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IL-33–Responsive Group 2 Innate Lymphoid Cells Are Regulated by Female Sex Hormones in the Uterus

Kathleen Bartemes,^{*,†} Chien-Chang Chen,^{*,†} Koji Iijima,^{*} Li Drake,^{*} and Hirohito Kita^{*,†}

Group 2 innate lymphoid cells (ILC2s) reside in multiple organs in the body, where they play roles in immunity, tissue homeostasis, and metabolic regulation. However, little is known about the regulatory mechanisms of ILC2s in different organs. Here, we identified ILC2s in the mouse uterus and found that they express cell surface molecules, including the IL-33 receptor, ST2, that are roughly comparable to those expressed by lung ILC2s. Both in vivo and in vitro treatment with IL-33 induced type 2 cytokine production in uterine ILC2s, suggesting that they respond to IL-33 in a manner similar to ILC2s in other organs. Importantly, uterine ILC2s were nearly absent in ovariectomized mice and were increased in wild-type mice by estrogen administration, whereas lung ILC2s were unaffected by both ovariectomy and estrogen administration. Likewise, a marked reduction in uterine ILC2s was observed in mice deficient in estrogen receptor α or estrogen receptor β . Furthermore, uterine ILC2s highly expressed estrogen receptor α , and in vitro culture of isolated uterine ILC2s with 17 β -estradiol modified expression of a number of genes. Finally, an increased prevalence in neonatal mortality was observed in litters from dams lacking the IL-33 receptor, ST2. Taken together, our findings indicate that unlike lung ILC2s, uterine ILC2s are regulated by female sex hormones, which may specialize them for specific physiological functions. *The Journal of Immunology*, 2018, 200: 000–000.

Innate lymphoid cells (ILCs) are the most recently identified immune cell type and contribute to inflammation, immunity, and the maintenance of tissue integrity and homeostasis (1). Groups 2 ILCs (ILC2s) respond to innate immunological stimuli and signals from stressed or injured cells, particularly cytokines that are produced by mucosal epithelium, such as thymic stromal lymphopoietin, IL-25, and IL-33 (2–6). Originally, ILC2s were described in type 2 immune responses that protect against helminth infections and drive airway inflammation implicated in allergic diseases (7). ILC2s are also involved in airway epithelium repair after viral infections through the production of the epidermal growth factor, amphiregulin (8). Recently, ILC2s have been shown to regulate thermogenesis from beige fat (9, 10), and to prevent metabolic syndrome and insulin resistance

(11, 12). Thus, ILC2s likely carry out pleiotropic functions beyond innate immunity that are specific to the organs in which they reside.

The female reproductive tract (FRT) is unique because it must accept a semiallograft fetus while protecting it against pathogens. Although a shift from Th1- to Th2-type adaptive immunity in the placental environment during pregnancy is considered critical for the preservation of the fetus (13), many gaps remain in our understanding of FRT immune regulation. Innate immune cells, such as NK cells, have been implicated in the successful pregnancy by establishing an immunosuppressive environment and promoting tissue growth (14, 15). Furthermore, NK cells have been shown to induce regulatory T cells that may promote immune tolerance in the fetal-maternal interface (16). IL-33 is present in the endothelium of the FRT, as well as in smooth muscle cells of the placenta and in CD14⁺ macrophages in the chorioamniotic membranes (17). IL-33 has been implicated in ovarian tissue homeostasis and folliculogenesis (18, 19), and its production by endometrial stromal cells may promote embryo implantation in mice (20).

Given the potential role of IL-33 in both the FRT and the pleiotropic functions of ILC2s, it is possible that IL-33–responsive ILC2s are present in the uterus. The aim of this study was to examine this possibility, as well as how these cells are regulated in the organ. We found a small but distinct population of ILC2s in the mouse uterus, which are activated by IL-33 to produce type 2 cytokines. Importantly, uterine ILC2s highly expressed the estrogen receptor α (ER α), and the number of uterine ILC2s, but not lung ILC2s, was controlled by female sex hormone levels. Furthermore, in vitro culture of isolated uterine ILC2s with estrogen modified expression of a number of genes. These observations suggest that sex hormones likely play critical roles in the regulation of ILC2s in an organ-specific manner, and that sex differences need to be considered when studying these cell types.

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K.B. and H.K. designed the studies and experiments, interpreted the data, and wrote the manuscript. K.B., C.-C.C., K.I., and L.D. performed the experiments and interpreted the data.

The microarray data presented in this article have been submitted to the ArrayExpress database (<https://www.ebi.ac.uk/arrayexpress>) under accession number E-MTAB-5789.

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The online version of this article contains supplemental material.

Abbreviations used in this article: E2, 17 β -estradiol; ER α , estrogen receptor α ; ER β , estrogen receptor β ; FRT, female reproductive tract; ILC, innate lymphoid cell; ILC2, group 2 ILC; Lin[−], lineage-negative; OVX, ovariectomized; P4, progesterone; qRT-PCR, quantitative RT-PCR; WT, wild-type.

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Materials and Methods

Mice

BALB/cJ (BALB/c, stock number 000651), C57BL/6J (C57BL/6, stock number 000664), *Esr1*^{-/-} (α ER^{-/-}, C57BL/6 background, stock number 004744), *Esr2*^{-/-} (β ER^{-/-}, C57BL/6 background, stock number 004745), and ovariectomized (OVX) or sham-operated BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The IL-5-reporter C.129S4(B6)-*Il5*^{tm1Ktk} (*Il5*^{Venus}) mice (21) and *ST2*^{-/-} mice (22) were gifts from Dr. K. Takatsu, Toyama University, Japan and Dr. A. McKenzie, Medical Research Council, London, U.K., respectively. The *Il5*^{Venus} and *ST2*^{-/-} mice (both on BALB/cJ background) were maintained in the Mayo Clinic animal facility. Male and female mice aged 8–12 wk were used in all the experiments. All animal experiments and handling procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee and performed according to their guidelines.

Reagents

Fluorescently labeled Abs against CD3 (145-2C11), CD25 (PC61), CD44 (IM7), CD16/CD32 (2.4G2), CD14 (rmC5-3), CD45R/B220 (RA3-6B2), Thy-1.2 (53-2.1), ICOS (7E.17G9), Sca-1 (D7), CD69 (H1.2F3), MHC class II (AMS-32.1), and Siglec-F (E50-2440) were purchased from BD Biosciences (San Jose, CA). The anti-T1/ST2 (DJ8) Ab was from MD Biosciences (St. Paul, MN). The Abs to CD127/IL-7R α (A7R34) and Gata3 (TWAJ) were from eBioscience (San Diego, CA). Recombinant mouse IL-7 and IL-33 were purchased from eBioscience and recombinant mouse IL-25 was from R&D Systems (Minneapolis, MN). The placebo, 17 β -estradiol (E2), and combination progesterone (P4)/E2 pellets were ordered from Innovative Research of America (Sarasota, FL) and E2 powder for tissue culture was purchased from Sigma-Aldrich (St. Louis, MO).

Flow cytometry and cytokine production analyses

Lungs, uteri, and femurs were harvested from naive wild-type (WT) or gene-deficient mice, as well as from those treated with IL-33 or IL-25. To obtain single-cell suspensions, lungs or uteri were homogenized using the gentleMACS dissociator (Miltenyi Biotec, San Diego, CA) in buffer containing a mixture of collagenases, (Liberase TM; Roche Diagnostics, Indianapolis, IN) as described previously (6). Single-cell suspensions of bone marrow were also obtained from femurs. For flow cytometry, single-cell suspensions were stained with Abs for lineage markers (CD3, CD14, CD16/CD32, and B220), CD25, and CD44, followed by analysis (BD FACS-Canto) or sorting (BD FACS Aria). Lineage-negative (Lin⁻) CD25⁺CD44^{hi} lymphocytes were identified as ILC2s (6). Permeabilization for intranuclear staining for Gata3 was performed by using a Foxp3/transcription factor staining kit (eBioscience). CountBright absolute counting beads (Life Technologies, Grand Island, NY) were added to calculate the absolute cell numbers in each sample.

For cultures, uterine single cells or sorted ILC2s (i.e., Lin⁻CD25⁺CD44^{hi} cells) were resuspended in RPMI 1640 medium supplemented with 50 μ M β -mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FBS (RPMI-10). Cells were then cultured at a concentration of $0.5\text{--}4 \times 10^5$ cells/ml, either with untreated medium or with an IL-33 (10 ng/ml) and IL-7 (10 ng/ml) treated medium, for 7 d at 37°C and 5% CO₂. Supernatants were analyzed for IL-5 and IL-13 using DuoSet ELISA kits (R&D Systems). In some experiments, uterine single-cell suspensions were obtained from *Il5*^{Venus} mice, cultured with or without IL-33 and IL-7 for 48 h, and examined via flow cytometry.

In vivo administration of cytokines and hormones

To examine the effects of IL-25 or IL-33 on ILC2s in vivo, IL-33 (400 ng), IL-25 (400 ng), or vehicle (PBS) was administered i.p. three times (on days 1, 2, and 3) to naive *Il5*^{Venus} mice. Lungs and uteri were collected 24 h after the final administration, and ILC2s were identified via flow cytometry as described above. To determine the effect of exogenous E2 on ILC2s in the lungs and uteri, timed-release E2 pellets (0.25 mg released over 60 d) were inserted between the ear and shoulder of naive BALB/c female mice. Additionally, to investigate the effect of loss and reconstitution of female sex hormones, timed-release pellets (40 mg P4 and 0.1 mg E2, released over 60 d) were inserted in OVX mice. Placebo pellets containing only matrix components were used as a control. Lungs and uteri were collected 2–3 wk after pellet placement and ILC2 numbers were determined via flow cytometry or homogenized in 0.5 ml PBS for cytokine analyses. The homogenates were centrifuged at $10,000 \times g$ at 4°C for 15 min, and protein concentrations in the homogenates were quantified using a Bio-Rad DC

Protein Assay kit. Subsequently, IL-33 and IL-5 levels were quantified by DuoSet and Quantikine ELISA kits, respectively (R&D Systems).

Gene microarray analysis and quantitative RT-PCR

For gene microarray analysis, ILC2s were sorted from single-cell suspensions of pooled lungs and uteri from 5 and 20 mice, respectively. ILC2s were cultured for 16 h in RPMI 1640 medium with 10% charcoal/dextran treated calf serum (HyClone, GE Healthcare Life Sciences, Logan, UT) with or without 100 ng/ml E2. Total RNA was purified from lung and uterine ILC2s with TRIzol and PureLink RNA Mini Kit columns (Thermo Fisher Scientific, Waltham, MA). mRNA was amplified using the GeneChip 3' IVT Pico Kit (Thermo Fisher Scientific) and probed via the GeneChip Mouse Genome 430 2.0 Array (Thermo Fisher Scientific). Microarray data have been deposited in the ArrayExpress database at the European Bioinformatics Institute of the European Molecular Biology Laboratory (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5789 (direct link <https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-5789>).

For quantitative RT-PCR (qRT-PCR), total RNA was purified from lung and uterine ILC2s with TRIzol and PureLink RNA Mini Kit columns as described above, and cDNA was reverse transcribed with iScript (Bio-Rad Laboratories, Hercules, CA). mRNA transcripts for *Esr1*, *Esr2*, *Bik*, and *Il1f9* were quantified by qRT-PCR using TaqMan Gene Expression Arrays and TaqMan Gene Expression Master Mix (Thermo Fisher Scientific), according to the manufacturer's instructions. Data was normalized to 18S transcript levels.

Mating of *ST2*^{-/-} mice

WT BALB/c and *ST2*^{-/-} female mice were mated with BALB/c and C57BL/6 male mice. The litter sizes and conditions at birth and 24 h postpartum were recorded.

Statistical analysis

Data are presented as means $\pm$ SEMs for either the number of mice or experiments, as indicated. In box-and-whisker plots, the ends of each box designate the first and third quartiles, the center line represents the median, and the whisker shows the tenth and ninetieth percentiles. Statistical analyses were performed using the Mann-Whitney *U* test or Fisher exact test, as appropriate for each set of experimental conditions. The *p* values < 0.05 were considered significant.

Results

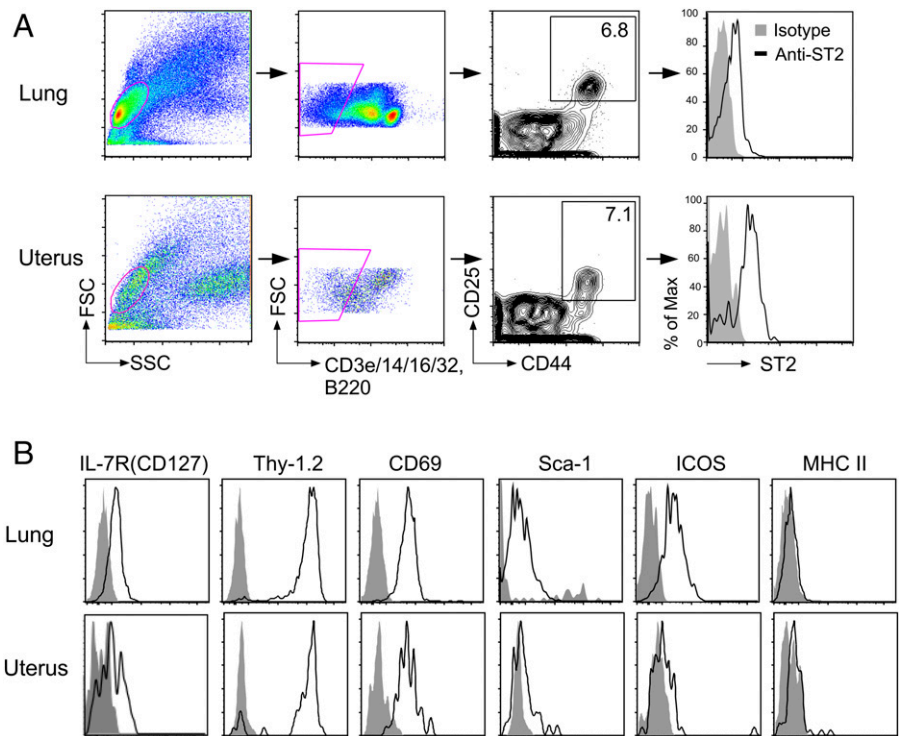
ILC2s are present in the uterus

Although the presence of ILC2s has been demonstrated in multiple organs (1), it is unclear whether ILC2s are localized to the uterus. To address this question, we obtained single-cell suspensions of lung and uterine tissues from naive BALB/c female mice, and stained them for ILC2s. We found that cells taken from the lung contained a distinct population of Lin⁻CD25⁺CD44^{hi} lymphocytes (Fig. 1A, top row), which has previously been identified as ILC2s (6). A similar population of lymphocytes was identified in the uterus (Fig. 1A, bottom row); ILC2s constituted $\sim$ 0.3% of the total uterine cells. Similar to lung ILC2s, uterine ILC2s expressed the IL-33 receptor ST2 and the canonical transcription factor Gata3 (Fig. 1A, Supplemental Fig. 1). A panel of other surface molecules was also expressed by both uterine and lung ILC2s, including CD127 (IL-7R), Thy-1.2, and CD69, but not MHC class II (MHC II) (Fig. 1B). In contrast, uterine ILC2s showed minimal expression of Sca-1 and ICOS, whereas both were highly expressed in lung ILC2s, suggesting that the tissue microenvironment may affect ILC2 phenotypes.

Uterine ILC2s respond to IL-33

A robust production of IL-5 and IL-13 in response to stimulation with IL-33 is a hallmark of ILC2s (6, 23). To examine whether uterine ILC2s share this capacity, we obtained uterine single-cell suspensions from naive IL-5 reporter *Il5*^{Venus} mice and stimulated the cells with IL-33 for 7 d in vitro. We found that the prevalence of ILC2s (i.e., Lin⁻CD25⁺CD44^{hi} cells) was higher in samples

FIGURE 1. ILC2s are present in the uterine tissue of naive mice. **(A)** Single-cell suspensions were prepared from the lungs (top panels) and uteri (lower panels) of naive BALB/c mice. ILC2s are identified as lineage (CD3, CD14, CD16/32, and B220)-negative (Lin^-) and $\text{CD25}^+\text{CD44}^{\text{hi}}$ lymphocytes, as described by Bartemes et al. (6). Right panel, The expression of the IL-33 receptor, ST2, is presented. Data are representative of >10 experiments showing similar results. **(B)** $\text{Lin}^- \text{CD25}^+\text{CD44}^{\text{hi}}$ cells identified in **(A)** were examined for the expression of selected cell surface molecules via flow cytometry. Gray filled histograms, isotype control; solid lines, indicated target.



treated with IL-33 than in those cultured in untreated medium (Fig. 2A). Furthermore, ~50% of the uterine ILC2s cultured with IL-33 tested positive for IL-5 Venus , whereas none of the ILC2s

cultured with untreated medium were positive. Additionally, all other uterine cell populations treated with IL-33 were negative for IL-5 (data not shown).

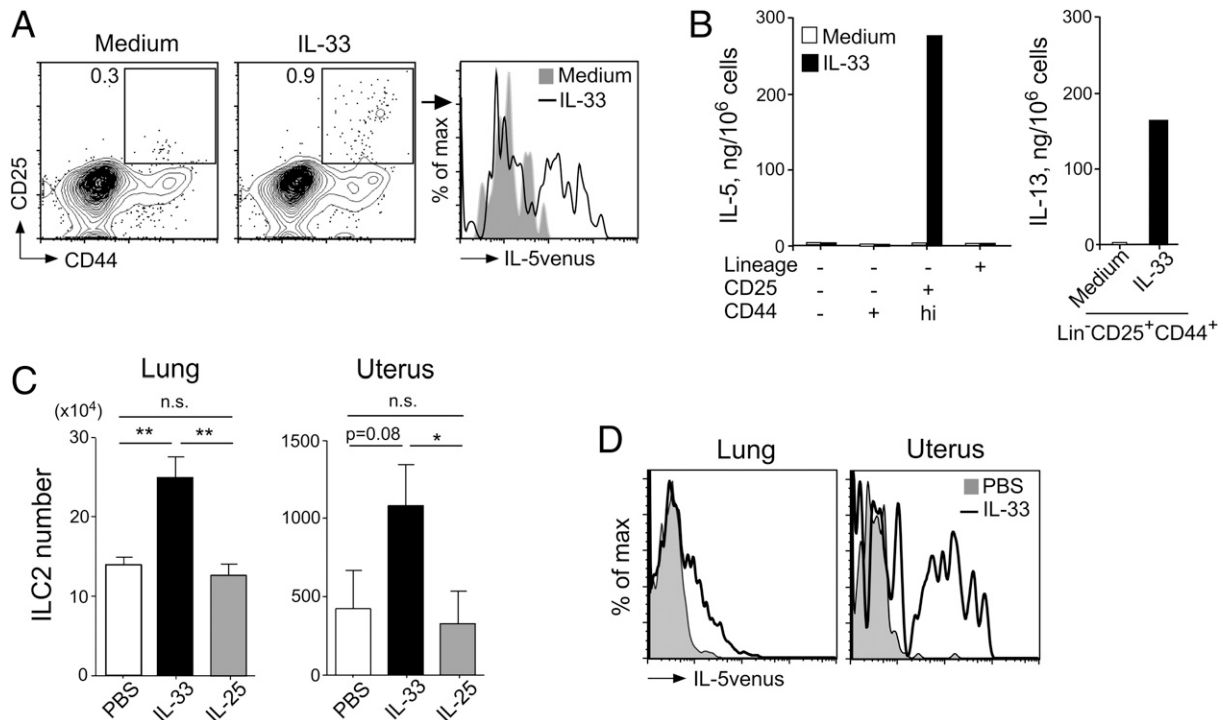


FIGURE 2. Uterine ILC2s are activated by IL-33 in vitro and in vivo. **(A)** Uterine single-cell suspensions from naive IL-5-reporter C.129S4(B6)- $\text{IL5}^{\text{tm1Krk}}$ ($\text{IL5}^{\text{Venus}}$) mice were cultured for 7 d with untreated medium or with IL-33 and IL-7 (each at 10 ng/ml). ILC2s were identified using the gating strategy outlined in Fig. 1A, and IL-5 Venus expression was examined by flow cytometry. **(B)** Three populations of Lin^- cells, including $\text{CD25}^- \text{CD44}^-$, $\text{CD25}^- \text{CD44}^+$, and $\text{CD25}^+ \text{CD44}^{\text{hi}}$, and lineage-positive cells were sorted from uterine, single-cell suspensions taken from naive BALB/c mice. Cells were cultured with medium alone or with IL-33 and IL-7 for 7 d. The levels of IL-5 (left panel) and IL-13 (right panel) in the supernatants were analyzed by ELISA. Data are representative of two experiments. **(C)** Naive $\text{IL5}^{\text{Venus}}$ mice were injected i.p. with PBS, IL-33, or IL-25 (400 ng per dose), once daily for 3 d. Twenty-four hours after the last injection, lungs and uteri were harvested and analyzed by flow cytometry. Data are presented as the mean $\pm$ SEM. $n = 5-6$ mice per group. * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney U test) between the groups indicated by horizontal lines. **(D)** Naive $\text{IL5}^{\text{Venus}}$ mice were treated as described in **(C)**, and IL-5 Venus expression was examined after gating on ILC2s. Gray filled histogram, PBS; solid line, IL-33. $n = 5-6$ mice per group.

To verify this observation, we isolated ILC2s from naive BALB/c uterine single-cell suspensions by cell sorting. We observed that treatment with IL-33 in vitro resulted in robust production of IL-5 and IL-13 (>100 ng/ 10^6 cells) by uterine ILC2s (Fig. 2B). Conversely, no other Lin[−] populations, including Lin[−]CD25[−]CD44[−] and Lin[−]CD25[−]CD44⁺, or lineage-positive cells, produced IL-5 in response to IL-33. The IL-5 levels produced by uterine ILC2s were comparable to, if not larger than, those produced by lung ILC2s (6).

We next examined whether uterine ILC2s respond to IL-33 in vivo. To this end, *IL5^{Venus}* mice were administered a 400-ng daily dose of IL-33 or IL-25 in PBS, or untreated PBS, for 3 d. IL-33 treatment significantly increased the number of lung ILC2s by $\sim 75\%$ (Fig. 2C, left panel, $p < 0.01$). Similarly, significantly more ILC2s were present in the uteri of IL-33-treated mice than in IL-25-treated mice ($p < 0.05$, Fig. 2C, right panel). Furthermore, IL-33 treatment resulted in expression of IL-5^{Venus} in ~ 25 and 50% of lung and uterine ILC2s, respectively (Fig. 2D). In contrast, IL-25 treatment did not affect the number of ILC2s (Fig. 2C) or IL-5^{Venus} expression (data not shown). Additionally, although IL-5 was below the level of detection in uterine tissue lysates from untreated mice, and was only minimally induced by IL-25

(3.9 ± 2.4 pg per 10 mg protein), IL-33 increased IL-5 levels to 535.3 ± 213.5 pg per 10 mg protein (mean $\pm$ SEM, $p < 0.05$, $n = 5$). Taken together, this series of in vitro and in vivo experiments shows that ILC2s in the uterus are responsive to IL-33 and produce large quantities of IL-5 and IL-13, similar to those in the lungs.

Estrogen increases ILC2s in the uterus, not in the lung

In our previous work (6), we observed sex differences in the prevalence of lung ILC2s. For example, lungs from naive female BALB/c mice contained an average of $8.5 \pm 0.6 \times 10^4$ ILC2s, whereas lungs from their male counterparts averaged $4.3 \pm 0.4 \times 10^4$ ILC2s (Fig. 3A, $p < 0.01$, $n = 16$). Male lungs were significantly larger (166 ± 5 mg) compared with female lungs (145 ± 3 mg, $p < 0.01$), suggesting that lung volume does not account for this difference. This observation, along with an emerging body of literature suggesting that IL-33 and ST2 play roles in the FRT (17–20), led us to hypothesize that female sex hormones, such as estrogen (E2) and P4, might affect ILC2 abundance in the lungs and uteri.

In support of this hypothesis, we found that loss of female sex hormones in OVX adult female BALB/c mice significantly de-

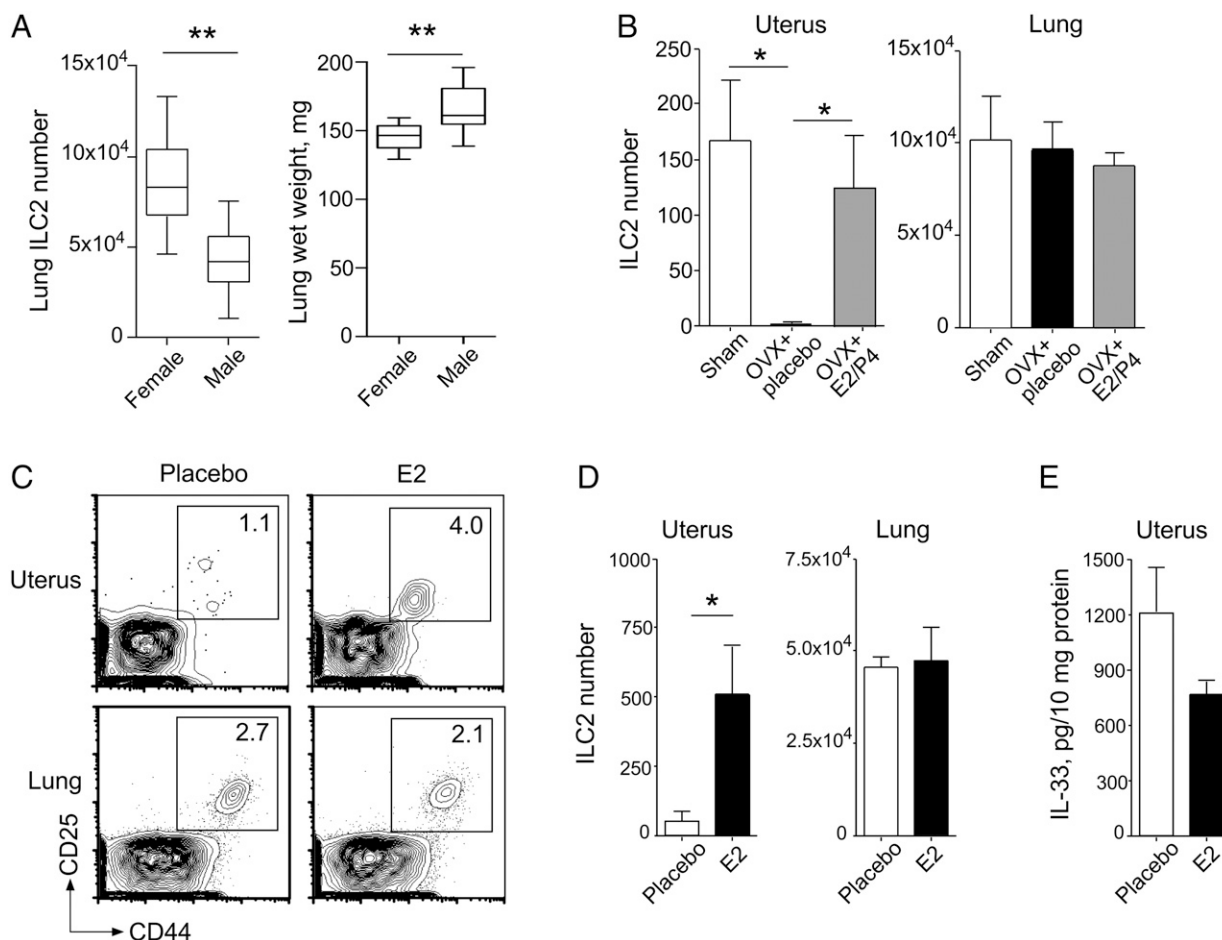


FIGURE 3. Female sex hormones regulate ILC2 numbers in the uterus. **(A)** Lungs were harvested from naive BALB/c female and male mice ($n = 16$ mice per group), weighed, and examined for ILC2s by flow cytometry, as described in Fig. 1A. The results are shown as box-and-whisker plots. $**p < 0.01$ (Mann–Whitney U test) between the groups indicated by horizontal lines. **(B)** Naive BALB/c mice were sham-operated or OVX. OVX mice were implanted with placebo pellets (OVX + placebo) or a combination of E2 and P4 pellets (OVX + E2/P4). ILC2s in the uteri and lungs were examined 3 wk after pellet implantation. Data are shown as the mean $\pm$ SEM ($n = 4$ mice per group). $*p < 0.05$, $**p < 0.01$ (Mann–Whitney U test) between the groups indicated by horizontal lines. **(C and D)** Naive BALB/c mice were implanted with placebo or estrogen (E2, 0.25 mg released over 60 d) pellets, and uterine and lung ILC2 numbers were determined 2 wk later. Representative FACS plots (C) and the summarized data (D) are shown ($n = 4$ mice per group). Data are shown as the mean $\pm$ SEM. $*p < 0.05$ (Mann–Whitney U test) between the groups indicated by horizontal lines. **(E)** The levels of IL-33 in uterine homogenates from (C) were analyzed by ELISA. Data are shown as the mean $\pm$ SEM ($n = 4$ mice per group).

creased uterine ILC2 numbers compared with female mice subjected to sham surgery (Fig. 3B). Reconstitution of E2 and P4 for 3 wk after OVX restored uterine ILC2 numbers. Interestingly, lung ILC2 numbers were not affected by OVX, consistent with recent observations by other investigators (24). Additionally, because OVX may affect hormones other than E2 and P4, we examined whether an increase in E2 alone was sufficient to affect ILC2s in otherwise normal mice. To this end, we placed timed-release E2 pellets s.c. in naive WT female mice. After 14 d of E2 exposure, the number of uterine ILC2s significantly increased by ~5-fold compared with mice exposed to placebo pellets (Fig. 3C, 3D). Lung ILC2 numbers were again not affected by E2 administration. Furthermore, no differences in uterine IL-33 levels were observed in mice treated with placebo or E2 (Fig. 3E). Taken together, these findings suggest that ILC2 numbers in the uterus are increased by female sex hormones, particularly E2, whereas those in the lungs remain unchanged.

Estrogen regulates ILC2s in the uterus

E2 signaling is mediated predominantly by ER α and estrogen receptor β (ER β) (25). Following E2 ligation and subsequent nuclear translocation, they function primarily as transcription factors and regulate expression of a number of genes (26). These receptors are differentially distributed throughout the body (26) and their downstream effects are likely determined by the tissue they are localized to and the binding of cofactors (26). We characterized the expression of estrogen receptors by ILC2s in uterine and lung tissues of WT female mice. Following FACS sorting and qRT-PCR, we found that *Esr1*, which encodes for ER α , was more

highly expressed by uterine ILC2s than lung ILC2s (Fig. 4A). In contrast, no apparent difference in the expression of *Esr2*, which encodes ER β , was observed between uterine and lung ILC2 populations. Moreover, ER α -deficient mice (α ER $^{-/-}$) showed hypoplastic uteri, whereas ER β -deficient mice (β ER $^{-/-}$) retained fully developed uteri (Fig. 4B), consistent with previous findings (27). No difference in lung size was observed between α ER $^{-/-}$ and β ER $^{-/-}$ mice (Fig. 4B).

By FACS analyses, we found that ILC2 numbers were significantly decreased in the uteri of α ER $^{-/-}$ and β ER $^{-/-}$ mice compared with WT C57BL/6 age-matched controls (Fig. 4C, 4D, $p < 0.01$). Additionally, the number of ILC2s in the lungs was decreased in α ER $^{-/-}$ mice compared with WT mice. In contrast, no significant change in the number of ILC2s was observed in the lungs and bone marrow of β ER $^{-/-}$ mice, suggesting that ER β signaling is likely critical for uterine ILC2s, but not for ILC2s in other organs. Similarly, ER α signaling may affect uterine ILC2s more strongly than those in the lungs.

To examine whether estrogen directly affects uterine ILC2s, we isolated ILC2s from both the lungs and uteri of naive BALB/c mice, cultured them for 16 h with E2, and analyzed their gene expression by microarray. At the baseline (i.e., without E2), over 14,000 genes were detectable in both uterine and lung ILC2s. Of those genes, 11,032 were common to ILC2s from both compartments, whereas 3407 or 3791 genes were expressed only in uterine or lung ILC2s, respectively. As expected, the genes for prototypic ILC2s, such as *Gata3*, *Rora*, *Il5*, and *Il7r*, were highly expressed by both uterine and lung ILC2s. Genes that represent other ILC subsets, such as *Tbx21* and *Rorc*, were minimally expressed by uterine or lung

FIGURE 4. Expression of the estrogen receptors is necessary for the accumulation of ILC2s in the uterus. **(A)** ILC2s were isolated from the uteri and lungs of BALB/c mice injected i.p. daily with IL-33 (400 ng per dose) for 3 d. The expression levels of *Esr1* and *Esr2* were examined by qRT-PCR. Box-and-whisker plots summarize the results from five (uterus) or eight (lungs) mice. $^{**}p < 0.01$ (Mann-Whitney *U* test) between the groups indicated by horizontal lines. **(B)** The wet weight of the uteri and lungs of WT C57BL/6, α ER $^{-/-}$, or β ER $^{-/-}$ mice. Data are shown as the mean $\pm$ SEM from six mice. $^{**}p < 0.01$ (Mann-Whitney *U* test) between the groups indicated by the horizontal line. **(C and D)** ILC2 numbers in the uteri, lungs, and bone marrow were examined in naive WT C57BL/6, α ER $^{-/-}$, or β ER $^{-/-}$ mice by flow cytometry. Representative FACS plots (C) and the summarized data from six (uteri, lungs) or three (bone marrow) mice per group (D) is shown. Data are shown as the mean $\pm$ SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ (Mann-Whitney *U* test) between the groups indicated by the horizontal line.

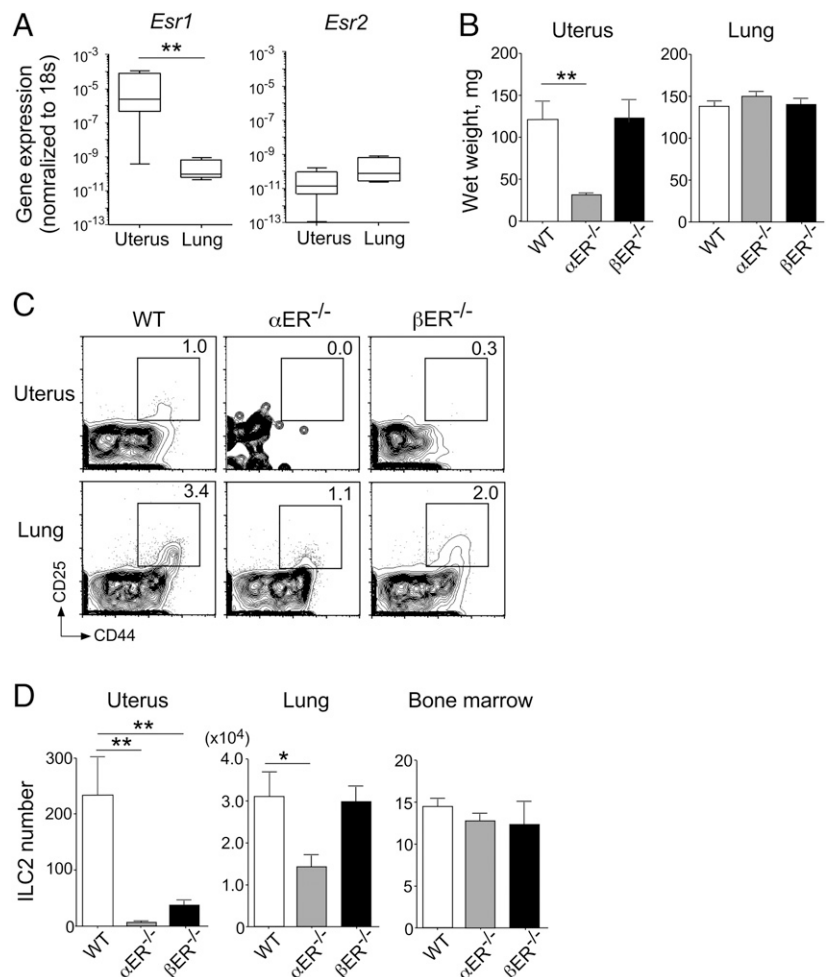
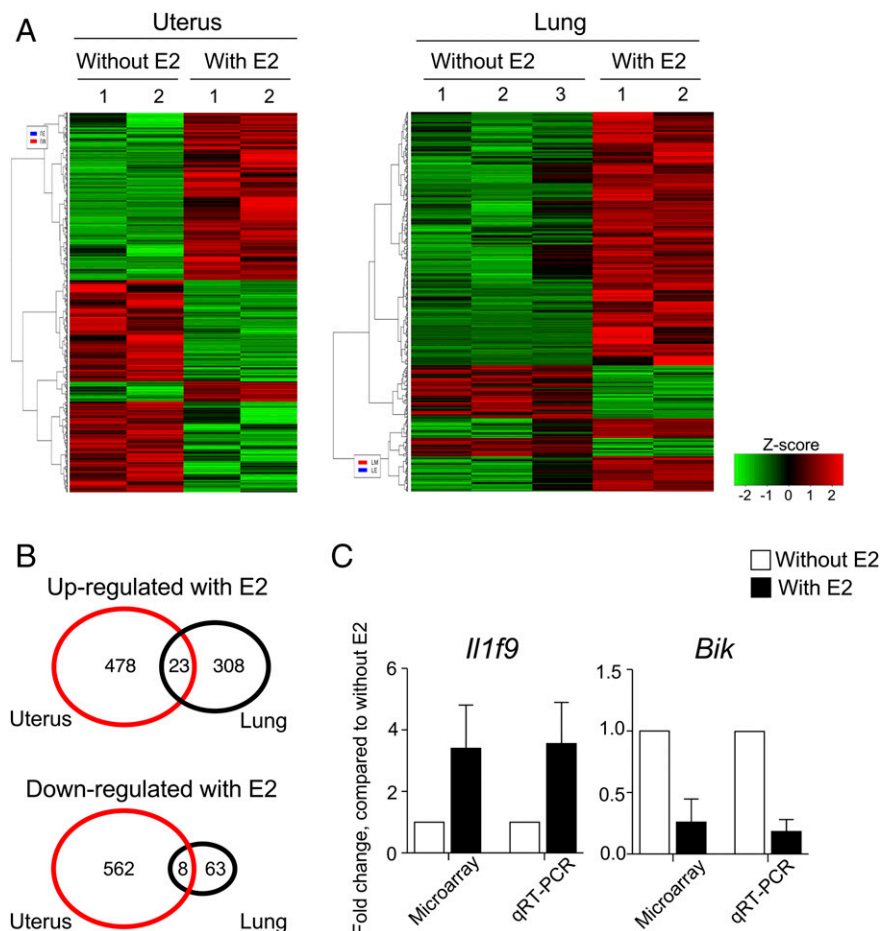


FIGURE 5. Gene microarray analysis of ILC2s cultured with E2. (A–C) ILC2s were isolated from the uteri and lungs of naive BALB/c mice and cultured with or without 100 ng/ml E2 for 16 h. mRNA was analyzed by microarray. (A) A heat map generated from hierarchical clustering of differentially expressed genes shows effects of E2 treatment on uterine (left panel) and lung (right panel) ILC2s. (B) The numbers of genes that were upregulated (upper panel) or downregulated (lower panel) significantly ($p < 0.05$) more than 2-fold by E2 treatment are shown for uterine and lung ILC2s. Numbers in the overlapping area indicate the genes shared by lung and uterine ILC2s. (C) Changes in expression of IL-36g (*Il1f9*, left panel) and *Bik* (right panel) mRNA by E2 treatment were determined by microarray analysis and qRT-PCR. Fold change was calculated relative to the transcript levels in cells cultured without E2. Data are shown as the mean $\pm$ SEM ($n = 2$ or 3).



ILC2s (data not shown). Furthermore, consistent with qRT-PCR (Fig. 4A), *Esr1* was highly expressed by uterine ILC2s compared with lung ILC2s (933 versus 13 U), whereas no apparent differences were observed in *Esr2* (47 versus 48 U).

When cultured with E2, both lung and uterine ILC2s altered expression of a number of genes (Fig. 5A). Overall, 2.5 times more genes were affected by E2 treatment in uterine ILC2s than in lung ILC2s (Fig. 5B). Few genes were regulated commonly by both uterine and lung ILC2s. Pathways analysis revealed E2-induced changes in the molecules involved in cellular assembly and organization and cell death and survival in both uterine and lung ILC2s, whereas the molecules involved in gene expression and cell cycle were relatively unique to uterine ILC2s and lung ILC2s, respectively (Table I). Furthermore, increased expression of the IL-1 family member *Il1f9* (IL-36g) and decreased expression of the proapoptotic gene *Bik* in E2-treated uterine ILC2s were confirmed by qRT-PCR (Fig. 5C). Taken together, these findings

suggest that estrogen and the estrogen receptor play a key role in steady-state accumulation of ILC2s in the uterus.

The IL-33/ST2 axis affects reproduction

As mentioned above, uterine ILC2s, which express the IL-33 receptor, ST2, are regulated by female sex hormones. Recently, an increase in fetal resorption was observed in allogeneic matings of mice deficient in inducible regulatory T cells (28). Therefore, to address the potential role of uterine ILC2s in reproduction, we mated *ST2*^{-/-} female mice with congenic BALB/c or allogeneic C57BL/6 males. No differences were observed in litter size between WT and *ST2*^{-/-} females, irrespective of whether the males expressed the same or different MHC haplotypes (Fig. 6). However, more pups were found dead within 24 h of birth from *ST2*^{-/-} mothers. Both the percentage of pups that died and the percentage of affected litters were higher in *ST2*^{-/-} BALB/c dams compared with WT BALB/c dams ($p < 0.01$ or $p < 0.05$). The use of male

Table I. Top molecular and cellular functions regulated by E2 in uterine and lung ILC2s

Uterine ILC2s			Lung ILC2s		
Function	<i>p</i> Value	No. of molecules	Function	<i>p</i> Value	No. of molecules
Gene expression	6.8×10^{-3} – 1.1×10^{-8}	217	Cellular assembly and organization	1.6×10^{-2} – 5.7×10^{-5}	73
Cellular assembly and organization	9.4×10^{-3} – 5.5×10^{-6}	187	DNA replication, recombination, and repair	1.6×10^{-2} – 6.6×10^{-5}	22
Cellular function and maintenance	8.1×10^{-3} – 5.5×10^{-6}	195	Cell death and survival	1.6×10^{-2} – 1.1×10^{-4}	96
Cell morphology	9.5×10^{-3} – 3.4×10^{-5}	218	Cell cycle	1.6×10^{-2} – 2.0×10^{-4}	70
Cell death and survival	9.4×10^{-3} – 5.0×10^{-5}	271	Posttranslational modification	1.6×10^{-2} – 2.3×10^{-4}	57

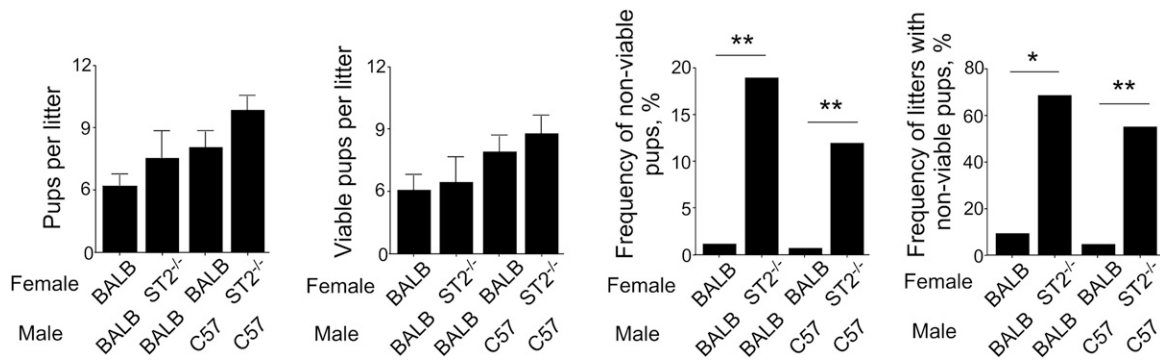


FIGURE 6. The IL-33/ST2 pathway affects viability of pups. WT BALB/c and ST2^{-/-} female mice were mated with WT BALB/c and C57BL/6 male mice. For each litter, the size and condition at birth and 24 h postpartum were recorded. The number of pups that were either dead at birth or died within 24 h after birth were counted as nonviable pups. Data are presented as mean $\pm$ SEM from nine or more litters or a pool of more than 58 pups in each group. * $p < 0.05$, ** $p < 0.01$ (Fisher exact test) between the groups indicated by the horizontal line.

mice with MHC haplotypes did not result in significant changes compared with controls. These findings suggest that the IL-33/ST2 pathway plays a role in creating a uterine environment in dams that is optimal for the growth and/or well-being of pups.

Discussion

In this study, we provide novel insight into immune cells in the FRT and reveal that ILC2s populate the uterus. Similar to those in other mucosal organs, uterine ILC2s express the IL-33 receptor, ST2, and produce a large quantity of IL-5 and IL-13 in response to IL-33 stimulation. Furthermore, uterine ILC2s express many of the cell surface molecules found on ILC2s in the lungs, such as CD127, Thy-1.2, and CD69. However, we did note that several molecules common to lung ILC2s were missing or minimally expressed in uterine ILC2s (Fig. 1). Particularly interesting was the difference in ICOS expression between uterine and lung ILC2s, as some ILC2 functions are diminished in the absence of ICOS signaling (29). These findings indicate that although uterine ILC2s share some features with ILC2s in other organs, they are likely regulated in an organ-specific manner.

Moreover, we found that female sex hormones, in particular estrogen, regulate the number of ILC2s in the uterus, but not lungs, as demonstrated by the studies with OVX mice, as well as with normal mice implanted with slow-release E2 pellets (Fig. 3). In agreement with this, uterine ILC2s highly express ER α at levels 10⁵-fold higher than that of lung ILC2s, although ER β expression is comparable (Fig. 4). In a cell, the ratio of ER α to ER β is generally an important determinant of the response to E2: an increased expression of ER β relative to ER α inhibits the E2 response (30). Intriguingly, Laffont et al. (24) recently reported that ILC2 numbers in the lungs are not affected by ovariectomy but are increased by ovariectomy. Therefore, although sex hormones modulate ILC2 homeostasis, their effects are likely to be dependent on the organs where they reside; ILC2s may be more sensitive to androgens in the lungs and to estrogen in the uterus. Indeed, we found that exposure of isolated uterine ILC2s to estrogen modulates their expression of a number of genes (Fig. 5, Table I). Nonetheless, we cannot rule out the possibility that the striking effects of estrogen on uterine ILC2s are due to the effects of estrogen on uterine tissue cells that may affect survival and/or proliferation of ILC2s by direct cell-to-cell interaction or by their products. In a pilot study, estrogen alone did not enhance survival of uterine ILC2s (data not shown). Generally, the complex interactions between epithelial cells, fibroblasts, and immune cells in response to female sex hormones likely create a unique environment optimal for both immunity and reproduction in the FRT (31).

Conversely, some noncytokine molecules, such as vitamins (e.g., vitamin A), hormones (e.g., vasoactive intestinal peptide), and neuropeptides (e.g., neuromedin U), can be recognized directly by ILC2s (12, 32, 33). Recent studies also show functional plasticity of ILC2s that is regulated by cytokines, such as IL-1 β , IL-4, and IL-12 (34, 35). Therefore, further studies on the mechanisms involved in tissue-specific regulation of ILC2s in the uterus and other organs will help us to understand better the biology of this unique cell type.

Another lingering question is the precise role of uterine ILC2s. Previously, ILC2s have been shown to promote protective immunity against helminth pathogens and to maintain tissue integrity and homeostasis (1). Therefore, it is possible that uterine ILC2s assist in altering the uterine immune environment to facilitate tolerance of an embryo or, in the event of an infection, help downregulate the type 1 responses that would be detrimental to the fetus (36). Specifically, uterine ILC2s might facilitate the M1 to M2 transition in macrophages, as M2 macrophages likely play protective roles in the FRT (31), and a similar role has been described in visceral adipose tissue ILC2s (11, 37). Additionally, because ILC2s can promote tissue repair by producing amphiregulin (8, 38), uterine ILC2s may contribute to the repair and rebuilding of uterine tissues during each estrus cycle. Finally, IL-33-stimulated ILC2s have been shown to be important for maintaining metabolic homeostasis in white adipose tissue (9–11). Loss of a potential homeostatic function of uterine ILC2s could have contributed to the detrimental outcome we observed for pups from ST2-deficient dams (Fig. 6). However, a significant limitation of this ST2-deficient model is that the lack of ST2 is not limited to ILC2s; other immune cells in the uterus that are responsive to IL-33, such as eosinophils, Th2 cells, and regulatory T cells (39, 40), may also contribute to the detrimental effects of ST2 deficiency. Future studies should investigate the role of uterine ILC2s in regulation and maintenance of the uterus using animals that are selectively deficient for ILC2s, when they become more widely available.

Finally, our observations with respect to the sex differences and hormonal regulation of ILC2s may be relevant to normal and pathological immune responses beyond the FRT. For example, asthma is more common in males before puberty and in females after puberty (41). Additionally, increased proportions of ILC2s were identified in the cord blood of neonatal boys compared with girls (42). These observations, in combination with the findings by others and us that lung ILC2s are more prevalent in female mice (Fig. 3) (24), suggest that ILC2s could potentially explain the sex differences associated with asthma. Moreover, sex-specific dif-

ferences in ILC2 responses may extend beyond the airway. For example, in a mouse model of multiple sclerosis, females exhibited a diminished protective ILC2 response in the nervous system compared with males (43). In conclusion, future studies on the interplay between sex hormones and ILCs that include multiple organs may be critical to understanding the sex differences in immune system diseases. Similarly, a more comprehensive analysis of the interaction between ILC2s, tissue parenchymal cells, and the microenvironment is required to elucidate the roles of ILC2s in normal biological processes and the pathophysiology of diseases in different organs.

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Disclosures

The authors have no financial conflicts of interest.

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SUPPLEMENTAL FIGURE

IL-33-responsive group 2 innate lymphoid cells are regulated by female sex hormones in the uterus

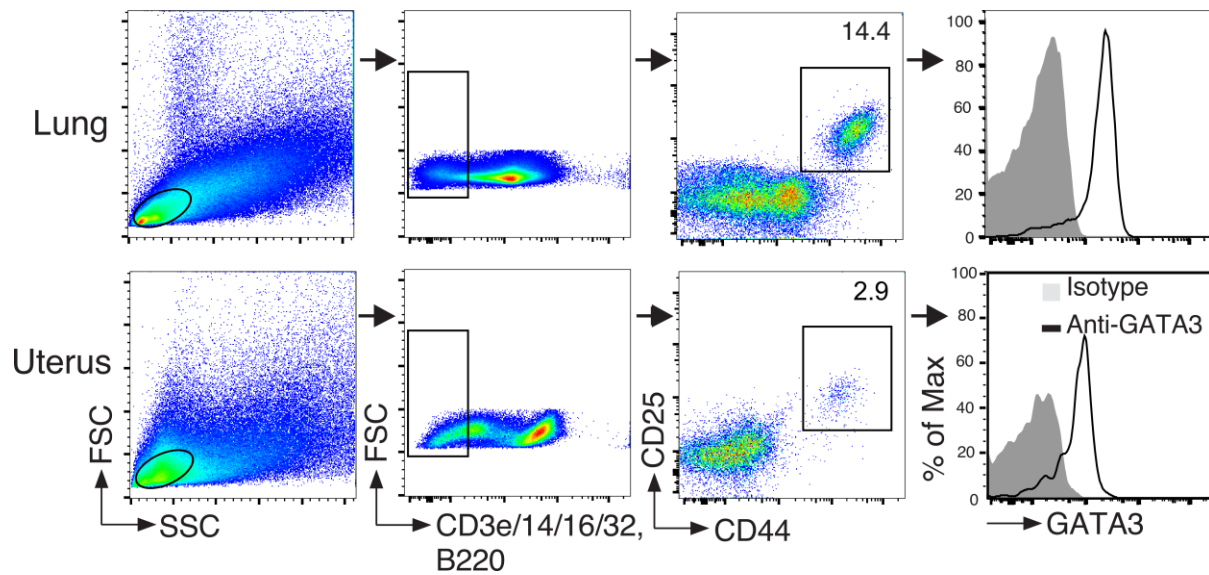
Running Title: 17 β -estradiol control of uterine ILC2s

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Supplemental Figure 1. Lung and uterine $\text{Lin}^- \text{CD25}^+ \text{CD44}^+$ ILC2s express the transcription factor Gata3.

Single-cell suspensions were prepared from the lungs (top panels) and uteri (lower panels) of naïve BALB/c mice. Cells were stained for cell surface molecules, followed by permeabilization of cellular and nuclear membranes as described in *Materials and Methods*, and staining for intracellular Gata3. Left panels: ILC2s are identified as lineage (CD3, CD14, CD16/32 and B220)-negative (Lin^-) and $\text{CD25}^+ \text{CD44}^{\text{hi}}$ lymphocytes, as described by Bartemes *et al.* (6). Right most panel: The intracellular expression of Gata3 in the ILC2 population is shown. Grey filled histograms, isotype control; solid line, Gata3.