

Androgen signaling negatively controls group 2 innate lymphoid cells

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Prevalence of asthma is higher in women than in men, but the mechanisms underlying this sex bias are unknown. Group 2 innate lymphoid cells (ILC2s) are key regulators of type 2 inflammatory responses. Here, we show that ILC2 development is greatly influenced by male sex hormones. Male mice have reduced numbers of ILC2 progenitors (ILC2Ps) and mature ILC2s in peripheral tissues compared with females. In consequence, males exhibit reduced susceptibility to allergic airway inflammation in response to environmental allergens and less severe IL-33-driven lung inflammation, correlating with an impaired expansion of lung ILC2s. Importantly, orchietomy, but not ovariectomy, abolishes the sex differences in ILC2 development and restores IL-33-mediated lung inflammation. ILC2Ps express the androgen receptor (AR), and AR signaling inhibits their differentiation into mature ILC2s. Finally, we show that hematopoietic AR expression limits IL-33-driven lung inflammation through a cell-intrinsic inhibition of ILC2 expansion. Thus, androgens play a crucial protective role in type 2 airway inflammation by negatively regulating ILC2 homeostasis, thereby limiting their capacity to expand locally in response to IL-33.

INTRODUCTION

Type 2 immune responses are characterized by the secretion of IL-4, IL-5, IL-9, and IL-13. This specific cytokine signature is crucial to control parasitic infection but is also associated with allergic reactions. These responses involve the coordinated action of group 2 innate lymphoid cells (ILC2s) and Th2 lymphocytes. The ILC2s are rapid and potent producers of the type 2 cytokines representing an early source of mediators responsible for the initiation of Th2-dependent immune responses and the rapid elimination of intestinal parasites (Eberl et al., 2015; Tait Wojno and Artis, 2016). Indeed, they are widely distributed at mucosal surfaces, where they can be activated by epithelial stress signals such as thymic stromal lymphopoietin, IL-25, and IL-33 (Schmitz et al., 2005; Bartemes et al., 2012; Barlow et al., 2013; Imai et al., 2013; Kim et al., 2013; Salimi et al., 2013). In response to these stimuli, ILC2s secrete large amounts of IL-5, IL-13, and IL-9. The secretion of IL-5 by ILC2s leads to the recruitment and activation of eosinophils and mast cells, whereas IL-13 activates goblet cells and mucus production by epithelial cells (Tait Wojno and Artis,

2016). Moreover, IL-13 production by ILC2s is determinant to mount an effective Th2 cell response by instructing DCs to prime Th2 cells in draining lymph nodes (Halim et al., 2014). ILC2s have therefore recently emerged as critical cells in the initiation of allergic inflammatory responses such as asthma or atopic dermatitis (Halim et al., 2012, 2014; Salimi et al., 2013). They also play a key role in protective immunity against parasitic helminth infection (Moro et al., 2010; Neill et al., 2010) and are associated with metabolic homeostasis (Molofsky et al., 2013). Besides cytokines, additional mediators including the arachidonic acid metabolites leukotriene D4 (LTD4) and prostaglandin D2 (PGD2) appear to be potent regulators of ILC2 function (Doherty et al., 2013; Tait Wojno et al., 2015). However, our current understanding of the homeostatic regulation of ILC2 is limited, and whether other endogenous factors, such as sex steroid hormones, also influence ILC2 responses is currently unknown.

Asthma is a hallmark of type 2 immune response-mediated disease causing chronic inflammation of the airways (Lambrecht and Hammad, 2015). Both clinical observations and murine models revealed that asthma incidence, prevalence, and severity differ according to sex (Townsend

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Abbreviations used: AR, androgen receptor; Cx, castrated; DHT, dihydrotestosterone; ER, estrogen receptor; HDM, house dust mite; ILC2, group 2 innate lymphoid cell; ILC2P, ILC2 progenitor; MGG, May-Grünwald-Giemsa; VAT, visceral adipose tissue.

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et al., 2012). Although males are more susceptible to asthma than females in childhood, the onset of puberty reverses that trend for most allergic disorders (Carey et al., 2007; Almqvist et al., 2008). The drop in asthma incidence observed in and around the time of puberty in males is suggestive of a protective action of male sex hormones (Carey et al., 2007; Almqvist et al., 2008). Along the same line, hypogonadism has been associated with enhanced susceptibility to asthma when compared with men with normal testosterone levels (Mulligan et al., 2006). Although these observations support a protective role for androgens in the susceptibility to allergic asthma, the underlying mechanisms responsible for this effect are unknown.

Based on the role of ILC2s in the induction of airway inflammation (Halim et al., 2012, 2014; Gold et al., 2014), we investigate whether ILC2s could be influenced by sex hormones. In this study, we have unraveled a novel level of regulation of ILC2 responses. Analysis of multiple tissues from both sexes revealed a significantly increased presence of ILC2s in females compared with males. Interestingly, this difference was not caused by an enhancing effect of estrogens in females, but rather to the inhibition of ILC2 maintenance and local expansion in males mediated by androgens. As a consequence, females developed exacerbated lung inflammation in response to house dust mite (HDM) extract or to IL-33 administration. Our results demonstrate that androgen receptor (AR) signaling exerts a critical cell-intrinsic role in inhibiting ILC2 differentiation and the development of ILC2-dependent type 2 inflammatory responses in response to IL-33.

RESULTS AND DISCUSSION

Males are less susceptible than females to allergic airway inflammation

In an experimental model of ovalbumin-induced asthma, airway inflammation is more severe in female mice than in male mice (Melgert et al., 2005). We first investigated whether such a sex bias was also observed in the more physiological setting of HDM-induced allergic airway inflammation, where the initial immunological challenge occurs in the lung in the absence of an adjuvant (Lambrecht and Hammad, 2015). Inflammatory leukocyte numbers found in bronchoalveolar lavage fluid, particularly eosinophils, were higher in female mice than in male mice (Fig. 1 A). This was correlated with enhanced inflammatory infiltrates in the lung of female mice (Fig. 1 B). A similar trend was observed for all the immunological parameters examined. Serum IgE and IgG1 concentrations were strongly up-regulated upon HDM challenge. This effect was more robust in female mice than in male mice, suggesting that exacerbated type 2 immunity preferentially developed in a female sex environment (Fig. 1 C). Indeed, the numbers of Th2 lymphocytes (Fig. 1 D) were markedly higher in the inflammatory lung tissues of female mice than in males. Thus, these data revealed a strong female sex bias for all the cardinal features of HDM-induced airway inflammation.

Sex bias in steady-state ILC2 numbers is controlled by male androgen hormone

As ILC2s have been previously reported to play a critical role in priming Th2 responses in the HDM-induced asthma model (Gold et al., 2014), we next examined whether the ILC2 distribution in various organs was subjected to sex differences at steady-state. In the lungs, the frequencies and total numbers of ILC2s were twofold higher in females than in males (Fig. 2 A). These differences in ILC2 numbers were accompanied with changes in the expression of phenotypic markers. ILC2s from male mice showed an increase in the expression of KLRG1 and IL-33 receptor (ST2; Fig. 2, B and C), whereas CD25, the high-affinity α chain of the IL-2 receptor, was similarly expressed between both sexes (Fig. 2 A). KLRG1 interactions with E-cadherin expressed on epithelium have been shown to alter ILC2 function and act as a suppressive mechanism to dampen down the ILC2 responses (Salimi et al., 2013). Thus, our data suggest that both developmental and phenotypic differences occur between sexes. Furthermore, these differences in ILC2 extended to other tissues in which ILC2 dominate such as the visceral adipose tissues (VATs; Fig. 2 D) and mesenteric lymph nodes (Fig. 2 E). In VATs, ILC2s are critical regulators of metabolic homeostasis, and their loss leads to adiposity and insulin resistance when animals are placed on a high-fat diet (Molofsky et al., 2013). Our results showing reduced ILC2 numbers in male VATs are compatible with the observation that males are more vulnerable to high-fat diet-induced weight gain in terms of onset or magnitude (Hwang et al., 2010).

We next investigated the mechanisms that might underpin these sex differences in ILC2s and determined whether endogenous estrogens and estrogen receptor (ER) signaling were involved in lung ILC2 up-regulation. In ovariectomized mice, we could not detect significant changes in the frequency of lung ILC2s (Fig. 2 F). To exclude any potential role of estrogen signaling on ILC2 development or maturation, we analyzed the presence of ILC2s in mice that specifically lack ER α expression in the hematopoietic lineages. We found similar frequencies of lung ILC2s between ER $\alpha^{\text{flox/flox}} \times \text{Tie2-Cre}$ mice and their ER $\alpha^{\text{flox/flox}}$ control littermates (Fig. 2 G). In contrast, a significant up-regulation in ILC2 frequency was observed in the lungs of castrated (Cx) males (Fig. 2 H), indicating that male androgen hormones, rather than estrogens, were involved in the homeostatic regulation of ILC2 at mucosal surfaces.

Male sex hormones blunt IL-33-driven ILC2 expansion and lung inflammation in vivo

Systemic administration of IL-33 induces the proliferation of ILC2 and secretion of cytokines such as IL-5 and IL-13, resulting in lung inflammation (Neill et al., 2010). We therefore examined whether sex differences exist in lung inflammation induced by administration of human IL-33_{95–270}, as described previously (Lefrançois et al., 2014). In this setting, IL-33 strongly increased the number and frequency of ILC2s

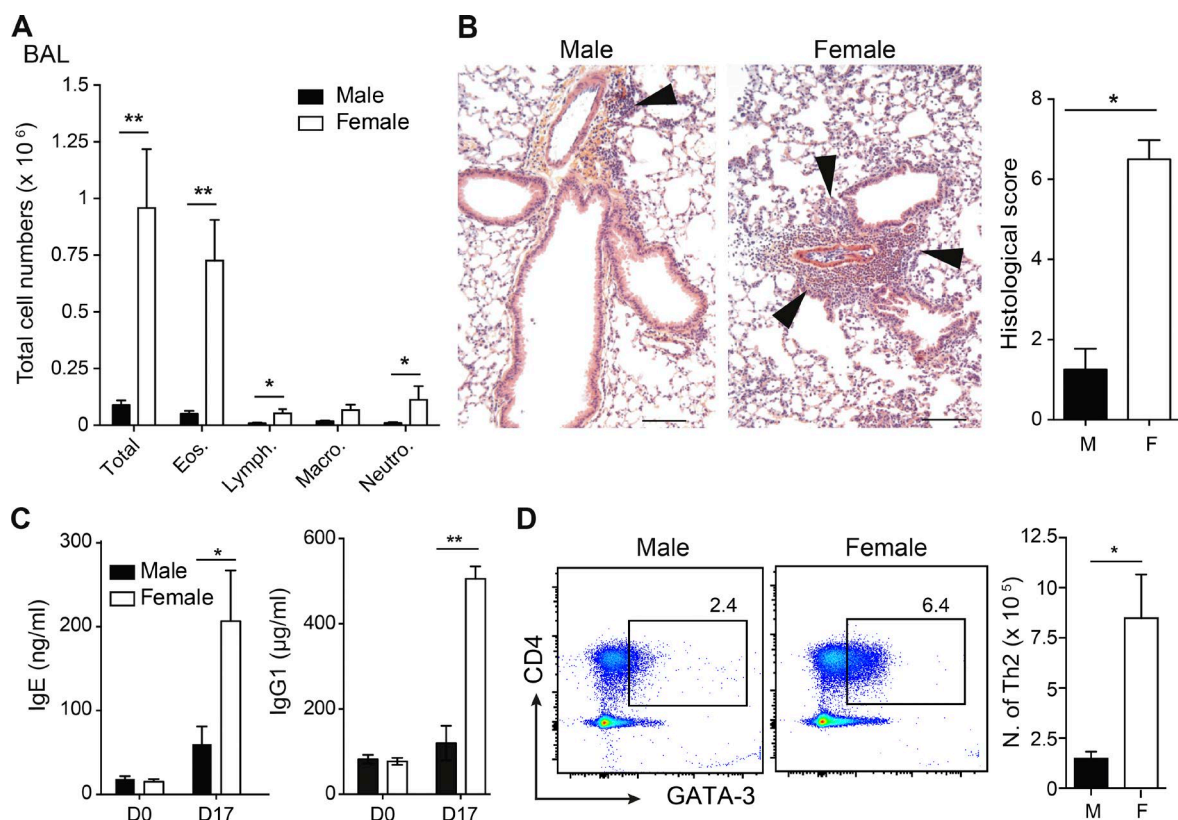


Figure 1. Male mice develop less severe HDM-induced allergic asthma than female mice. (A) Quantification of leukocyte populations present in the bronchoalveolar lavage fluid of HDM-challenged male or female mice 17 d after treatment using MGG staining. The total number of each leukocyte subset is shown ($n = 5$ mice/group). (B) Lung tissue sections from HDM-treated male and female mice stained with H&E (left; bars, 100 μ m) and quantification of lung inflammation (left [arrowheads] and right) showing the mean \pm SEM. H&E-stained representative sections of five mice/group were analyzed. (C) Quantification of serum concentration of IgE and IgG1 by ELISA at the indicated time points ($n = 5$ mice/group). (D) Enumeration of lung-infiltrating Th2 lymphocytes (singlet, live, CD45⁺CD4⁺GATA-3⁺). Histogram shows the total number of Th2 cells ($n = 5$ mice/group). Error bars indicate the mean \pm SEM. Comparison between groups was calculated using the unpaired Student's *t* tests. *, $P \leq 0.05$; **, $P < 0.01$. Data are representative of two independent experiments. F, female; M, male.

in the lungs of treated mice compared with PBS-injected control mice (Fig. 3, A and B). ILC2 frequencies and numbers were however approximately twofold higher in female mice than in male mice. The frequency of lung ILC2s that positively stained with dead cell-discrimination dye and/or Annexin V was also determined in control and IL-33-treated mice (Fig. S1). As expected, the numbers of dead cells and apoptotic cells were significantly increased in lung ILC2s from IL-33-treated mice as compared with untreated controls (Fig. S1). However, no differences in cell death and apoptosis were observed between lung ILC2s isolated from female or male mice, both at steady state or upon IL-33 administration (Fig. S1). Thus, enhanced apoptosis and/or impaired survival does not seem to account for the sex differences in ILC2s at steady state and during inflammation.

Interestingly, Cx males responded as intact females, indicating that endogenous male sex hormones act as critical regulators of IL-33-driven ILC2 expansion in vivo (Fig. 3, A and B). In IL-33-injected mice, there was a marked up-regulation (>10 -fold) in the frequency of IL-5⁺IL-13⁺

ILC2s as compared with control mice (Fig. 3 C). Despite the lack of significant differences in the proportion of cytokine-producing ILC2s among males, females, and Cx males (Fig. 3 C), the absolute numbers of IL-5⁺IL-13⁺ ILC2s were dramatically increased in females and Cx males compared with intact male mice (Fig. 3 D). This exacerbated ILC2 response was correlated with a massive infiltration of inflammatory cells around the bronchi and pulmonary vessels (Fig. 3 E). The histological scores in females were significantly higher than in intact males. This difference was abrogated in Cx males, in which disease scores were similar to females (Fig. 3 F). Collectively, these results show that ILC2-mediated lung inflammation is more severe in female mice than male mice and that orchietomy abolishes these differences.

Androgens restrain ILC2 development at steady state

As ILC2s develop from the ILC2 progenitor (ILC2P) in the bone marrow (Hoyler et al., 2012), we compared ILC2P frequency between sexes. The absolute number and proportion

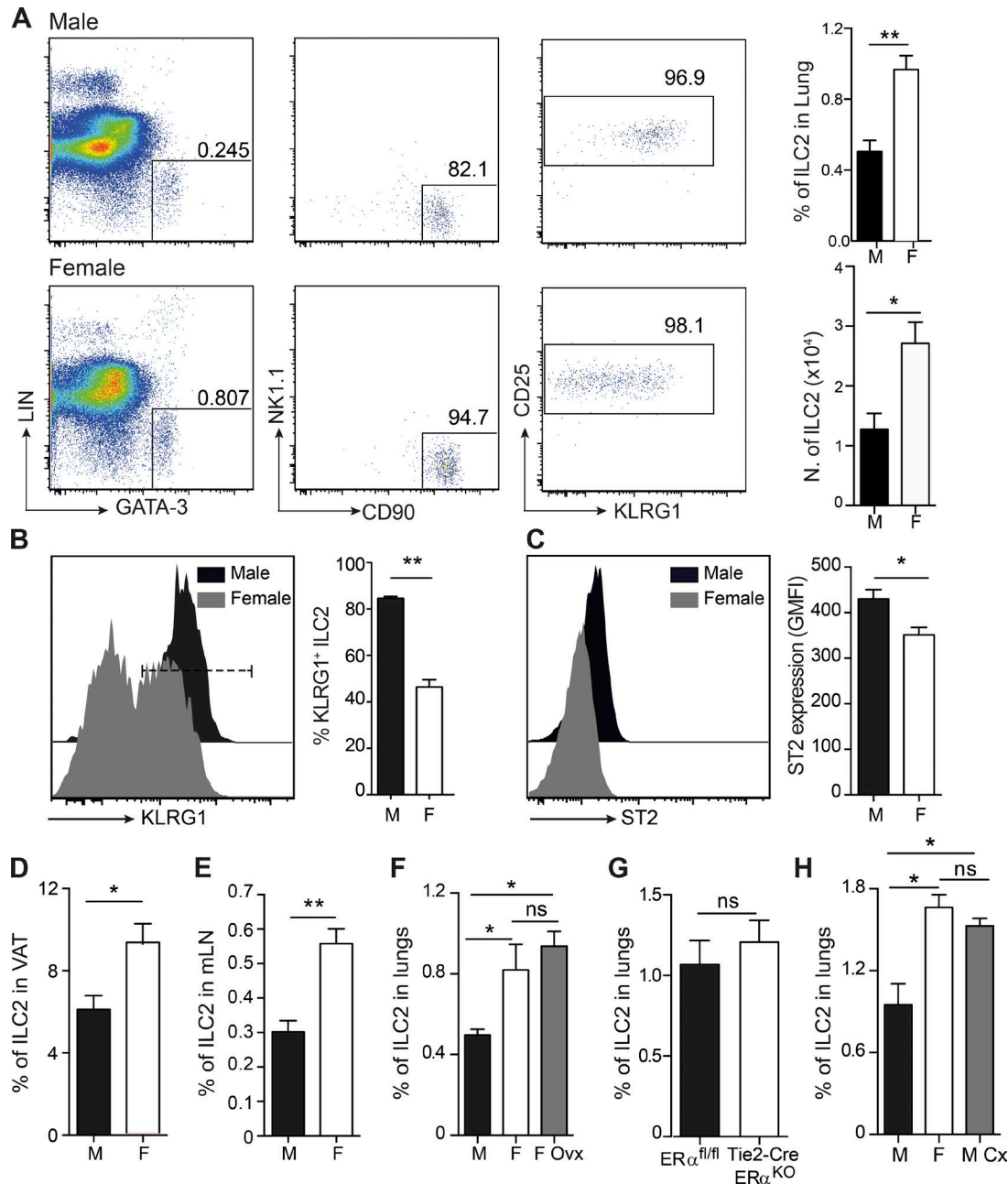


Figure 2. Male hormones inhibit ILC2 development at steady state. (A) Flow cytometry analysis of lung ILC2s defined by lack of expression of lineage marker (LIN: CD3e, TCR β , CD11b, CD19, B220, Gr1, CD11c) and NK1.1, and expression of GATA3, CD90, and CD25 in naive male and female mice (pregate: singlet, live, CD45⁺ cells). Representative dot plots (left) are shown together with results from one representative experiment out of eight. Bar graphs show data as means \pm SEM ($n = 4$ mice/group). (B and C) Expression of KLRG1 (B) and ST2 (C) on ILC2s. Histograms show the frequency of KLRG1-expressing ILC2s and the geometric mean fluorescence intensity of ST2 on ILC2s ($n = 5$ mice/group). Data are representative of six experiments. (D and E) ILC2 frequency in VAT and mesenteric lymph nodes (mLNs) of male and female mice. (F) ILC2 frequency in the lung of male, female, and ovariectomized (Ovx) female mice. (G) ILC2 frequency in the lung of ER $\alpha^{fl/fl}$ \times Tie2-Cre female mice and their littermate control ER $\alpha^{fl/fl}$ mice. (H) ILC2 frequency in the lung of male, female, and Cx male mice. (D–H) Bar graphs show data as means \pm SEM ($n = 5$ mice/group). Data are representative of two to four experiments. Comparison between groups was calculated using the unpaired Student's t tests. *, $P < 0.05$; **, $P < 0.01$; ns, not significant. F, female; M, male.

of ILC2Ps (identified as Lin[−]Sca-1⁺CD127⁺CD25⁺) were two- to threefold higher in females than in males (Fig. 4, A and B), whereas the total cell numbers in the bone marrow

were similar between both sexes (not depicted). Thus, the increased number and frequency of ILC2s in peripheral tissues in female mice was correlated with a rise in ILC2Ps in the

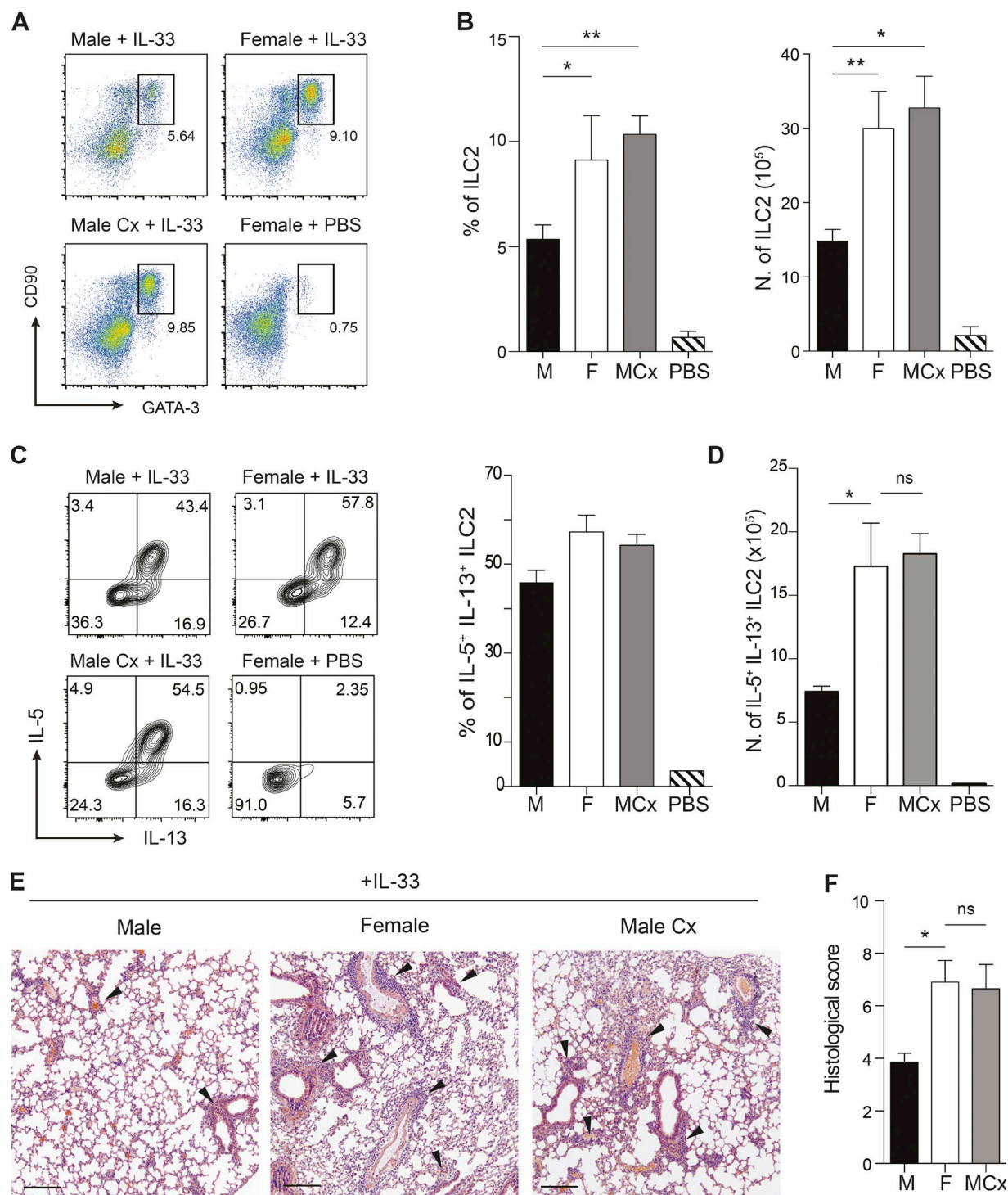


Figure 3. Male castration abolishes sex differences in IL-33-mediated lung inflammation. Male, female, and Cx male mice were injected i.p. with huIL-33 (4 μ g/mouse/day) on days 0, 1, 4, 5, and 6 and analyzed on day 7. Control mice i.p. received PBS. (A) Flow cytometry staining of pulmonary ILC2s (CD90⁺GATA-3⁺) pregated on singlet, live, CD45⁺, CD3⁻, CD19⁻, NK1.1⁻ cells. (B) Frequency and total number of ILC2s in the lungs. (C and D) Intracellular expression of IL-5 and IL-13 in lung ILC2s from the indicated mice. (C and D) Frequency (C) and total number (D) of ILC2s producing IL-5 and IL-13 are shown. (E) H&E staining of 4- μ m lung sections (bars, 200 μ m). Arrowheads indicate sites of leukocyte infiltration. (F) Histological score of lung inflammation. Data from five mice/group are shown. Comparison between groups was calculated using the unpaired Student's *t* tests. Error bars indicate the mean and SEM. *, *P* \leq 0.05; **, *P* \leq 0.01; ns, not significant. Data are representative of two independent experiments. F, female; M, male.

bone marrow. Contrasting with the lack of differential expression of CD25 between male and female tissue-resident ILC2s, ILC2Ps from female mice expressed a significantly higher level of IL2R α than ILC2Ps from male mice (Fig. 4 C). To test whether sex-linked factors affect ILC2 proliferation at steady state, we examined intracellular expression of Ki67 in ILC2Ps and lung ILC2s isolated from male or female mice. Whereas Ki67⁺ cells were detected at low frequency in ILC2Ps and ILC2s from male mice (ranging from 1–5%), a significantly higher proportion of ILC2Ps (Fig. 4 D) and lung ILC2s (Fig. 4 E and Fig. S2) positively stained for Ki67 in female mice (7–15%). As male sex hormones, rather than estrogens, were shown to negatively control ILC2s (Fig. 2), we measured the expression profile of sex-steroid hormone receptors. Accordingly, ILC2Ps primarily expressed transcripts encoding for AR, whereas those encoding for the ER (*Esr-1* or *Esr-2*) genes were almost undetectable (Fig. 4 F). To test whether androgen signaling influences ILC2 development, ILC2Ps were cultured with IL-33 in the absence or presence of dihydrotestosterone (DHT) or the AR antagonist flutamide (Fig. 4, G and H). Interestingly, addition of DHT to the culture inhibited ILC2 differentiation after 10 d (Fig. 4 H). On the contrary, flutamide increased the numbers of ILC2 (~1.3–1.7-fold) that differentiated after 7 to 10 d of culture (Fig. 4 H). Of note, KLRG1 expression on developing ILC2 was down-regulated in flutamide-treated cultures (Fig. 4 I), indicating that AR-signaling controls KLRG1 expression in male ILC2 as shown in Fig. 2. Thus, androgen actively represses ILC2 development through AR-signaling in ILC2Ps.

AR negatively controls IL-33-driven ILC2 expansion and lung inflammation

To evaluate the role of the AR in hematopoietic cells on the sex bias in ILC2-mediated lung inflammation, we generated bone marrow chimeras. Lethally irradiated C57BL/6 mice, from either sex, were engrafted with bone marrow cells from WT or AR-deficient (AR^{KO}) littermate male mice, and lung inflammation induced by IL-33 injection was monitored as described in Fig. 3. The absence of AR in the hematopoietic compartment abolished the sex differences in the total numbers of inflammatory cells, including eosinophils, in bronchoalveolar lavage fluid (Fig. 5 A). Lung-infiltrating cells were significantly reduced in WT chimeric males compared with females, but this sex difference was absent in AR-deficient bone marrow chimeras (Fig. 5 B). ILC2 numbers were higher in the lungs of male mice engrafted with bone marrow cells from AR^{KO} rather than WT mice (Fig. 5 C). KLRG1 expression was again significantly down-regulated on AR-deficient ILC2s, confirming that the differences observed between males and females in ILC2 development and phenotype are dependent on AR signaling (Fig. 5 D), in agreement with the *in vitro* data in Fig. 4 I. This enhanced responsiveness to IL-33-driven lung inflammation in AR-deficient males was further confirmed by histological evaluation (Fig. 5, E and F). In contrast, AR deficiency in hematopoietic cells had no

significant impact on lung inflammation in female chimeric mice (Fig. 5, E and F). To determine whether the deficiency in ILC2-driven lung inflammation observed in AR^{KO} male was cell intrinsic, mixed bone marrow chimera experiments were performed. Lethally irradiated CD45.1 male mice were transplanted with an equal mixture of bone marrow cells from CD45.1 WT and CD45.2 AR^{KO} mice. After 6 wk of reconstitution, the ratio between CD45.1⁺ (WT) and CD45.1^{neg} (AR^{KO}) lung ILC2s was examined after IL-33 injection (Fig. 5, G and H). We observed a significant deficiency in the proportion of WT ILC2s (CD45.1⁺) compared with ILC2s derived from AR^{KO} progenitors (CD45.1^{neg}; Fig. S3 A), resulting in a WT/AR^{KO} ILC2 ratio <0.2 (Fig. 5 G). For comparison, the WT/AR^{KO} ratios for total CD45⁺ lung infiltrating cells or lung eosinophils were >0.7 (Fig. 5 G and Fig. S3 C), yet the frequencies of Ki67⁺ cells and dead cells were similar between WT and AR-deficient ILC2s (Fig. S3, B and D). Interestingly, in agreement with results in Fig. 5 D, the reduced expression of KLRG1 in AR-deficient ILC2s was maintained in the mixed chimeras (Fig. 5 H). Altogether, these results indicate that endogenous androgens signal through hematopoietic ARs in a cell-intrinsic manner to control the level of ILC2 responsiveness in the lung. As ILC2 survival and proliferation were not affected by AR deficiency, these results suggest a major effect of androgen signaling on the regulation of tissue-resident ILC2 numbers at steady-state.

The prevalence of asthma is consistently reported to be higher in boys than in girls (Carey et al., 2007; Almqvist et al., 2008). This pattern changes during adolescence, where the onset of asthma becomes much less prevalent in males than in females. However, the mechanism underpinning this transition is unknown. Our results indicate that androgen-mediated regulation of ILC2-dependent Th2 responses to environmental allergens could contribute to this switch around puberty. At steady-state, we observed a strong sex bias in ILC2 numbers, with an increased representation of ILC2s in various tissues, including lung, fat, and mesenteric lymph nodes. This was associated with a strong up-regulation of ILC2Ps in the bone marrow of female compared with male mice. Accumulating evidence indicates that male androgens are immunosuppressive, whereas female estrogens may promote innate and adaptive immunity (Markle and Fish, 2014). We excluded a positive impact of estrogens in this sex bias, as ovariectomy or selective ablation of *Esr-1* gene in the hematopoietic compartment had no effect on the number and phenotype of lung ILC2s at steady state. In contrast, orchiectomy abolished this sex difference, indicating that endogenous androgens may negatively regulate ILC2 development and/or expansion *in vivo*. Analysis of sex hormone receptor expression in ILC2Ps demonstrated a selective expression of *Ar* gene mRNA, whereas those encoding for the ERs *Esr-1* or *Esr-2* were barely detected. This observation is in agreement with recent studies showing that *Ar* is highly expressed in tissue-resident ILC2s and has been classified as a prototypic ILC2 signature gene (Robinette et al., 2015), highly conserved

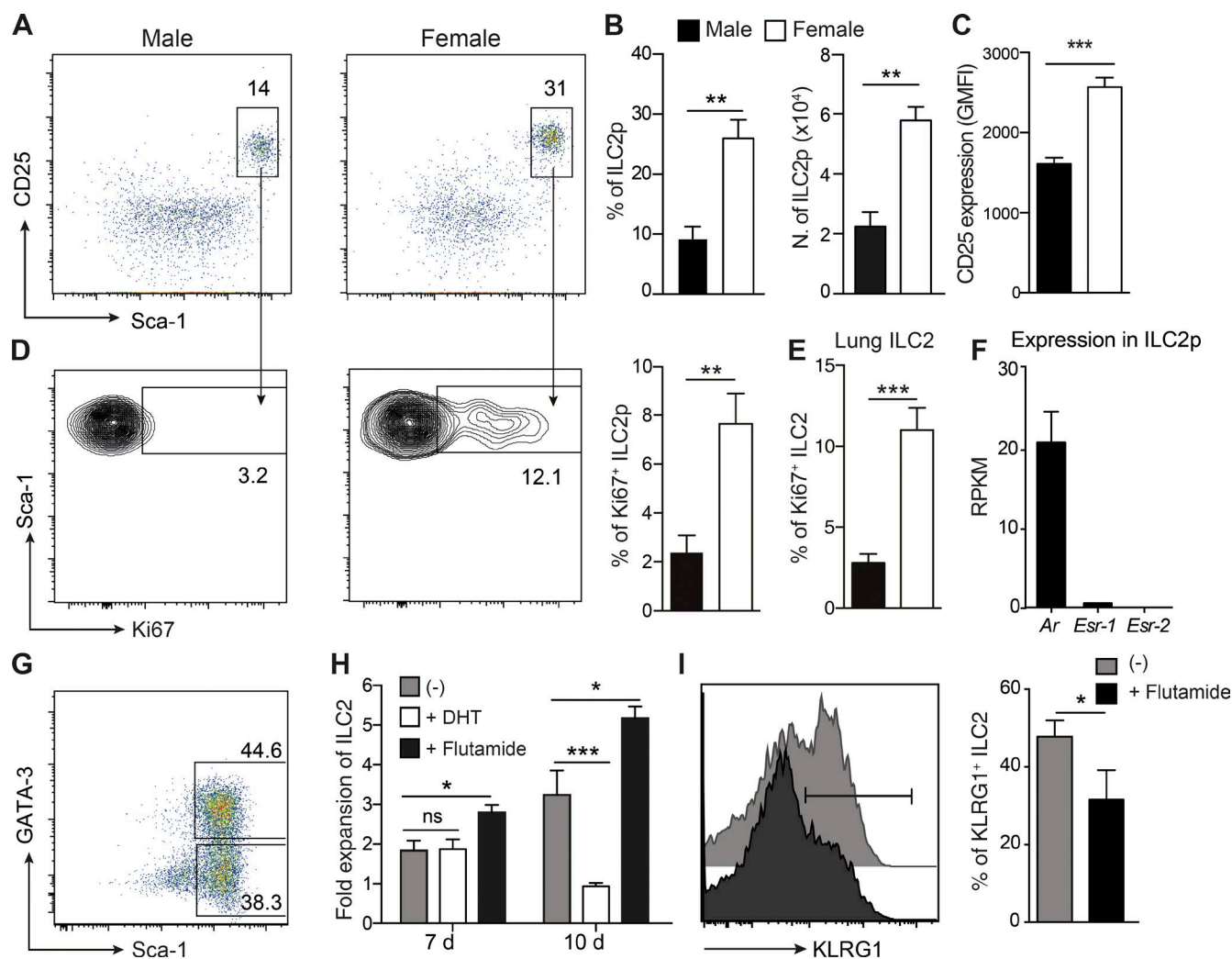


Figure 4. ILC2P numbers are reduced in male mice, whereas AR blockade promoted ILC2 development in vitro. (A) Flow cytometry analysis of ILC2Ps in bone marrow cells from male and female B6 mice. ILC2Ps (CD25⁺Sca-1⁺) were gated as SingletLiveLin⁺CD127⁺. (B) Frequency and total number of ILC2Ps between male and female mice. (C) Geometric mean fluorescence intensity (GMFI) of CD25 on ILC2Ps. (D) Frequency of Ki67⁺ ILC2Ps in the bone marrow. (E) Frequency of Ki67⁺ ILC2s in the lung ($n = 4-5$ mice/group). Results are representative of three independent experiments performed. (F) Expression (RPKM [reads per kilobase of exon model per million mapped read values]) of sex hormone receptor mRNA expression in female ILC2Ps by RNA sequencing. (G-I) In vitro differentiation of ILC2Ps on OP9-DL1 feeder cells in complete medium supplemented with mouse IL-33 in the presence or absence of 1 nM DHT or 100 nM of the AR antagonist flutamide. (G) Flow cytometric analysis of ILC2s after 7 d of differentiation. (H) Quantification of ILC2 expansion expressed as the fold increase as compared with ILC2P input in quadruplicate cultures. (I) Representative histograms (left) and quantification (right) of KLRG1 expression on differentiated ILC2s in quadruplicate cultures. Data representative of three to four experiments are shown. Error bars indicate the mean \pm SEM. Comparison between groups was calculated using the unpaired Student's *t* tests. *, $P \leq 0.05$; **, $P < 0.01$; ***, $P < 0.001$. F, female; M, male.

during evolution (Vivier et al., 2016). In favor of a unique role for the male sex hormone androgen in the regulation of ILC2-dependent responses, we showed that orchietomy or AR deficiency in hematopoietic cells both abolished all phenotypic changes in IL-33-mediated lung inflammation. These results strongly suggest that ligand-induced activation of AR within ILC2s is the main signaling pathway contributing to the sex differences in this model. Indeed, we provided evidence for AR-dependent regulation of IL-33-driven ILC2 differentiation and expansion in vitro and demonstrated using

mixed chimeras the cell-intrinsic requirement of AR for the inhibition of IL-33-mediated ILC2 expansion in vivo.

Although previous studies suggested that female sex hormones might contribute to the sex differences in allergic asthma (Keselman and Heller, 2015), our results are the first to establish that male androgen hormones protect from ILC2-driven lung inflammation. Developing ILC2s themselves or their progenitors seems to represent the direct target of androgens. Cell-intrinsic AR signaling in ILC2Ps appears to promote the development of KLRG1^{hi} ILC2s, which are less

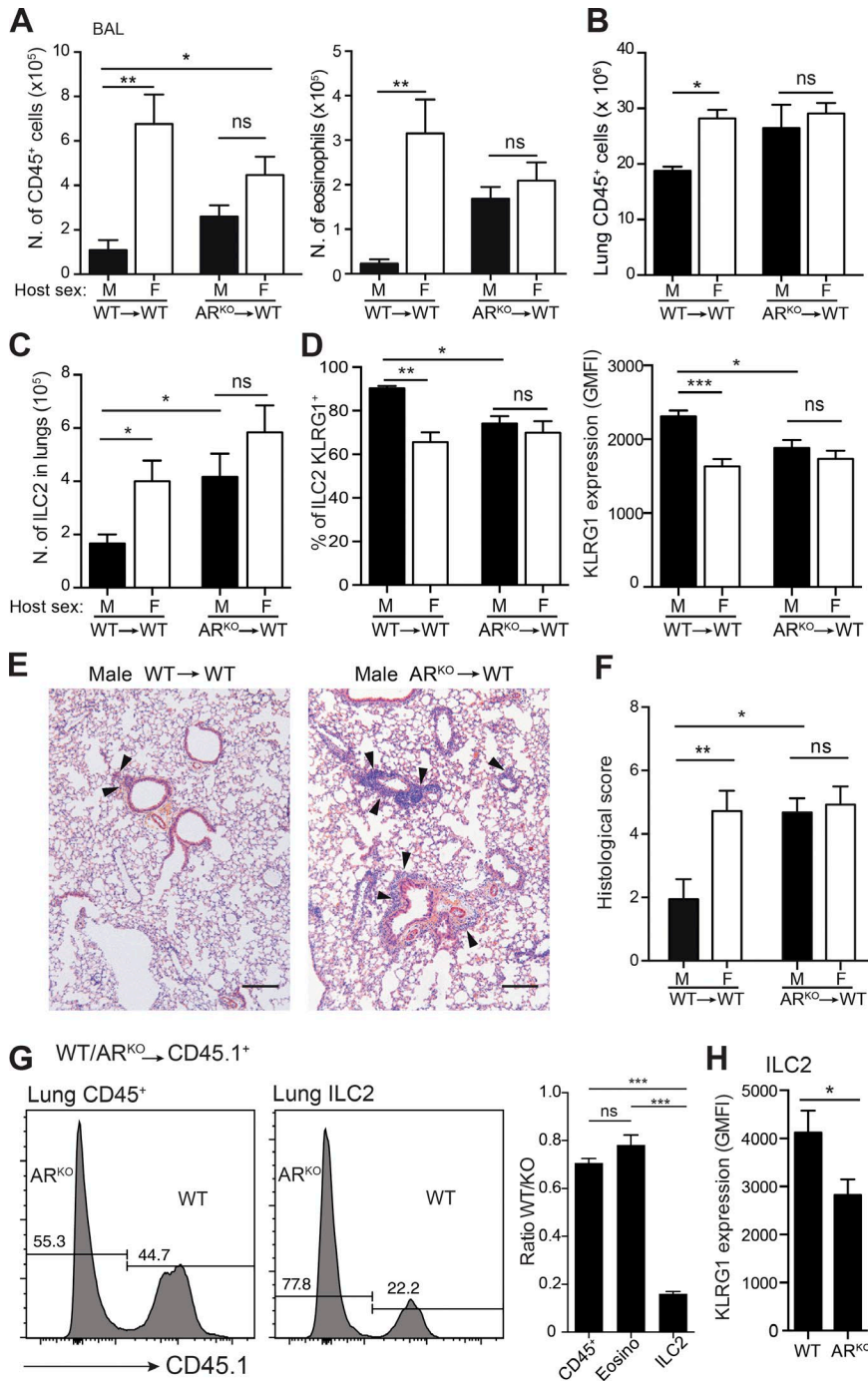


Figure 5. AR deficiency abrogates sex differences in ILC2-dependent lung inflammation. Bone marrow chimeras reconstituted with cells either from AR^{KO} male B6 mice or their littermate WT controls were injected with huIL-33 as in Fig. 3 at 8 wk after reconstitution, and the inflammatory response was analyzed. (A) Quantification of total leukocyte populations (MGG staining; left) and eosinophils (right) present in the bronchoalveolar lavage fluid of IL-33-injected male or female chimeric mice as indicated. (B) Quantification of leukocytes infiltrating the lungs of chimeric mice. (C) Total numbers of ILC2s (SingletLiveCD45⁺Lin[−]CD90⁺GATA3⁺). (D) Frequency of KLRG1-expressing ILC2 (left) and geometric mean fluorescence intensity (GMFI) of KLRG1 on ILC2s. (E) H&E staining of lung tissue sections from chimeric mice (bars, 100 μ m). Arrowheads indicate sites of leukocyte infiltration. (F) Histological score of lung inflammation. Data from five to six mice/group are shown and are representative of two experiments performed. (G) Lethally irradiated CD45.1⁺ male recipient hosts were i.v. reconstituted with an equal mixture of CD45.1⁺ WT and CD45.2⁺ AR^{KO} bone marrow cells. At 6 wk after reconstitution, mice were i.p. injected with huIL-33 as described in Fig. 3, and the relative frequency of WT and AR-deficient lung ILC2 was assessed as shown in Fig. S3. The ratio of CD45.1⁺ to CD45.2⁺ cells in total lung-infiltrating CD45⁺ cells, eosinophils (CD45⁺Siglec-F⁺), and ILC2s are shown. (H) Geometric mean fluorescence intensity of KLRG1 on ILC2. Data from five to seven mice/group are shown and are representative of two experiments performed. Comparison between groups was calculated using the unpaired Student's *t* test or ANOVA. Error bars indicate the mean and SEM. *, *P* \leq 0.05; **, *P* < 0.01; ***, *P* < 0.001; ns, not significant.

frequent in female mice or Cx males. These cells, however, are unlikely to represent the KLRG1^{hi} inflammatory ILC2 population, which is mobilized by IL-25 or infection, as these cells, unlike steady-state ILC2s, lacked ST2 and did not proliferate in response to IL-33 (Huang et al., 2015). Thus, we propose that androgen signaling may directly regulate the development of natural ILC2s. However, as very few ILC2s develop from bone marrow progenitors in adult mice (Gasteiger et al., 2015), we believe that most of the sex differences observed in

the IL-33-driven inflammation model are already imprinted by the preexisting sex bias in tissue-resident ILC2s reflected in the steady-state numbers. Enhanced CD25 expression in female ILC2Ps correlated with an increased frequency of proliferating (Ki67⁺) cells, suggesting that greater sensitivity to IL-2 in female ILC2Ps could promote ILC2 seeding in nonlymphoid tissues during ontogeny. This hypothesis is however not supported by recent findings demonstrating that CD25-deficient tissue-resident ILC2s proliferated to a similar

extent as WT ILC2s in bone marrow chimeric mice, both at steady state and during acute helminth infection (Gasteiger et al., 2015). Although the environmental cues that orchestrate the sex bias in tissue-resident ILC2 at steady-state still need to be identified, we cannot exclude that androgen signaling might also act by altering the IL-2/CD25-dependent regulation of ILC2 effector function during inflammation (Gasteiger et al., 2015).

AR is a ligand-dependent transcription factor, which could directly or indirectly regulate the expression of key transcription factors or molecules involved in the maintenance of ILC2Ps or essential for their differentiation into ILC2s. Such specific downstream targets of ARs in ILC2Ps remain to be identified. Alternatively, AR signaling may regulate the IL-33 signaling machinery via the down-regulation of NF- κ B or AP-1 pathways, resulting in reduced cell proliferation. Studies of the T cell differentiation pathways suggested that testosterone inhibited CD4 T cell differentiation by up-regulating the phosphatase Ptpn1, which dephosphorylates Tyk2, the upstream kinase responsible for IL-12-induced Stat-4 phosphorylation, thereby inhibiting Th1 polarization (Kissick et al., 2014). Whether altered IL-33 signaling occurs in androgen-stimulated ILC2s will require further investigation.

Our study suggests that androgen therapy could be useful to protect against allergic diseases through its inhibitory action on ILC2s. Androgen replacement therapy is currently used for the treatment of subjects with deficient testicular function, and administration of dehydroepiandrosterone, a weak androgen with fewer virilizing side effects, has shown some beneficial actions in the treatment of allergic asthma in humans (Shah, 2004; Wenzel et al., 2010) and mice (Liou and Huang, 2011). Understanding further how AR signals in ILC2s or their progenitors may provide new therapeutics or downstream targets for the treatment of allergic diseases while minimizing the side effects of androgen therapy.

MATERIALS AND METHODS

Mice

Female and male C57BL/6JRJ (B6) mice were purchased from the Centre d'Élevage R. Janvier or the Walter and Eliza Hall Institute of Medical Research. Congenic C57BL/6-Ly5.1 (CD45.1) mice were purchased from Charles River. Mice were used at 8–12 wk old unless otherwise stated. Bilateral orchiectomy and ovariectomy were performed in anesthetized 4–5-wk-old mice. Mice were then rested for a period of 5–6 wk before use. Mice selectively lacking ER α in the hematopoietic compartment (ER $\alpha^{\text{fl/fl}}$ \times Tie2-Cre) have been described elsewhere (Lélu et al., 2011). Mice lacking AR have been described previously (Sato et al., 2004). AR $^{\text{L-/+}}$ females on a B6 background, bearing one AR-null allele, were bred with WT B6 males to produce AR $^{\text{KO}}$ (AR $^{\text{L-/-}}$) and AR $^{+/y}$ male mice. AR $^{\text{L-/+}}$ and ER $\alpha^{\text{fl/fl}}$ mice were provided by S. Kato (Iwaki Meisei University, Iwaki, Japan) and P. Chambon (IGBMC, Illkirch-Graffenstaden, France), respectively. For the generation of irradiation bone marrow chimeras, mice

were γ -irradiated (11 Gy, ^{137}Cs source) and engrafted with 5×10^6 to 10×10^6 bone marrow cells/mouse. Mice were used 6–8 wk after reconstitution. Mice were housed in specific pathogen-free conditions and handled in accordance with the Animal Care and Use of Laboratory Animal guidelines of the French Ministry of Research (study approval number 05187.01), the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes guidelines and were approved by the Walter and Eliza Hall Institute Animal Ethics Committee.

HDM-induced allergic asthma and IL-33 injection

HDMs (*Dermatophagoides pteronyssinus* extracts; Greer Laboratories) were used to induce allergic airway inflammation. Mice were sensitized intranasally with 1 μg HDM extracts on day 0 and subsequently challenged with 10 μg /mouse on days 7–11. Six days after the last challenge, lungs, bronchoalveolar lavage fluid, and serum were collected for flow cytometry, histological analysis, and ELISA. Human recombinant IL-33 injections were performed as described previously (Lefrançois et al., 2014), with some modification. In brief, mice were treated intraperitoneally with 4 μg recombinant human IL-33 $_{95-270}$ for two consecutive days and then left untreated for 2 d and injected again for three consecutive days. 24 h after the last injection, bronchoalveolar lavage fluid and lungs were collected for flow cytometry and histological analyses. Recombinant human IL-33 $_{95-270}$ was produced as described previously (Lefrançois et al., 2014).

Tissue preparation

Lungs were cut into small fragments and digested for 30 min at 37°C with 1 mg/ml Collagenase III (Worthington Biochemical Corporation) and 200 μg /ml DNase I (Sigma-Aldrich). Red blood cells were lysed by treatment with hypotonic solution and then filtered. Perigonadal adipose tissue was used as representative VAT. Adipose tissue was finely dissected with a scalpel blade and digested in 3 ml PBS containing 0.2 mg/ml Collagenase III (Worthington Biochemical Corporation) and 4% BSA at 37°C for 30 min with gentle agitation. Digests were filtered through 70- μm sterile cell strainers and centrifuged at 800 g for 15 min to enrich for immune cells in stromal vascular fractions. Single-cell suspensions were blocked with PBS containing 5 μg /ml anti-CD16/CD32 (2.4G2) and stained for 30 min on ice with fluorophore-conjugated antibodies. The following antibodies, purchased from BD, were used for the identification and purification of ILC2s: CD19 (ID3), B220 (RA3-6B2), CD3 (145-2C110), CD4 (GK1.5), CD11b (M1/70), CD11c (HL3), Gr-1 (RB6-8C5), TCR β (H57-597), NKp46 (29A1.4), NK1.1 (PK136), CD45.1 (A20), CD45.2 (104), CD117 (2B8), CD127 (A7R34), Sca1 (E13-161.7), KLRG1 (2F1), Thy1.2 (30H12), and ST2 (DJ8 or RMST2-2). Intracellular staining was performed using the Transcription Factor Staining Buffer Set (eBioscience) and monoclonal antibodies to Gata-3 (TWAJ), IL-13 (eBio13A), and IL-5 (TRFK5). Intracellular cytokine staining for IL-13 and IL-5

was performed after stimulation for 4 h with 50 ng/ml PMA and 100 ng/ml ionomycin in the presence of 1 μ g/ml Brefeldin A. Ki67-staining analysis (BD) and Annexin V staining analysis (eBioscience) were performed according to the manufacturer's protocol. Cells were analyzed using a Fortessa (BD), and FlowJo software (Tree Star) was used for analysis. Flow cytometric sorting was performed with a FACS Aria (BD).

ILC2 differentiation

To induce ILC2 differentiation *in vitro*, cell-sorted ILC2Ps from female bone marrow cells were cultured for 7 d in α -MEM complete medium (10% heat-inactivated FCS) on OP9-DL1 with 10 ng/ml each of SCF, IL-7, and IL-33. 5- α DHT (Sigma-Aldrich) or the AR antagonist flutamide (Sigma-Aldrich) were diluted in methanol, respectively at 10^{-2} M and 10^{-3} M, to generate a stock solution and then added at the indicated concentration on day 0, +4, and +7 of culture.

Hormone receptor expression determined by RNA sequencing

RNA was isolated from *ex vivo* ILC2Ps from female WT bone marrow using the QIAGEN RNeasy Micro kit. Libraries were generated using the Illumina Truseq RNA sample preparation kit following the manufacturer's instructions and subjected to a transcriptome 75-bp paired-end sequencing on an Illumina Next-Seq instrument. Sequence reads were aligned to the GRCm38/mm10 build of the *Mus musculus* genome using the Subread aligner (Liao et al., 2013). Only uniquely mapped reads were retained. Genewise counts were obtained using featureCounts (Liao et al., 2014). RNA sequencing data have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus and are accessible under accession number GSE97354.

Lung histology and May-Grünwald-Giemsa (MGG) staining

Lung tissue was fixed in 10% buffered formalin for 24 h and then placed in ethanol 70% before embedding in paraffin. 4- μ m sections were stained with hematoxylin and eosin (H&E). Histological disease scores from 0 to 3 were attributed based on the severity of peribronchial, perivascular, and interstitial immune cell infiltration, together with thickening of peribronchial epithelium, resulting in a maximum score of 12. MGG staining of BAL cells were performed using a standard procedure. All scores were attributed on a blinded basis.

ELISA

For serum IgG1 quantification, rat anti-mouse IgG1 (LO-MG1-13; AbD Serotec) antibody was used for coating and rat anti-mouse/HRP (LO-MK-1; AbD Serotec) was used for the secondary detection step. For IgE, rat anti-mouse IgE (LO-ME-3; Serotec) antibody was used for coating. Biotin-conjugated rat anti-mouse IgE mAb (BD) and streptavidin-HRP conjugate (GE Healthcare) were used for detection. Quantification standards were established using mouse IgG1 and IgE mAb (Serotec).

Statistical analysis

For all experiments, the difference between two groups was calculated with the Student *t* test using GraphPad Software Prism 4. ANOVA and Bonferroni's test were used for multiple comparisons (*, $P \leq 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant). All graphs show mean and SEM.

Online supplemental material

Fig. S1 shows the assessment of cell death and apoptosis in lung ILC2s at steady state and after IL-33 administration. Fig. S2 shows the gating strategy for Ki67 staining in lung ILC2s. Fig. S3 show the gating strategies for the assessment of lung-infiltrating cells (ILC2s and eosinophils) in CD45.1 (WT)/CD45.2 (AR^{KO}) mixed bone marrow chimeras.

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Author contributions: S. Laffont, E. Blanquart, M. Savignac, C. Cénac, G.T. Belz, L. Pelletier, C. Seillet, and J.-C. Guéry designed the research, performed experiments, and analyzed the data. G. Laverny and D. Metzger provided samples from AR^{KO} mice and contributed to the design and interpretation of experiments. J.-P. Girard provided reagents and expertise for experiments with IL-33. C. Seillet and J.-C. Guéry designed the conceptual framework of the study and wrote the paper with the help of the other co-authors.

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