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Article type : Original Article: Asthma and Lower Airway Disease

Title: Estrogen receptor- α signaling increases allergen-induced IL-33 release and airway inflammation

Short Running Title: ER- α signaling increases IL-33 and airway inflammation

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Acknowledgements:

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14491](#)

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This project was supported by the National Institute of Health (HL122554, AI121420, HL122554S1). The authors would like to thank Paul Bryce (Northwestern University, Chicago, IL) for the IL-33eGFP mice.

Conflicts of interest:

The authors have no conflict of interest in relation to this work.

Author contributions:

JYC was responsible for design of the study, performed the experiments, statistical analysis and drafting of the manuscript. RS, VDG, JBD, JAY, and HF performed the experiments, data acquisition and analyzed the data. WZ and JZ provided reagents and advice on experimental design. VVP was the pathologist that scored the histopathology slides. HK provided the hBE33 cells, experimental design input, and edited the manuscript. DCN helped with design, supervised the research and wrote the manuscript. All authors have read and approved the final manuscript.

Abstract

Background: Group 2 innate lymphoid cells (ILC2) are stimulated by IL-33 to increase IL-5 and IL-13 production and airway inflammation. While sex hormones regulate airway inflammation, it remained unclear if estrogen signaling through estrogen receptor- α (ER- α , *Esr1*) or ER- β (*Esr2*) increased ILC2-mediated airway inflammation. We hypothesize that estrogen signaling increases allergen-induced IL-33 release, ILC2 cytokine production, and airway inflammation.

Methods: Female *Esr1*^{-/-}, *Esr2*^{-/-}, wild-type (WT), and IL33^{fl/fl}eGFP mice were challenged with *Alternaria* extract (Alt Ext) or vehicle for 4 days. In select experiments, mice were administered tamoxifen or vehicle pellets for 21 days prior to challenge. Lung ILC2, IL-5 and IL-13 production, and BAL inflammatory cells were measured on day 5 of Alt Ext challenge model. Bone marrow from WT and *Esr1*^{-/-} female mice was transferred (1:1 ratio) into WT female recipients for 6 weeks followed by Alt Ext challenge. hBE33 cells and normal human bronchial epithelial cells (NHBE) were pre-treated with 17 β -estradiol (E2), propyl-pyrazole-triol (PPT, ER- α agonist) or diarylpropionitrile (DPN, ER- β agonist) before allergen challenge to determine IL-33 gene expression and release, extracellular ATP release, DUOX-1 production, and necrosis.

Results: Alt Ext challenged *Esr1*^{-/-}, but not *Esr2*^{-/-}, mice had decreased IL-5 and IL-13 production, BAL eosinophils, and IL-33 release compared to WT mice. Tamoxifen decreased IL-5 and IL-13 production and BAL eosinophils. IL-33eGFP⁺ epithelial cells were decreased in Alt Ext challenged *Esr1*^{-/-} mice compared to WT mice. 17 β -E2 or PPT, but not DPN, increased IL-33 gene expression, release, and DUOX-1 production in hBE33 or NHBE cells.

Conclusion: ER- α signaling increased IL-33 release and ILC2-mediated airway inflammation.

Keywords: allergic airway inflammation, estrogen receptor alpha, IL-33 release, ILC2, sex disparity

Introduction

Women have increased prevalence of allergic diseases, including asthma, compared to men (1). Sex hormones regulate inflammatory pathways involved in asthma pathogenesis (2), and fluctuations in sex hormones are frequent for women during their menstrual cycle, pregnancy, and menopause (3–9). Approximately 30–40% of women with asthma report pre-menstrual or peri-menstrual worsening of asthma, with increased use of inhaled short acting beta agonist, decreased morning peak expiratory flow rates, and increased healthcare utilization for asthma (3,4). During pregnancy, asthma exacerbations are increased in women with more severe phenotypes of asthma, but no changes in asthma symptoms and asthma medication use were reported in interviews from pregnant women with mild to severe asthma (9). During menopause, there are variable findings in asthma symptoms and control depending on severity of asthma and/or obesity status of women (6,8,9). Combined, these clinical and epidemiological studies showed fluctuations in ovarian sex hormones, including estrogen and progesterone, altered asthma symptoms and exacerbations, highlighting the importance of defining how ovarian sex hormones increase airway inflammation.

Allergic asthma is the most common phenotype of asthma and is characterized by increased type 2 inflammation, airway hyperresponsiveness (AHR), and mucus production (10). Type 2 cells, including Th2 and group 2 innate lymphoid cells (ILC2), produce IL-4, IL-5, and IL-13, leading to increased eosinophil recruitment, IgE production, AHR, and mucus production (10). ILC2 do not require antigen presentation. Rather, ILC2 are stimulated by IL-33, TSLP, and IL-25, in an antigen independent manner, to robustly produce IL-5 and IL-13 as well as IL-4, IL-9, and amphiregulin (11–13). ILC2 are increased in the bronchoalveolar lavage (BAL) fluid and the circulation of patients with asthma compared to healthy controls (13–16). Further, women with moderate to severe asthma have increased circulating ILC2 compared to men with moderate to severe asthma (17), suggesting that sex hormones regulate ILC2 function.

IL-33 is the critical alarmin cytokine for increasing IL-5 and IL-13 production from ILC2 (18), and anti-IL33 therapeutics are currently in Phase II clinical trials for asthma (19). Multiple genome wide association studies showed that *IL33* and its receptor, ST2 (*IL1RL1*), were strongly associated with asthma (20–23), and that increased IL-33 in the BAL fluid inversely correlated to lung function in patients with asthma (19). Under homeostatic conditions, IL-33 is sequestered in the nucleus to prevent an unprovoked inflammatory response (24). In humans, IL-33 is predominantly expressed in the endothelial and epithelial cells of the lung, but in mice, IL-33 is expressed in the endothelial cells and alveolar type II pneumocytes of the lung (25,26). Upon exposure to allergens, IL-33 is quickly released, within 15-60 minutes, and stimulates ILC2 and other cells to secrete type 2 cytokines (17,27). IL-33 release occurs through necrosis of the cell or by active secretion that involves increased P2 purinergic receptor activation, extracellular accumulation of ATP, and activation of NADPH oxidase dual oxidase 1 (DUOX1) (28,29). Once released, IL-33 binds to a full-length ST2 to initiate downstream signaling and production of IL-5 and IL-13 from multiple type 2 cells, including ILC2 and CD4⁺ Th2 cells (30,31).

We recently showed that gonadectomized female mice (lacking estrogen and progesterone) had decreased IL-5⁺ and IL-13⁺ ILC2 after allergen challenge (17). Testosterone and androgen receptor (AR) signaling were also reported to attenuate ILC2 proliferation and cytokine production as well as ILC2-mediated airway inflammation (17,32–34). However, the role of estrogen signaling on IL-33 release, cytokine production from ILC2, and ILC2-mediated airway inflammation remained unclear. Estrogen canonical signaling involves estrogen binding to ER- α and ER- β , encoded by *Esr1* and *Esr2*, respectively. The estrogen-ER complex then binds to estrogen response element sequences or other DNA-binding proteins to regulate gene transcription (35). Estrogen signaling through the nuclear receptors, ER- α and ER- β , increased OVA or allergen-induced eosinophil infiltration, IL-5 and IL-13 cytokine expression, IgE production, and airway hyperresponsiveness (36). ER- α and ER- β are expressed to varying degrees in different cell types (35). Bone marrow ILC progenitor cells do not express ER- α and ER- β by qPCR (33), but ER- α and ER- β is

expressed in murine lung and uterine ILC2 (37). Based on these findings that gonadectomized female mice have decreased allergen-induced lung ILC2 and that ERs are expressed on lung ILC2, we hypothesized that ER- α signaling increased allergen-induced ILC2 cytokine production and airway inflammation. Surprisingly, our results showed that ER- α signaling increased IL-33 production and Alt Ext-induced airway inflammation, but ER- α signaling had no direct effect on ILC2 proliferation and cytokine expression.

Materials and Methods

Animals

Wild type (WT) C57BL/6J or BALB/c, *Esr1*^{-/-} (stock number 004744) and *Esr2*^{-/-} (stock number 004745) mice were purchased from Jackson Laboratory or bred in house. IL33^{fl/fl}eGFP were a kind gift from Paul Bryce, PhD (Northwestern University), and these mice are now commercially available at Jackson Laboratory (stock number 030619). Female mice aged 8-10 weeks old were used for experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee at Vanderbilt University and conducted according to the Care and Use of Laboratory Animals guidelines.

Alt Ext challenge

Mice were anesthetized with isoflurane and challenged intranasally with 7.5 µg in 75 µL of Alt Ext (Lot 338869; Greer) or vehicle (PBS) for 4 consecutive days as previously described (17). At various time points after the last challenge, mice were sacrificed for endpoint analysis.

ELISA

Following manufacturer's instructions, cytokine levels were measured by DuoSet and Quantikine ELISA kits (R&D Systems). Any value below the lower limit of detection was assigned half the value of the lowest detectable standard.

Bronchoalveolar lavage (BAL)

BAL was performed by instilling 800 µL of saline through a tracheostomy tube and gentle withdrawal of fluid through a 1 mL syringe. Cells from BAL were fixed to a slide and stained using a commercially available Three-Step Stain kit (Richard-Allen Scientific; ThermoFisher). Eosinophils, neutrophils, lymphocytes, or monocytes were identified and categorized using light microscopy as previously described (32).

Histopathology

To measure airway inflammation, lungs were perfused, inflated and instilled with 800 μ L of neutral-buffered formalin overnight at room temperature. Lungs were transferred to 70% ethanol and paraffin embedded. Tissue sections (5 μ m) were stained with hematoxylin and eosin (H&E) or Periodic Acid-Schiff (PAS) stain, and slides were quantified and scored according to following scale by a pathologist blinded to the experimental groups to score inflammation. The scoring system for H&E staining was a 0 to 3 scoring system: 0 indicated no inflammatory cells; 1, a few inflammatory cells; 2, increased accumulation of inflammatory cells; and 3, abundant accumulation of inflammatory cells. The scoring system for PAS- staining was: 0, no PAS-positive cells; 1, <5% PAS-positive cells; 2, 5% to 10% PAS-positive cells; 3, 10% to 25% PAS-positive cells; and 4, >25% PAS-positive cells.

Flow Cytometry and cell sorting

For single cell lungs suspensions, lung tissue was minced and digested with 1 mg/mL collagenase (Sigma-Aldrich) and 0.02 mg/mL DNase I (Sigma-Aldrich) in RPMI with 10% FBS for 30 min at 37°C as previously described (17,37). To inactivate the enzymatic digestion, 1 μ M EDTA was added to the sample, and the sample was filtered through a 70- μ m strainer to remove debris from single cell suspension. A red blood cell lysis was performed per manufacturer's instructions (Biolegend), and the total number of viable cells was determined by counting on a hemocytometer using Trypan blue exclusion dye. Two to five million cells were then used for flow cytometry assays. Cells were stained with a fixable viability dye (Ghost Dye UV450; Tonbo Biosciences), blocked using an anti-mouse FcR (CD16 and CD32) antibody, and surface stained with lineage cell detection antibody cocktail (Miltenyi), anti-CD3, anti-CD127, anti-CD90, anti-CD45, anti-ST2, anti-ICOS and anti-CD25. Cells were then stained with APC-Cy7 streptavidin, permeabilized with Foxp3/ Transcription Factor Staining Buffer kit (Tonbo Biosciences), and intracellularly stained with anti-IL-5 and anti-IL-13 (see Table 1). In select experiments using IL33eGFP mice, single cell lung suspensions were stained with anti-CD45, anti-EpCAM, anti-CD146, anti-CD11b, anti-CD11c, anti-Fc ϵ R1, and anti-c-kit antibodies (see Table 1). Epithelial cells were defined as CD45- IL-33eGFP+ EpCAM+ cells and endothelial cells were defined as CD45- IL-33eGFP+

CD146+ cells. All flow cytometry was conducted on a BD LSR II flow cytometer and analyzed using FlowJo software.

For cell sorting experiments, lungs were harvested, homogenized, and digested into a single cell suspension as described above. Lineage negative (Lin-) cells were enriched by negative selection using a lineage cell depletion kit (Miltenyi) as previously described (17,27).

Enriched Lin- cells were then blocked with an anti-mouse FcR antibody and surface stained with a biotin-labelled lineage cell detection cocktail (Miltenyi) and anti-mouse antibodies against CD3, CD45, CD25 and CD127 (see Table 1). Cells were stained with FITC streptavidin and DAPI viability dye. ILC2, defined as Lin-, CD45+, CD127+, CD25+ cells, were sorted on a BD FACS Aria III and plated at 2,000 cells/well in 96-well round bottom plates. ILC2 were stimulated with rhIL-2 (100U; NIH) and rmIL-33 (10 ng/mL; Peprotech) for 5 days. In selected experiments, ILC2 were also treated with 1 nM propyl-pyrazole-triol (PPT) or vehicle (DMSO).

Administration of tamoxifen or vehicle pellets

At 6-8 weeks of age, 60-day slow-release pellets from Innovative Research of America containing tamoxifen (5 mg) or vehicle were subcutaneously implanted into WT female mice. Three weeks after implantation, Alt Ext challenge protocol was conducted.

Bone Marrow Chimeras

Bone marrow was harvested from the femurs of age matched WT C57BL/6J female mice (CD90.1+) and *Esr1*^{-/-} female mice (CD90.2+) and mixed at a 1:1 ratio. Eight- to 10-week old heterozygous CD90.1+ CD90.2+ C57BL/6J female recipient mice were lethally irradiated (11 Gy) using a cesium irradiator. Four hours after the irradiation, one million of the mixed bone marrow cells (at a 1:1 ratio) were adoptively transferred into the lethally irradiated female CD90.1+ CD90.2+ C57BL/6J recipient mice via retro-orbital injection. After 6 weeks of BM engraftment, female recipient mice were challenged intranasally with 7.5 µg in 75 µL of Alt Ext or vehicle (PBS) for 4 consecutive days. Twenty-four hours after last intranasal

challenge, lungs were harvested to assess donor ILC2 frequencies by flow cytometry. ILC2 were defined as viable, Lineage negative (Lin-), CD45+, ST2+ ICOS+ cells. Residual cells remaining from the recipient mice (CD90.1+ CD90.2+) were excluded from analysis.

Culturing human bronchial epithelial (hBE33) and normal human bronchial epithelial (NHBE) cells

hBE33 cells are immortalized human primary airway epithelial cells that were engineered to stably express IL-33 (38). Cells were maintained in Bronchial Life media supplemented with defined growth factors, antibiotics, and retinoic acid (Lifeline Cell Technology). NHBE cells were obtained from Lonza, and NHBE cells were cultured in submerged conditions using PneumaCult™-Ex Plus Medium (StemCell Technologies) plus hydrocortisone, penicillin, and streptomycin on 0.03 mg/mL type 1 rat collagen-coated tissue cultured treated flasks. At 80% confluency, hBE33 or NHBE cells were pre-treated 10^{-15} to 10^{-12} M 17β -estradiol (E2), 1-100 nM PPT, 1-100 nM diarylpropionitrile (DPN), or vehicle (ethanol or DMSO) for 6 hours followed by 1 hour challenge with Alt Ext (30 μ g/mL), house dust mite (HDM; 30 μ g/mL Greer Lot 360923), or vehicle (PBS). Culture supernatants and cells were collected to measure IL-33 protein expression by ELISA or gene expression by qPCR.

Western blotting

Cells were lysed using RIPA buffer containing protease inhibitor (Roche), 2 μ M phenylmethylsulfonyl fluoride (PMSF) and 10 μ M dithiothreitol (DTT). Protein concentrations were determined by bicinchoninic acid (BCA) assay (Pierce) and 30 μ g of total protein for each sample was run on a 4-20% SDS-Page gel (Bio-Rad). After electrophoresis, samples were transferred to a nitrocellulose membrane (Whatman). Membranes were blocked and incubated with an anti-human DUOX-1 antibody (Abcam) at 1:500 dilution or anti-human actin antibody at 1:2000 dilution followed by an IRDye secondary antibodies (LI-COR) at 1:10000 dilution. Bands were visualized using Li-Cor Odyssey Infrared Imaging System and densitometry was determined using Image Studio Lite.

Lactate dehydrogenase (LDH) assay

Culture supernatants and lysed cells were collected for analysis of LDH using CytoTox96 non-radioactive cytotoxicity assay (Promega). Assay was performed and the percent of viable cells was determined by calculating $100\% - \%$ cytotoxic cells per manufacturer's directions.

ATP assay

Extracellular ATP was measured following manufacturer's instructions from BioAssay Systems (Hayward, CA) in culture supernatants from 0-30 minutes after Alt Ext challenge.

qPCR

Total RNA was extracted from NHBE cells using the RNeasy Mini Kit (Qiagen). cDNA was normalized to 50 ng of total RNA and created using SuperScript IV First-Strand Synthesis System (ThermoFisher). Gene expression was determined using Taqman primers or SYBR Green and primers purchased from Applied Biosystems as previously described (17). Relative expression was normalized to housekeeping gene *GAPDH*.

Statistical analysis

Data were normally distributed as determined by D'Agostino-Pearson omnibus K2 normality test. *p* values were calculated by using unpaired Student's *t*-test or one-way ANOVA with Tukey post hoc test. Values of $p < 0.05$ were considered significant.

Results

ER- α deficiency, but not ER- β deficiency, decreased Alt Ext-induced airway inflammation

ILC2 are important in allergic airway inflammation (10,13–16), and we hypothesized that estrogen signaling increased allergen-induced ILC2 cytokine production and airway inflammation. To test our hypothesis, WT female, *Esr1*^{-/-} female, and *Esr2*^{-/-} female mice were intranasally challenged with 7.5 μ g of *Alternaria alternata* extract (Alt Ext) for 4 consecutive days (Figure 1A). Sensitivity to fungal allergens, including *Alternaria alternata*, is a significant cause of allergic asthma and asthma morbidity is increased when *Alternaria alternata* spore counts are high (39–43). Lungs and BAL fluid were collected 24 hours following the last challenge. IL-5 and IL-13 protein expression were measured in whole lung homogenates, and Alt Ext challenged *Esr1*^{-/-} female mice had decreased lung IL-5 and IL-13 protein expression compared to WT female mice and *Esr2*^{-/-} female mice (Figure 1B-C). IL-4 protein expression was also measured, but Alt Ext challenge did not increase IL-4 protein expression compared to PBS in this short time course (data not shown). The number of inflammatory cells was also measured in BAL fluid. Alt Ext challenged *Esr1*^{-/-} female mice had decreased number of macrophages, eosinophils, and neutrophils in the BAL fluid compared to WT female and *Esr2*^{-/-} female mice (Figure 1D-F). As expected with this short Alt Ext challenge model (27), the number of lymphocytes in the BAL fluid was minimal with no significant difference between WT and *Esr1*^{-/-} mice (Figure 1G). No statistical differences in lung IL-5 and IL-13 protein expression or the numbers of BAL inflammatory cells were detected between Alt Ext challenged WT and *Esr2*^{-/-} female mice (Figure 1B-G), suggesting that lack of ER- α deficiency, but not ER- β deficiency, decreased Alt Ext-induced airway inflammation.

Next, we determined airway inflammation and mucus production by histopathology in WT and *Esr1*^{-/-} mice. Alt Ext challenged *Esr1*^{-/-} mice had decreased airway inflammation, as measured by H&E staining, compared to Alt Ext challenged WT mice (Figure 1H-I). Mucus production, as measured by PAS staining, was also decreased in Alt Ext challenged *Esr1*^{-/-}

compared to WT mice (Figure 1H, J). These data provide additional support that lack of ER- α signaling decreases airway inflammation and mucus production.

ER- α deficiency decreased Alt Ext induced lung ILC2

To continue to test our hypothesis, we next determined the number of Alt Ext-induced IL-5+IL-13+ ILC2 in the lungs of WT and *Esr1*^{-/-} female mice 24 hours after the final Alt Ext challenge. Single cell lung suspensions were restimulated with PMA, ionomycin, and golgi-stop and flow cytometry was conducted with gating strategies shown in Figure 2 and Supplemental Figure 1. Alt Ext increased the percentage and total numbers of lung ILC2 compared to PBS (vehicle alone), and Alt Ext challenged *Esr1*^{-/-} female mice had decreased percentage and numbers of ST2+ ICOS+ ILC2 compared to Alt Ext challenged WT female mice (Figure 2A-C). The percentage of IL-5+ IL-13+ ILC2 was not changed between WT and *Esr1*^{-/-} Alt Ext challenged mice, but the number of IL-5+ IL-13+ ILC2 was decreased in *Esr1*^{-/-} mice compared to WT mice (Figure 2D-E). Further, no differences in IL-5 and IL-13 mean fluorescent intensity was determined (Figure 2F-G), suggesting that ER- α signaling is important in increasing ILC2 numbers but not IL-5 and IL-13 cytokine production from ILC2. We also determined mean fluorescent intensity for ILC2 surface markers, ST2, ICOS, CD25, and CD127, and found no differences between Alt Ext challenged WT and *Esr1*^{-/-} mice (Supplemental Figure 1B-E). Figures 1-2 combined showed that ER- α deficiency decreased the number of Alt Ext-induced lung ILC2, leading to decreased IL-5 and IL-13 protein expression in the lung.

Estrogen increases Alt Ext-induced airway inflammation

Esr1^{-/-} female mice have increased serum testosterone concentrations compared to WT and *Esr2*^{-/-} female mice (34), and we confirmed these findings in our mice with serum testosterone levels of 1545.4 + 233.8 pg/mL in *Esr1*^{-/-} female mice compared to 56.7 + 25.4 pg/mL in WT female mice. Testosterone signaling through the AR attenuates ILC2-mediated airway inflammation (17,34,44), and this could be a reason for the decreased IL-5 and IL-13 production from ILC2. However, we had previously shown that Alt Ext challenged

gonadectomized female mice, lacking estrogen and progesterone with low serum testosterone levels, also had decreased IL-5⁺ ILC2 and airway eosinophils (17). These results in gonadectomized female mice suggest that estrogen and/or progesterone signaling increases ILC2 cytokine production and airway inflammation. To determine if estrogen signaling increased Alt Ext-induced ILC2-mediated airway inflammation, we administered slow release tamoxifen (5 mg), a selective ER modulator, or vehicle pellets to WT female mice. Twenty-one days after pellet administration, mice were challenged with Alt Ext and lungs and BAL fluid were harvested. IL-5 and IL-13 protein expression was significantly decreased in whole lung homogenates from Alt Ext challenged, tamoxifen treated mice compared vehicle treated mice (Figure 3A-B). Alt Ext-induced infiltration of eosinophils was also significant decreased in female mice administered tamoxifen pellets compared to female mice administered vehicle pellets (Figure 3C-F). These results show that estrogen signaling is important for increasing ILC2-mediated airway inflammation.

ER- α signaling had no direct effect on ILC2 production of IL-5 and IL-13

To determine if estrogen signaling had a direct effect on ILC2 cytokine production, we conducted a mixed BM chimera model where BM from WT female mice (CD90.1) and *Esr1*^{-/-} female mice (CD90.2) was mixed at a 1:1 ratio and adoptively transferred into lethally irradiated WT recipient female mice (CD90.1/CD90.2). The WT recipient female mice have testosterone levels similar to WT female mice (approximately 50 pg/mL). Therefore, the direct role of ER- α signaling on Alt Ext-induced ILC2 cytokine production can be determined. As shown in Figure 4A, six weeks after the BM transfer, female recipient mice were challenged with Alt Ext for 4 days and lungs were harvested 1 day after the final challenge to determine the number of WT (CD90.1) and *Esr1*^{-/-} (CD90.2) ILC2 in the lungs of the recipient mice (Figure 4B). The percentage of ILC2 and the percentage of IL-5⁺, IL-13⁺, and IL-5⁺ IL-13⁺ ILC2 from WT and *Esr1*^{-/-} female mice was similar (Figure 4C-F). Further, ST2 surface expression on ILC2 from WT and *Esr1*^{-/-} female mice was similar (Figure 4G). These data suggest that ER- α signaling had no direct effect on ILC2 potentiation or cytokine expression.

To confirm these results, we sorted ILC2 from WT and *Esr1*^{-/-} female mice and stimulated with IL-33 and IL-2 to induce IL-5 and IL-13 protein expression. No differences in IL-5 and IL-13 protein expression was determined in ILC2 from WT and *Esr1*^{-/-} female mice (Supplemental figure 2A-B). In separate experiments, ILC2 proliferation was determined by pre-loading the cells with CellTrace Violet dye prior to IL-33 and IL-2 stimulation, and ILC2 from WT and *Esr1*^{-/-} female mice had similar proliferation (Supplemental figure 2C-D). To further evaluate the direct effect of ER-α signaling on ILC2, we administered the ER-α agonist, PPT (1 nM), or vehicle (DMSO) at the time of stimulation with IL-33 and IL-2. Direct addition of PPT showed no significant differences in IL-5 and IL-13 protein expression in ILC2 culture supernatants from WT and *Esr1*^{-/-} mice (Supplemental figure 2E-F). The combined results from the bone marrow mixed chimera experiment and the *in vitro* ILC2 culture experiments showed that ER-α signaling does not directly increase ILC2 proliferation or production of IL-5 and IL-13. However, Alt Ext challenged *Esr1*^{-/-} mice and tamoxifen treated, Alt Ext challenged female mice had attenuated numbers of ILC2 and eosinophils in the airway after Alt Ext challenge (Figures 1-3), suggesting that ER-α signaling may regulate ILC2 cytokine expression and airway inflammation by increasing release of a ILC2 stimulatory cytokines, such as IL-33, TSLP, or IL-25.

ER-α deficiency decreased Alt Ext-induced IL-33 release

IL-33 is an alarmin that is critical for increasing IL-5 and IL-13 production from ILC2, where IL-25 and TSLP enhance IL-33-mediated cytokine production from ILC2 (18). Therefore, we challenged WT, *Esr1*^{-/-}, and *Esr2*^{-/-} female mice with Alt Ext and assessed IL-33 release in the BAL fluid after 1 hour, the peak of IL-33 release (38). Alt Ext challenged *Esr1*^{-/-} female mice had decreased IL-33 protein expression in BAL fluid compared to WT female mice, but no significant difference in IL-33 protein expression was detected between WT and *Esr2*^{-/-} female mice (Figure 5A). TSLP production in whole lung homogenates was also measured and there were no differences in Alt Ext-induced TSLP production between WT, *Esr1*^{-/-} and *Esr2*^{-/-} female mice (data not shown). ER-α deficiency decreasing Alt Ext-induced IL-33 release was surprising as previous findings showed that gonadectomized female mice,

lacking both estrogen and testosterone, had no change in Alt Ext-induced IL-33 release compared to hormonally intact, Alt Ext challenged WT female mice (17). Therefore, we wanted to further determine the cell source of Alt Ext-induced IL-33 and the role of ER signaling on IL-33 release.

IL-33 is sequestered and stored in the nucleus prior to being released (24), and we also wanted to determine if estrogen signaling increased IL-33 production after Alt Ext challenge. To do this, we challenged IL33eGFP, *Esr1*^{-/-}xIL33^{fl/fl}eGFP, and *Esr2*^{-/-}xIL33^{fl/fl}eGFP female mice with Alt Ext and determined the cell source of IL-33 using the gating strategy shown in Supplementary Figure 3. Total IL-33eGFP⁺ cells were increased in the lungs of Alt Ext challenged IL33eGFP female mice compared to *Esr1*^{-/-}xIL33^{fl/fl}eGFP (Figure 5B). In humans, lung IL-33 is predominantly expressed and secreted by bronchial epithelial and endothelial cells, but in mice, IL-33 is predominantly expressed by endothelial and alveolar type II pneumocytes (25,26). Therefore, we next determined if IL-33eGFP expression in epithelial cells (CD45- EpCAM⁺), endothelial cells (CD45- CD146⁺) or CD45⁺ leukocytes. IL-33eGFP was predominantly expressed in epithelial cells, and IL-33eGFP was decreased in epithelial cells of Alt Ext challenged *Esr1*^{-/-}xIL33^{fl/fl}eGFP compared to IL33^{fl/fl}eGFP female mice (Figure 5C-D). No differences in IL-33eGFP⁺ total cells, epithelial cells, or endothelial cells were determined in IL33^{fl/fl}eGFP female and *Esr2*^{-/-}xIL33^{fl/fl}eGFP female mice.

ER- α signaling increases IL-33 expression and release from human airway epithelial cells

Our data in mouse models of Alt Ext-induced airway inflammation suggested that estrogen signaling is important in regulating ILC2-mediated inflammation by increasing IL-33 production. However, it was unclear if 17 β -E2 directly induced IL-33 production. To determine this, we utilized a human airway epithelial cell line (hBE33 cells) that is engineered to express IL-33 (38). hBE33 cells were pre-treated with 17 β -E2 (10^{-15} to 10^{-12} M) or vehicle (ethanol) for 6 hours prior to Alt Ext challenge. One hour after Alt Ext challenge, IL-33 was measured by ELISA in cell culture supernatants. 17 β -E2 pre-treatment

significantly increased IL-33 release from hBE33 cells (Figure 6A). To determine if 17 β -E2 was signaling through ER- α and/or ER- β signaling, hBE33 cells were pre-treated with the ER- α agonist, PPT (1-100 nM), the ER- β agonist, DPN (1-100 nM), or vehicle (DMSO) for 6 hours prior to Alt Ext challenge. PPT, but not DPN, increased IL-33 release from Alt Ext challenged hBE33 cells (Figure 6B-C). Previous studies showed that IL-33 is released via a necrotic (passive) or active pathways (29,38). Therefore, we also determined if 17 β -E2 increased hBE33 cell necrosis by conducting an LDH assay and calculating the percentage of viable cells. As expected, hBE33 cells challenged with Alt Ext had increased cell cytotoxicity compared to PBS challenged cells, but pre-treatment with 17 β -E2 had no effect on hBE33 cell necrosis (Figure 6D). Next, we analyzed if 17 β -E2 altered IL-33 active release by measuring extracellular ATP and DUOX-1 expression, known intermediates in IL-33 active release (28). 17 β -E2 pre-treatment had no effect on extracellular ATP release (Figure 6E) but did increase DUOX-1 expression (Figure 6F), suggesting that 17 β -E2 increased IL-33 release in hBE33 cells by increasing DUOX-1. As mentioned earlier, Alt Ext challenged gonadectomized female did not have decreased IL-33 release compared to Alt Ext challenged, hormonally intact female mice (17). Gonadectomized female mice lack both estrogen and progesterone, and so we pre-treated hBE33 cells with progesterone (10⁻¹²M) prior to Alt Ext challenge and determined that progesterone significantly decreased Alt Ext-induced *IL33* expression (Supplemental Figure 4).

Primary, submerged NHBE cells were also pre-treated with 17 β -E2, PPT, or DPN for 6 hours prior to allergen challenge and *IL33* expression was determined by qPCR. Alt Ext significantly increased *IL33* expression compared to PBS challenge, and 17 β -E2 or PPT pre-treatment increased Alt Ext-induced *IL33* expression in NHBE cells (Figure 7A-B). DPN pre-treatment did not further increase Alt Ext-induced *IL33* expression (Figure 7C). Alt Ext is an important aeroallergen (39–43), but other aeroallergens, including HDM, also stimulate allergic airway inflammation. Therefore, NHBE cells were pre-treated with 17 β -E2, PPT, or DPN as before and challenged with 30 μ g/mL HDM for 1 hour. HDM challenged cells increased *IL33* expression compared to PBS challenged cells, and 17 β -E2 and PPT further

increased HDM-induced *IL33* expression (Figure 7D-E). DPN had no effect on HDM-induced *IL33* expression (Figure 7F). Progesterone pre-treatment also significantly decreased HDM-induced *IL33* expression in hBE33 cells (Supplemental Figure 4B). Combined, these data showed that ER- α signaling increased and progesterone decreased Alt Ext and HDM-induced *IL33* expression in NHBE cells.

Protease containing allergens, like Alt Ext and HDM, increase production of TSLP and IL-8 (*CXCL8*) in NHBE cells (45). Therefore, we also measured *CXCL8* and *TSLP* expression from NHBE cells after Alt Ext or HDM challenge. 17β -E2, PPT, and DPN significantly increased Alt Ext-induced *CXCL8* expression (Supplemental Figure 5A-C). Alt Ext did not increase *TSLP* expression in NHBE cells at the time points measured, and pre-treatment with 17β -E2, PPT, or DPN did not significantly increase *TSLP* (Supplemental Figure 5D-F). HDM challenge did not increase *CXCL8* expression, and pre-treatment with 17β -E2, PPT, or DPN did not significantly increase *CXCL8* expression (Supplemental Figure 5G-I). However, 17β -E2 signaling through ER- β further increased HDM-induced *TSLP* expression in NHBE cells (Supplemental Figure 5J-L). Combined, these data showed that 17β -E2 signaling through ER- α increased *IL33* expression, but that 17β -E2 signaling was also important in increasing allergen-induced *CXCL8* and *TSLP* expression in primary human airway epithelial cells.

Discussion

After puberty, women have increased prevalence of asthma compared to men (1), suggesting sex hormones are important in asthma pathogenesis. Lung ILC2 initiate allergic airway inflammation in response to a protease containing aeroallergens (46), while ILC2 in uterine and adipose tissues maintain pregnancy and homeostasis, respectively (37,47). Previous studies reported a female predominance in lung ILC2-mediated airway inflammation, and that AR signaling attenuated ILC2 cytokine production and airway inflammation (17,32,33,48). However, the mechanisms by which estrogen signaling increased ILC2-mediated airway inflammation remained unclear.

Esr1 and *Esr2* mRNA are expressed at low levels in mouse lung ILC2 (37), and in this study, we confirmed that *Esr1*^{-/-} mice had decreased airway inflammation (34). Kadel et al showed increased androgens in *Esr1*^{-/-} mice were responsible for decreased ILC2 cytokine production and airway inflammation (34). We expanded upon these findings showing that inhibition of estrogen signaling (with tamoxifen) decreased ILC2-mediated airway inflammation. Further, we showed that ER- α signaling did not have a direct effect on ILC2, rather 17 β -E2 signaling through ER- α increased IL-33 leading to increased ILC2-mediated airway inflammation.

Our findings using the 4 day Alt Ext model are discordant with previous findings showing that ER- α deficiency did not decrease OVA-induced type 2 cytokine production or eosinophil infiltration into BAL fluid but did reduce AHR (49). OVA sensitization and challenge does not induce robust ILC2 accumulation in the lung like protease-containing aeroallergens (including Alt Ext) (50), and the timeframe for OVA sensitization and challenge is much longer than our model. Therefore, the differences in allergens and timeframes are likely responsible for the discordant findings.

Tamoxifen administration decreased IL-5 and IL-13 protein expression in the lung as well as decreased BAL eosinophils. Our findings align with previous data in atopic dermatitis showing

that tamoxifen decreased OVA-induced ear swelling, infiltration of CD4⁺ T cells, CD8⁺ T cells, mast cells, and CD11c⁺ cell into the dermis and epidermis, and IgE production in a mouse model of dermatitis (51). An additional study showed that tamoxifen administration to granulocytes increased apoptosis (52), suggesting that tamoxifen may also reduce Alt Ext-mediated airway inflammation by inducing apoptosis of eosinophils. In this study, we did not determine apoptotic state of eosinophils, or neutrophils, in BAL fluid after tamoxifen administration, so this could be a potential mechanism for the observed decreases in airway eosinophilia.

ER- α signaling did not directly enhance ILC2 proliferation or cytokine production, expanding upon our previous findings showing 17 β -estradiol administration to gonadectomized female or male mice did not increase lung ILC numbers or IL-5 and IL-13 protein expression (17). In this study, we determined that ER- α signaling increased IL-33 release from hBE33 and *IL33* expression from NHBE airway epithelial cells; however, progesterone decreased *IL33* expression in NHBE cells. Lack of estrogen and progesterone signaling in our Alt Ext-challenged gonadectomized female mice is likely why no change was observed in Alt Ext-induced IL-33 and TSLP production compared to hormonally intact, sham-operated female mice (17). Additional studies have also shown administration of estrogen increased Muc5AC and Muc5B mucin protein expression and mucous production while progesterone decreased cilia beat and mucociliary clearance (53–55). Therefore, estrogen and progesterone have distinct impacts on allergen induced airway inflammation, mucus production, and AHR.

Recently, it was reported that the ER- α agonist (PPT) or ER- β agonist (DPN) increased *IL33* mRNA expression in BEAS-2B cells, a human bronchial epithelial cell line (56). Our results extend these findings and show that 17 β -E2 and ER- α agonist pre-treatment increased Alt Ext-induced IL-33 release and expression from a human airway epithelial cell line as well as from primary NHBE cells. These results were surprising since airway epithelial cells predominantly express ER- β , with lower expression levels of ER- α (54). However, the pre-treatment with the ER- β agonist, DPN, had no effect on Alt Ext-induced IL-33 release and

IL33 expression, but did increase Alt Ext-induced *CXCL8* expression and HDM-induced *TSLP* expression in NHBE cells, suggesting differential ER signaling pathways for allergen-induced cytokine production in airway epithelial cells.

Under normal conditions, IL-33 is sequestered in the nucleus to prevent an unprovoked inflammatory response (24). After an insult, including exposure to extract of the fungal aeroallergen *Alternaria alternata*, IL-33 is released rapidly primarily through necrosis (17,27). While we did observe increased IL-33 release in hBE33 cells pre-treated with 17 β -E2 or PPT, there was no increase in cell cytotoxicity. However, *DUOX1* was increased in hBE33 cells pre-treated with 17 β -E2, and *DUOX1* is important in upregulated IL-33 and *CXCL8* expression (28,57). ER- α signaling enhances *DUOX1* in airway epithelial cells, providing a potential mechanism for ER- α signaling increasing *IL33* and *CXCL8* expression in NHBE.

Perimenstrual worsening of asthma for women suggests that fluctuations in ovarian hormones may be important in regulating airway inflammatory responses (58). IL-33 is a critical cytokine in both the innate and adaptive allergic responses. BAL levels of IL-33 inversely correlated to lung function in patients with asthma (59–61), and anti-IL33 and anti-IL-33R therapeutics are currently in clinical trials for asthma and other allergic diseases. Our data imply that estrogen signaling through ER- α is important in IL-33 production and release from airway epithelial cells and highlight the important of ER- α signaling on allergic airway inflammation. Furthermore, our data suggest that ER- α signaling may prevent IL-33 degradation by proteases once secreted or increase active secretion of IL-33, leading to increased IL-33 in culture supernatants. Our data implies a novel function of ER- α signaling effect on IL-33 secretion and degradation and allergic airway inflammation. These data may provide potential mechanism to explain the sex disparity in asthma after puberty. Understanding these mechanisms is needed to personalize asthma therapeutics at various life stages for women and men with asthma.

Word Count: 5289

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Tables:

Table 1: Anti-Mouse Flow antibodies

Antibody	Host species	Clone	Fluorophore	Manufacturer
Ghost Dye™ UV 450				Tonbo Biosciences
Rat anti-mouse CD16/32	Rat	2.4G2	purified	BD Biosciences
Lineage Cell Detection Cocktail	Rat		biotin	Miltenyi
anti-mouse CD11b	Rat	M1/70	PE-Cy7	Biolegend
anti-mouse CD11c	Armenian hamster	N418	PE-Cy5	BD Biosciences
anti-mouse CD127	Rat	A7R34	PE-Cy5	eBioscience
anti-mouse CD146	Rat	ME-9F1	APC	eBioscience
anti-mouse CD25	Rat	PC61.5	Alexa Fluor 488	eBioscience
anti-mouse CD3	Rat	17A2	biotin	eBioscience
anti-mouse CD45	Rat	30-F11	Alexa Fluor 700	eBioscience
anti-mouse CD90.1	Rat	OX-7	BV510	BD Biosciences
anti-mouse CD90.2	Rat	53-2.1	BV786	BD Biosciences
anti-mouse cKit	Rat	ACK2	Alexa Fluor 700	eBioscience
anti-mouse EpCAM	Rat	G8.8	eFluor 450	eBioscience
anti-mouse FcεR1	Armenian hamster	MAR-1	PE	eBioscience
anti-mouse ICOS	Armenian hamster	C398.4A	PE-Cy7	eBioscience
anti-mouse/human IL-13	Rat	eBio13A	PE	eBioscience
anti-mouse/human IL-5	Rat	TRFK5	APC	BD Biosciences
anti-mouse ST2	Rat	RMST2-2	PerCP-eFluor 710	eBioscience
Lineage Cell Depletion				Miltenyi
Kit, mouse				

Figure legends:

Figure 1: *Esr1* deficiency decreased Alt Ext-induced IL-5 and IL-13 protein expression and infiltration of inflammatory cells into the lung

A. Experimental design of Alt Ext (7.5 µg) or vehicle intranasal challenge protocol. Endpoints were taken 24 hours following final Alt Ext challenge. **B-C.** IL-5 and IL-13 protein expression in lung homogenates. **D-G.** Inflammatory cells in BAL fluid. **H-J.** Representative sections at 10x magnification and quantification of H&E and PAS staining to detect airway inflammation in lung sections on 48 hours after final challenge. Black arrowheads denote PAS+ staining. Data are mean ± SEM, n=5-15 mice; * p<0.05, ANOVA with Tukey post-hoc analysis.

Figure 2: *Esr1* deficiency decreased Alt Ext-induced ILC2 in the lung.

Lungs were harvested 24 hours following last Alt Ext challenge and restimulated with PMA, ionomycin, and golgi-stop to detect IL-5 and IL-13+ ILC2. **A.** Representative dot plots of cytokine producing ILC2 in lungs of mice. Dot plots are pre-gated on Lin- CD45+ CD90+ CD127+ cells. **B.** Percentage of lung ILC2 from viable cells. **C.** Total number of lung ILC2. **D-E.** Percentage and total number of IL-5+ IL-13+ ILC2. **F-G.** Mean fluorescence intensity (MFI) of IL-5 and IL-13 in ILC2. Data are mean ± SEM, n=5-8 mice; * p<0.05, ANOVA with Tukey post-hoc analysis (B and C) and *t*-test (D-E).

Figure 3: Estrogen signaling increased Alt Ext-induced ILC2 cytokine production and inflammatory cell infiltration in the lung. WT female mice were administered subcutaneous Tamoxifen (5mg) or vehicle pellets for 21 days and were challenged with Alt Ext protocol. **A-B.** IL-5 and IL-13 protein expression in lung homogenates. **C-F.** Inflammatory cells in BAL fluid. Data are mean ± SEM, n=3-5 mice; * p<0.05, ANOVA with Tukey post-hoc analysis.

Figure 4: *Esr1* signaling had no intrinsic effect on Alt Ext-induced ILC2 cytokine production. **A.** Experimental design of mixed BM chimera experiments with a 1:1 BM

mixture from WT (CD90.1) and *Esr1*^{-/-} (CD90.2) female mice transferred into lethally irradiated WT (CD90.1/CD90.2) female recipient mice. After 6 weeks of reconstitution, recipient mice were challenged with Alt Ext protocol. Recipient mouse lungs were harvested 24 hours following last challenge and restimulated with PMA, ionomycin, and golgi-stop to detect IL-5 and IL-13⁺ ILC2. **B.** Representative dot plots showing gating strategy with ILC2 defined as viable, Lin⁻, CD45⁺, ICOS⁺, ST2⁺ cells. **C-E.** Percent of ILC2 and IL-5⁺, IL-13⁺, or IL-5⁺ IL-13⁺ ILC2 in lungs. **F.** ST2 MFI in lung ILC2. Data are mean ± SEM, n=9 mice; * p<0.05, ANOVA with Student *t*-test.

Figure 5: *Esr1* deficiency decreased IL-33 production in lung cells after Alt Ext challenge. **A.** IL-33 protein expression in BAL fluid 1 hour after last Alt Ext or vehicle challenge. **B-D.** Lungs were harvested 24 hours following last challenge and IL33eGFP was measured by flow cytometry. Number of IL-33eGFP⁺ cells in total lung cells, epithelial (CD45-IL-33eGFP+EpCAM⁺) and endothelial (CD45-IL-33eGFP+CD146⁺) cell subsets. Data are mean ± SEM, n=6-9 mice; * p<0.05, ANOVA with Tukey post-hoc analysis.

Figure 6: *Esr1* signaling increased IL-33 release from Alt Ext challenged hBE33 cells. A human bronchial epithelial cell line that expresses IL-33 (hBE33) was pre-treated for 6 hours with 17β-E2, ER agonists, or vehicle followed by an allergen challenge with Alt Ext or vehicle. **A.** IL-33 protein expression in hBE33 cells treated with 17β-E2 or vehicle (ethanol). **B-C.** IL-33 protein expression in hBE33 cells treated with PPT (ER-α agonist), DPN (ER-β agonist) or vehicle (DMSO). **D.** Analysis of cell viability measured by LDH assay. **E.** Extracellular ATP concentrations in cell supernatants at various time points after challenge. **F.** Relative intensity of DUOX1 protein levels 1 hour after challenge with β-actin serving as a loading control. Lanes shown are from lanes 2-4 and 6-8 of the same blot. Data are mean ± SEM, n=4 wells combined from 2 experiments; * p<0.05, ANOVA with Tukey post-hoc analysis.

Figure 7: *Esr1* signaling increased IL-33 expression from Alt Ext challenged NHBE cells. Normal human bronchial epithelial (NHBE) cells were pre-treated for 6 hours with 17 β -E2, ER agonists, or vehicle followed by an allergen challenge (Alt Ext, HDM or vehicle). **A.** Relative quantification of *IL33* expression in NHBE cells pre-treated with 17 β -E2 or vehicle (ethanol) followed by Alt Ext or vehicle challenge. **B-C.** Relative quantification of *IL33* expression in NHBE cells pre-treated with PPT, DPN or vehicle followed by Alt Ext or vehicle challenge. **D.** Relative quantification of *IL33* expression in NHBE cells treated with 17 β -E2 or vehicle (ethanol) followed by HDM or vehicle challenge. **E-F.** Relative quantification of *IL33* expression in NHBE cells treated with PPT, DPN or vehicle followed by HDM or vehicle challenge. All relative expressions were normalized to housekeeping gene, *GAPDH*. Data are mean $\pm$ SEM, n=4 wells combined from 2 independent experiments; * p<0.05, ANOVA with Tukey post-hoc analysis.

Figure 1

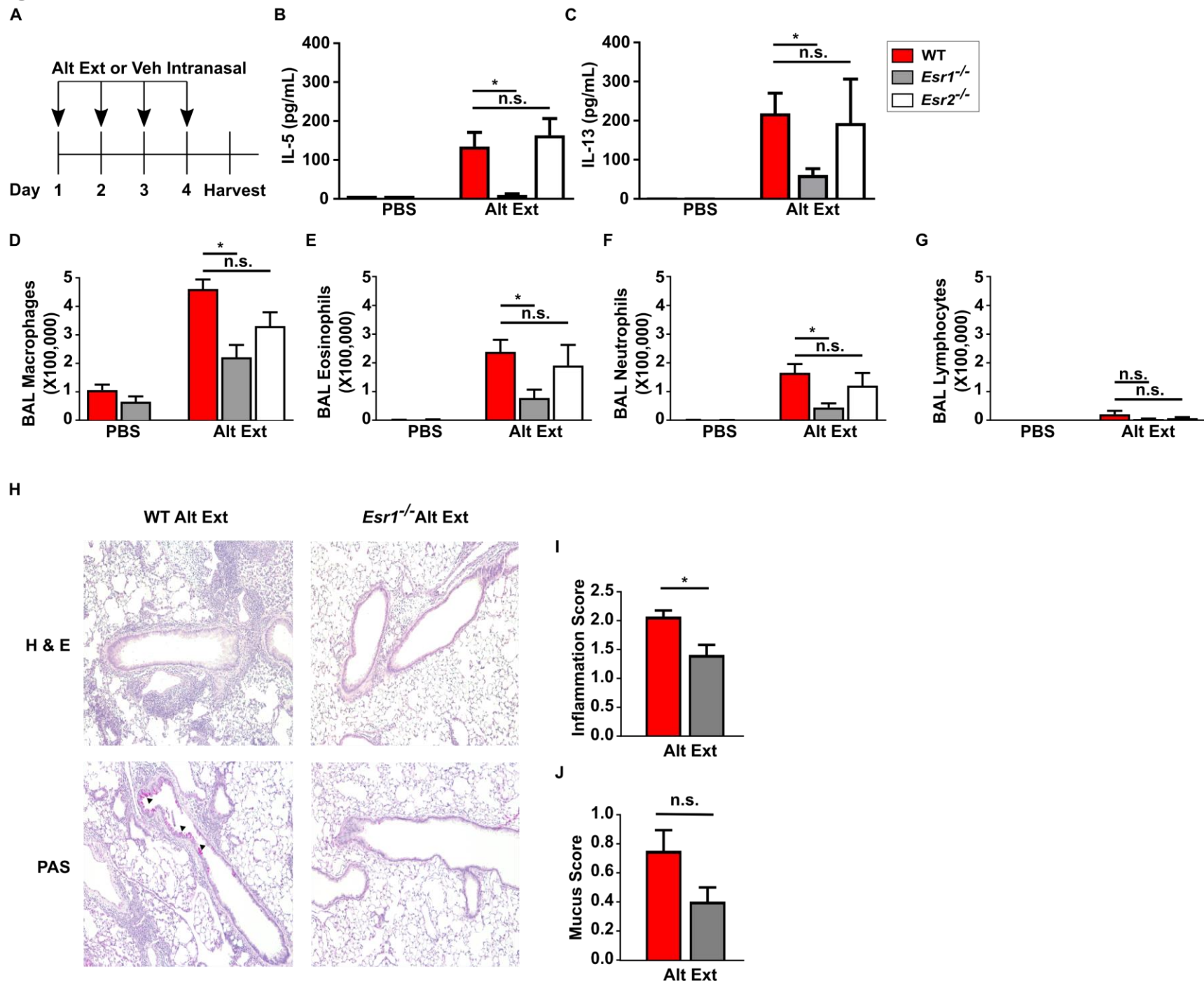
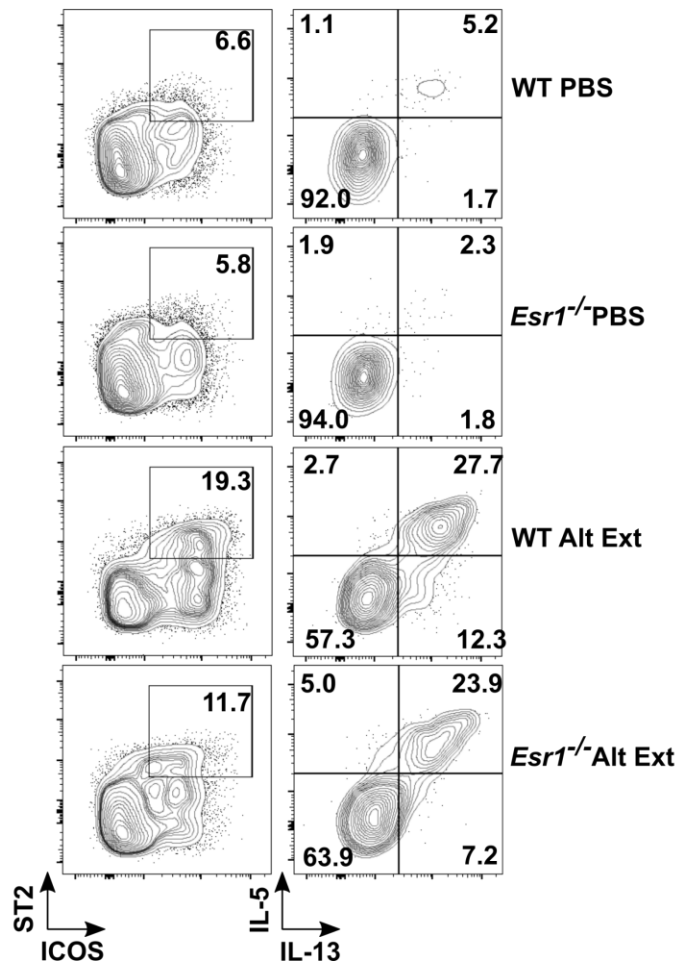
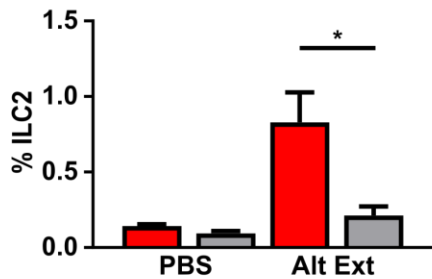


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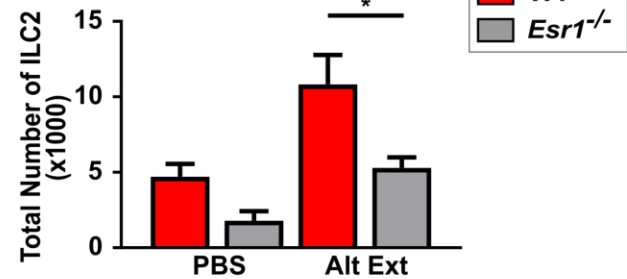
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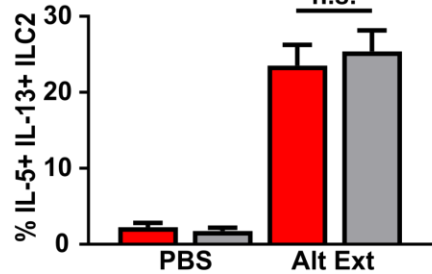
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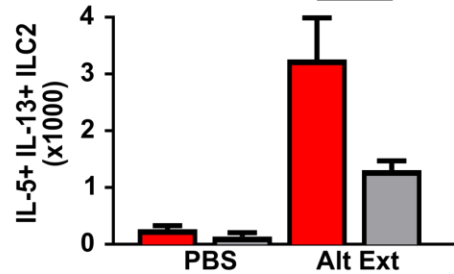
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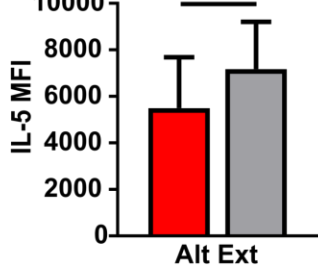
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E



F



G

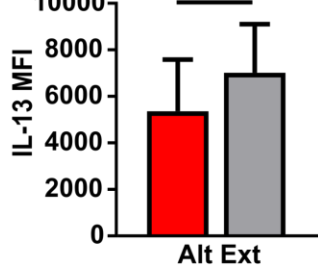
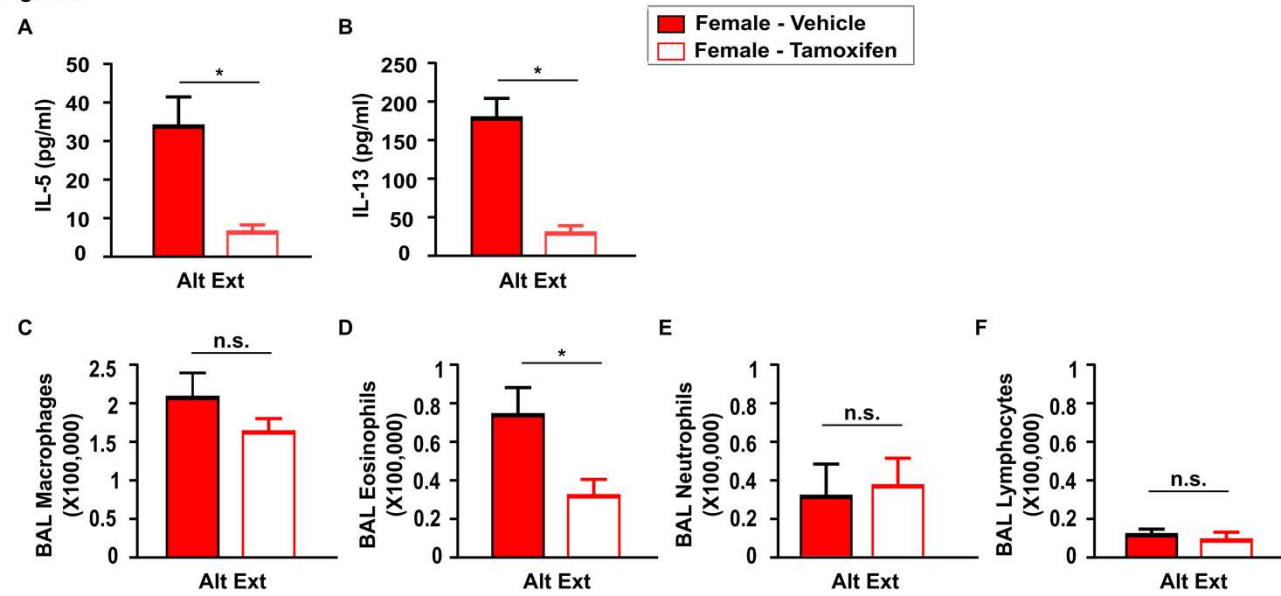


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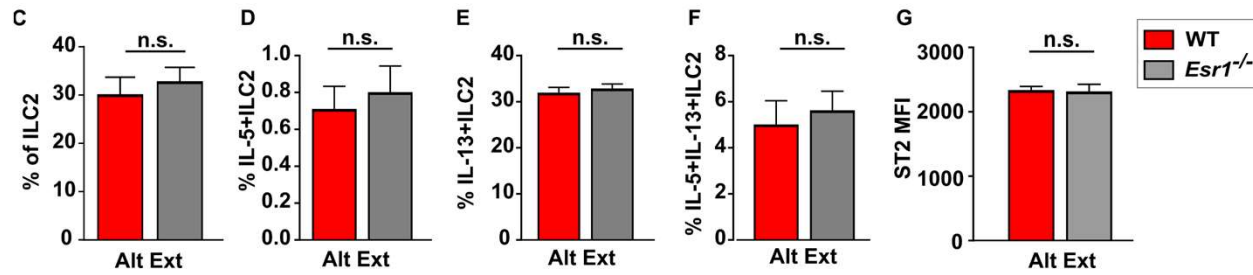
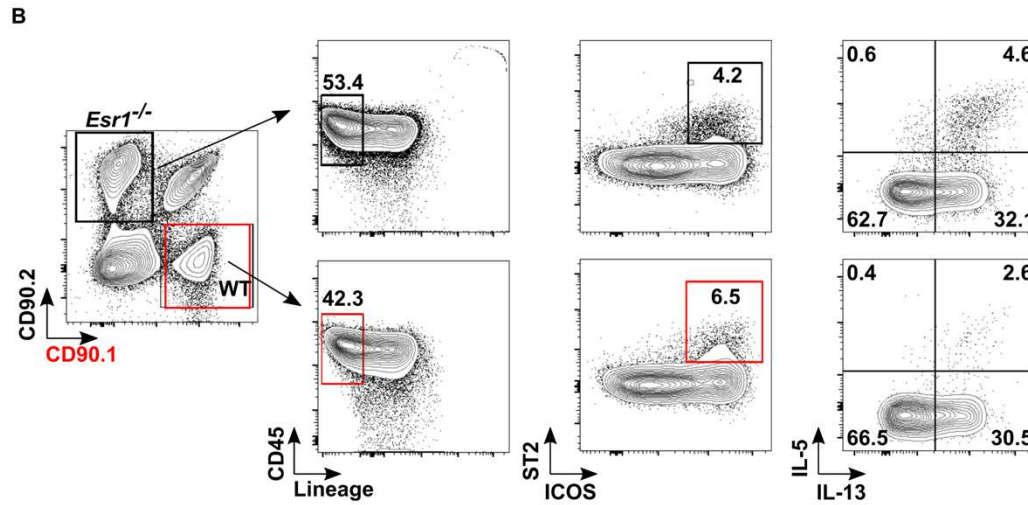
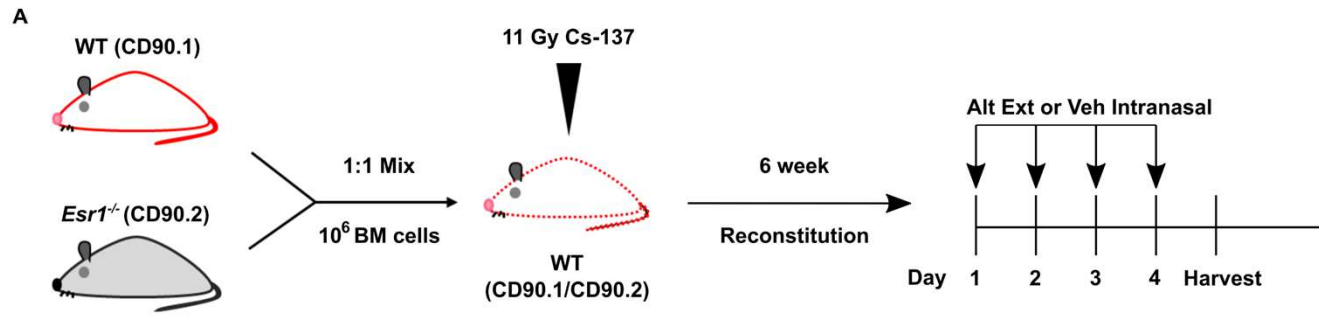
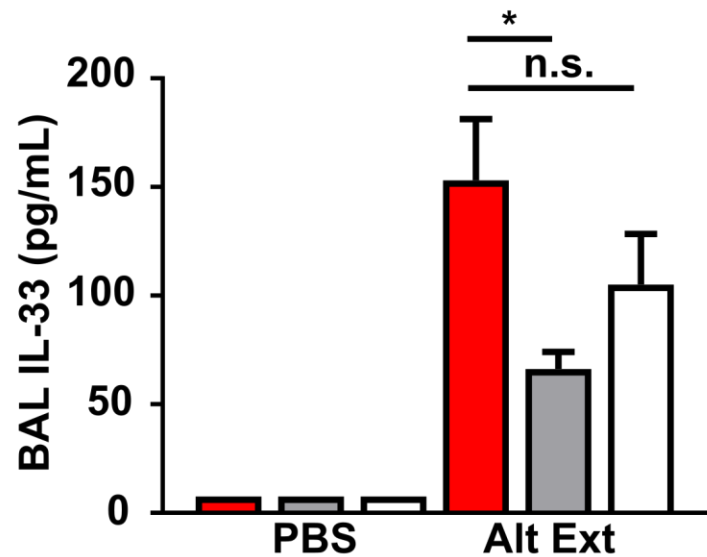
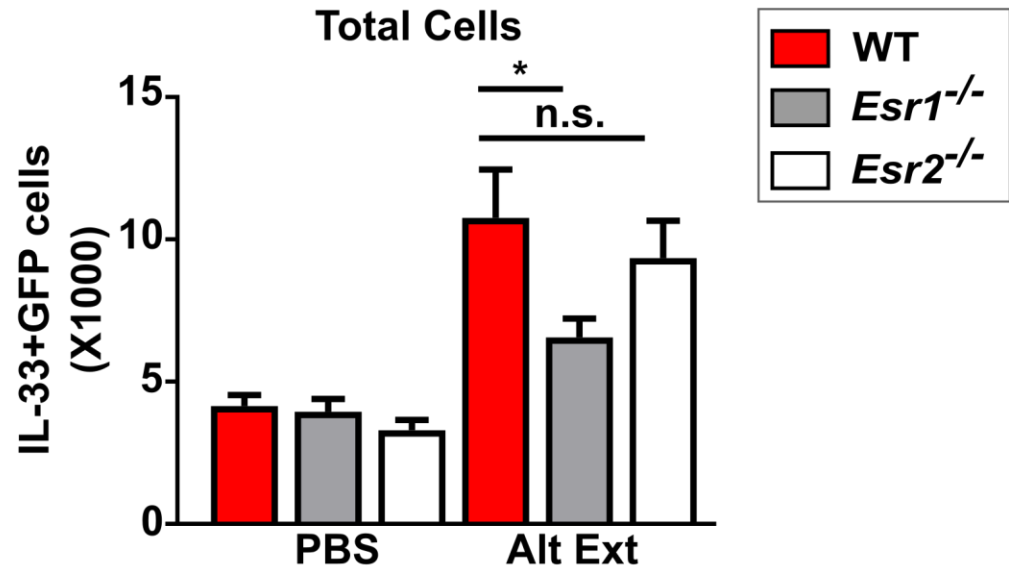


Figure 5

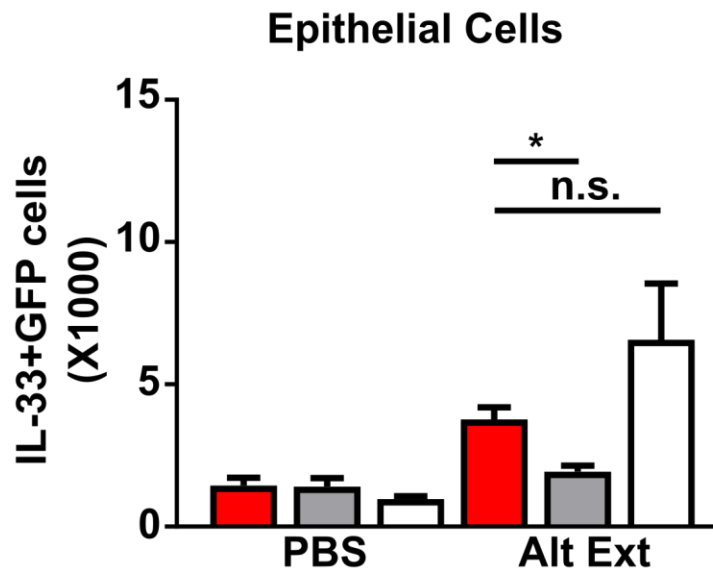
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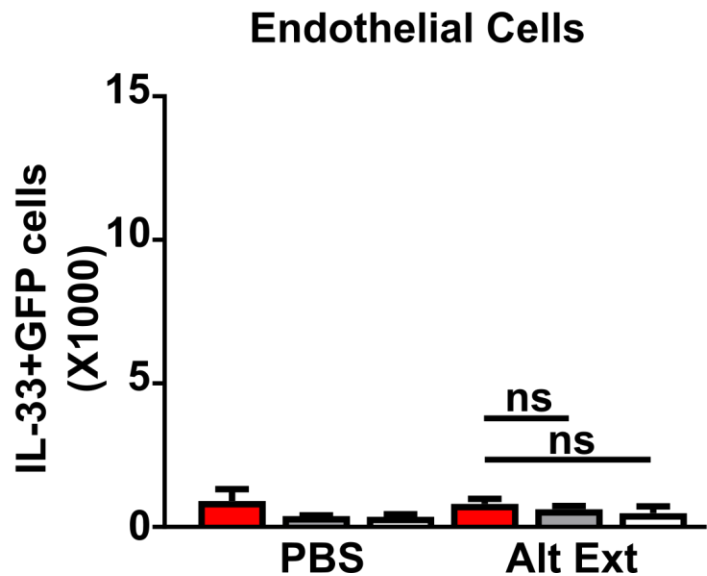


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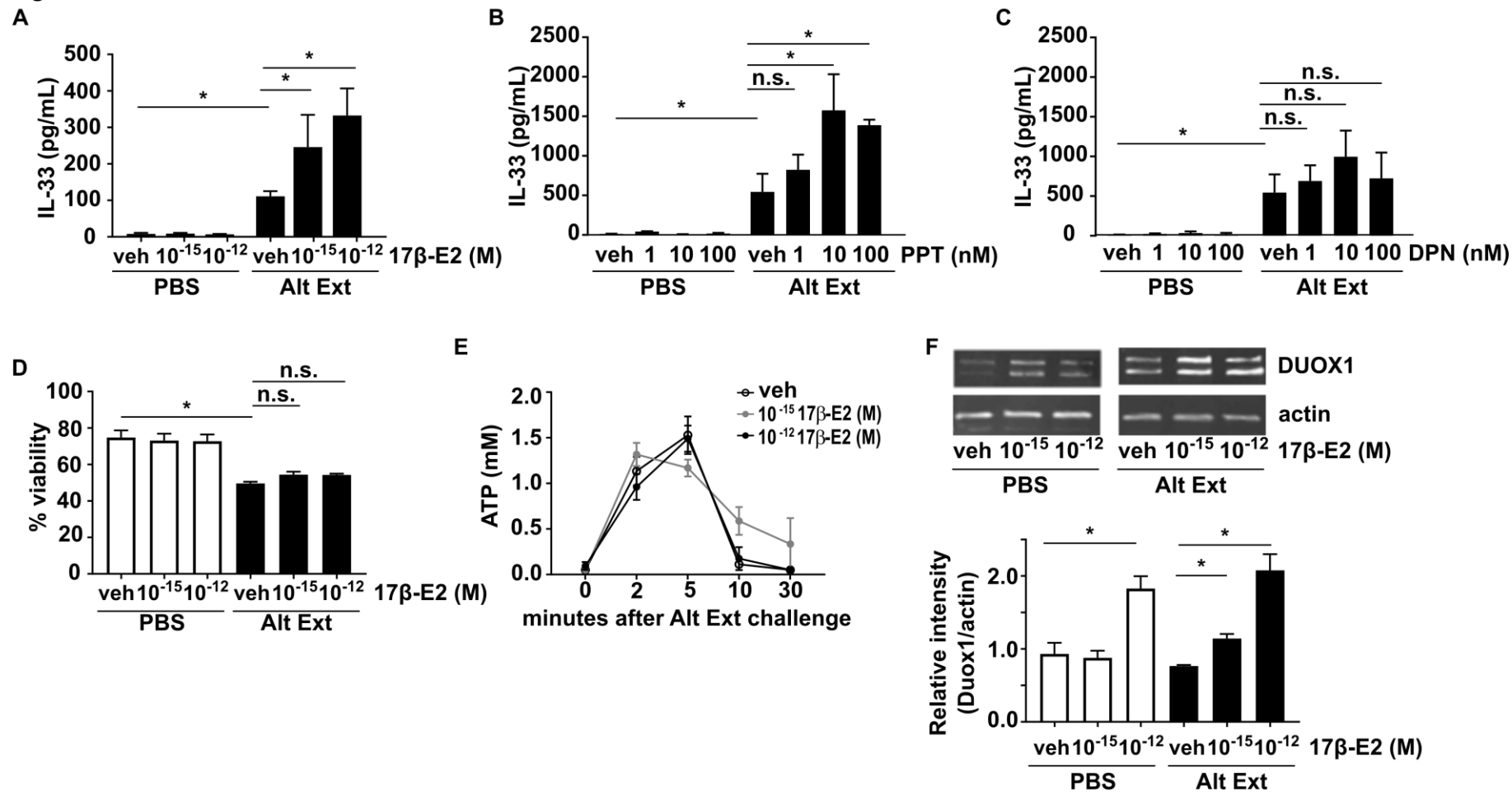
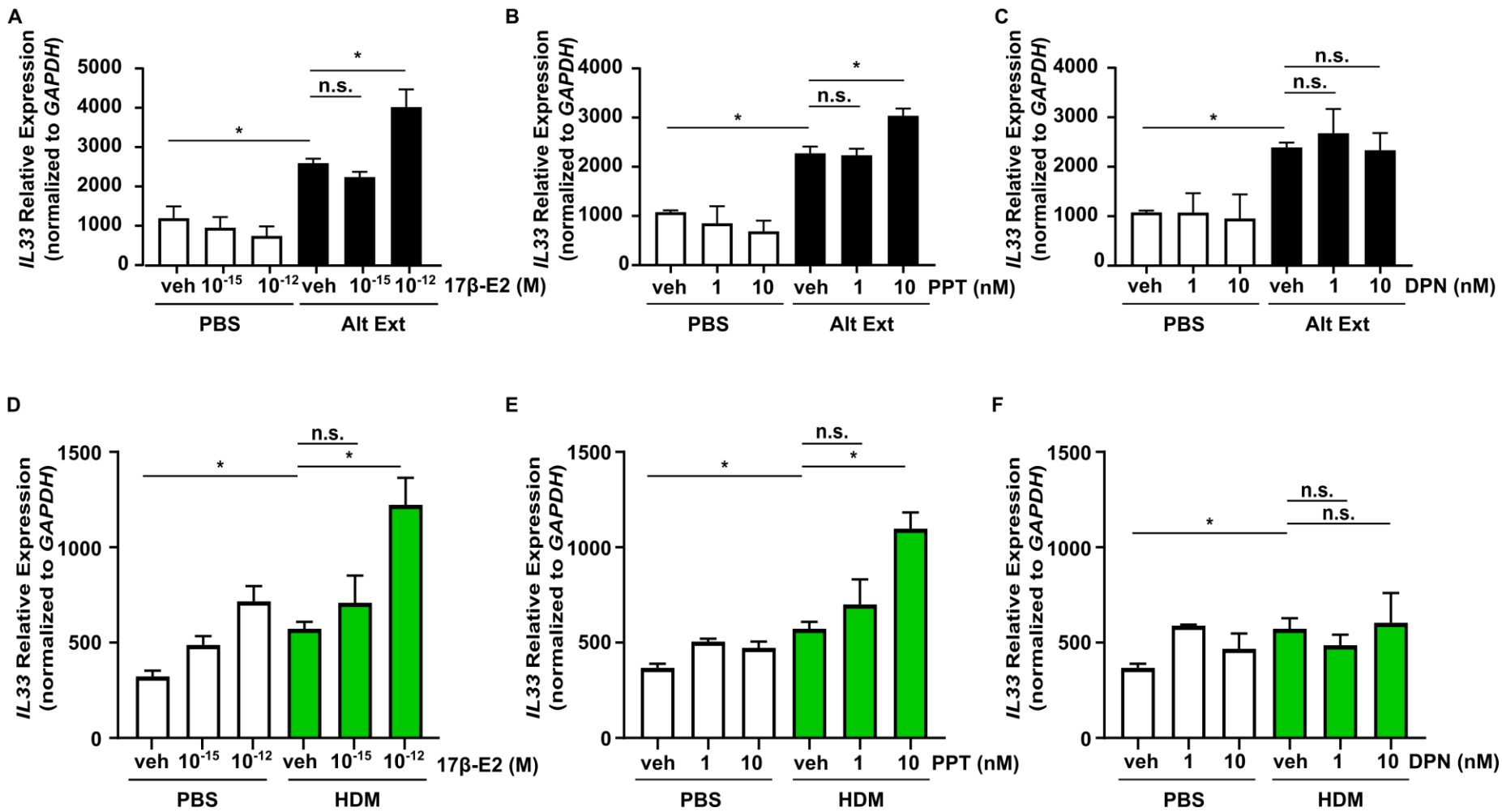
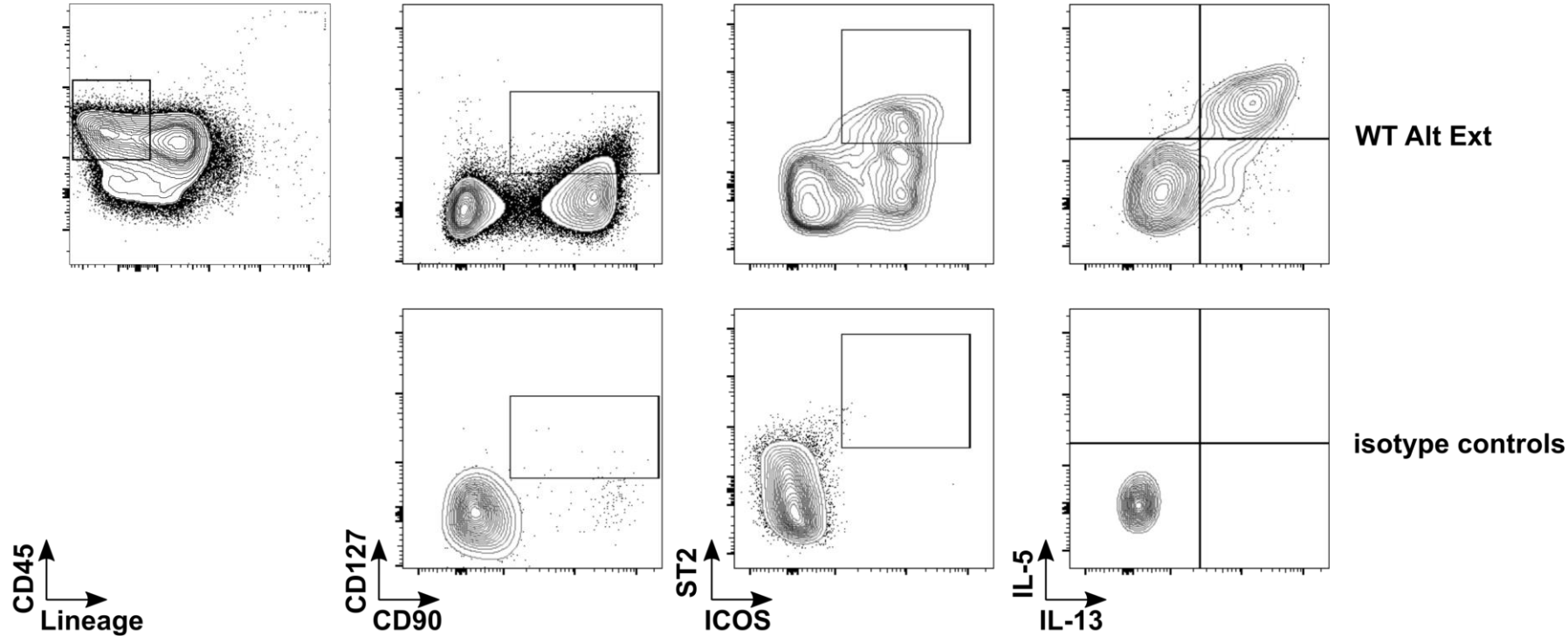


Figure 7

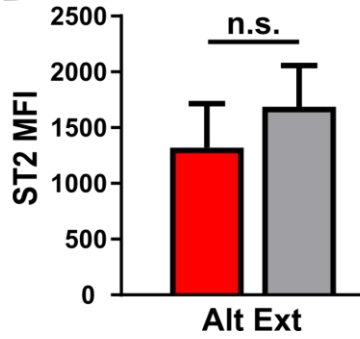


Supplemental Figure 1

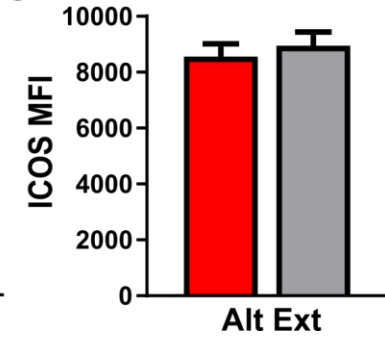
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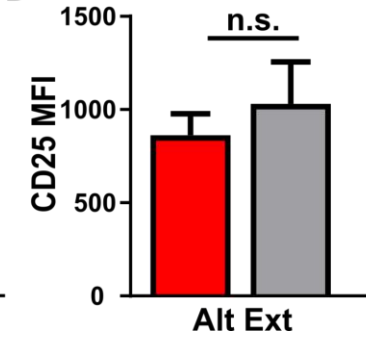
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