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Androgen signaling negatively controls type 2 innate lymphoid cells

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Running title: Evidence for sex-bias in ILC2 development

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Summary

At the onset of adolescence, asthma becomes less prevalent in males than in females, suggesting a protective role of male sex hormones. Here, Laffont *et al.* show that androgens negatively control ILC2-development and ILC2-driven lung inflammation in male mice.

Abstract

Prevalence of asthma is higher in women than in men, but the mechanisms underlying this sex bias are unknown. Group 2 innate lymphoid cells (ILC2) are crucial 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 (ILC2p) and mature ILC2 in peripheral tissues compared to 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 ILC2. Importantly, orchiectomy, but not ovariectomy, abolishes the sex differences in ILC2 development and restores IL-33-mediated lung inflammation. ILC2p express the androgen receptor (AR) and AR-signaling inhibits their differentiation into mature ILC2. 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 the type 2 innate lymphoid cells (ILC2) and the T helper (Th) 2 lymphocytes. The ILC2 are rapid and potent producers of the type 2 cytokines representing a critical 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 (TSLP), IL-25 and IL-33 (Barlow et al., 2013; Bartemes et al., 2012; Imai et al., 2013; Kim et al., 2013; Salimi et al., 2013; Schmitz et al., 2005). In response to these stimuli, ILC2 secrete large amounts of IL-5, IL-13 and IL-9. The secretion of IL-5 by ILC2 leads to the recruitment and activation of eosinophils and mast cells, while IL-13 activates goblet cells and mucus production by epithelial cells (Tait Wojno and Artis, 2016). Moreover, IL-13 production by ILC2 is critical to mount an effective Th2 cell response by instructing dendritic cells (DC) to prime Th2 cells in draining lymph nodes (Halim et al., 2014). ILC2 have therefore recently emerged as critical cells in the initiation of allergic inflammatory responses such as asthma or atopic dermatitis (Halim et al., 2012; Halim et al., 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; 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 et al., 2012). While males are more susceptible to asthma than females in childhood, the onset of puberty reverses that trend for most allergic disorders (Almqvist et al., 2008; Carey et al., 2007). 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 (Almqvist et al., 2008; Carey et al., 2007). Along the same line, hypogonadism has been associated with

enhanced susceptibility to asthma when compared to 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 ILC2 in the induction of airway inflammation (Gold et al., 2014; Halim et al., 2012; Halim et al., 2014), we investigate whether ILC2 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 ILC2 in females compared with males. Interestingly, this difference was not due to 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 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 ovalbumine-induced asthma, airway inflammation is more severe in female 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 house dust mite (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 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 as compared to male, 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 feature of HDM-induced airway inflammation.

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

As ILC2 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 ILC2 were 2-fold higher in females than in males (Fig. 2 A). These differences in ILC2 numbers were accompanied with changes in the expression of phenotypic markers. ILC2 from male mice showed an increase in the expression of KLRG1 and IL33 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 the different sexes. Furthermore, these differences in ILC2 extended to other tissues in which ILC2 dominate such as the visceral adipose tissues (VAT) (Fig. 2 D) and mesenteric lymph nodes (Fig. 2 E). In VAT, ILC2 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 VAT are compatible with the observation that male are more vulnerable to high-fat diet-induced weight gains in terms of onset or magnitude (Hwang et al., 2010).

We next investigated the mechanisms that might underpin these sex differences in ILC2 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 ILC2 (Fig. 2 F). To exclude any potential role of estrogen signaling on ILC2 development or maturation, we analyzed the presence of ILC2 in mice that specifically lack ER α expression in the hematopoietic lineages. We found similar frequencies of lung ILC2 between ER $\alpha^{\text{flox/flox}}$ \times Tie2-Cre mice and their ER $\alpha^{\text{flox/flox}}$ control littermates (Fig. 2 G). By contrast, a significant up-regulation in ILC2 frequency was observed in the lungs of castrated males (Cx) (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-33₉₅₋₂₇₀, as described (Lefrancais et al., 2014). In this setting, IL-33 strongly increased the number and frequency of ILC2 in the lungs of treated mice compared with PBS-injected control mice (Fig. 3 A and B). ILC2 frequencies and numbers were however ~2-fold higher in female over male mice. The frequency of lung ILC2 which 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 ILC2 from IL-33-treated mice as compared to untreated controls (Fig. S1). However, no differences in cell death and apoptosis were observed between lung ILC2 isolated from female or male mice, both at steady state or upon IL-33 administration (Fig. S1). Thus, enhanced apoptosis and/or impaired survival do not seem to account for the sex differences in ILC2 at steady state and during inflammation.

Interestingly, castrated 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⁺ ILC2 as compared to control mice (Fig. 3 C). Despite lack of significant

differences in the proportion of cytokine producing ILC2 between males, females and castrated males (Fig. 3 C), the absolute numbers of IL-5⁺IL-13⁺ ILC2 were dramatically increased in females and castrated 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 castrated males, in which disease scores were similar to females (Fig. 3 F). Taken together, these results show that ILC2-mediated lung inflammation is more severe in female than male mice, and that orchiectomy abolishes these differences.

Androgens restrain ILC2 development at steady state

As ILC2 develop from the ILC2 progenitor (ILC2p) in the bone marrow (Hoyler et al., 2012), we compared ILC2p frequency between both sexes. The absolute number and proportion of ILC2p (identified as Lin⁻CD117^{low}Sca-1⁺CD127⁺CD25⁺) were 2 to 3-fold higher in females than in males (Fig. 4 A and B), while the total cell numbers in the bone marrow were similar between both sex (not depicted). Thus, the increased number and frequency of ILC2 in peripheral tissues in female mice was correlated with a rise in ILC2p in the bone marrow. Contrasting with the lack of differential expression of CD25 between male and female tissue-resident ILC2, ILC2p from female mice significantly expressed higher level of IL2R α as compared to male ILC2p (Fig. 4 C). To test whether sex-linked factors affect ILC2 proliferation at steady state, we examined intracellular expression of Ki67 in ILC2p and in lung ILC2 isolated from male or female mice. Whereas Ki67⁺ cells were detected at low frequency in ILC2p and ILC2 from male mice (ranging from 1-5 %), a significantly higher proportion of ILC2p (Fig. 4 D) and lung ILC2 (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 ILC2 (Fig. 2), we measured the expression profile of sex-steroid hormone receptors. Accordingly, ILC2p primarily expressed transcripts encoding for androgen receptor (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, ILC2p were cultured with IL-33 in 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 days (Fig. 4 H). On the contrary, Flutamide increased the numbers of ILC2 (~1.3-1.7 fold) that differentiated after 7 to 10 days of culture (Fig. 4 H). Of note, KLRG-1 expression on developing ILC2 was down-regulated in Flutamide-treated

cultures (Fig. 4 I), indicating that AR-signaling controls KLRG-1 expression in male ILC2 as shown in Fig. 2. Thus, androgen signaling actively represses ILC2 development through AR-signaling in ILC2p.

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

To evaluate the role of 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 wild-type 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 wild type chimeric males compared with females, however 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 wild-type mice (Fig. 5 C). KLRG1 expression was again significantly down-regulated on AR-deficient ILC2 confirming that the differences observed between males and females on 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). By contrast, hematopoietic expression of AR-deficiency 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 weeks of reconstitution, the ratio between CD45.1⁺ (WT) and CD45.1^{neg} (AR^{KO}) lung ILC2 were examined following IL-33 injection (Fig. 5 G and H). We observed a significant deficiency in the proportion of WT ILC2 (CD45.1⁺) compared with ILC2 derived from AR^{KO} progenitors (CD45.1^{neg}) (Fig. S3 A), resulting in a WT/AR^{KO} ILC2 ratio below 0.2 (Fig. 5 G). For comparison, the WT/AR^{KO} ratios for total CD45⁺ lung infiltrating cells or lung eosinophils were above 0.7 (Fig. 5G and Fig. S3C). Yet, the frequencies of Ki67⁺ cells and dead cells were similar between WT and AR-deficient ILC2 (Fig. S3 B and D). Interestingly, in agreement with results in Fig. 5 D, the reduced expression of KLRG1 on AR-deficient ILC2 was maintained in the mixed chimeras (Fig. 5 H). Altogether, these results indicate that

endogenous androgens signal through hematopoietic AR 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 (Almqvist et al., 2008; Carey et al., 2007). This pattern changes during adolescence where the onset of asthma becomes much less prevalent in male than in female. 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 ILC2 in various tissues, including lung, fat and mesenteric lymph nodes. This was associated with a strong up regulation of ILC2p in the bone marrow of female compared with male mice. Accumulating evidence indicate 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 ILC2 at steady state. By 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 ILC2p demonstrated a selective expression of *Ar* gene mRNA, whereas those encoding for estrogen receptors *Esr-1* or *Esr-2* were barely detected. This observation is in agreement with recent reports 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 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 orchiectomy 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 ILC2 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 ILC2 themselves or their progenitors seem to represent the direct target of androgens. Cell-intrinsic AR signaling in ILC2p appears to promote the development of KLRG1^{hi} ILC2, which are less frequent in female mice or castrated 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 ILC2, 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 ILC2. However, as very few ILC2 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 pre-existing sex-bias in tissue-resident ILC2 reflected in the steady-state numbers. Enhanced CD25 expression in female ILC2p correlated with an increased frequency of proliferating (Ki67⁺) cells, suggesting that greater sensitivity to IL-2 in female ILC2p could promote ILC2 seeding in non-lymphoid tissues during ontogeny. This hypothesis is however not supported by recent findings demonstrating that CD25-deficient tissue-resident ILC2 proliferated to a similar extent as WT ILC2 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 signalling 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 ILC2p or essential for their differentiation into ILC2 cells. Such specific downstream targets of AR in ILC2p remain to be identified. Alternatively, AR signaling may directly or indirectly 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 investigations.

Our study suggests that androgen therapy could be useful to protect against allergic diseases through its inhibitory action on ILC2. Androgen replacement therapy is currently

used for the treatment of subjects with deficient testicular function, and administration of dihydroepiandrosterone (DHEA), a weak androgen with less virilizing side effects, has shown some beneficial actions in the treatment of allergic asthma in human (Shah, 2004; Wenzel et al., 2010) and in mouse (Liou and Huang, 2011). Understanding further how AR signals in ILC2 or their progenitors may provide new therapeutics or downstream targets for the treatment of allergic diseases, while minimizing side effects of androgen therapy.

MATERIALS AND METHODS

Mice. Female and male C57BL/6J (B6) mice were purchased from the Centre d'Elevage R. Janvier (Le Genest St. Isle, France) or the Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). The congenic C57BL/6-Ly5.1 (CD45.1) mice were purchased from Charles River (Saint-Germain Nuelles, France). Mice were used at 8-12 weeks old unless otherwise stated. Bilateral orchiectomy and ovariectomy were performed in anesthetized 4-5 week-old mice. Mice were then rested for a period of 5-6 weeks before use. Mice selectively lacking ER α in the hematopoietic compartment (ER $\alpha^{\text{fl/fl}}$ x Tie2-Cre) have been described elsewhere (Lelu et al., 2011). Mice lacking AR have been described previously (Sato et al., 2004). AR^{L/+} females on a B6 background, bearing one AR-null allele, were bred with wild-type B6 males to produce AR^{KO} (AR^{L/y}) and AR^{+y} male mice. For the generation of irradiation bone marrow chimeras, mice were γ -irradiated (11 Gy, ¹³⁷Cs source) and engrafted with 5 to 10 $\times 10^6$ bone marrow cells/mouse. Mice were used 6-8 weeks 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 (NHMRC) 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.

House dust mite induced allergic asthma and IL-33 injection. HDM (*Dermatophagoides pteronyssinus* extracts, Greer Laboratories, NC, USA) was used to induce allergic airway inflammation. Mice were sensitized intranasally with 1 μ g HDM extracts on day 0 and were subsequently challenged with 10 μ g/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 (huR) IL-33 injections were performed as described with some modification (Lefrancais et al., 2014). Briefly, mice were treated intraperitoneally with 4 μ g recombinant human IL-33₉₅₋₂₇₀ for two consecutive days, then left untreated for two days and injected again for 3 consecutive days. Twenty-four hours after the last injection, bronchoalveolar lavage fluid and lungs were collected for flow cytometry and histological analyses. Recombinant human IL-33₉₅₋₂₇₀ was produced as described in (Lefrancais et al., 2014).

Tissue Preparation. Lungs were cut into small fragments and digested for 30 min at 37°C with Collagenase III (1 mg/ml; Worthington), DNase I (200 µg/ml; Sigma). Red blood cells were lysed by treatment with hypotonic solution then filtered. Perigonadal adipose tissue was used as representative visceral adipose tissue. Adipose tissue was finely dissected with a scalpel blade and digested in 3 ml of phosphate buffered saline (PBS) containing Collagenase III (0.2 mg/ml; Worthington) and 4% BSA at 37°C for 45 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 Biosciences, were used for the identification and purification of ILC2 cells: CD19 (ID3), B220 (RA3-6B2), CD3 (145-2C110), CD4 (GK1.5), CD11b (M1/70), CD11c (HL3), Gr-1 (RB6-8C5), TCRb (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 following stimulation for 4 h with PMA (50 ng/ml) and ionomycin (100 ng/ml), in the presence of Brefeldin A (1 µg/ml). **Ki67-staining analysis (BD Pharmingen) and Annexin V staining analysis (eBioscience) were performed according to the manufacturer's protocol.** Cells were analyzed using a Fortessa (BD Biosciences), and FlowJo software (Tree Star) was used for analysis. Flow cytometric sorting was performed with a FACS Aria (BD Biosciences).

ILC2 differentiation. To induce the ILC2 differentiation *in vitro*, cell-sorted ILC2p from female bone marrow cells were cultured for 7 days 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 dihydrotestosterone (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-Seq. RNA was isolated from *ex vivo* ILC2p from female wild-type 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 were 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).

Lung histology and MGG staining. Lung tissue was fixed in 10% buffered formalin for 24 h and then placed in ethanol 70% before embedding in paraffin. Sections (4 μ m) were stained with hematoxylin and eosin. 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 peri-bronchial epithelium, resulting in a maximum score of 12. May-Grünwald-Giemsa staining (MGG) 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, Serotec) Ab was used for coating and rat anti-mouse/HRP (LO-MK-1) was used for the secondary detection step, all purchased from Serotec. For IgE, rat anti-mouse IgE (LO-ME-3, Serotec) Ab was used for coating. Biotin-conjugated rat anti-mouse IgE mAb (BD Pharmingen) and streptavidin–HRP conjugate (Amersham) 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 Prism 4 (GraphPad Software, La Jolla, CA). ANOVA and Bonferroni's test were used for multiple comparisons. All graphs show mean and SEM. *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

Online supplemental material

Fig. S1 shows the assessment of cell death and apoptosis in lung ILC2 at steady state and after IL-33 administration. Fig. S2 shows the gating strategy for Ki67-staining in lung ILC2. Fig. S3 show the gating strategies for the assessment of lung infiltrating cells (ILC2 and eosinophils) in CD45.1 (WT)/CD45.2(AR^{KO}) mixed bone marrow chimeras.

AUTHOR CONTRIBUTIONS

S.L., E.B., M.S., C.C., G.T.B., L.P., C.S. and J.C.G. designed the research, performed experiments and analyzed the data; G.L., D.M. provided samples from AR^{KO} mice, and contributed to the design and interpretation of experiments. J.P.G. provided reagents and expertise for experiments with IL-33. C.S. and J.C.G. designed the conceptual framework of the study and wrote the paper with the help of the other co-authors.

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REFERENCES

- Almqvist, C., M. Worm, B. Leynaert, and G.A.L.E.N.W.P.G. working group of. 2008. Impact of gender on asthma in childhood and adolescence: a GA2LEN review. *Allergy* 63:47-57.
- Barlow, J.L., S. Peel, J. Fox, V. Panova, C.S. Hardman, A. Camelo, C. Bucks, X. Wu, C.M. Kane, D.R. Neill, R.J. Flynn, I. Sayers, I.P. Hall, and A.N. McKenzie. 2013. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. *J Allergy Clin Immunol* 132:933-941.
- Bartemes, K.R., K. Iijima, T. Kobayashi, G.M. Kephart, A.N. McKenzie, and H. Kita. 2012. IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *J Immunol* 188:1503-1513.
- Carey, M.A., J.W. Card, J.W. Voltz, S.J. Arbes, Jr., D.R. Germolec, K.S. Korach, and D.C. Zeldin. 2007. It's all about sex: gender, lung development and lung disease. *Trends Endocrinol Metab* 18:308-313.
- Doherty, T.A., N. Khorram, S. Lund, A.K. Mehta, M. Croft, and D.H. Broide. 2013. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol* 132:205-213.
- Eberl, G., M. Colonna, J.P. Di Santo, and A.N. McKenzie. 2015. Innate lymphoid cells. Innate lymphoid cells: a new paradigm in immunology. *Science* 348:aaa6566.
- Gasteiger, G., X. Fan, S. Dikiy, S.Y. Lee, and A.Y. Rudensky. 2015. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science* 350:981-985.
- Gold, M.J., F. Antignano, T.Y. Halim, J.A. Hirota, M.R. Blanchet, C. Zaph, F. Takei, and K.M. McNagny. 2014. Group 2 innate lymphoid cells facilitate sensitization to local, but not systemic, TH2-inducing allergen exposures. *J Allergy Clin Immunol* 133:1142-1148.
- Halim, T.Y.F., R.H. Krauß, A.C. Sun, and F. Takei. 2012. Lung Natural Helper Cells Are a Critical Source of Th2 Cell-Type Cytokines in Protease Allergen-Induced Airway Inflammation. *Immunity* 36:451-463.
- Halim, T.Y.F., C.A. Steer, L. Mathä, M.J. Gold, I. Martinez-Gonzalez, K.M. McNagny, A.N.J. Mckenzie, and F. Takei. 2014. Group 2 Innate Lymphoid Cells Are Critical for the Initiation of Adaptive T Helper 2 Cell-Mediated Allergic Lung Inflammation. *Immunity* 40:425-435.
- Hoyler, T., C.S. Klose, A. Souabni, A. Turqueti-Neves, D. Pfeifer, E.L. Rawlins, D. Voehringer, M. Busslinger, and A. Diefenbach. 2012. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity* 37:634-648.
- Huang, Y., L. Guo, J. Qiu, X. Chen, J. Hu-Li, U. Siebenlist, P.R. Williamson, J.F. Urban, Jr., and W.E. Paul. 2015. IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat Immunol* 16:161-169.
- Hwang, L.L., C.H. Wang, T.L. Li, S.D. Chang, L.C. Lin, C.P. Chen, C.T. Chen, K.C. Liang, I.K. Ho, W.S. Yang, and L.C. Chiou. 2010. Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. *Obesity (Silver Spring)* 18:463-469.
- Imai, Y., K. Yasuda, Y. Sakaguchi, T. Haneda, H. Mizutani, T. Yoshimoto, K. Nakanishi, and K. Yamanishi. 2013. Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proc Natl Acad Sci U S A* 110:13921-13926.

- Keselman, A., and N. Heller. 2015. Estrogen Signaling Modulates Allergic Inflammation and Contributes to Sex Differences in Asthma. *Front Immunol* 6:568.
- Kim, B.S., M.C. Siracusa, S.A. Saenz, M. Noti, L.A. Monticelli, G.F. Sonnenberg, M.R. Hepworth, A.S. Van Voorhees, M.R. Comeau, and D. Artis. 2013. TSLP Elicits IL-33-Independent Innate Lymphoid Cell Responses to Promote Skin Inflammation. *Science translational medicine* 5:170ra116.
- Kissick, H.T., M.G. Sanda, L.K. Dunn, K.L. Pellegrini, S.T. On, J.K. Noel, and M.S. Arredouani. 2014. Androgens alter T-cell immunity by inhibiting T-helper 1 differentiation. *Proc Natl Acad Sci U S A* 111:9887-9892.
- Lambrecht, B.N., and H. Hammad. 2015. The immunology of asthma. *Nat Immunol* 16:45-56.
- Lefrancais, E., A. Duval, E. Mirey, S. Roga, E. Espinosa, C. Cayrol, and J.P. Girard. 2014. Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proc Natl Acad Sci U S A* 111:15502-15507.
- Lelu, K., S. Laffont, L. Delpy, P.E. Paulet, T. Perinat, S.A. Tschanz, L. Pelletier, B. Engelhardt, and J.C. Guery. 2011. Estrogen receptor alpha signaling in T lymphocytes is required for estradiol-mediated inhibition of Th1 and Th17 cell differentiation and protection against experimental autoimmune encephalomyelitis. *J. Immunol.* 187:2386-2393.
- Liao, Y., G.K. Smyth, and W. Shi. 2013. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res* 41:e108.
- Liao, Y., G.K. Smyth, and W. Shi. 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30:923-930.
- Liou, C.J., and W.C. Huang. 2011. Dehydroepiandrosterone suppresses eosinophil infiltration and airway hyperresponsiveness via modulation of chemokines and Th2 cytokines in ovalbumin-sensitized mice. *J Clin Immunol* 31:656-665.
- Markle, J.G., and E.N. Fish. 2014. Sex matters in immunity. *Trends Immunol* 35:97-104.
- Melgert, B.N., D.S. Postma, I. Kuipers, M. Geerlings, M.A. Luinge, B.W. van der Strate, H.A. Kerstjens, W. Timens, and M.N. Hylkema. 2005. Female mice are more susceptible to the development of allergic airway inflammation than male mice. *Clin Exp Allergy* 35:1496-1503.
- Molofsky, A.B., J.C. Nussbaum, H.-E. Liang, S.J. Van Dyken, L.E. Cheng, A. Mohapatra, A. Chawla, and R.M. Locksley. 2013. Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. *J Exp Med* 210:535-549.
- Moro, K., T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J.-I. Furusawa, M. Ohtani, H. Fujii, and S. Koyasu. 2010. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 463:540-544.
- Mulligan, T., M.F. Frick, Q.C. Zuraw, A. Stemhagen, and C. McWhirter. 2006. Prevalence of hypogonadism in males aged at least 45 years: the HIM study. *Int J Clin Pract* 60:762-769.
- Neill, D.R., S.H. Wong, A. Bellosi, R.J. Flynn, M. Daly, T.K.A. Langford, C. Bucks, C.M. Kane, P.G. Fallon, R. Pannell, H.E. Jolin, and A.N.J. McKenzie. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464:1367-1370.
- Robinette, M.L., A. Fuchs, V.S. Cortez, J.S. Lee, Y. Wang, S.K. Durum, S. Gilfillan, M. Colonna, and C. Immunological Genome. 2015. Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. *Nat Immunol* 16:306-317.
- Salimi, M., J.L. Barlow, S.P. Saunders, L. Xue, D. Gutowska-Owsiak, X. Wang, L.C. Huang, D. Johnson, S.T. Scanlon, A.N.J. McKenzie, P.G. Fallon, and G.S. Ogg. 2013. A role

- for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med* 210:2939-2950.
- Sato, T., T. Matsumoto, H. Kawano, T. Watanabe, Y. Uematsu, K. Sekine, T. Fukuda, K. Aihara, A. Krust, T. Yamada, Y. Nakamichi, Y. Yamamoto, T. Nakamura, K. Yoshimura, T. Yoshizawa, D. Metzger, P. Chambon, and S. Kato. 2004. Brain masculinization requires androgen receptor function. *Proc Natl Acad Sci U S A* 101:1673-1678.
- Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T.K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D.M. Gorman, J.F. Bazan, and R.A. Kastelein. 2005. IL-33, an Interleukin-1-like Cytokine that Signals via the IL-1 Receptor-Related Protein ST2 and Induces T Helper Type 2-Associated Cytokines. *Immunity* 23:479-490.
- Shah, S. 2004. European Respiratory Society--14th Annual Congress. Drug highlights. 4-8 September 2004, Glasgow, UK. *IDrugs* 7:914-916.
- Tait Wojno, E.D., and D. Artis. 2016. Emerging concepts and future challenges in innate lymphoid cell biology. *J Exp Med* 213:2229-2248.
- Townsend, E.A., V.M. Miller, and Y.S. Prakash. 2012. Sex differences and sex steroids in lung health and disease. *Endocr Rev* 33:1-47.
- Vivier, E., S.A. van de Pavert, M.D. Cooper, and G.T. Belz. 2016. The evolution of innate lymphoid cells. *Nat Immunol* 17:790-794.
- Wenzel, S.E., C.B. Robinson, J.M. Leonard, and R.A. Panettieri, Jr. 2010. Nebulized dehydroepiandrosterone-3-sulfate improves asthma control in the moderate-to-severe asthma results of a 6-week, randomized, double-blind, placebo-controlled study. *Allergy Asthma Proc* 31:461-471.
- Wojno, E.D., L.A. Monticelli, S.V. Tran, T. Alenghat, L.C. Osborne, J.J. Thome, C. Willis, A. Budelsky, D.L. Farber, and D. Artis. 2015. The prostaglandin D(2) receptor CTRH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. *Mucosal Immunol* 8:1313-1323.

Abbreviations used:

AR, androgen receptors; Cx, castrated; HDM, house dust mite; ER, estrogen receptor; ILC, innate lymphoid cell(s); ILC2p, innate lymphoid cell type 2 progenitor; Ovx, Ovariectomised; VAT, visceral adipose tissue

FIGURE LEGENDS

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 days after treatment using May-Grünwald-Giemsa staining (MGG). 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 hematoxylin & eosin (left panels, Scale bar, 100 μ m) and quantification of lung inflammation (left panel arrowheads, and right panel) showing the mean \pm S.E.M. H&E show representative sections of 5 mice/group 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; ***, p<0.001. Data are representative of 2 independent experiments.

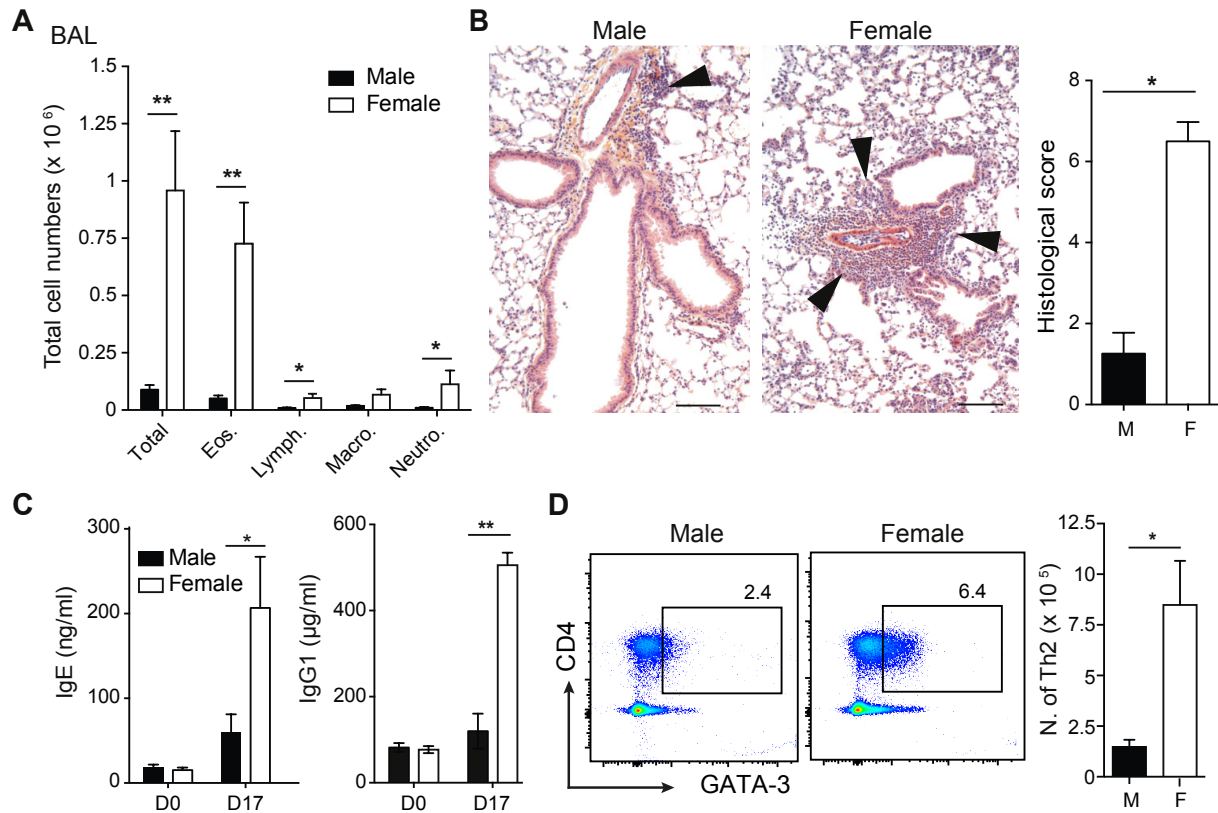
Figure 2. Male hormones inhibit ILC2 development at steady-state. (A) Flow cytometry analysis of lung ILC2 defined by lack of expression of lineage marker (LIN: CD3 ϵ , TCR β , CD11b, CD19, B220, Gr1, CD11c) and NK1.1, and expression of GATA3, CD90 and CD25 in naïve male and female mice (pre-gate: Singlet, Live, CD45⁺ cells). Representative dot plots (left panels) are shown together with results from one representative experiment out of 8. Bar graphs show data as means \pm SEM (n=4 mice/group). (B) Expression of KLRG1 and (C) ST2 on ILC2. Histograms show the frequency of KLRG1-expressing ILC2 and the geometric mean fluorescence intensity of ST2 on ILC2 (n=5 mice/group). Data are representative of 6 experiments. (D, E) ILC2 frequency in visceral adipose tissue (VAT) and mesenteric lymph nodes (mLN) 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 $\alpha^{fl/fl}$ x Tie2-Cre female mice and their littermate control ER $\alpha^{fl/fl}$ mice. (H) ILC2 frequency in the lung of male, female and castrated male mice (Cx). (D-H) Bar graphs show data as means \pm SEM (n=5

mice/group). Data are representative of 2 to 4 experiments. Comparison between groups was calculated using the unpaired Student's t tests. * $p < 0.05$; **, $p < 0.01$; ns, not significant.

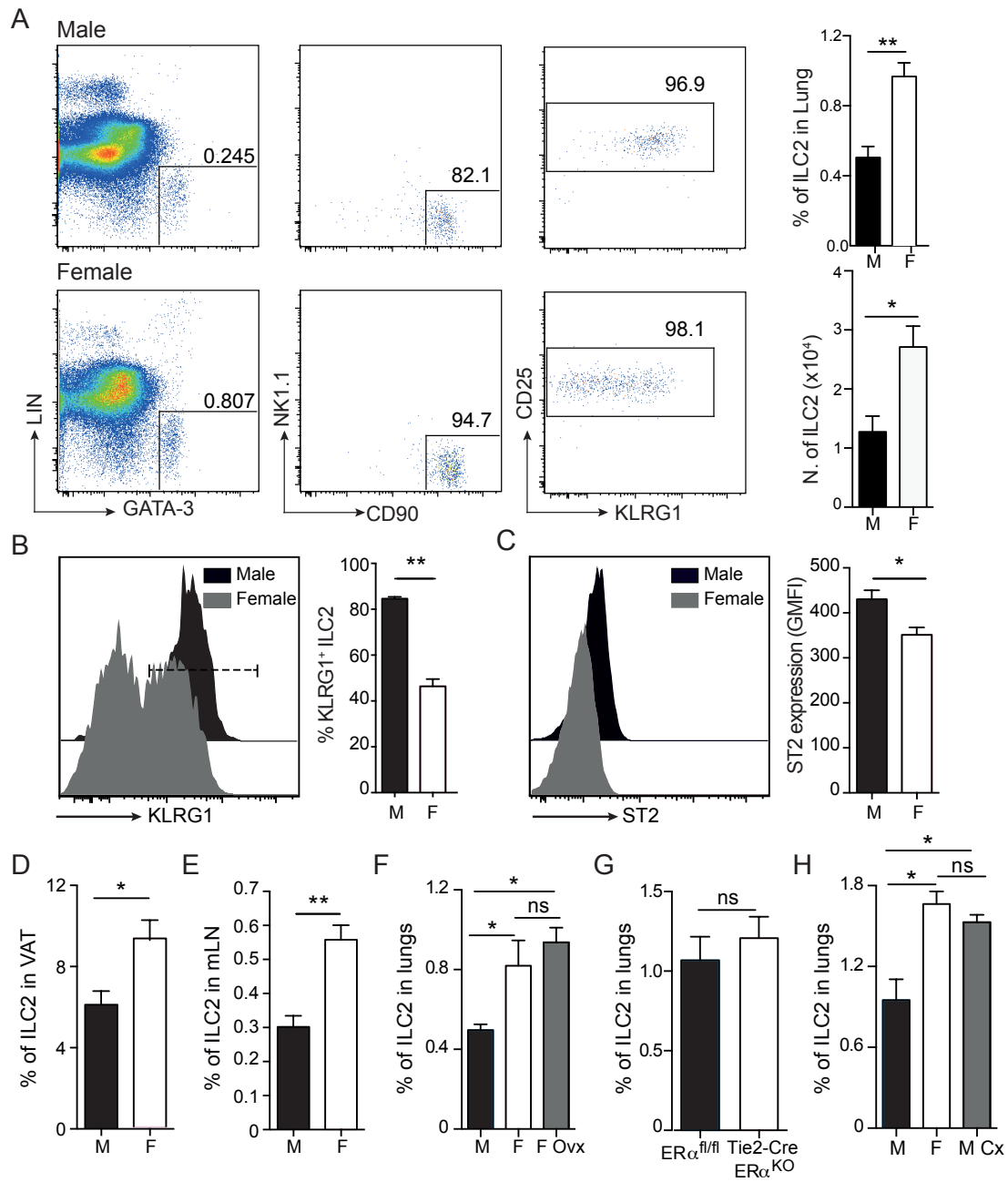
Figure 3. Male castration abolishes sex differences in IL-33-mediated lung inflammation. Male, female and castrated (Cx) male mice were injected i.p. with huIL-33 (4 $\mu\text{g}/\text{mouse}/\text{day}$) on day 0, 1, 4, 5 and 6 and analyzed on day 7. Control mice i.p. received PBS. (A) Flow cytometry staining of pulmonary ILC2 ($\text{CD90}^+\text{GATA-3}^+$) pre-gated on Singlet, Live, CD45^+ , CD90^+ , CD3^- , CD19^- , NK1.1^- cells. (B) Frequency and total number of ILC2 in the lungs. (C, D) Intracellular expression of IL-5 and IL-13 in lung ILC2 from indicated mice. Frequency (C) and total number (D) of ILC2 producing IL-5 and IL-13 are shown. (E) Hematoxylin and eosin staining of 4 μm lung sections (scale bar, 200 μm). Arrowheads indicate sites of leukocyte infiltration. (F) Histological score of lung inflammation. Data from 5 mice/group are shown. Comparison between groups was calculated using the unpaired Student's t tests. Error bars indicate the mean and SEM. * $p \leq 0.05$; **, $p < 0.01$; ns, not significant. Data are representative of 2 independent experiments.

Figure 4. ILC2p numbers are reduced in male mice while AR-blockade promoted ILC2 development *in vitro*. (A) Flow cytometry analysis of ILC2p in BM cells from male and female B6 mice. ILC2p ($\text{CD25}^+\text{Sca-1}^+$) were gated as SingletLiveLin $^-$ CD127 $^+$. (B) Frequency and total number of ILC2p between male and female mice. (C) Geometric mean fluorescence intensity of CD25 on ILC2p. (D) Frequency of Ki67 $^+$ ILC2p in the BM. (E) Frequency of Ki67 $^+$ ILC2 in the lung. (n=4-5 mice/group). Results are representative of 3 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 ILC2p by RNA-seq. (G-I) *In vitro* differentiation of ILC2p on OP9-DL1 feeder cells in complete medium supplemented with mouse IL-33 in presence or absence of dihydrotestosterone (DHT, 10 nM) or the AR-antagonist Flutamide (100 nM). (G) Flow cytometric analysis of ILC2 after 7 days of differentiation. (H) Quantification of ILC2 expansion expressed as the fold increase as compared to ILC2p input. (I) Representative histograms (left) and quantification (right) of KLRG1 expression on differentiated ILC2 in quadruplicate cultures. Data representative of 3-4 experiments are shown. Error bars indicate the mean \pm SEM. Comparison between groups was calculated using the unpaired Student's t tests. * $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

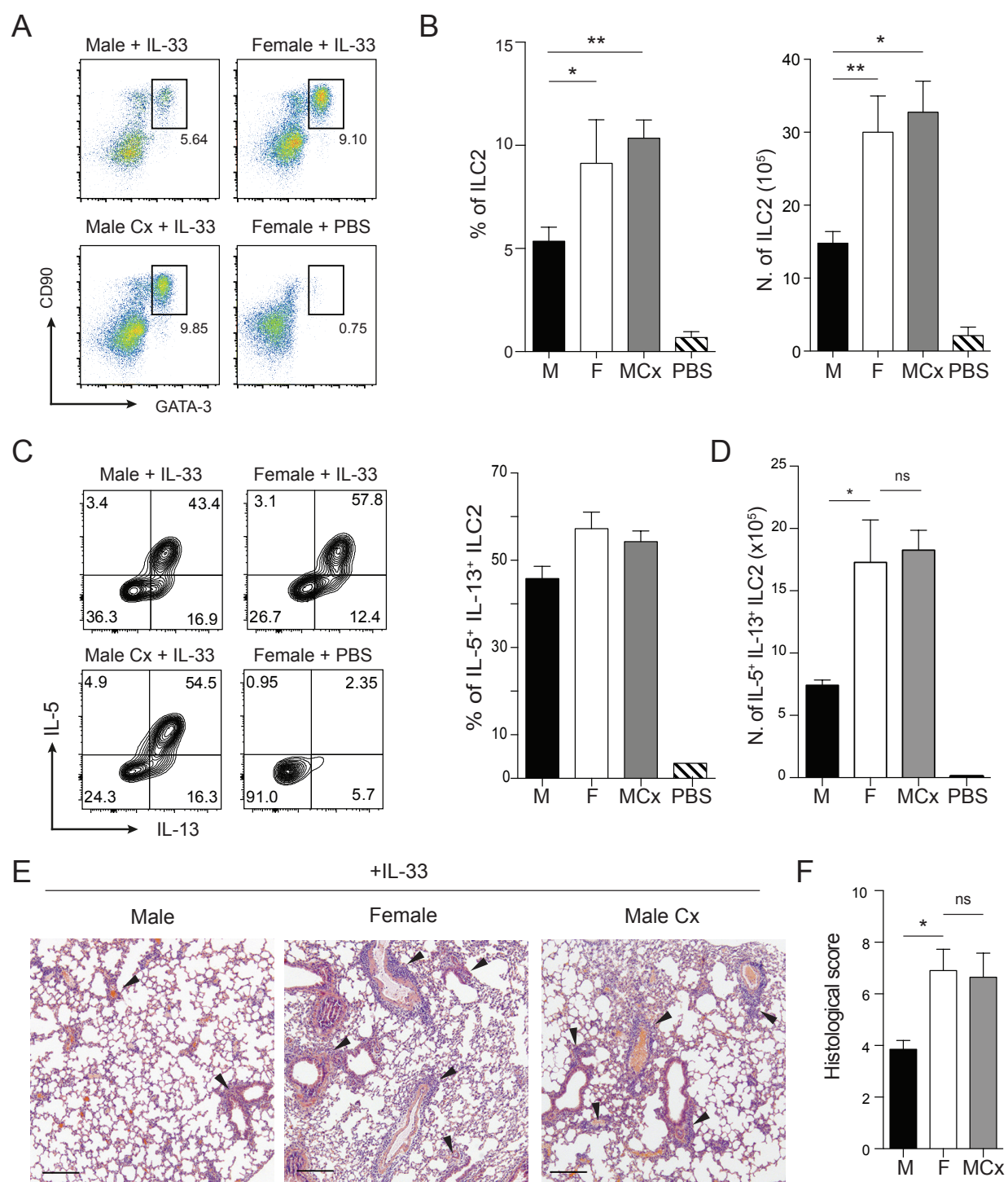
Figure 5. AR-deficiency abrogates sex differences in ILC2-dependent lung inflammation. Bone marrow chimeras reconstituted with cells either from AR^{KO} male B6 mice or their littermate WT controls were injected with huIL-33 as in Fig. 3 at 8 weeks after reconstitution and the inflammatory response was 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 ILC2 (SingletLiveCD45⁺Lin⁻CD90⁺GATA3⁺). (D) Frequency of KLRG1-expressing ILC2 (left) and geometric mean fluorescence intensity of KLRG1 on ILC2. (E) Hematoxylin & eosin staining of lung tissue sections from chimeric mice (scale bar, 100 μ m). Arrowheads indicate sites of leukocyte infiltration. (F) Histological score of lung inflammation. Data from 5-6 mice/group are shown and are representative of 2 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 weeks post-reconstitution, mice were i.p. injected with huIL-33 as described above 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 ILC2 are shown. (H) Geometric mean fluorescence intensity of KLRG1 on ILC2. Data from 5-7 mice/group are shown and are representative of 2 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.



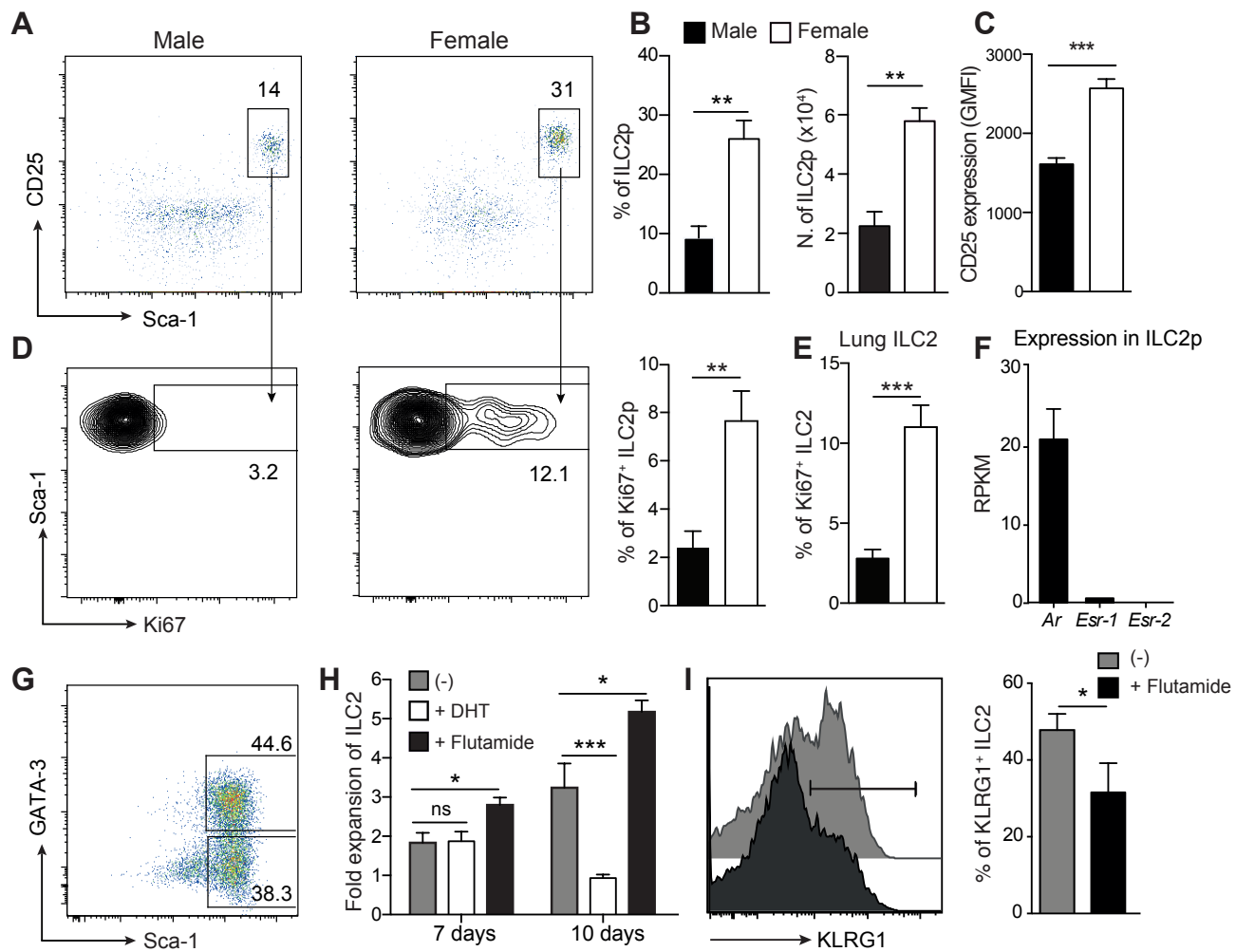
Laffont et al. Figure 1.



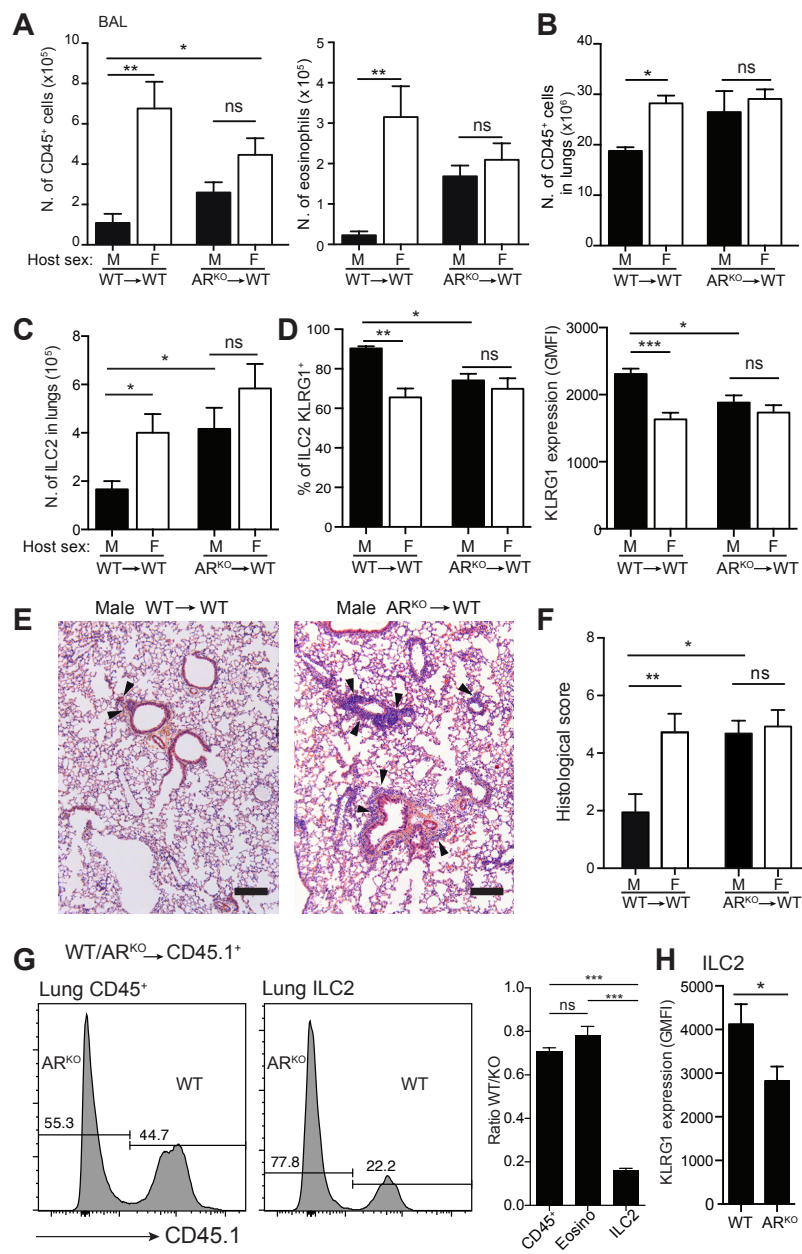
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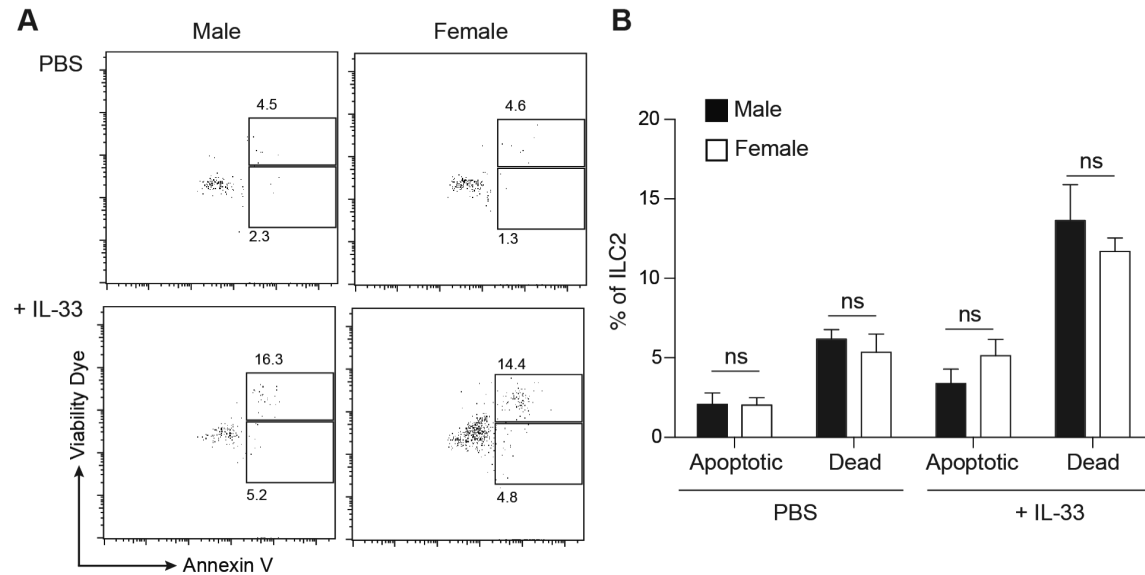


Figure S1. Sex does not influence cell death and apoptosis of lung ILC2. Male and female mice were i.p. injected with PBS or huIL-33 (4 μ g/mouse/day) on day 0, 1, 4 and analyzed on day 5. (A) Lung ILC2 were gated on SingletLive Lin⁻(CD3, CD19, B220, CD4, TCR β , DX5, CD11c, CD11b, Gr1), CD90⁺, Sca1⁺ cells and assessed for dead-cell-discrimination dye and annexin V staining. (B) The frequency of apoptotic (ViabilityDye⁻ AnnexinV⁺) and dead cells (ViabilityDye⁺ AnnexinV⁺) within ILC2 are shown from 5 mice/group and expressed as mean \pm SEM. Comparison between groups was calculated using the unpaired Student's t test. ns, not significant.

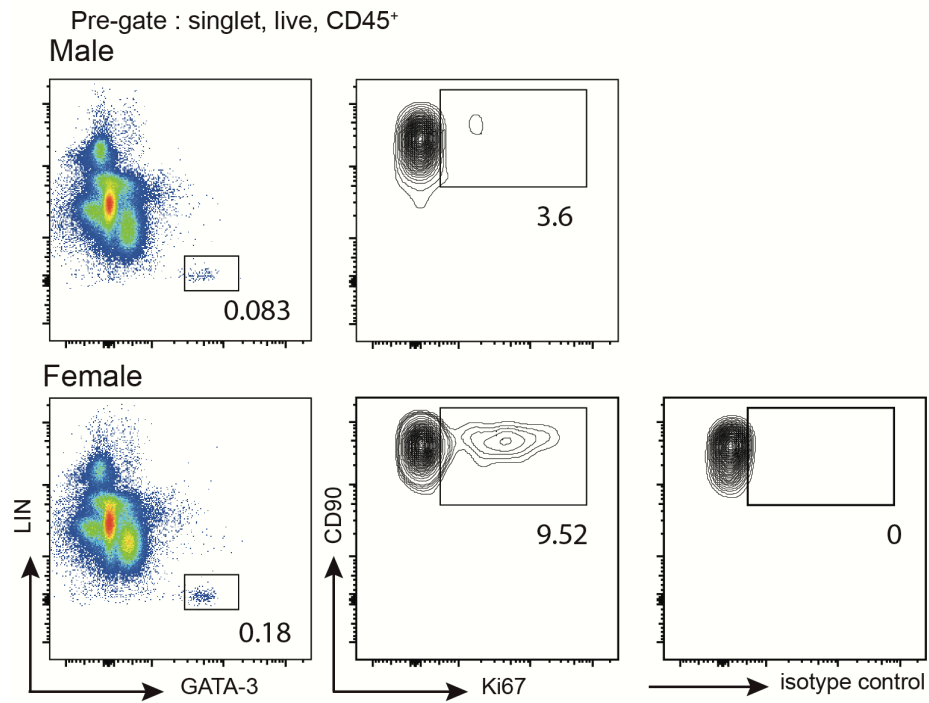


Figure S2. Gating strategy for of Ki67 staining of lung ILC2 from male and female mice. ILC2 from the lung of male and female mice were identified as SingletLiveCD45⁺Lin⁻ (CD3, CD19, B220, CD4, TCR β , DX5, CD11c, CD11b, Gr1), and GATA-3⁺CD90⁺ cells. The frequency of Ki67⁺ cells in ILC2 was determined as shown.

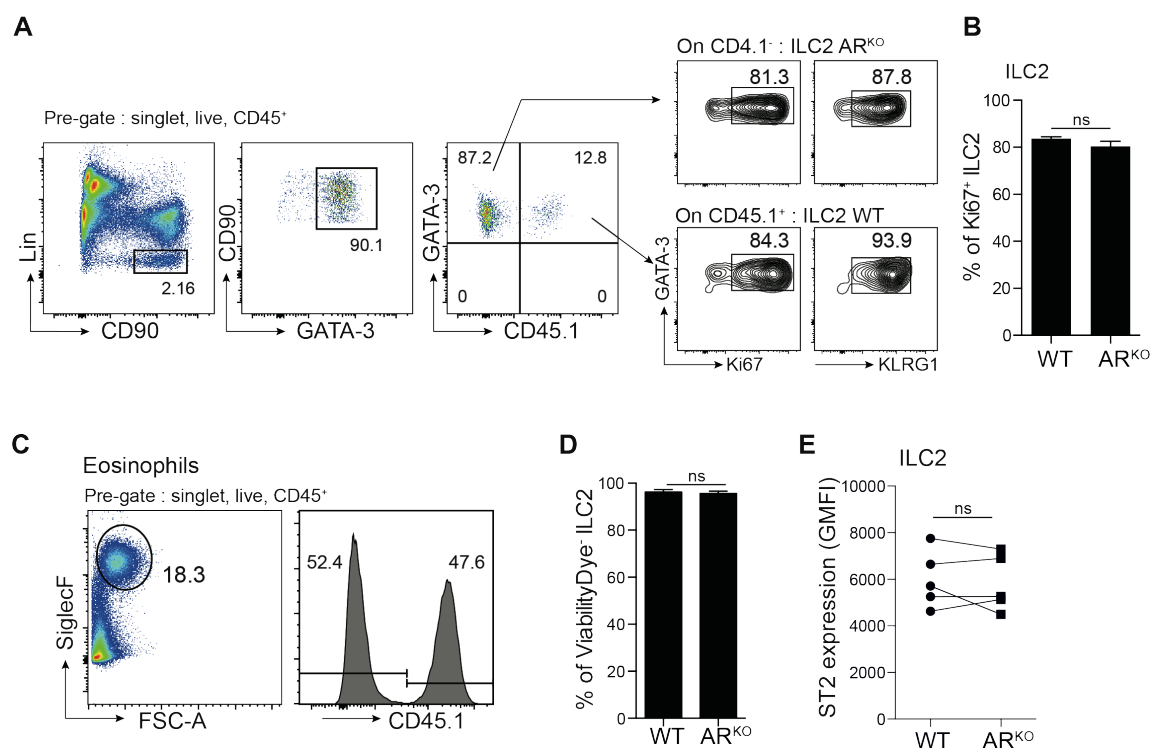


Figure S3. Gating strategies for the analysis of mixe chimera transplant experiments.

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 weeks post-reconstitution, mice were i.p. injected with huIL-33 as described in Fig. 3. (A) Gating strategy for analysis of pulmonary ILC2 and ILC2 expression of KLRG1 and Ki67. (B) Frequency of Ki67⁺ lung ILC2 that developed from CD45.1⁺ (WT) and CD45.1⁻ (AR^{KO}) bone marrow cells. (C) Gating strategy for the analysis of lung eosinophils. (D) Quantification in the lung of live (CD45.1⁺) WT or (CD45.1⁻) AR^{KO} ILC2 gated as in (A). (E) Geometric mean fluorescence intensity of ST2 staining on lung WT or AR^{KO} ILC2. Data are from 5 mice per group and are representative of two experiments.