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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. ImmunoHorizons, 2018, 2: 74–86.

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 (Il-1rl1, also termed ST2), Sca-1, IL-2R/CD25, and ICOS, and they require high levels of GATA-3 for their development, maintenance, and function (23–25). ILC2s produce IL-5, IL-9, IL-13, and amphiregulin in response to the alarms 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– and 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, Tcf1, Ets1, and Bcl11b 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 ILC2s 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 ILC2s 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-Esr1tm1Nile/J) (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 (Esr1fl/fl) (47) were backcrossed to C57BL/6 mice for 10 generations. IL-7Rα-Cre-Esr1fl/fl (or -Esr1+/−)-Rosa26-stopYFP mice were generated by breeding either conditional (fl/fl) or wild-type (WT; +/+ ) alleles of Esr1 to mice bearing IL-7Rα promoter-driven Cre recombinase (provided by Dr. H.R. Rodewald) (48) and the YFP reporter B6.129X1-Gt(Rosa)
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26So<sup>tm1(EYFP)Cos/J</sup> (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 µg/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 I45-2C11), CD5 (53-1.3), TCRγ (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. After gating lineage (Lin<sup>−</sup>) cells, fluorochrome-conjugated mAbs specific for CD45.2 (30-F1) ST2 (DJ8 or RMST2-2), CD90.2 (30-H12), CD25 (PC61.5), IL-7Rα (A7R34), KLRG1 (2F1/KLRG1), and GATA-3 (LS5–823) were used to characterize lung ILC2s. Other Abs specific for Sca-1 (D7), αβ (DATK32), PLZF (9F12), PD-1 (J43), Ki67 (B56), IL-5 (TRFK5), IL-13 (ebio13A), Siglec-F (E50–2440), CD24 (M1/69) and MHC class II (M5/114.15.2) were purchased from BD Biosciences, BioLegend, 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<sup>+</sup> ILC2s is indicated by †, and in KLRG1<sup>+</sup> 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<sup>+</sup> cells (Fig. 1A), lungs of female mice (~12–14 wk old) contained higher numbers of ST2<sup>+</sup>GATA-3<sup>+</sup> 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<sup>+</sup> (Fig. 1D). At 3 wk of age, female and male mice lungs contained similar numbers of ILC2s, and they were uniformly KLRG1<sup>+</sup> (Fig. 1E). By 12 wk of age, numbers of KLRG1<sup>+</sup> ILC2s in females were significantly increased relative to males, and KLRG1<sup>−</sup> ILC2s began to accumulate. As females aged, numbers of KLRG1<sup>−</sup> ILC2s continued to increase and by 20 wk, approximately equal fractions of ILC2s were KLRG1<sup>−</sup> and KLRG1<sup>+</sup>. In contrast, most male ILC2s were KLRG1<sup>+</sup> and the numbers of KLRG1<sup>+</sup> and KLRG1<sup>−</sup> ILC2s in males remained constant between 3 and 20 wk of age (Fig. 1E).

Next we determined whether lung (KLRG1<sup>+</sup> or KLRG1<sup>+</sup>) 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⁺ and KLRG1⁻ ILC2s 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 ERα 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 (5–30-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-7Rα-cere−/− mice). Cre activity in lung ILC2s was confirmed using a YFP reporter in the Rosa26 locus (Fig. 2B). IL-7Rα-cere−/− mice were analyzed as WT controls. The numbers and frequency of lung ILC2s (both KLRG1+ and KLRG1−) were not significantly different between IL-7Rα-cere−Esr1β/β and IL-7Rα-cere−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+) 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.

FIGURE 2. Elevated androgens in global ERα−/− mice decrease lung ILC2 numbers and diminish the KLRG1− ILC2 subset.

(A) Total numbers of lung ILC2s (with KLRG1+ and KLRG1− subsets indicated by black or white sections, respectively, in the stacked bars) in WT females and males and in ERα−/− females and males. The data shown were compiled from four independent experiments (total n = 8–12 per group) with mice of ages 14–20 wk. The data were evaluated using a one-way ANOVA with a Sidak multiple comparison test for either KLRG1+ and KLRG1− ILC2s, and significance is denoted for KLRG1− ILC2s by # and for KLRG1+ ILC2s by §. §§ p, 0.01, #### p, 0.0001. (B) Representative gating of lung ILC2s (Lin−CD90+ST2+CD25+) in IL-7Rα-cere−Esr1β/β-Rosa26-stopYFP mice. Also shown is YFP expression in the gated ST2+CD25+ cells (open histogram) compared with the same cells in mice lacking IL-7Rα-cere− (filled histogram). (C) Total number of lung ILC2s in IL-7Rα-cere−Esr1β/− mice and IL-7Rα-cere−Esr1+/− controls of age 16 wk. The data shown are representative of three independent experiments (n = 3–4 per group) with mice of ages 12–16 wk. Significance was evaluated using a one-way ANOVA with a Sidak multiple comparison test. (D and E) Representative FACS plots and compilation of the frequency of lung KLRG1+ and KLRG1− ILC2s in multiple WT and ERα−/− females and males with or without ovariectomy (OVX) or castration (CX). Uterine weight was measured in intact and ovarioctomized WT and ERα−/− females to confirm the ovarioctomy; Supplemental Fig. 1D). The data shown are compiled from two to three independent experiments (n = 3–7 per group) with mice of ages 16–19 wk. The data were evaluated using a one-way ANOVA with a Sidak multiple comparison test, except that a Mann–Whitney U test was used for the control and castrated ERα−/− males in (E). The significance is denoted for KLRG1− ILC2s by # and for KLRG1+ ILC2s by §. §§ p, 0.01, #### p, 0.0001. (F) Percentage of Ki67+ ILC2s in naive and castrated (CX) WT males. Symbols indicate individual mice of age 16 wk compiled from three independent experiments. The data were evaluated using a Mann–Whitney U test. **p < 0.01.
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 ovarioctomized females because this reduces the high levels of both androgens and estrogens in female global ERα−/− mice (53, 55). Indeed, ovarioectomy 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 KLRG1+ 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 high levels of androgens in WT males and ERα−/− females and males lead to a decrease in the total numbers of ILC2s, especially reducing the KLRG1− ILC2 subset.

**Lung KLRG1− ILC2s are functional upon ex vivo stimulation**

The absence of KLRG1 on ILC2s has been associated with functional immaturity in BM ILC2s (23). Therefore, we assessed the functional capacity of the abundant subset of lung KLRG1− ILC2s in females. Both the KLRG1+ and KLRG1− ILC2 subsets were capable of producing IL-5 and IL-13 upon ex vivo stimulation with PMA/ionomycin (Fig. 3A, 3B, Supplemental Fig. 1E). Investigation of sex differences in type 2 cytokine production showed that KLRG1− ILC2s were the predominant cytokine producers in mice, whereas both KLRG1+ and KLRG1− ILC2s produced cytokines in females (Fig. 3A, 3B). In females, KLRG1+ and KLRG1− ILC2s were equally capable of IL-5 and IL-13 production upon PMA/ionomycin stimulation (Supplemental Fig. 1F).

**Elevated androgens decrease cytokine production from lung ILC2s**

Next, we determined whether ILC2s in ERα−/− mice showed reduced cytokine production, and whether this was due to elevated androgens. ERα−/− ILC2s in both sexes showed reduced capacity for IL-5 production upon ex vivo stimulation (Supplemental Fig. 1G). Taken together with the reduced numbers of ILC2s, the total number of ILC2s capable of producing IL-5 was significantly lower in ERα−/− compared with WT mice (Fig. 3C). IL-5 production by lung ILC2s in naïve mice regulates eosinophil numbers in homeostasis (56, 57). In agreement with the reduced capacity of ERα−/− ILC2s to produce IL-5, lungs of male and female ERα−/− mice contained fewer eosinophils (Fig. 3D). WT female ILC2s 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 ovarioectomy (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 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 ovarioectomy 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 present in WT females lead to an abundant and functional KLRG1− ILC2 subset.
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 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.

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FIGURE 4. Sex differences in BM ILC2 phenotypes are regulated by androgens.  

(A) Shown are representative FACS plots of BM ILC2s, identified as Lin$^{-}IL-7R^{+}CD90^{+}Sca-1^{+}ST2^{+}$ 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 were 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$^{+}$ 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$^{-}IL-7R^{+}CD90^{+}GATA-3^{+}$) 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, §§, p < 0.001. (D and E) The frequency of KLRG1$^{+}$ BM ILC2s was compared between control and gonadectomized (ovariectomy [OVX] or castration [CX]) WT and ER$^{a-/-}$ 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 ER$^{a-/-}$ 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 ER$^{a-/-}$ (dotted line) mice, relative to binding of an isotype control mAb (shaded histogram). The (Continued)
orchidectomy of Eρ−/− 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 Eρ−/− 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, Supp. Fig. 2C). Eρ−/− 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 Eρ−/− 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, maintenance, and function (23–25). Indeed, male BM Eρ−/− 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 Eρ−/− mice and WT mice (Fig. 4H). Orchidectomy of male global Eρ−/− mice restored BM ILC2 numbers and their GATA-3 expression to WT levels (Fig. 4I, Supplemental Fig. 2C). Taken together, these data suggest that elevated androgens in male Eρ−/− mice lead to BM ILC2s that have only partially upregulated GATA-3, which may explain their reduced numbers and functional capacity. Female Eρ−/− 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 Eρ−/− females were insufficient to reduce GATA-3 expression.

**Elevated androgens in mice with global Eρ−/− 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 Eρ−/− 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 Eρ−/− 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 ILC2s in male Eρ−/− mice is associated with a decreased frequency of ILC2s (Supplemental Fig. 2C). This suggests that ILC2s 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 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 Eρ−/− 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, Eρ−/−, and castrated (CX) Eρ−/− 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 levels of AR than ERα.
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 did lead 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 progression of the ILCP 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 postnatal 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 Ki67 marker of proliferation and express lower levels of the IL-2Rα/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 ILC2s in fetal or adult lungs has not been reported, and we were not able to identify PLZF+ ILC2s 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 androgens may regulate the fetal or neonatal ILC 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 ILCP 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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