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A Major Population of Functional KLRG1− ILC2s in Female Lungs Contributes to a Sex Bias in ILC2 Numbers

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ABSTRACT

Humans show significant sex differences in the incidence and severity of respiratory diseases, including asthma and virus infection. Sex hormones contribute to the female sex bias in type 2 inflammation associated with respiratory diseases, consistent with recent reports that female lungs harbor greater numbers of GATA-3− dependent group 2 innate lymphoid cells (ILC2s). In this study, we determined whether sex hormone levels govern sex differences in the numbers, phenotype, and function of ILC2s in the murine lung and bone marrow (BM). Our data show that lungs of female mice harbor significantly greater ILC2 numbers in homeostasis, in part due to a major subset of ILC2s lacking killer-cell lectin like receptor G1 (KLRG1), a population largely absent in male lungs. The KLRG1− ILC2s were capable of type 2 cytokine production and increased with age after sexual maturity, suggesting that a unique functional subset exists in females. Experiments with gonadectomized mice or mice bearing either global or lymphocyte restricted estrogen receptor α (Esr1) deficiency showed that androgens rather than estrogens regulated numbers of the KLRG1− ILC2 subset and ILC2 functional capacity in the lung and BM, as well as levels of GATA-3 expression in BM ILC2s. Furthermore, the frequency of BM PLZF+ ILC precursors was higher in males and increased by excess androgens, suggesting that androgens act to inhibit the transition of ILC precursors to ILC2s. Taken together, these data show that a functional subset of KLRG1− ILC2s in females contributes to the sex bias in lung ILC2s that is observed after reproductive age.

INTRODUCTION

Significant sex differences in the incidence and severity of respiratory diseases, including asthma and virus infection, are present in the human population, with women and girls often experiencing stronger immune responses and greater morbidity after puberty (1–3). Sex disparities, including sex hormone levels, may regulate the type 2 immune mechanisms that predispose to inflammatory lung diseases such as asthma but also promote tissue repair after respiratory virus infection (4–7). Estrogens and androgens act via the nuclear hormone receptors estrogen receptor (ER) α, ERβ, and androgen receptor (AR) to modulate the development and functional responses of lymphoid and myeloid immune cells (8–10). Indeed, murine models of allergic asthma show that sex hormones act on multiple immune cell types to promote the increased type 2 inflammation observed in females (11–13). Recent studies have shown that sex hormone levels regulate tissue-resident populations of group 2 innate lymphocytes (ILC2s) in homeostasis. AR activity and endogenous androgens reduce numbers of ILC2s in the lung and visceral adipose tissue of...
male mice (14, 15), whereas ERα activity and endogenous estrogens increase numbers of ILC2s in the female uterus (16). The ER and AR control epigenetic and transcriptional programs through their activity as ligand-dependent DNA-binding factors (9, 10). Thus, new information about the cellular and molecular mechanisms by which sex hormones regulate the differentiation or function of ILC2s will lead to a greater understanding of sex biases in type 2 immune diseases such as allergic asthma.

ILC2s are rare but potent innate cells that participate in type 2 immune responses in allergic asthma and pulmonary infection in humans and murine models (17, 18). ILCs are characterized by lymphoid morphology and IL-7Rα expression, yet they do not bear Ag receptors and are distinct from B, T, or myeloid cells (19, 20). Similar to Th1, Th2, and Th17 cell subsets, ILCs are subdivided into ILC1, ILC2, and ILC3 subsets based on transcription factors required for their development and effector cytokines produced (21, 22). Lung ILC2s are identified within the CD90^−/IL-7Rα^−/TCR^− fraction by expression of the IL-33 receptor (Il1rl1, also termed ST2), Sca-1, IL-2R/CD25, and Icos, and they require high levels of Gata3 for their development, maintenance, and function (23–25). ILC2s produce IL-5, IL-9, IL-13, and amphiregulin in response to the alarmins IL-33, IL-25, and thymic stromal lymphopoietin released from damaged epithelial cells (26).

Lung-resident ILC2s are well studied in murine models of asthma and infection, where they have been linked to initiation or perpetuation of type 2 responses and tissue repair (27–31). Increased numbers of IL-5^−/IL-13^−-producing ILC2s in females were observed in models of allergic lung inflammation, suggesting that baseline sex differences in ILC2 numbers contribute to the magnitude of the inflammatory response (15, 32). ILC2s may contribute to human disease pathology as they are expanded in the blood, respiratory tract, or skin of individuals with asthma, chronic rhinosinusitis, idiopathic pulmonary fibrosis, and atopic dermatitis (18, 33). Importantly, sex differences in human blood ILC2s in asthma patients were recently reported (15).

Killer cell lectin-like receptor G1 (KLRG1) is commonly used as a marker of mature tissue ILC2s. Also expressed by NK and CD8^+ T cells, KLRG1 is an ITIM-containing inhibitory receptor that binds the ligands E-, R-, and N-cadherin (34, 35). Human ILC2 function is inhibited upon binding to E-cadherin in vitro (36), suggesting that KLRG1^+ ILC2s might exhibit greater functional responses in the presence of E-cadherin expressing lung epithelial cells. However, KLRG1^+ lung ILC2s appear to be functional in vivo, so the physiological role of KLRG1 on ILC2s remains unclear. A substantial pool of KLRG1^− ILC2s is present in the bone marrow (BM). Whereas one report suggested these cells are ILC2 precursors due to their differentiation to KLRG1^+ ILC2s upon adoptive transfer (23), others have shown that BM ILC2s are an important source of type 2 cytokines and mobilized from BM during IL-33–induced inflammation (37, 38). The effects of sex hormones on the functional capacity of KLRG1^− ILC2s in the BM or lungs have not been described.

Adoptive transfer studies have shown that ILC2s can arise from IL-7Rα^−/common lymphoid progenitors or lymphoid-primed multipotent progenitors and require ID2 and the cytokine receptor common γ-chain (IL-2Rγ) for development (19, 22, 39). The transcription factors Gata3, Rora, Tcfl, Ets1, and Bel1l1 guide ILC2 differentiation, and a subset of these are maintained at high levels in mature ILC2s and are required for survival or function in tissues (40). BM ILC precursors (ILCPs) expressing the transcription factor Plzf and surface PD-1 differentiate into the ILC subsets but not NK cells (41, 42). However, parabiosis experiments with adult mice showed that most tissue ILC2s are not circulating or replenished via blood in homeostasis, suggesting the existence of a tissue-resident precursor (43). Plzf^+ precursors with the same phenotype and developmental capacity of BM ILC2s have been identified in fetal liver and small intestine, suggesting that ILC2s seed fetal tissues and contribute to the wave of tissue ILCs that develops in situ after birth (41, 44, 45). Thus, the relative importance of ILCPs present in fetal tissues and adult BM in either homeostasis or inflammation remains unclear. Although sex differences in ILC2 numbers were reported (14–16), we lack information about the possible sex hormone–mediated regulation of the ILCPs or ILC2-promoting transcription factors such as GATA-3.

In this study, we provide new insights into the sex differences in the numbers and function of lung and BM ILC2s and ILCPs in homeostasis, with a focus on the KLRG1^− ILC2 subset in females. Our data show that a prominent population of functional KLRG1^− ILC2s accumulates in lungs of females after they have reached reproductive age but is largely absent in males. Experiments involving gonadectomized mice and mice bearing either global or lymphocyte restricted ERα (Esr1) deficiency showed that numbers of KLRG1^− ILC2s in lung and BM are decreased by androgens but not estrogens. We show in the present study that the frequency of PLZF^+ BM ILCPs is elevated in males and is further increased by excess androgens, suggesting that male levels of androgens reduce ILC2 numbers by decreasing the transition of ILCPs to ILC2s. This is consistent with the reduced GATA-3 levels in BM ILC2s exposed to high levels of androgens. Taken together, our new data show that female lungs harbor significantly higher numbers of lung ILC2s in part due to a functional KLRG1^− subset that increases after reproductive age, is negatively regulated by androgens, and is largely absent in male lungs.

MATERIALS AND METHODS

**Mice**

Female and male C57BL/6 mice were purchased from The Jackson Laboratory. Heterozygous mice bearing a disrupted Esr1 allele (B6.129-Esr1^tm1Ksk^N10) (46) were purchased from The Jackson Laboratory and bred to yield Esr1^+/− and Esr1^−/− mice at the Oklahoma Medical Research Foundation. Mice bearing a conditional allele of Esr1 (Esr1^fl/fl^) (47) were backcrossed to C57BL/6 mice for 10 generations. Il7ra^−/Cre-Esr1^fl/fl^ (or -Esr1^−/−^-Rosa26-stop^YFP mice were generated by breeding either conditional (fl/fl) or wild-type (WT; +/+) alleles of Esr1 to mice bearing Il7ra^−/Cre promoter-driven Cre recombinase (provided by Dr. H.R. Rodewald) (48) and the YFP reporter B6.129X1-Gt(Rosa)
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26Sor^tm1(EYFP)C3/J (The Jackson Laboratory) (49). Age-matched females and males were compared, with WT and mutant mice either littermates or taken from the same breeding colony. Ovariectomy and orchidectomy were performed at the Oklahoma Medical Research Foundation on mice 4–5 wk after birth, and their lungs were harvested 8–12 wk after surgery. All experiments were approved by the Oklahoma Medical Research Foundation Institutional Animal Care and Use Committee.

**Tissue preparation**

Lung single cells were prepared as described previously with modifications (50). Briefly, lungs were perfused, minced, and incubated in 100 µg/ml each of Liberase (Roche) and DNAse I (Roche) at 37°C for 1 h in RPMI 1640 medium with 2% newborn calf serum. RBCs were then lysed with ACK buffer (BD Biosciences). A single-cell suspension was prepared by passing the tissue through a 70-μm filter. BM cells were isolated from both hind limbs, and lineage depletion was performed in some experiments using a hematopoietic progenitor (stem) cell enrichment kit (BD Biosciences) according to the manufacturer’s directions, with addition of an anti-CD5 mAb to the lineage mixture.

**Ex vivo stimulation to measure cytokine production**

Lung lymphocytes were enriched with 40% Percoll (GE Healthcare Life Sciences) in some experiments as described (51). Single-cell suspensions of lung cells and BM cells were stimulated with PMA (500 ng/ml) and ionomycin (30 ng/ml) in complete medium (RPMI 1640 plus 10% FCS) for 5 h. Monesin (1 µl/ml; BD Biosciences) was added for the last 2.5 h. Buffers optimized for intracellular cytokine staining or intranuclear staining (when also detecting GATA-3) were used to identify IL-5 and IL-13 production by ILC2s.

**Flow cytometry**

Complete gating strategies for lung or BM cells are shown in Supplemental Figs. 1A, 2A, and 2G. Dead cells were identified with a fixable Zombie Aqua cell stain kit according to the manufacturer’s instructions (BioLegend). Surface staining was performed by incubating cells with mAbs on ice for 15 min after 5 min of anti-CD16/32 treatment. Buffers and protocols for intranuclear staining (eBioscience/Thermo Fisher Scientific) and intracellular staining (BD Biosciences) were used according to the manufacturers’ instructions. Samples were acquired on an LSR II instrument and analyzed using FlowJo software (version 9.8.5). Lung ILC2s were identified after gating on a singlet, live population using mAbs specific for lineage markers CD3 (clone 145-2C11), CD5 (S3-L3), TCRβ (H57-597), B220 (RA3-6B2), CD11b (M1/70), CD11c (N418), TER19 (TER-119), Gr-1 (RB6-8C5), CD19 (MB19-1), and NK1.1 (PK136) purchased from BioLegend or Tonbo Biosciences, and Thermo Fisher Scientific.

**Statistical analysis**

Comparison between two groups was performed using an unpaired t test or a Mann–Whitney U test using Prism 7 software. A two-way ANOVA followed by a Sidak multiple comparison test or a one-way ANOVA followed by a Tukey multiple comparison test was performed for multiple group comparisons. A p value < 0.05 was considered significant. Significance is indicated by *, except that the significance of differences in KLRG1+ ILC2s is indicated by †, and in KLRG1− ILC2s by ‡, when analyzed separately but presented in stacked box plots. All graphs show mean and SEM.

**RESULTS**

**Male and female mice show differences in the number and phenotype of lung ILC2s in homeostasis**

Despite having significantly lower numbers of CD45+ cells (Fig. 1A), lungs of female mice (~12–14 wk old) contained higher numbers of ST2+ GATA-3+ ILC2s (Fig. 1B, 1C, Supplemental Fig. 1A) compared with age-matched males. To determine the age at which the sex difference in lung ILC2s arises, we analyzed male and female lungs between 3 and 20 wk of age. No sex difference in the numbers of lung ILC2s was observed at 3 wk after birth (Supplemental Fig. 1B). In contrast, females showed a significant increase in ILC2 numbers after acquisition of reproductive capacity (by 12 wk of age) and older (Supplemental Fig. 1B). These data suggest that age-associated factors such as sex hormones regulate ILC2 numbers in the lung in homeostasis.

Upon further investigation of the phenotypic differences between male and female lung ILC2s, we observed that a significant fraction (in number and frequency) of female ILC2s lacked KLRG1, whereas male ILC2s were primarily KLRG1+ (Fig. 1D). At 3 wk of age, female and male mice lungs contained similar numbers of ILC2s, and they were uniformly KLRG1+ (Fig. 1E). By 12 wk of age, numbers of KLRG1− ILC2s in females were significantly increased relative to males, and KLRG1− ILC2s began to accumulate. As females aged, numbers of KLRG1− ILC2s continued to increase and by 20 wk, approximately equal fractions of ILC2s were KLRG1− and KLRG1+. In contrast, most male ILC2s were KLRG1+, and the numbers of KLRG1+ and KLRG1− ILC2s in males remained constant between 3 and 20 wk of age (Fig. 1E).

Next we determined whether lung (KLRG1+ or KLRG1−) ILC2s from males and females (age 14–16 wk) showed differential expression of markers associated with ILC2 function and proliferation (Fig. 1F). Female ILC2s had significantly higher expression (mean fluorescence intensity [MFI]) of CD25 (IL-2R), which promotes ILC2 proliferation and type 2 cytokine secretion. Consistent with this, a significantly higher fraction of female ILC2s was positive for the Ki67 chromosomal marker associated with an active phase of the cell cycle, indicating that a greater
number were proliferating. IL-33R/ST2 expression was higher in male ILC2s. However, the expression of the transcription factor GATA-3 was not significantly different in female or male ILC2s. In females, the KLRG1+2 and KLRG1− subsets showed no significant differences in expression of CD25, Ki67, ST2, or GATA-3 (Fig. 1F).

Taken together, these data show that once mice reach reproductive age, females harbor significantly higher numbers of lung ILC2s, which is associated with increased markers of proliferation. Notably, the fraction of female KLRG1− ILC2s continues to increase with age. Thus, KLRG1 may not be a definitive marker of mature tissue ILC2s, because expression of KLRG1 is dependent on sex and age in naive mice and is not associated with a distinct proliferative capacity or GATA-3 expression level.

**Elevated androgens in global ERα−/− mice decrease lung ILC2 numbers and diminish the KLRG1− ILC2 subset**

Our data show that sex differences in the numbers and phenotype of lung ILC2s in homeostasis arise after sexual maturity, suggesting a role for sex hormones. We initially hypothesized that ERs activity regulates lung ILC2s, and we determined ILC2 numbers in a murine model of global ERα deficiency. ERα−/− mice of both sexes showed a significant decrease in the total numbers of lung ILC2s (Fig. 2A). Whereas a reduction in KLRG1− ILC2s primarily contributed to the decrease in ERα−/− males, the numbers of
KLRG1− ILC2s were significantly lower in ERα−/− females as compared with WT females (Fig. 2A). Although these data suggested regulation by ERα, it is well documented that both sexes of ERα−/− mice have significantly (~5 to 30-fold) higher levels of serum estradiol and testosterone compared with WT mice (52–55). To avoid this variable and to restrict ERα (Esr1) deficiency to lymphocytes, including ILC2s, we bred mice lacking ERα only in IL-7Rα+ cells (IL-7Ra−cre−Esr1+/− mice). Cre activity in lung ILC2s was confirmed using a YFP reporter in the Rosa26 locus (Fig. 2B). IL-7Ra−cre−Esr1+/− mice were analyzed as WT controls. The numbers and frequency of lung ILC2s (both KLRG1+ and KLRG1−) were not significantly different between IL-7Ra−cre−Esr1+/− and IL-7Ra−cre−Esr1−/− mice (Fig. 2C, Supplemental Fig. 1C), indicating that ERα acting in lymphoid cells does not regulate ILC2 numbers. To determine whether endogenous estrogens act indirectly via other nonlymphoid cell types to regulate ILC2s, we performed ovariectomy to remove a major source of systemic estrogens. Ovariectomy of WT females at 4–5 wk of age did not alter the frequency of lung ILC2s (both KLRG1+ and KLRG1−) present at 16–20 wk of age (Fig. 2D). These experiments show that lymphocyte-restricted loss of ERα or loss of estrogen through ovariectomy of WT females did not alter numbers of KLRG1+ and KLRG1− ILC2 subsets.
We next sought to understand the reduction in ILC2s in female and male global ERα−/− mice. We hypothesized that higher levels of androgens including testosterone in ERα−/− mice might explain our results. To test this, we ovariectomized females because this reduces the high levels of both androgens and estrogens in female global ERα−/− mice (53, 55). Indeed, ovariectomy of ERα−/− females led to a dramatic increase in frequency of total lung ILC2s that was primarily driven by an increase in KLRG1− ILC2s (Fig. 2D, Supplemental Fig. 1D).

To confirm the effect of androgens on ILC2 numbers, we performed orchidectomy (castration) of WT and global ERα−/− males at 4–5 wk of age to remove the major source of androgens (55), and we analyzed the mice at 16 wk of age. Orchidectomy led to a significant increase in the frequency of KLRG1− ILC2s in WT males (Fig. 2E). This correlated with a significant increase in the percentage of Ki67+ ILC2s in orchidectomized WT males (Fig. 2F). Orchidectomized ERα−/− mice also showed a dramatic increase in the frequency of lung ILC2s (both KLRG1− and KLRG1+). Taken together, these data show that compared with WT females, the frequency of lung ILC2s (both KLRG1+ and KLRG1−) was primarily driven by an increase in KLRG1− ILC2s (Fig. 2F, Supplemental Fig. 1D).

To further test the role of androgens in reducing ILC2 numbers, we performed orchidectomy of WT and ERα−/− female mice (Fig. 2E). This correlated with a significant increase in the percentage of Ki67+ ILC2s in orchidectomized female ERα−/− mice (55), and we analyzed the mice at 16 wk of age. orchidectomy of female ERα−/− mice showed a subtle but significant increase in the frequency of KLRG1+ ILC2s in BM. Both sexes of ERα−/− mice showed a subtle but significantly higher frequency of cytokine-positive cells (IL-5+ or IL-5+IL-13+) than did WT male ILC2s after ex vivo stimulation (Fig. 3E), suggesting that androgens reduce ILC2 cytokine production capacity. Consistent with this, female ERα−/− ILC2s showed decreased capacity for cytokine production, and this was reversed by ovariectomy (Fig. 3E). Similarly, orchidectomy of WT and ERα−/− males increased the cytokine production capacity of ILC2s (Fig. 3F).

Taken together, these experiments show that elevated androgens in WT males and ERα−/− females and males lead to a minor but significant reduction in the capacity of lung ILC2s to produce IL-5 and IL-13; however, the major effect of androgens is to reduce ILC2 numbers and decrease the KLRG1− subset. In contrast, our data suggest that the lower levels of androgens present in WT females lead to an abundant and functional KLRG1− ILC2 subset.

Sex differences in BM ILC2 phenotypes are regulated by androgens

ILC2s in BM have been described as an immature precursor reservoir that lacks KLRG1 and gives rise to functional KLRG1− ILC2s upon adoptive transfer (23). However, others have reported that BM ILC2s are functional in disease models (37, 38). In view of their potential importance, we determined whether BM ILC2s showed sex differences in number, phenotype, or function. Although the frequency of Sca-1+ST2+GATA-3+ ILC2s increased after reproductive age, we did not observe a sex difference in frequency at any age (Fig. 4B, Supplemental Fig. 2A). However, male but not female BM contained a prominent subset of KLRG1− ILC2s that increased after reproductive age (between 12 and 20 wk) (Fig. 4A, 4B).

Both the KLRG1− and KLRG1+ subsets in males were capable of producing IL-5 upon ex vivo stimulation with PMA/ionomycin, and the total fraction of IL-5–producing BM ILC2s did not differ in males and females (Fig. 4C), indicating that KLRG1 expression does not promote or diminish functional capacity in this assay. ILC2s isolated from the BM or lung of the same mice showed no difference in capacity to produce IL-5, despite sex differences in KLRG1 expression (Fig. 4C). However, BM ILC2s produced negligible IL-13 compared with lung ILC2s, suggesting that they may not have full functional capacity (Supplemental Fig. 1E).

To determine whether sex hormones were responsible for the increase in KLRG1− BM ILC2s in males, we performed ovariectomy or orchidectomy of WT females and males. Because gonadectomy alters bone structure and numbers of immune cells recovered from the BM (58, 59), in this study we report ILC2 frequency of live cells rather than their total number. No significant difference in the frequency of KLRG1− BM ILC2s was observed in ovariectomized WT females compared with controls (Fig. 4D). Consistent with this, the frequency of KLRG1− BM ILC2s in IL-7Rα−cre-Esr1fl/fl mice did not differ from that in IL-7Rα−cre-Esr1fl/+ mice (Supplemental Fig. 2B). In contrast, orchidectomy dramatically decreased the frequency of KLRG1− BM ILC2s, suggesting that androgens promote this subset (Fig. 4E). Next, we used global ERα−/− mice to determine whether elevated androgen levels increase the frequency of KLRG1− ILC2s in BM. Both sexes of ERα−/− mice had a significantly higher frequency of KLRG1− BM ILC2s compared with WT mice (Fig. 4D, 4E). Ovariectomy or
FIGURE 3. KLRG1− ILC2s are functional and elevated androgens decrease cytokine production from lung ILC2s. (A and B) Lung cells were stimulated ex vivo with PMA/ionomycin for 5 h in the presence of monesin. Shown are representative FACS plots of IL-5 or IL-13 expression by KLRG1+ and KLRG1− lung ILC2s from a 16-wk-old WT female and male (pregate: Lin−CD90−GATA-3−ST2−). The data from unstimulated lung cells are shown in Supplemental Fig. 1E. The percentage of IL-5+ and the percentage of IL-13+ KLRG1+ and KLRG1− ILC2s gated as shown in (A) and (B) were compiled from multiple mice. The data are representative of three independent experiments (n = 3–4 per group) with mice of ages 14–16 wk. The significance of data was evaluated using a Mann–Whitney U test, and is denoted for KLRG1− ILC2s by # and for KLRG1+ ILC2s by §. #, § p < 0.05. (C) Total frequency of IL-5+ ILC2s (gating shown in Supplemental Fig. 1E) of WT and ERα−/− females and males after stimulation of lung lymphocytes enriched using a 40% Percoll gradient. Symbols indicate individual mice of ages 9–16 wk compiled from two to four independent experiments. The data were evaluated using a Mann–Whitney U test. ***p < 0.001. (D) Total number of lung eosinophils identified as MHC class II−CD11b+CD24+Siglec-F+ cells in the lungs of WT and ERα−/− males and females. Symbols indicate individual mice compiled from two to four independent experiments. The data were evaluated using a t test. **p < 0.01. (E and F) The normalized percentages of lung ILC2s either single positive for IL-5 or double positive for IL-5 and IL-13 are shown for intact and gonadectomized (ovariectomy [OVX] or castration [CX]) WT and ERα−/− mice of both sexes. Symbols indicate individual mice of ages 14–20 wk compiled from three independent experiments. For each experiment, the data from each group were normalized to the average value for WT females in (E) or for the WT males in (F). Significance was evaluated using a Mann–Whitney U test. *p < 0.05, **p < 0.01.
FIGURE 4. Sex differences in BM ILC2 phenotypes are regulated by androgens.

(A) Shown are representative FACS plots of BM ILC2s, identified as Lin−IL-7Rα+CD90+Sca-1+ST2+ as gated in Supplemental Fig. 2A, in a 14-wk-old WT female and male. (B) The total frequency of BM ILC2s (divided as KLRG1+ and KLRG1−) in WT female and male mice at different ages. The data are compiled from six independent experiments (total n = 4–10 per age group). The data were evaluated using a two-way ANOVA with a Sidak multiple comparison test, and significance is denoted for KLRG1− in WT female and male mice at different ages. The data were evaluated using a two-way ANOVA with a Sidak multiple comparison test, and significance is denoted for KLRG1− ILC2s by # and for KLRG1+ ILC2s by §. #, p < 0.05, ##, p < 0.01, ####, p < 0.0001.

(C) Shown are representative FACS plots of IL-5+ BM ILC2s (pregate: Lin−IL-7Rα+CD90+GATA-3+) from a WT female and male. BM and lung cell suspensions were incubated for 5 h with PMA/ionomycin in the presence of monesin. The percentage of IL-5+ KLRG1+ and KLRG1− ILC2s in BM and lungs was compiled from two independent experiments (n = 5–6 per group) with mice of age 16 wk. The data were evaluated using a Mann-Whitney U test, and significance is denoted for KLRG1− ILC2s by # and for KLRG1+ ILC2s by §. ##, ##, p < 0.01. (D and E) The frequency of KLRG1+ BM ILC2s was compared between control and gonadectomized (ovariectomy [OVX] or castration [CX]) WT and ERα2/2 mice of both sexes. Symbols indicate the individual mice of ages 14–20 wk compiled from three independent experiments. The data were evaluated using a one-way ANOVA with a Sidak multiple comparison test. *, p < 0.05, **, p < 0.01, ****, p < 0.0001.

(F) The frequency of KLRG1− BM ILC2s was compared between naive and castrated (CX) WT and ERα−/− males. Symbols indicate individual mice of ages 11–20 wk compiled from five independent experiments. The data were evaluated using a one-way ANOVA with a Sidak multiple comparison test. *, p < 0.05.

(G) A representative FACS plot of GATA-3 levels in BM ILC2s in 15- to 17-wk-old male WT (solid line) and ERα−/− (dotted line) mice, relative to binding of an isotype control mAb (shaded histogram). The (Continued)
orchidectomy of ERα−/− females and males decreased the frequency of KLRG1− BM ILC2s (Fig. 4D, 4E). These experiments show that androgens rather than estrogens increase numbers of KLRG1− BM ILC2s.

Despite an increased frequency of KLRG1− ILC2s in BM, male ERα−/− mice showed a reduction in frequency of total BM ILC2s, as observed in the lung, due to a decrease in the KLRG1− subset (Fig. 4F, Supplemental Fig. 2C). ERα−/− ILC2s in BM also were less capable of IL-5 production compared with WT males (Supplemental Fig. 2D). In view of these numerical and functional deficits, we hypothesized that ERα−/− ILC2s in BM might express lower GATA-3 levels than do WT ILC2s. Whereas all differentiating ILCs express a low level of GATA-3, only ILC2s upregulate GATA-3 to a higher level, which is needed for development, and function (23–25). Indeed, male BM ERα−/− ILC2s (both KLRG1+ and KLRG1− subsets) contained lower levels of GATA-3 (measured upon intracellular staining as MFI) than did WT ILC2s (Fig. 4G, 4H). Non-ILC2s in the Lin−CD90+IL-7Rα+ST2+Sca-1− gate expressed comparably low levels of GATA-3 in ERα−/− mice and WT mice (Fig. 4H). Orchidectomy of male global ERα−/− mice restored BM ILC2s numbers and their GATA-3 expression to WT levels (Fig. 4I, Supplemental Fig. 2C). Taken together, these data suggest that elevated androgens in male ERα−/− mice lead to BM ILC2s that have only partially upregulated GATA-3, which may explain their reduced numbers and functional capacity. Female ERα−/− mice showed a significant increase in the frequency of KLRG1− BM ILC2s but not total BM ILC2s or GATA-3 levels (Supplemental Fig. 2E, 2F), suggesting that the increased testosterone levels in ERα−/− females were insufficient to reduce GATA-3 expression.

**Elevated androgens in mice with global ERα−/− deficiency lead to the accumulation of PLZFhi ILCPs in BM**

BM ILC2s (Lin−IL-7Rα+α4β7−; PLZF−PD-1−) can differentiate into all ILC subsets upon adoptive transfer or in vitro culture (41, 42). To determine effects of sex differences and elevated androgens on ILC2s, we determined their frequency in lineage-depleted BM of male and female WT and ERα−/− mice (Fig. 5A, 5B, Supplemental Fig. 2G). The frequency of PLZF−PD-1− ILC2s within the IL-7Rα+α4β7− fraction of Lin− BM (Fig. 5A) and within the total Lin− BM pool (Fig. 5B) was significantly higher in males than females. Similarly, the ILC2 frequency was higher in male global ERα−/− mice compared with WT males (Fig. 5A, 5B), and this was reduced by orchidectomy (Fig. 5A, 5C). Thus, higher frequencies of ILC2s in BM correlate with increased levels of androgens. However, a higher ILC frequency in males does not lead to more BM ILC2s in males relative to females (Fig. 4B). Indeed, the ratio of BM ILC2s to ILCPs is significantly higher in WT females than in WT males (Fig. 5D). Furthermore, the elevated frequency of ILCPs in male ERα−/− mice is associated with a decreased frequency of ILC2s (Supplemental Fig. 2C). This suggests that ILCPs accumulate in males because higher levels of androgens partially inhibit the transition of ILCPs to ILC2s.

**DISCUSSION**

Sex hormones acting via ER or AR regulate the development and/or functional responses of multiple types of innate immune cells (9, 10). Despite this, there are few examples of significant sex differences in numbers of innate immune cells in homeostasis. Prior reports have shown that female mice have higher numbers of lung-resident ILC2s, including a prominent subset lacking KLRG1 (14, 15). In this study, we have shown that the KLRG1− subset is a very minor population at 3 wk of age in both sexes, but increases after reproductive age (by 8–12 wk) in females but not in males. In older (5 mo) mice, the KLRG1− subset often comprises half of the ILC2 population in female lungs and is capable of producing type 2 cytokines. Investigation of the role of sex hormones and ERs showed that the KLRG1− subset is most prominent when androgen levels are low (as in normal females) but does not depend on endogenous estrogens or ERs signaling. Our data are consistent with recent reports that lung ILC2 numbers in males are attenuated by AR signaling (14, 15). The presence of a functional lung-resident KLRG1− ILC2 subset in females may alter the magnitude or quality of type 2 inflammation upon allergen or pathogen insult to the respiratory tract.

In contrast to an earlier report that KLRG1− ILC2s in the BM are immature precursors (23), we found that the female lung KLRG1− subset was capable of producing IL-5 and expressed comparable levels of GATA-3 and markers of proliferative capacity to the KLRG1− subset. Similarly, the KLRG1− ILC2s in BM of both sexes also were functional. KLRG1 is an ITIM-containing inhibitory receptor, also expressed by NK and CD8+ T cells, which mediates inhibitory effects through recruitment of SHIP-1 and SHP-2, leading to defective AKT phosphorylation (60, 61). The ligands for KLRG1 are E-, R-, and N-cadherin (34, 35), but the effect of E-cadherin–KLRG1 interactions on ILC2 function in epithelial tissue is unclear. Human skin ILC2 cytokine production

MFI values are indicated. (H) Shown are normalized GATA-3 MFI values for the KLRG1+ ILC2s, KLRG1− ILC2s, and non-ILC2s (defined as Lin−IL-7Rα−CD90+ Sca-1− ST2−) in BM of multiple WT and ERα−/− males. Symbols indicate individual mice of ages 10–16 wk compiled from four independent experiments. The data were normalized to the average value for the GATA-3 MFI in the WT male ILC2s in each experiment. Significance was evaluated using a one-way ANOVA with a Sidak multiple comparison test. ***p < 0.001, ****p < 0.0001. (I) Shown are the GATA-3 MFI values in total BM ILC2s of WT, ERα−/−, and castrated (CX) ERα−/− mice. Symbols indicate individual mice of ages 15–17 wk compiled from two independent experiments. The data were normalized to the average GATA-3 MFI on BM ILC2s of WT male mice in each experiment. Significance was evaluated using a one-way ANOVA with a Sidak multiple comparison test. **p < 0.01. Iso, isotype control.

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is inhibited upon binding to E-cadherin in vitro (36), suggesting that KLRG1− ILC2s might exhibit greater functional responses in the presence of E-cadherin–expressing lung epithelial cells. Conversely, KLRG1+ ILC2s may have defects in interactions with epithelial cells that would reduce their function. It is also possible that KLRG1− ILC2s convert to KLRG1+ cells during inflammation. Although our data show that the lung and BM-resident KLRG1− and KLRG1+ subsets have comparable functional capacity upon PMA/ionomycin stimulation, KLRG1 may regulate ILC2 function in response to physiological or environmental triggers in tissues. If so, the presence of a major KLRG1− ILC2 subset in females may alter ILC2-dependent cellular interactions in homeostasis and in inflammatory environments.

The age dependence of the ILC2 sex bias suggests that KLRG1− ILC2s accumulate in females due to sex hormone–related factors. Our analyses of WT females and males coupled with gonadectomy show that lung ILC2 numbers and the KLRG1− subset are unaffected by loss of estrogens after ovariectomy but are significantly decreased in the presence of the higher serum levels of androgens present in adult males (~8–10 ng/ml) relative to females (~0.05 ng/ml) (55). The higher levels of androgens in males also were associated with a minor reduction in capacity for type 2 cytokine production, as judged by an assay of ex vivo stimulation with PMA/ionomycin. The ability of elevated androgens to decrease ILC2 numbers, including the KLRG1− subset, and their functional responses was amplified in both sexes of globally Erst-deficient mice, which contain ~5 to 30-fold higher levels of serum androgens produced by the ovary and testes (53–55). Ovariectomy of ERα−/− females and orchidectomy of ERα−/− males restored ILC2 numbers and function to WT levels. These data are consistent with recent reports showing that males have lower lung ILC2 numbers, and that the sex bias is a product of testosterone and AR signaling in males rather than ER signaling in females (14–16). However, factors governing the increase in the KLRG1− subset as females age remain unclear, because estrogens appear not be involved and testosterone levels are already low. Our data are consistent with the possibility that the KLRG1− subset converts into the KLRG1+ subset as females age and/or that extended exposure to male levels of androgens is necessary to maintain KLRG1 expression.

Among the ILCs in mice, ILC2s are the predominant type in diverse organs, including the lung, small intestine, uterus, and adipose tissue, where they have a role in protective immunity during infection as well as in regulation of metabolism and tissue integrity during homeostasis (18, 56). Interestingly, these ILC2 populations are differentially regulated by sex hormones depending on their location. Whereas male sex, AR activity, and elevated androgens reduce ILC2 numbers in the lung, visceral adipose tissue, and the mesenteric lymph node (14, 15) (Fig. 2A), estrogens and ERα increase ILC2 numbers in the uterus (16). Consistent with this, ILC2s in the BM and small intestine express higher levels of AR than ERα, whereas those in the uterus express higher

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**FIGURE 5. Elevated androgens in mice with global ERα deficiency lead to the accumulation of PLZFhi ILC2s in BM.**

(A) Shown are representative FACS plots of PLZF+ PD-1+ ILC2s of 9- to 16-wk-old WT and ERα−/− female and male mice and a castrated (CX) ERα−/− mouse after lineage depletion and gated as shown in Supplemental Fig. 2G (pregate: Lin−IL7Ra+α4β1+ cells). (B) The frequency of ILC2s in lineage-depleted BM of multiple WT and ERα−/− female and male mice. Symbols indicate individual mice of ages 8–10 wk compiled from three independent experiments. The data were evaluated using a one-way ANOVA with a Sidak multiple comparison test. *p < 0.05, **p < 0.001. ****p < 0.0001. (C) The frequency of ILC2s in lineage-depleted BM of multiple intact or castrated WT and ERα−/− male mice. Symbols indicate individual mice of ages 8–16 wk compiled from three independent experiments. The data were evaluated using a one-way ANOVA with a Sidak multiple comparison test. **p < 0.01. (D) The ratio of ILC2s to ILC2p in BM of multiple WT female and male mice is shown. Symbols indicate individual mice of ages 10–15 wk compiled from three independent experiments. The data were evaluated using a t test. ***p < 0.001.

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levels of ERα than AR (14, 16) (http://www.1mmgen.org). Although we found that IL-7Rα-restricted Esr1 deficiency did not alter lung ILC2 numbers, treatment of lung ILC2s in vitro with 100 ng/ml estradiol led to changes in gene expression (16), suggesting that both androgens and estrogens may modulate lung ILC2 function.

ILCPs in BM differentiate to ILC2s upon adoptive in vivo transfer or in vitro culture on OP-9 stromal cells (41). Sex differences in ILCP numbers or GATA-3 expression have not been reported. Through comparisons of intact and orchidectomized mice, our data show that elevated serum testosterone levels in WT and ERα−/− males (reported to be ~8–20 ng/ml) lead to increased numbers of the PLZF−PD-1+ ILCPs in adult BM. However, despite increased testosterone levels in ERα−/− females (~2 ng/ml) relative to WT females (~0.05 ng/ml) (55), ILCP numbers did not differ. The ILCP accumulation in males suggests that a threshold level of androgen is needed to attenuate the transition of ILCPs to ILC2s. Consistent with this, the ratio of BM ILC2s to ILCPs is significantly higher in females. We also show in the present study that elevated androgens in ERα−/− males reduce GATA-3 levels in BM ILC2s to a level that is intermediate between that of ILC precursors and WT ILC2s, and this effect is alleviated by orchidectomy. Thus, androgens may act to inhibit the upregulation of GATA-3 to levels needed for the ILCP to efficiently differentiate into ILC2s. This partial block of GATA-3 expression by elevated androgens suggests that the epigenetic effect of AR activity is needed to fully open the locus or to recruit key coactivators. Although reduced in number, lung ILC2s in ERα−/− mice do not show reduced levels of GATA-3 relative to WT mice, suggesting that ILC2s cannot survive in the lung unless they express high amounts of GATA-3.

The pool of lung ILC2s in homeostasis may initially be expanded by new differentiation from lung-resident ILCPs in the postnataal period and later maintained by homeostatic proliferation. Androgens may attenuate one or both of these pathways. We and others (14, 15) found that adult male lung ILC2s show a reduced frequency of the Kit67 marker of proliferation and express lower levels of the IL-2R/CD25 that augments proliferation and survival. Our data also suggest that elevated androgens attenuate the transition of ILCPs to ILC2s.

However, it is currently unclear how BM ILCPs contribute to the noncirculating tissue-resident pools of mature ILC2s in adults. Murine parabiosis experiments showed that in adults ~95% of ILC2s do not apparently circulate in blood or replenish from blood-borne precursors, although small numbers of ILC precursors were noted in blood (43). Similarly, rare ILC precursors and ILC2s were identified in human blood (62). Most tissue ILC2s are thought to arise from precursors seeded into tissues before birth, and there is a rapid rise of mature ILC2s in lungs and small intestine in the first weeks after birth (39, 44, 45). ILCPs with the same phenotype as those in adult BM were identified in fetal liver (41) and fetal small intestine (44). The presence of ILCPs in fetal or adult lungs has not been reported, and we were not able to identify PLZF+ ILCPs in lungs of either 3- or 19-wk-old mice (S.K. data not shown).

The phenotypic similarity of fetal liver and adult BM ILCPs raises the possibility that the androgens may regulate the fetal or neonatal ILC2 pool, as we have shown for the adult BM pool. Although both sexes are exposed to high levels of maternal estrogens in utero, male fetuses express higher levels of testosterone produced by developing testes (63, 64). Additionally, in the first few weeks after birth, human and rodent males have a “mini puberty” with testosterone levels that approach those of adults (63, 65, 66). Although a sex difference in ILC2 numbers is not yet apparent at 3 wk, it is possible that the elevated testosterone levels in fetal and neonatal males lead to epigenetic imprinting of ILCPs or developing ILC2s. This early AR activity may modulate their later gene expression program upon exposure to adult levels of androgens, perhaps ultimately leading to reduced upregulation of GATA-3 and reduced proliferation or survival of lung ILC2s.

Androgens also attenuate the expansion or functional responses of ILC2s that occur upon lung inflammation. Males show reduced numbers of ILC2s producing IL-5 in murine models of lung allergy (15, 32), indicating that the sex bias in ILC2s is maintained during respiratory inflammation. This is consistent with the increased incidence and severity of asthma in women and girls after puberty (2). Furthermore, the preferential expansion or function of ILC2s during influenza virus infection results in increased airway hyperresponsiveness in murine models (29, 30), and increased ILC2 activity in females may exacerbate the asthma symptoms often observed after influenza virus infection. Therefore, a greater understanding of the molecular mechanisms by which androgens regulate lung ILC2s may lead to new strategies for therapy of type 2-mediated immune diseases of the respiratory tract.

DISCLOSURES

The authors have no financial conflicts of interest.

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