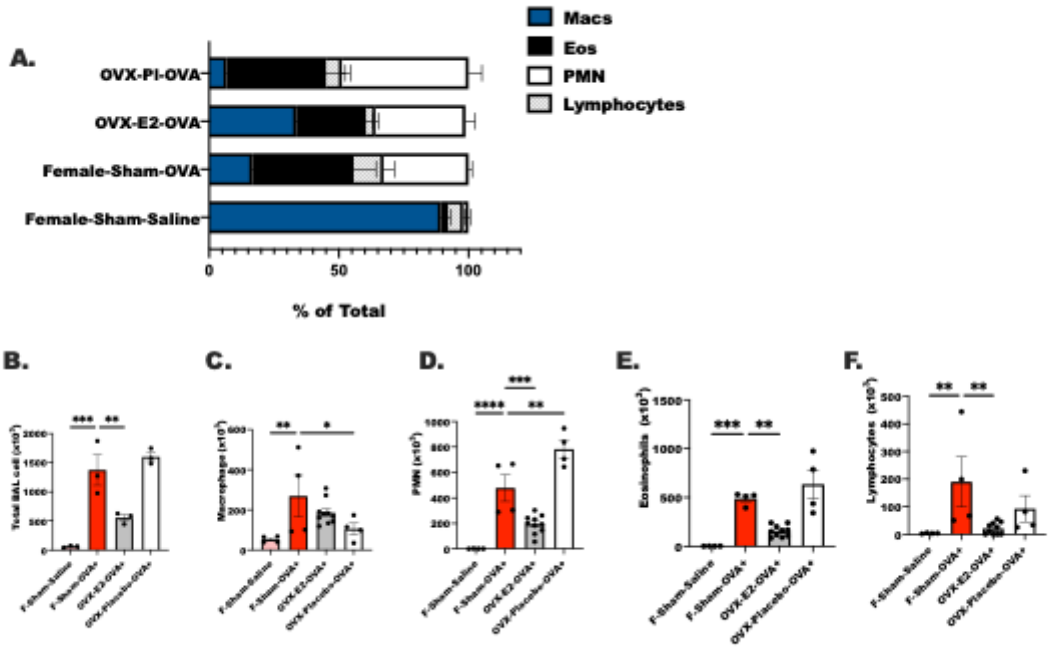

Figure 3

Total immune cell infiltrate is reduced in BAL fluid from allergen-challenged, ovarectomized, estrogen-treated animals compared to F-sham, allergen-treated females. Animals were treated as in Figure 1. Cytospins were prepared from BAL fluids collected immediately following methacholine challenges. Total BAL return volume and numbers of total cells (B) were recorded and applied to cytospin slides at 300 x g for 10 min. Slides were stained with Giemsa and 2-4 fields were assessed per slide. 200 cells per field were counted as (C) macrophages, (D) neutrophils, (E) eosinophils or (F) lymphocytes. **A.** Percentages of each cell population quantified are shown. **B-F** are the counts of cells determined by multiplying the percentages by the total cells recovered. Data are representative of 3 independent experiments with 3-8 animals per group. One-Way ANOVA was used to determine statistical differences across groups; Bonferroni post-test were used to determine statistical differences between groups.

Figure 4.

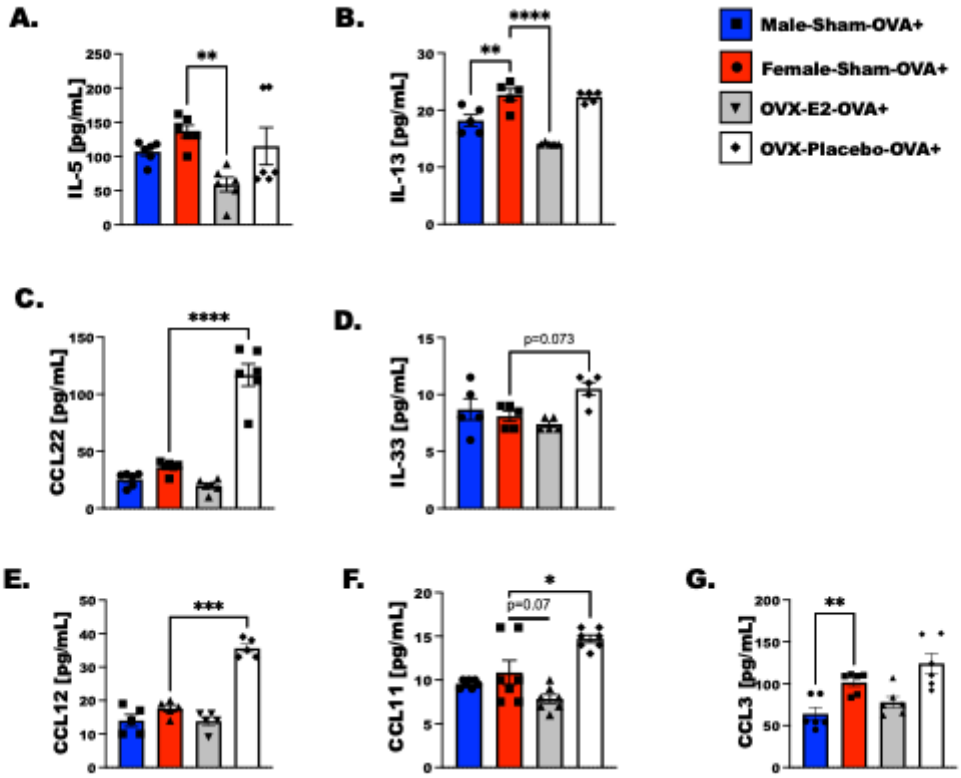

Figure 4

Allergic inflammatory cytokines are released at different rates in OVX-E2 treated animals compared to intact female-sham mice treated with airway allergen. 6 week-old male and female, sham-operated (M-Sham, F-Sham), ovariectomized with subcutaneous 17β -E2 pellets (OVX-E2), and ovariectomized with a placebo pellet (OVX-Pl) (OVX-pl) were sensitized with OVA-alum and challenged with intranasal OVA for 5 consecutive days. Animals were euthanized by overdosing with ketamine and bronchoalveolar lavage fluid was collected and cleared by centrifugation (500 x g) prior to applying to ELISA plates for analysis. (A) IL-5, (B) IL-13, (C) CCL22, (D) IL-33, (E) CCL12, (F) CCL11 and (G) CCL3 are shown as pg/mL. Statistical significance was determined as in figure 3.

Figure 5.

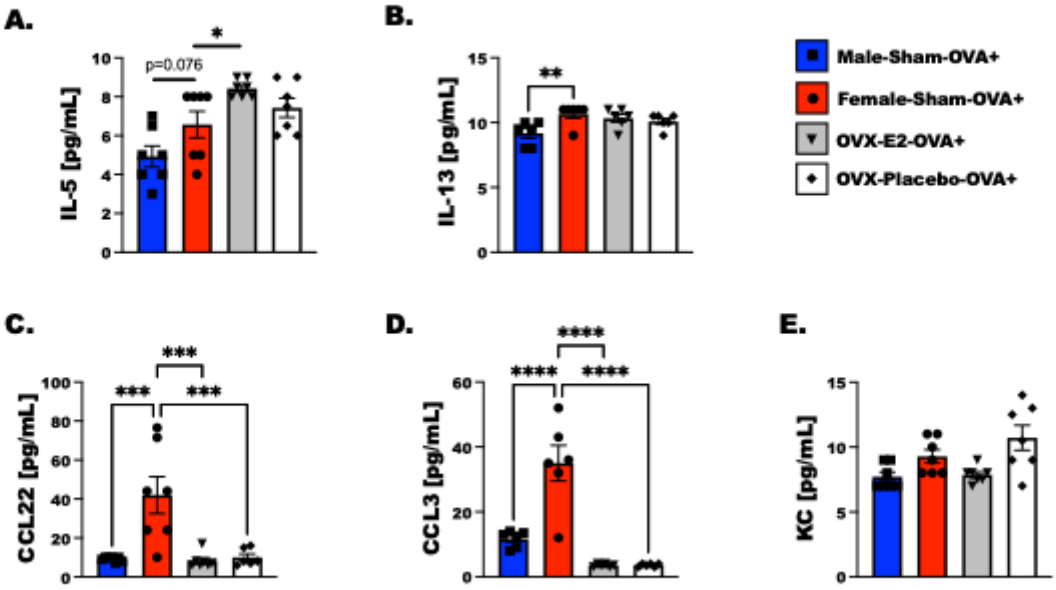

Figure 5

Circulating cytokines and chemokines are selectively altered with steady-state estrogen treatment. Groups of mice were prepared as in Figure 4. **A.** and **B.** Circulating cytokines were determined by ELISA in serum that was diluted 1:5 for all groups. (A) IL-5 and (B) IL-13 cytokines were detected, and (**C-E**) circulating chemokines (C) CCL22, (D) CCL3 and (E) KC are shown. The data are shown as mean ± SEM. Data are representative of 3 independent experiments. Comparison between groups were determined by One-Way ANOVA. Statistical significance was determined at $P < 0.05$ (indicated by *) and trends ($P < 0.1$) are reported as exact P-values.

Figure 6.

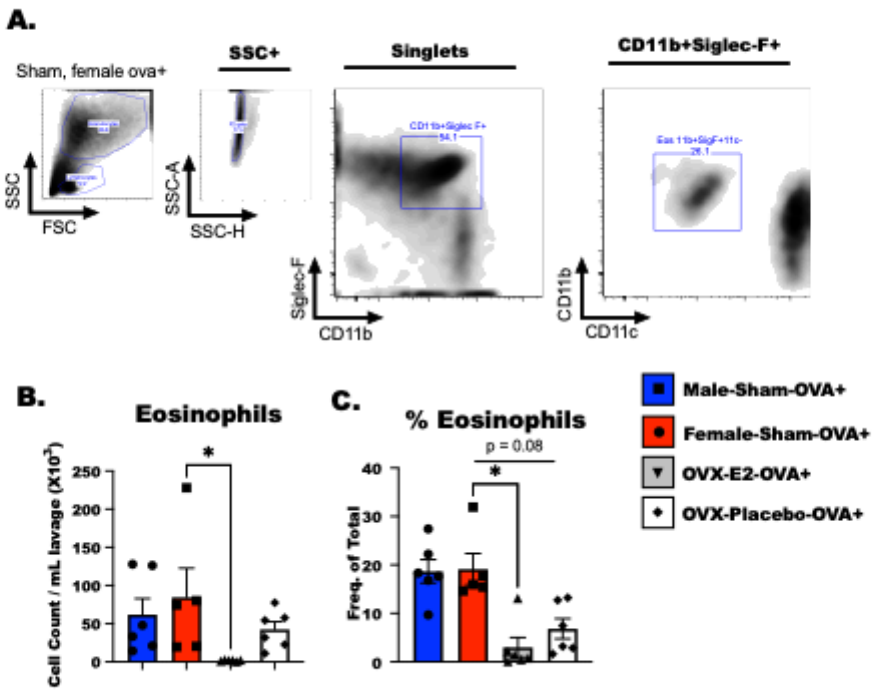

Figure 6

Estrogen treatment reduces eosinophils in BAL of OVA sensitized and challenged ovarectomized mice. A. Flow cytometry gating strategy for eosinophils defined as live, singlet, CD11b Siglec-F CD11c cells. **B,C**. Eosinophil concentrations (B) and frequency (D) in lavage are shown for all groups. Bar graphs show the data as mean ± SEM (n= 10 mice/group). The data represent 6 independent experiments. Statistical significance was determined as in Figure 3. * indicates $p<0.05$; trends are reported as exact p values.

Figure 7.

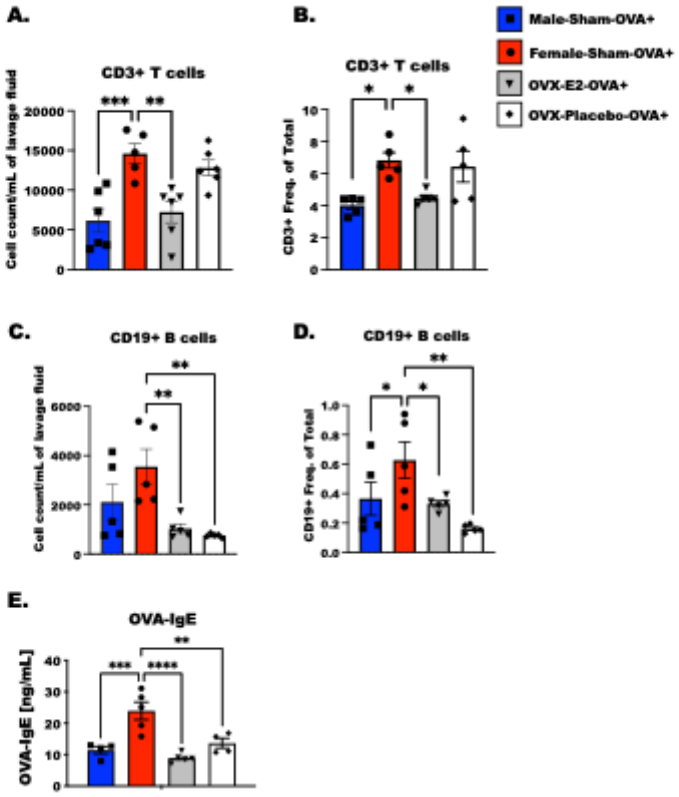

Figure 7

Estrogen reduces airway T cell and B cells in BAL fluid collected from ovarectomized animals compared to female sham-allergen challenged mice. Groups of animals were prepared as in Figure 4-6. Bronchoalveolar lavage fluid was collected immediately following euthanasia. **A-D.** By Flow cytometry total CD3+ and CD19+, T and B cells, respectively, were determined following treatment with 5 consecutive daily i.n. OVA administration. A and B. Counts of cells per mL of returned lavage fluid and percentages of the total cells acquired are shown for T cells (A and B), and B cells (C and D). **E.** OVA-Specific IgE was also measure in serum that was diluted 1:5 by ELISA. Results are representative of two separate experiments with 4-6 animals included per group. As before, statistical significance was determined using One-Way ANOVA with appropriate post-tests as in previous figures.

Figure 8.

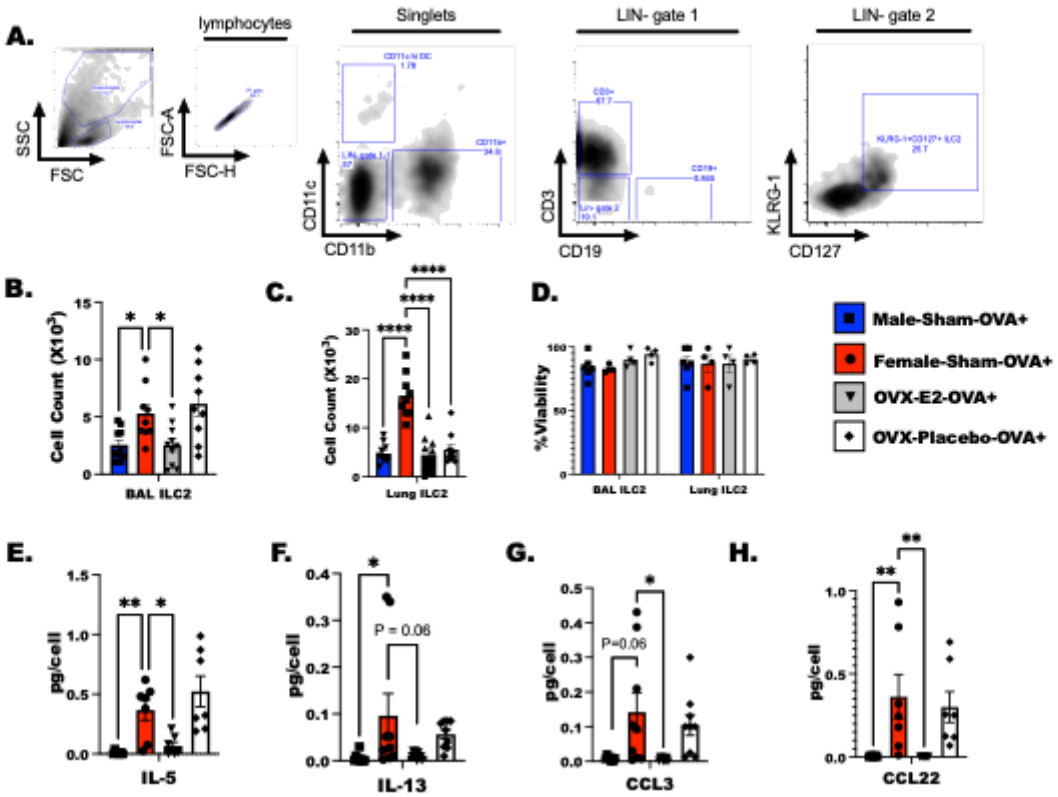

Figure 8

In vivo estrogen-treatment reduces ILC2 numbers and dampens the IL-33 responses of ILC2. A. Flow cytometry gating strategy of ILC2 defined as live, singlet cells lacking lineage markers (LIN: CD11b, CD11c, CD3, CD19) and expressing KLRG-1 and CD127. **B and C**. Counts of ILC2 in the (B) BAL and (C) lungs were quantified. **D.** Viability was assessed for ILC2 in bronchoalveolar lavage (BAL) fluid and lungs during flow cytometry assessment. The data are shown as means ± SEM and are representative of 3 independent experiments. **E-H.** Cytokine expression by lung ILC2 cultured with IL-2, IL-7 with and without IL-33 for 3 days were determined by ELISA. (E) IL-5, (F) IL-13, (G) CCL3, and (H) CCL22 are displayed as pg/cell based on ILC2 count determined at the beginning of culture. Results are representative of two separate experiments with 4-6 animals included per group. As before, statistical significance was determined using One-Way ANOVA with appropriate post-tests as in previous figures. Error bars denote means ± SEM.