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Oestrogen amplifies pre-existing atopy-associated Th2 bias in an experimental asthma model

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Abstract

Background: The prevalence and severity of asthma, particularly the most common (atopic) form of the disease, increase amongst females but not males after puberty, and asthma activity also changes throughout the menstrual cycle and during pregnancy. The contribution of female sex hormones to asthma pathogenesis is incompletely understood.

Objective: To obtain insight into the role of oestrogen (E2) in experimental atopic asthma, and guide future research on sex-related variations in atopic asthma susceptibility/intensity in humans.

Methods: We utilized an experimental model comprising rat strains expressing dichotomous Th2-high vs Th2-low immunophenotypes exemplified by eosinophilia, mirroring differences between human atopics/non-atopics. We compared the efficiency of Th2-associated immunoinflammatory mechanisms, which differed markedly between the two strains, and between sexes in the Th2-high strain, and determined the effects of E2 administration on these differences.

Results: Unique to the Th2-high strain, eosinophil: neutrophil ratios in the airways at baseline and following sensitization/aeroallergen challenge were logfold higher in females relative to males, and this was reflected by higher baseline blood eosinophil numbers in females. Pretreatment of Th2-high males with E2 abrogated this sex difference by selectively boosting Th2-associated genes in the airways and eosinophilia, but was without corresponding effect in the Th2-low strain. In contrast, parallel E2 effects on myeloid and lymphoid cell populations were relatively modest.

Conclusions and Clinical Relevance: E2 acts to amplify the eosinophilic component of pre-existing Th2-high immunophenotype, possibly acting at the level of the common eosinophil/neutrophil precursor in bone marrow to preferentially drive eosinophil differentiation. Constitutive granulocyte profiles in which the balance between eosinophils and neutrophils is skewed towards eosinophils have been identified in independent cohort studies as markers of asthma risk, and these findings suggest that more detailed studies on the role of E2 in this context, and in relation to asthma pathogenesis in post-pubertal females in particular, appear warranted.

KEYWORDS

allergic asthma, atopy, eosinophils, neutrophils, oestrogen

Lauzon-Joset, Mincham, Strickland and Leffler joint first and senior authors.

1 | INTRODUCTION

Before puberty, allergic asthma is more common in males than females but this ratio reverses after puberty when females display both a higher prevalence and severity of both allergic and non-allergic asthma.[1-4] This has been linked to several factors such as different pulmonary physiology, female expression of X-linked genes and association with female-specific obesity.[5] There is also a clear link with immune function and expression of female sex hormones such as oestrogen (E2), which modulates several aspects of the immune system.[6] However, how these mechanisms operate in vivo is poorly understood.

Eosinophils play a central role in parasite defence and allergic inflammation. In allergic airway inflammation, eosinophils are recruited to the airways through IL-5 signalling, which is preceded by local IL-25 and IL-33 production by airway epithelial and other innate immune cells in the airways.[7] Increased bone marrow output of eosinophils is commonly observed in atopic/asthmatic individuals,[8] resulting in increased blood and airway numbers. In the general population in which circulating eosinophil numbers are relatively low, males display increased number of circulating eosinophils compared to females; however, amongst individuals with child-onset atopic asthma in whom eosinophilia is a hallmark of their clinical phenotype, eosinophils appear elevated in females compared to males.[9] In cohort studies, increased levels of circulating eosinophils are also associated with risk of atopic asthma or asthma severity.[10,11] A potential role for E2 as an amplifier of eosinophil responses in females first emerged in the 1960s through studies demonstrating E2-induced recruitment of eosinophils into rat uterine tissue.[12] Since then, E2 contribution to airway infiltration and associated Th2-biased inflammation has been observed in both mouse- and rat-based experimental models.[13-15] However, there are also conflicting results, suggesting that E2, in some models, has a dual role and may induce anti-inflammatory responses[15] or reduce airway hyperresponsiveness.[16] In a model of peritoneal inflammation, E2 also appeared to reduce eosinophil infiltration through increased apoptosis.[17] A complicating factor in interpreting findings from experimental models is the concentration-dependent effects of E2, notably that low concentrations likely are immune-enhancing, whereas high levels (such as those encountered in pregnancy) can potentially selectively antagonize some aspects of host defence whilst other aspects of immunity are enhanced.[18] Thus, discrepancies between studies may relate to differences in E2 delivery systems, local vs systemic concentrations and continuous vs periodical administration.

Support for a potential stimulatory role for E2 in human asthma extends beyond the demonstration of post-pubertal increases in prevalence in females[1-4] and includes additional findings relating to adults, notably the increased risk for asthma symptom onset during the luteal phase of the menstrual cycle when serum E2 levels commonly spike,[19] and the worsening of asthma during pregnancy that occurs in up to one third of affected women, usually those with the most severe form of disease.[20] In experimental and human in vitro studies, female sex hormones have been shown to affect a range of immune cell subsets including development of dendritic cells (DC) and B cells,[21,22] T cell responsiveness[23,24] and altered balance in Th1/Th2 immunity.[25,26] However, it is not clear how these mechanisms contribute to increased asthma symptoms in vivo.

In the present study, we used an experimental animal model system to test the hypothesis that E2 contributes to allergic airway pathology in females susceptible to atopic disease. This system utilizes two rat strains, which express highly dichotomous $Th2^{high}$ (Brown Norway, BN) vs $Th2^{low}$ (Piebald Virol Glaxo, PVG) immunophenotypes that more closely model the extremes of the human IgE-responsiveness spectrum, relative to that seen across mouse strains. In this model, BN females and E2-treated males displayed increased Th2 bias as evident from increased eosinophil levels in circulation and airways compared to control BN males. The effects of E2 appeared dependent on underlying Th2 bias as they were not replicated in the $Th2^{low}$ PVG rat strain that lacks Th2 bias. This study suggests that E2 modulates the balance of eosinophils and neutrophils and given the association of eosinophil levels with asthma, E2 may contribute to increasing the risk of asthma development in females.

2 | METHODS

2.1 | Experimental animals

Brown Norway and PVG rats were bred at the Telethon Kids Institute. These strains were selected based on their contrasting susceptibilities to allergic airway inflammation,[27] viz. highly susceptible BN exhibiting the $Th2^{high}$ (human atopy-like) immunophenotype, vs resistant PVG manifesting a $Th2^{low}$ (non-atopic equivalent) immunophenotype. Animals were randomly allocated to an experimental group at the age of 7-9 weeks. All experiments were approved by the local ethics committee reference #AEC270 and #AEC284, which adhere to the national guidelines for the use of research animals in Australia. All animals were humanely killed by an intraperitoneal injection of Lethabarb (Virbac) administered under isoflurane-induced anaesthesia.

2.2 | Oestrogen administration and OVA stimulation

Slow-releasing 17β-oestradiol (E2) pellets at 0.25 mg/60 days or sham pellets (Innovative Research of America) were inserted subcutaneously into the back of isoflurane anaesthetized male rats. One week following E2 pellet insertion, male, female or E2-treated male rats were sensitized to ovalbumin (OVA) through an intraperitoneal injection of 0.1 mg OVA (Sigma) in Alu-Gel (Serva) under light isoflurane-induced anaesthesia. Two weeks following sensitization, rats were exposed to 0.1% aerosolized OVA for 30 minutes using a Ultraneb nebulizer (DeVilbiss). Animals were in general killed 24 hours following OVA challenge. Serum levels of free E2 were assessed 3 weeks following pellet insertion using the Estradiol EIA (Cayman Chemical). Testosterone levels in BN rat were measured using the Architect 2nd Generation Testosterone Test at PathWest

(Perth, WA) and the Testosterone Kit (Cayman Chemical) according to the manufacturer's instructions.

2.3 | Bronchoalveolar lavages

Bronchoalveolar lavages (BAL) were collected immediately following euthanasia by twice flushing the airways with 8 mL of 140 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L Na_2HPO_4, 5 mmol/L NaH_2PO_4 and 10 mmol/L glucose (GKN buffer). Samples were kept on ice before cells were pelleted, and supernatant was stored at −20°C. For the cell pellet, erythrocytes were lysed using 140 mmol/L NH_4Cl in 17 mmol/L Tris-HCl pH 7.2 and remaining cells were washed with GKN buffer. Following cell counting, 50 000 cells were spun onto a Superfrost microscope slide (Thermo Scientific) and stained with Rapid Diff Stain Kit (Australian Biostain) as per the manufacturer's instructions. The remaining cells were suspended in TRIzol (Thermo Fisher) and stored at −80°C until further use. Differential count was performed blinded on at least 300 cells per slide and sample. Levels of eosinophil cationic protein (ECP) in BAL supernatant were analysed, according to the manufacturer's instructions using an ECP ELISA (FineTest).

2.4 | Blood cell counts

Blood was collected through tail vein puncture or cardiac puncture prior to euthanasia into a heparinized microtube. For blood cell counts, whole blood was analysed using a BC-5000 Vet Auto Hematology Analyser (Mindray). For peripheral blood mononuclear cell (PBMC) isolation, 1 mL of heparinized blood was layered onto 3 mL Lymphoprep (Axis Shield). After centrifugation at 800 *g* for 20 minutes, the mononuclear layer was transferred to a new tube and washed with PBS before stained for flow cytometry as per below.

2.5 | Total and specific IgE

OVA-specific IgE was analysed using passive cutaneous anaphylaxis as previously described.[28] Total IgE was analysed by ELISA. Mouse anti-rat IgE (#MCA193; Bio-Rad) at 5 μg/mL or BSA was coated onto Maxisorp plates (Nunc) in PBS, washed in PBS supplemented with 0.05% Tween-20, blocked in 1% BSA, washed and incubated with 5% rat serum in PBS or IgE standard (#PRP07A; Bio-Rad). Following incubation, plates were washed and incubated with HRP-conjugated mouse anti-rat kappa-lambda chain (#MCA1296; Bio-Rad), washed and developed using TMB Core reagent (Bio-Rad) according to the manufacturer's instructions.

2.6 | Gene expression analysis

Total RNA was extracted from BAL cells stored in TRIzol, using RNeasy columns (Qiagen). RNA (250 ng/sample) was reverse-transcribed to cDNA using QuantiTect RT Kit (Qiagen). To assess gene expression, QuantiTect primers for *CCL17*, *CCL22*, *CCL24*, *CCR3* and *IL5αR* (Qiagen) or *GATA3* (F: TGGGCTGTACTACAAACTCCACAA R: GCGCATCATGCACCTTTTT, Sigma) were used in the QuantiFast SYBR Green PCR System (Qiagen) and detected on a CFX96 qPCR System (Bio-Rad). Expression was interpolated from primer-specific standards and displayed as normalized to mean expression in the three experimental groups.

2.7 | Tissue digestion

Airway draining lymph nodes (ADLN) and trachea were dissected out, mechanically disintegrated and digested as previously[29] with collagenase IV (Worthington) and DNase I (Sigma-Aldrich) in GKN buffer supplemented with 10% FCS for 30 minutes (ADLN) or 90 minutes (trachea) at 37°C, mixed thoroughly to extract cells and filtered through a nylon membrane into a single-cell suspension before stained for flow cytometry as per below.

2.8 | Flow cytometry

Freshly isolated cells (1×10^6) were stained with either a dendritic cell panel: Class II-PerCP (BD), CD11b-V450 (BD), CD172-PE (BD), CD103-Biotin (Serotec), CD45RA-APC-Cy7 (BD), CD86-FITC (BD) and CD4-APC (BD) followed by Strep-V500 (BD); or T cell panel: CD3-FITC (BD), CD4-PE-Cy7 (BD), CD8-PerCP (BD), CD25-biotin (BD) and CD45RA-APC-Cy7 (BD) followed by intracellular staining using FoxP3 staining solution (eBioscience) followed by FoxP3-PE (eBioscience) and Ki67-AF700 (BD) at 4°C before acquisition on a LSR Fortessa (BD Biosciences) and analysed using FlowJo 10 (FlowJo) as previously described.[29]

2.9 | Statistical analysis

All experimental groups consisted of at least three animals from two or more independent experiments. Calculations for statistical significance of difference were based on number of groups and normality of data using either Student's *t* test, Mann-Whitney, ANOVA followed by Fisher's LSD test or the Kruskal-Wallis followed by Dunn's uncorrected post-test. All statistics was calculated using Prism 8.0.2 (GraphPad).

3 | RESULTS

3.1 | Males and females differ in airway immune composition at baseline

The BN rat strain is frequently used in allergy studies, including our own previously published findings,[27,29] due to its constitutive $Th2^{high}$

immune response phenotype.[30] To assess whether male and female BNs differed in degrees of Th2 bias, we assessed airway immune cell composition at baseline. Bronchoalveolar lavage (BAL) of similarly housed rats demonstrated a significantly increased number of immune cells in females relative to males (Figure 1A). The increase in total number of cells in BAL appeared across the four cell types examined (macrophages, neutrophils, eosinophils and lymphocytes; Figure 1B), and this sex bias was mostly marked for eosinophils (Figure 1C). Additionally, the BN females displayed significantly higher blood eosinophil numbers (Figure 1D) and total IgE titres (Figure 1E) compared to their male counterparts. Moreover, the proportion of BAL eosinophils correlated strongly with both blood eosinophils (Figure 1F) and total IgE serum levels (Figure 1G). To assess whether these male/female differences in expression of Th2-associated markers were a general phenomenon in rats, we then assessed numbers of eosinophils in $Th2^{low}$ PVG rats. In this strain, no significant difference in airway eosinophil numbers was observed between males and females, which were also considerably lower compared to the BN strain (Figure 1H; BN male data included for direct comparison). This suggests that the difference in male/female airway eosinophils in the BN strain may be related to their underlying constitutive $Th2^{high}$ immunophenotype (see further discussion below).

3.2 | Th2-biased airway cell recruitment at baseline in females is driven by oestrogen

Oestrogen has previously been shown to increase eosinophil infiltration in airways following allergen exposure in rats.[13] We accordingly hypothesized that low endogenous oestrogen production in male BN rats may be responsible for their reduced levels of baseline airway eosinophil recruitment relative to their female BN counterparts. To test this hypothesis, we implanted E2-releasing pellets into male BN rats. Three weeks following implantation, pellets generated serum E2 concentrations that were comparable to female levels (35 ± 19 pg/mL vs 32 ± 16 pg/mL) and were significantly higher compared to male levels (11 ± 5 pg/mL; Figure 2A). Of note, the levels of E2 were physiologically active as observed by reduced weight gain in treated males compared to untreated (Figure 2B), as previously documented.[31] As anticipated from their counter-regulation, we also observed that E2 administration significantly reduced levels of total testosterone in males to levels below females (Figure 2C and Figure S1A). Analysis of BAL showed that E2-treated male rats displayed a female-like airway phenotype with increased number of BAL cells compared to untreated males (Figure 2D), and increase in cell numbers across macrophages, neutrophils, eosinophils and lymphocytes (Figure S1B), as expected from the male/female baseline difference, the main difference appeared in the proportion of eosinophils, which was increased also compared to females (Figure 2E). The increase in both airway neutrophils and eosinophils in E2-treated males suggests that E2 administration impacts the common neutrophil/eosinophil progenitor; however, given the higher proportional increase in eosinophils, E2 administration may preferentially skew progenitor differentiation towards generation of eosinophils. In addition to modulating airway eosinophil levels, E2 administration also increased the total serum IgE levels (Figure 2F), further suggesting that E2 may contribute to driving the elevated systemic Th2 response observed in female BN rats.

3.3 | Oestrogen induces a female-like response to allergen in male rats

The data above established that E2 is a likely contributor to systemic Th2 bias in BN females. To assess how E2 modulates specific allergen responses, OVA-AlOH was used to specifically sensitize male, female or E2-treated males to a defined allergen (Figure 3A). In this model, sensitization induced OVA-specific IgE, but no difference was observed in levels between groups following sensitization (Figure 3B). Sensitization did not affect total BAL cell numbers (Figure S2A) or proportions of macrophages, neutrophils or eosinophils; an increase in proportion of lymphocytes was, however, observed (Figure S2B). Following allergen (OVA) challenge, an increase in BAL cells was observed in males and females, whereas no additional increase from the elevated baseline was observed in E2-treated males (Figure 3C). OVA challenge of OVA-sensitized rats increased the proportion of neutrophils, eosinophils and lymphocytes in males and females (Figure S2C), and in E2-treated males, a trend towards increased proportion of neutrophils was also observed (Figure S2C). Moreover, numbers of BAL macrophages and eosinophils following OVA exposure in female and E2-treated males were significantly increased compared to males (Figure 3D). Females also displayed increased number of lymphocytes compared to males (Figure 3D). No difference between the groups was observed in the number of neutrophils. To elucidate the mechanism of increased recruitment of eosinophils at baseline, we measured expression of Th2- and eosinophil-related genes in the BAL cells (Figure 3E). Pre-challenge, females and E2-treated males expressed significantly elevated levels of DC-derived cytokines CCL17 and CCL22, the main Th2-associated transcription factor GATA3, eosinophil attractant eotaxin-2/CCL24 and eosinophil markers such as CCR3 and IL5αR compared to males (Figure 3E). Following OVA challenge, GATA3 expression was significantly up-regulated in both and males and females. There was also a trend for up-regulation of the other genes across the pathway in the males (Figure 3E). As expected, CCR3 and IL5αR correlated with proportion of eosinophil in the BAL pre- and post-challenge, whereas GATA3 expression did not (Figure S2D-F). As expected, levels of eosinophil granule proteins were also significantly elevated in females and E2-treated males (Figure 3F), suggesting that the eosinophils that are recruited release their granular content. Together, these data suggest that E2 administration leads to continuous activation of the Th2 pathway in the airways resulting in eosinophil recruitment. Once exposed to an allergen, this pathway is up-regulated in male airways and remains active in females and E2-treated males.

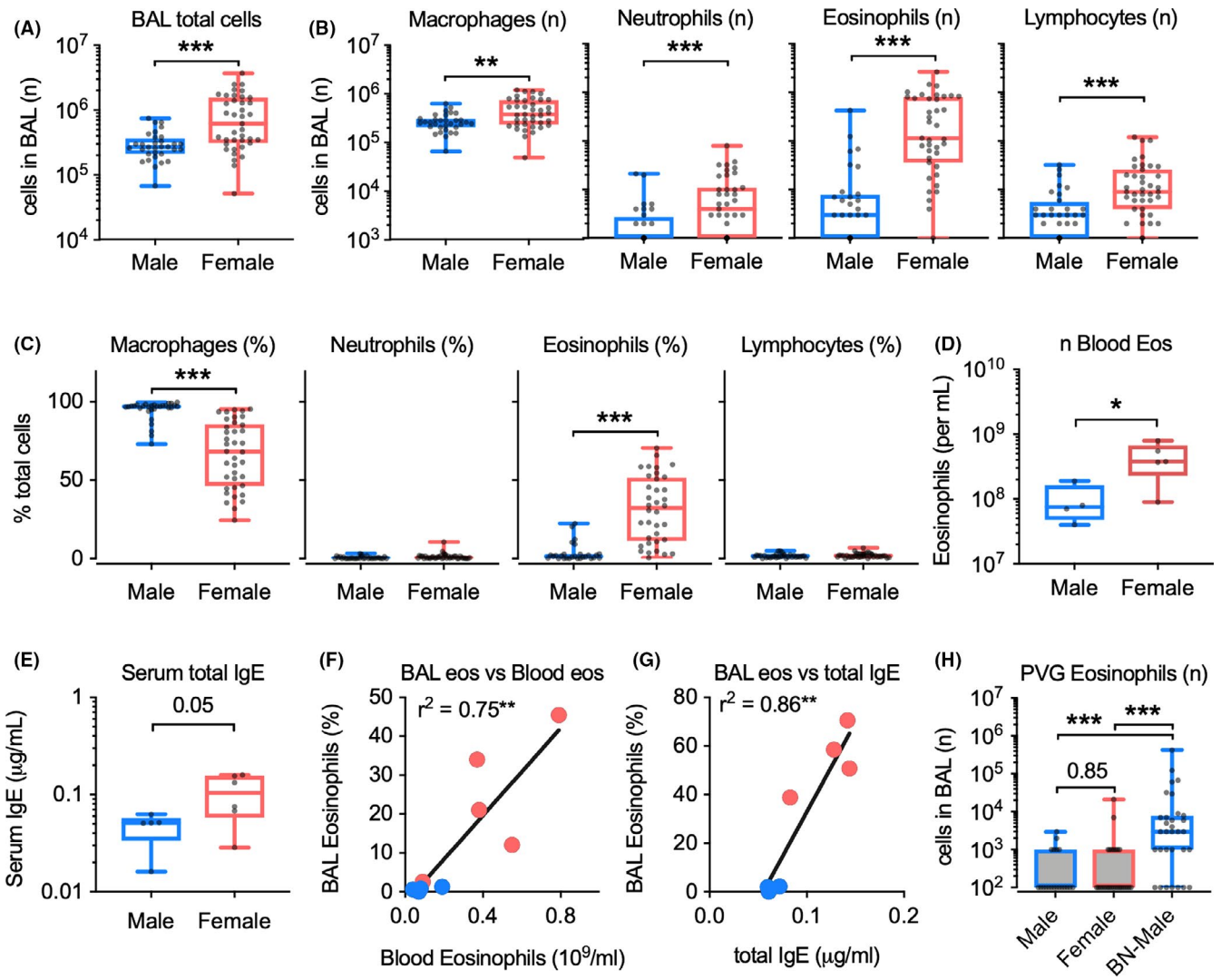

FIGURE 1 A-C, Total cells (A) and number (B) and proportion (C) of macrophages, neutrophils, eosinophils and lymphocytes in BAL from naïve male and female BN rats. D, Number of blood eosinophils in naïve male and female BN rats. E, Serum levels of total IgE in naïve male and female BN rats. F-G, Correlation of BAL eosinophils, blood eosinophils (F) and serum levels of total IgE (G) in naïve male and females BN rats. H, Number of eosinophils in BAL from naïve male and female PVG rats and male BN rats. Data are displayed as individual data points. Box and whiskers display median, quartiles and range. Significance of difference between groups is displayed as *, $P < .05$; **, $P < .01$; ***, $P < .001$ and calculated using the Mann-Whitney in A-B and E-F, Student's *t* test in C or Kruskal-Wallis test followed by Dunn's uncorrected post-test in D. Correlation in G-H was calculated using Pearson's test

The composition of the granulocyte population, in particular the balance between eosinophils and neutrophils, may contribute to clinical airway disease. Female BNs displayed a significantly increased Eos/Neut ratio compared to males both at baseline (Figure 4A) and after sensitization/aerosol challenge (Figure 4B), and E2 administration to male rats increased Eos/Neut ratios to similar levels as females. In contrast, this difference between male and females was not observed in the $Th2^{low}$ PVG strain, which, in general, displayed a neutrophil-dominated airway environment (Figure 4C). Moreover, in the PVG strain E2 administration did not appear to alter the Eos/Neut ratio although serum levels of E2 after treatment were slightly higher compared to E2-treated BN males (Figure S3A) and a similar decrease in free testosterone levels was observed (Figure S3B).

E2 has also been reported to impact other immune cells in addition to eosinophils,[6] and analysis of gene expression suggested that E2 may also impact DC activation. We therefore assessed the impact of E2 on DC and T cells by flow cytometry (Figure S4A-C) of single-cell preparations from airways, ADLN and PBMC at baseline and following OVA exposure. Employing an exploratory approach, discoveries were identified using Student's t test and corrected for multiple comparisons using a two-stage step-up method[32] across tissues at baseline and following OVA challenge. Although the analysis did not identify any consistent effect of E2 on T cell or DC proportions at baseline (Figure S5A-B), following OVA challenge, a monocyte/granulocyte population ($MHC\text{-}II^{-}/CD172^{hi}/CD11b^{hi}$) and two immature cDC ($MHC\text{-}II^{lo}$ and $MHC\text{-}II^{int}/CD11b^{+}/CD45RA^{-}$) subsets responded equivalently to OVA in the airways across the three

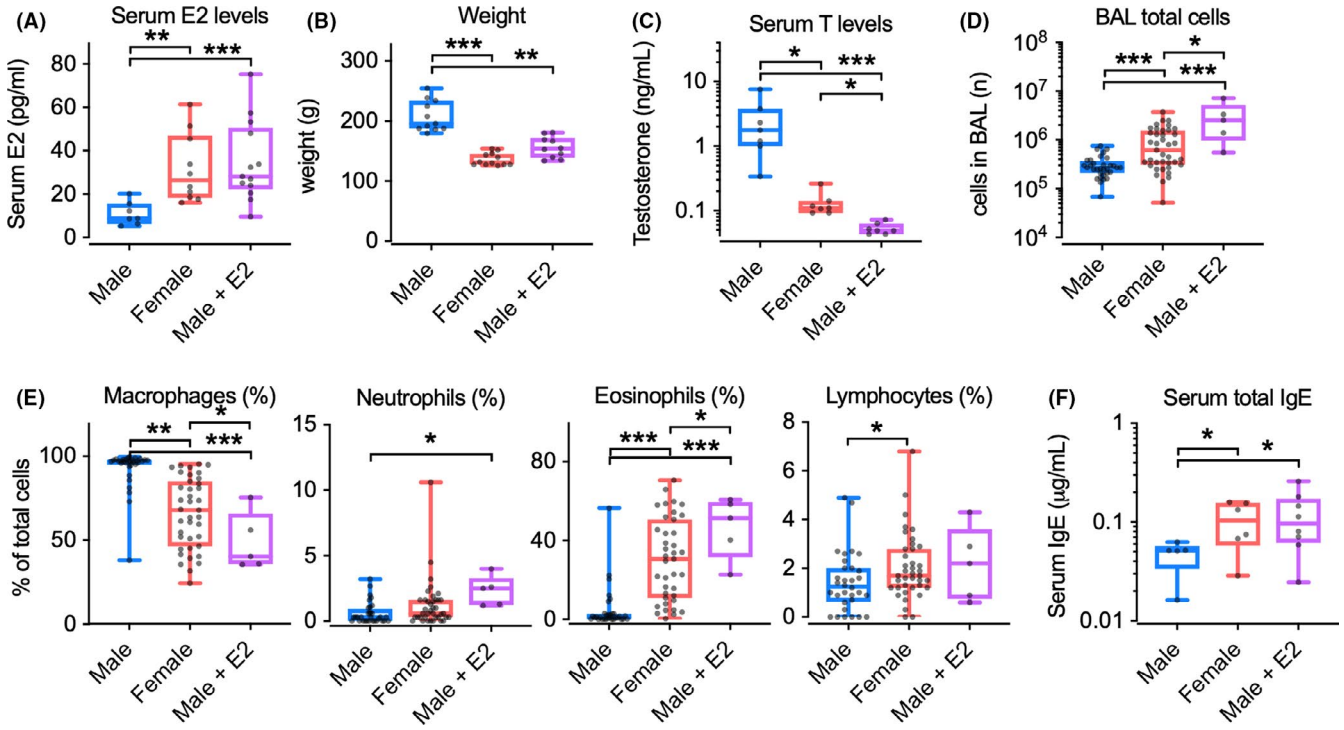

FIGURE 2 A-F, Serum E2-levels (A), weight (B), serum total testosterone levels (C), number of cells in BAL (D), proportion of macrophages, neutrophils, eosinophils and lymphocytes in BAL (E) and serum levels of total IgE (F) at baseline in BN males, females and E2-treated males. Data are displayed as individual data points. Box and whiskers display median, quartiles and range. Significance of difference between groups is displayed as *, $P < .05$; **, $P < .01$; ***, $P < .001$ and calculated using the Kruskal-Wallis test followed by Dunn's uncorrected post-test in A-D and F and ANOVA followed by Fisher's LSD post-test in E

groups (Figure 5A). In E2-treated males, mature MHC-II^{hi} cDC also responded with increased expression of the activation marker CD86 (Figure 5B). Further, across all groups, OVA challenge induced proliferation of CD4$^+$ Treg in the airways, but interestingly, only in males did OVA challenge also induce proliferation of CD8$^+$/CD25$^+$ T effector cells (Figure 5C). Additional findings that included inconsistent observations across the groups or findings relating to less-defined subsets are displayed in Figure S6. In general, these findings suggest that in the ADLN, OVA challenge induces more changes to cell proportions in males compared to females. Some of those changes are due to differences at baseline, and some also appear to be inhibited in E2-treated males (Figure S6A-C); however, further investigation is necessary to determine the immunological impact of these responses.

4 | DISCUSSION

In the present study, we have utilized the allergy-susceptible Th2^{high} BN rat strain, in which the strength of the systemic and airway Th2 bias at baseline is markedly higher in females than in males, and we demonstrate that these sex-dependent differences appear to be modulated by E2 administration. Importantly, these effects of E2 were not replicated in the Th2^{low} PVG strain, suggesting that additional cofactor(s) operating within the Th2^{high} background are required to permit expression of the Th2-promoting potential of E2. The main clinical effect of E2 in this regard appears to relate to increased generation of eosinophils, which in human studies are associated with an increased risk of allergic asthma and airway hyperresponsiveness in atopic individuals of both sexes.[10,11] However, it is noteworthy that asthmatic females frequently present with higher eosinophil counts compared to asthmatic males.[9] Moreover, a recent longitudinal study on the US Hutterite community demonstrated that atopy-associated asthma symptoms have increased in females but not males since the 1990s,[33] and the lack of an increase in asthma prevalence in the males hints that a female-specific factor lowers disease threshold in individuals with high-atopic bias, mirroring the pattern seen here in the Th2^{high} BN strain.

We additionally observed that the differences in baseline Th2 bias between males and females in the BN strain are characterized by increased expression of Th2-associated genes in the airways, particularly related to DC cytokine production, GATA3 expression and airway eosinophil infiltration in response to airway challenge with an allergen to which they were pre-sensitized. Systemically, we also observed increased blood eosinophils and elevated total IgE levels in females compared to males. This sex-dependent gradient (particularly in BAL eosinophil levels) again appears driven by E2 administration, as the sex differences are abolished following E2 pretreatment of the males. Oestrogen has previously been shown to increase infiltration of eosinophils following OVA sensitization and challenge in several experimental models,[13-15] and our data suggest that this may

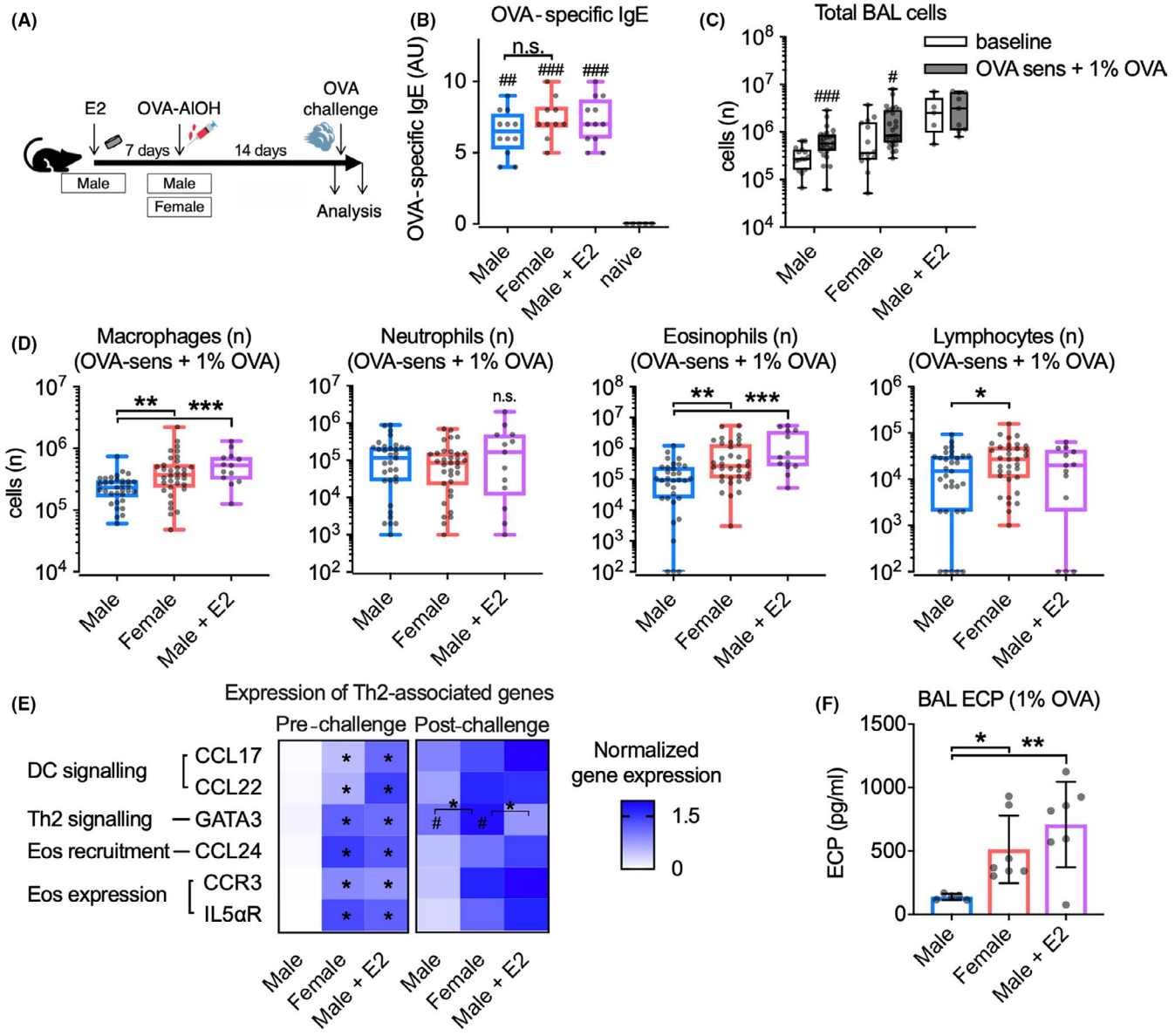

FIGURE 3 A, Schematic overview of the experimental model. B, OVA-specific IgE in OVA-AlOH–sensitized male, female and E2-treated male BN rats. C-D, Number of total cells in BAL (C) and number of macrophages, neutrophils, eosinophils and lymphocytes (D) at baseline or OVA-AlOH–sensitized and OVA-exposed male, female and E2-treated male BN rats. E, Heatmap of gene expression, normalized to mean expression pre- and post-OVA exposure. F, Levels of ECP in BAL supernatant following OVA challenge. Data in B-D and F are displayed as individual data points. In E, data are displayed as normalized gene expression to the mean for each gene from n = 3-4 per experimental group. Box and whiskers display median, quartiles and range. Significance of difference between groups is displayed as $^{\#/*}$, $P < .05$; $^{\#\#/**}$, $P < .01$; $^{\#\#\#/***}$, $P < .001$, where $^{\#}$ indicates differences compared to naïve/baseline controls, and * indicates differences as indicated or compared to males in E. Significance of difference is calculated using ANOVA followed by Fisher's LDS test in B, E and F, multiple Mann-Whitney tests in C and Kruskal-Wallis test followed by Dunn's uncorrected post-test in D

be mediated through increased GATA3 expression, presumably by airway Th2 cells or type 2 innate lymphoid cells (ILC2) as previously described.[34] It does remain unclear whether these effects are mediated directly by E2 or indirectly through the concurrent decrease in testosterone levels. Activation of estrogen receptors (α and β) in bronchial epithelial cells has been shown to increase IL-33 expression[35] and may drive Th2 inflammation. In addition, testosterone also directly inhibits ILC2s in mice[36] and it is possible that a similar mechanism operates in this model. However, as we also observe an impact on DC cytokine production, it is likely that several mechanisms are involved. In contrast to our findings, several studies involving both mesenchymal and immune cell populations also reported inhibitory effects on inflammation.[14,37,38] Differential experimental models, tissues of interest and E2 dose are likely explaining these discrepancies.

The BN strain is highly susceptible to Th2 inflammation and sensitizes to OVA in the absence of adjuvant.[29] Our findings suggest that the Th2-enhancing effects of E2 are dependent on an elevated

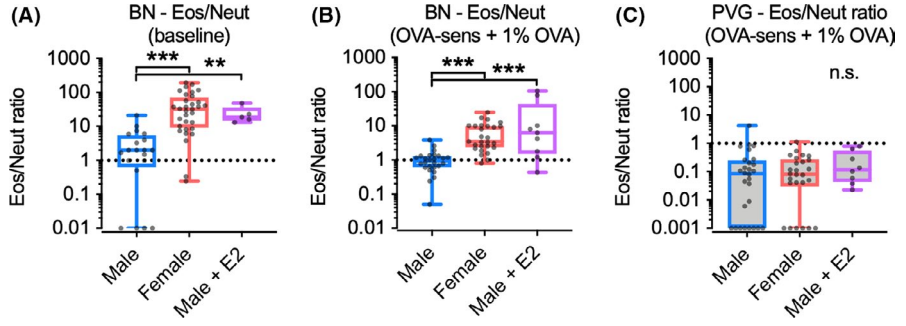

FIGURE 4 A-C, Ratio of number of eosinophils to neutrophils in BAL at baseline (A) or OVA-AlOH–sensitized and OVA–exposed male, female and E2-treated male BN rats (B) and PVG rats (C). Data are displayed as individual data points. Box and whiskers display median, quartiles and range. Significance of difference between groups is displayed as **, $P < .01$; ***, $P < .001$ and calculated using Kruskal-Wallis test followed by Dunn's uncorrected post-test

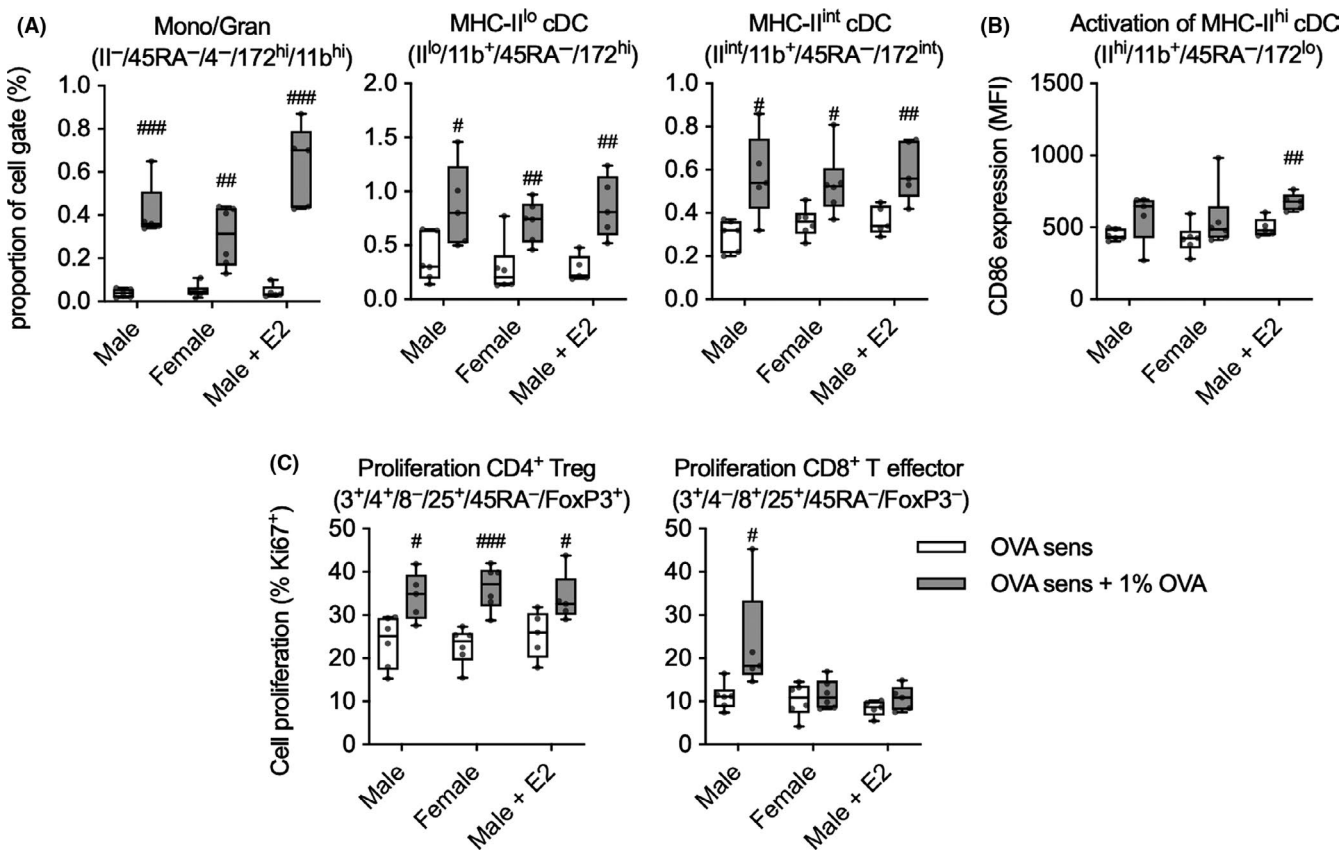

FIGURE 5 A, Proportion of monocytes/granulocytes, and MHC-II^{lo}–expressing and MHC-II^{int}–expressing airway cDC in OVA-sensitized male, female and E2-treated males pre- and post-OVA challenge. B, Expression of CD86 on MHC-II^{hi}–expressing airway cDC in OVA-sensitized male, female and E2-treated males pre- and post-OVA challenge. C, Proliferation, as indicated by expression of Ki67 in CD4$^+$ Treg and CD8$^+$ Teff cells in airways of OVA-sensitized male, female and E2-treated males pre- and post-OVA challenge. Data are displayed as individual data points, and box and whiskers display median, quartiles and range. Significance of difference between pre- and post-OVA challenge within groups is displayed as $^{\#}$, $P < .05$; $^{\#\#}$, $P < .01$; $^{\#\#\#}$, $P < .001$ and calculated using Student's *t* test

baseline Th2 bias as observed in the BN strain and in atopic individuals. Indeed, total serum IgE titres were higher in the females than in the males, and this difference was again abolished by E2 treatment. However, E2 exposure in male rats did not appear to translate into higher OVA-specific IgE titres. This is most likely due to the exaggerated strength of OVA sensitization in the presence of AlOH adjuvant, which generates a saturated IgE response in both male and female BN rats and thus clouds subtle differences in responses following adjuvant-free sensitization, and this possibility is currently under investigation.

Several experimental models have reported a host of immunological effects of E2 including a role in DC maturation,[21,39,40] effects on CD103$^+$ DC[41] and macrophage polarization[42] in the airways. Some of these effects are directly mediated through estrogen receptors [43] that

are expressed widely across cell populations in the immune system. In this study, we set out to assess some of these effects in the BN strain using flow cytometry. Analysis of the DC populations pre- and post-airway challenge did not identify any consistent contrasts in allergen-specific DC responses between male and females, although a small effect of E2 was observed in OVA-induced activation of mature cDC. The absence of effects may relate to a lack of sufficiently targeted markers in our flow cytometry panels or that our method did not allow analysis of DC that specifically interacted with OVA. It may also relate to the stable administration of E2 compared to the fluctuating levels in females as part of their oestrous cycle or the length of E2 administration. In addition to effects on DC, oestrogen may also influence T cell development[44] and the balance of CD4/CD8 activation.[45] Although $CD4^+$ Tregs were responding to OVA across all groups, only in males were $CD8^+$ T effector cells observed to respond. As $CD8^+$ and $CD4^+$ T cell populations can potentially cross-regulate each other, notably via the effects of Treg cells that are highly active in rats, the absence of CD8 activation in females and E2-treated males may result in excessive CD4 activation and subsequent Th2 inflammation in these groups, but this possibility was not tested.

In contrast to these relatively subtle sex-related variations within lymphoid and myeloid cell populations, log-scale male/female differences were observed within corresponding granulocyte populations in this model. Notably, unique to the $Th2^{high}$ BN strain, eosinophil:neutrophil ratios were markedly higher in the females, and this sex difference was again abolished by E2 pretreatment of the males, resulting in selective expansion of the eosinophil component of their granulocyte populations. In relation to possible underlying mechanism(s) mediating this effect, it is known that eosinophils and neutrophils are derived from a common $CD34^+$ progenitor in bone marrow,[46] and the ultimate contribution of each cell type to overall granulocyte output into the circulation is determined via the mix of competing cytokine/chemokine:receptor signals impacting on the progenitors. These include contributions from *inter alia* IL-5, GM-CSF, CXCL-12 and IL-3,[46] and we posit that E2 may directly or indirectly influence this process via influencing ligand delivery to, and/or corresponding receptor expression on, the $CD34^+$ progenitors, and these possibilities will be tested in follow-up studies.

Regardless of the mechanism(s) underlying E2 modulation of eosinophil:neutrophil balance, data from human studies have linked eosinophil-high/neutrophil-low phenotype comparable to that described above in BN females with enhanced risk for atopic asthma, firstly amongst atopics across a general community cohort[11] and secondly at population-wide level in studies contrasting the asthma-resistant Amish vs asthma-susceptible Hutterites[10] amongst whom the most affected subjects are females.[33] More detailed elucidation of the factors controlling expression of this E2-associated eosinophil-high/neutrophil-low phenotype may provide novel insight into asthma pathogenesis, particularly in post-pubertal females amongst whom a clearly defined but poorly understood clinical subgroup exists, which is characterized by late-onset and severe/chronic symptomatology,[47] and during pregnancy, which is associated with worsening of asthma in up to one third of affected women.[48]

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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