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Targeting androgen signaling in ILC2s protects from IL-33–driven lung inflammation, independently of KLRG1

Eve Blanquart, PhD,^a Audrey Mandonnet, BSc,^a Marion Mars, MSc,^a Claire Cenac, MSc,^a Nina Anesi, MSc,^a Pascale Mercier, MSc,^b Christophe Audouard, BSc,^c Stephane Roga, BSc,^b Gilberto Serrano de Almeida, PhD,^d Charlotte L. Bevan, PhD,^d Jean-Philippe Girard, PhD,^b Lucette Pelletier, MD, PhD,^a Sophie Laffont, PhD,^{a*} and Jean-Charles Guéry, PhD^{a*} *Toulouse, France; and London, United Kingdom*

Background: Allergic asthma is more severe and frequent in women than in men. In male mice, androgens negatively control group 2 innate lymphoid cell (ILC2) development and function by yet unknown mechanisms.

Objectives: We sought to investigate the impact of androgen on ILC2 homeostasis and IL-33–mediated inflammation in female lungs. We evaluated the role of androgen receptor (AR) signaling and the contribution of the putative inhibitory receptor killer cell lectin-like receptor G1 (KLRG1).

Methods: Subcutaneous pellets mimicking physiological levels of androgen were used to treat female mice together with mice expressing a reporter enzyme under the control of androgen response elements and mixed bone marrow chimeras to assess the cell-intrinsic role of AR activation within ILC2s. We generated KLRG1-deficient mice.

Results: We established that lung ILC2s express a functionally active AR that can be *in vivo* targeted with exogenous androgens to negatively control ILC2 homeostasis, proliferation, and function. Androgen signaling upregulated KLRG1 on ILC2s, which inhibited their proliferation on E-cadherin interaction. Despite evidence that KLRG1 impaired the competitive fitness of lung ILC2s during inflammation, KLRG1 deficiency neither alters *in vivo* ILC2 numbers and functions, nor did it lead to hyperactive ILC2s in either sexes.

Conclusions: AR agonists can be used *in vivo* to inhibit ILC2 homeostatic numbers and ILC2-dependent lung inflammation through cell-intrinsic AR activation. Although androgen signals in ILC2s to upregulate KLRG1, we demonstrate that KLRG1 is dispensable for androgen-mediated inhibition of pulmonary ILC2s. (*J Allergy Clin Immunol* 2021;■■■■:■■■■-■■■■.)

Key words: Sex differences in asthma, ILC2, androgen signaling, lung inflammation, KLRG1

Asthma is a chronic inflammation of the airways primarily mediated by exacerbated type 2 immune responses.^{1,2} Sex differences in asthma prevalence and phenotypes have been well described.³⁻⁵ Asthma is more common in males from birth until puberty^{5,6} but becomes more prevalent^{3,7} and more severe^{4,8,9} in women after puberty. Group 2 innate lymphoid cells (ILC2s) have recently emerged as central mediators of allergic asthma through their capacity to promote and enhance adaptive T_H2 immunity.² Indeed, the top susceptibility genes identified in genome-wide association studies from patients with asthma ranked first with ILC2 biology (*IL33*, the IL-33 receptor *IL1RL1*, *RORA*, *IL2RB*, and *IL13*).^{10,11} Furthermore, genetic polymorphisms associated with asthma have been reported to localize within gene regulatory elements in ILC2s.¹² Together, these studies in humans suggest that ILC2s most probably play a pathogenic role in patients with allergic asthma. Indeed, the numbers and the activation status of ILC2 are increased in samples (sputum, blood) from pediatric and adult patients with asthma compared with controls.¹³

Sex bias in ILC2 numbers and functional properties both at steady state and during IL-33 or allergen-driven inflammation have been recently reported by several independent studies.¹⁴⁻¹⁶ Reduced numbers of pulmonary ILC2s at steady state in male mice were associated with the presence of ILC2s expressing the killer cell lectin-like receptor G1 (KLRG1), whereas pulmonary ILC2s from female mice have a prominent population of functional KLRG1-negative ILC2s.^{14,16} Cell-intrinsic androgen receptor (AR) signaling was required for both KLRG1 upregulation on ILC2 and inhibition of ILC2 expansion in response to IL-33 *in vivo*.¹⁴ This robust phenotypic difference between male and female pulmonary ILC2s is context specific and vanishes on allergen challenge or IL-33–mediated lung inflammation, resulting in upregulation of KLRG1 to similar extent in both sexes.¹⁴ KLRG1 interacts with E-cadherins on epithelial cells,¹⁷ and transduces inhibitory signals on ligand binding.¹⁷⁻¹⁹ However, although the inhibitory effect of KLRG1/E-cadherin interactions was reported *in vitro* with human skin ILC2 in atopic

From ^aInstitut Toulousain des Maladies Infectieuses et Inflammatoires (INFINITY), ^bInstitut de Pharmacologie et de Biologie Structurale, and ^cCentre de Biologie du Développement, Université de Toulouse, CNRS, UPS, Toulouse; and ^dthe Department of Surgery & Cancer, Imperial College, London.

*These authors contributed equally to this work.

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Corresponding author: Sophie Laffont, PhD, or Jean-Charles Guéry, PhD, Institut Toulousain des Maladies Infectieuses et Inflammatoires (INFINITY), INSERM U1291, Centre Hospitalier Universitaire Purpan, Place du Dr Baylac, 31024 Toulouse Cedex 3, France. E-mail: sophie.laffont-pradines@inserm.fr. Or: jean-charles.guery@inserm.fr.

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Abbreviations used

AR:	Androgen receptor
ARE-Luc:	Androgen response element-luciferase
BAL:	Bronchoalveolar lavage
DHEA:	Dehydroepiandrosterone
DHT:	5 α -dihydrotestosterone
ILC2:	Group 2 innate lymphoid cell
ILC2P:	ILC2 progenitor
indel:	Insertion/deletion
KLRG1:	Killer cell lectin-like receptor G1
NK:	Natural killer
Ovx:	Ovariectomized
WT:	Wild-type

dermatitis,²⁰ the *in vivo* relevance of this pathway remains to be established. Given the central role of androgen signaling in the expression of KLRG1, considering the sex as a biological variable in investigating the *in vivo* relevance of KLRG1 on ILC2 biology is therefore of critical importance.

In the present study, we provide the proof of concept that *in vivo* administration of AR agonists can inhibit ILC2-dependent inflammatory response in female mice through cell-intrinsic AR signaling. We further examined whether KLRG1 expression could affect the competitive fitness of individual pulmonary ILC2s and control the sex bias in ILC2s at steady state and on allergen insult to the respiratory tract using newly developed KLRG1-deficient mouse models.

METHODS

Fully detailed methods are described in this article's [Methods](#) section in the Online Repository at www.jacionline.org.

Mice

C57BL/6JRJ (B6) and Rag2^{-/-} B6 mice were purchased from the Centre d'Élevage R. Janvier (Le Genest St. Isle, France). Mice were used at 8- to 12-week-old unless otherwise stated. Mice selectively lacking AR have been described previously.^{14,21} Heterozygous female on B6 background were bred to wild-type (WT) B6 males to produce AR^{KO} male mice (AR^{L-/-}). IL-33^{-/-} mice on a C57BL/6J background (B6-IL-33G1^{IST10946B6-Tigm-Girard}) have been previously described.²² For the generation of irradiated bone marrow chimeras, mice were γ -irradiated (11 Gy, ¹³⁷Cs source). The day post-irradiation, mice were reconstituted with 10 \times 10⁶ cells from the specified bone marrow. Mice were used 8 weeks after reconstitution. Ovariectomy or orchidectomy was performed in anesthetized 4- to 5-week-old mice. All mice were housed in specific pathogen-free conditions. All animals were handled according to the Animal Care and Use of Laboratory Animal guidelines of the French Ministry of Research (study approval no. 15236), which act in compliance with Directive 2010/63/EU. All animal studies were designed in accordance with the 3R (Replacement, Reduction, and Refinement).

***In vivo* testosterone treatment**

Twenty-milligram pellets were lab-made with 90% cholesterol (Sigma C3045) and 10% testosterone or DHT (5 α -dihydrotestosterone) (as described in this article's [Methods](#) section in the Online Repository). Placebo pellets were made of cholesterol only. Two pellets were subcutaneously implanted under general anesthesia 10 to 15 days before sacrifice, resulting in the release of plasma testosterone levels ranging from 2.11 to 3.9 ng/mL from day 7 to 15. This dose range corresponds to physiological amounts of androgens found in male mice, which varied from 3 to 11 ng/mL.²³

IL-2, IL-33, and papain treatment

IL-2/anti-IL-2 complexes were injected intraperitoneally every 2 days for 6 days in Rag2^{-/-} mice. One dose of complexes consisted of 1 μ g mL-2 (Peprotech, Rocky Hill, NJ) and 5 μ g of anti-IL-2 mAb (JES6 clone). Mice were analyzed on day 7. Human recombinant IL-33 injections were given as described with some modification.²⁴ Briefly, mice were treated intraperitoneally with 4 μ g recombinant human IL-33₉₅₋₂₇₀ (huIL-33) for 2 consecutive days, then left untreated for 2 days, and injected again for 3 consecutive days. Twenty-four hours after the last injection, bronchoalveolar lavage (BAL) fluids and lungs were collected for flow cytometry and histological analyses. Recombinant human IL-33₉₅₋₂₇₀ was produced as described elsewhere.²⁴ Mice were intranasally (i.n.) sensitized with 50 μ g papain extracts on day 0, 1, and 2. The day after the last challenge, lungs and BAL were collected for flow cytometry and histological analysis.

Tissue preparation

Lungs were cut into small fragments and digested for 30 minutes at 37°C with Collagenase III (1 mg/mL; Worthington, Lakewood, NJ) and DNase I (200 μ g/mL; Roche, Basel, Switzerland). Cells were filtered using a cell strainer after red blood cell lysis. Viability was assessed using fixable viability dye eFluor506 (ebioscience, San Diego, Calif). Single-cell suspensions were blocked with PBS containing 5 μ g/mL anti-CD16/CD32 (2.4G2) and stained for 30 minutes at 4°C with fluorophore-conjugated antibodies. For the list of antibodies used in the study, see this article's [Methods](#) section in the Online Repository.

Statistical analysis

For all experiments, the difference between 2 groups was calculated with the Mann-Whitney test. Kruskal-Wallis test with the original false discovery rate (FDR) method of Benjamini and Hochberg was used for multiple comparisons. For matching data, a mixed-effect analysis with the original FDR method of Benjamini and Hochberg was used. We used GraphPad Prism 8 (GraphPad Software, La Jolla, Calif). All graphs show mean and SEM.

RESULTS**Androgen treatment limits pulmonary ILC2 numbers and blunts IL-33-mediated lung inflammation in female mice**

Because ILC2 maintenance in adult lung tissue is largely independent of circulating bone marrow ILC2 progenitors,²⁵⁻²⁷ we investigated whether short-term exogenous androgen supplementation could directly inhibit ILC2 numbers in lung tissues of adult female mice. Ten-day treatment with testosterone, which is *in vivo* converted into DHT, the active AR-binding form, was sufficient to reduce by half lung ILC2 frequencies and total numbers in ovariectomized (Ovx) female mice (Fig 1, A-C; see Fig E1, A, in this article's Online Repository at www.jacionline.org). ILC2 numbers in androgen-treated female mice were now similar to those in age-matched male mice. Independent studies have reported that KLRG1 upregulation on ILC2s is a hallmark of androgen-specific effects on lung ILC2s at steady state.^{14,16} In testosterone-treated female mice, we observed that frequencies of KLRG1⁺ ILC2s were increased to similar levels to those observed in males (Fig 1, D and E). KLRG1 expression levels on lung ILC2s followed the same trend (Fig 1, F). Because testosterone can also be converted into 17 β -estradiol *in vivo*, and to make sure that the observed effects were mediated through AR signaling, female mice were treated with DHT. As in testosterone-treated mice, a marked reduction in lung ILC2s associated with an increased expression of KLRG1 was found in DHT-treated mice (see Fig E2, A and B, in this article's Online

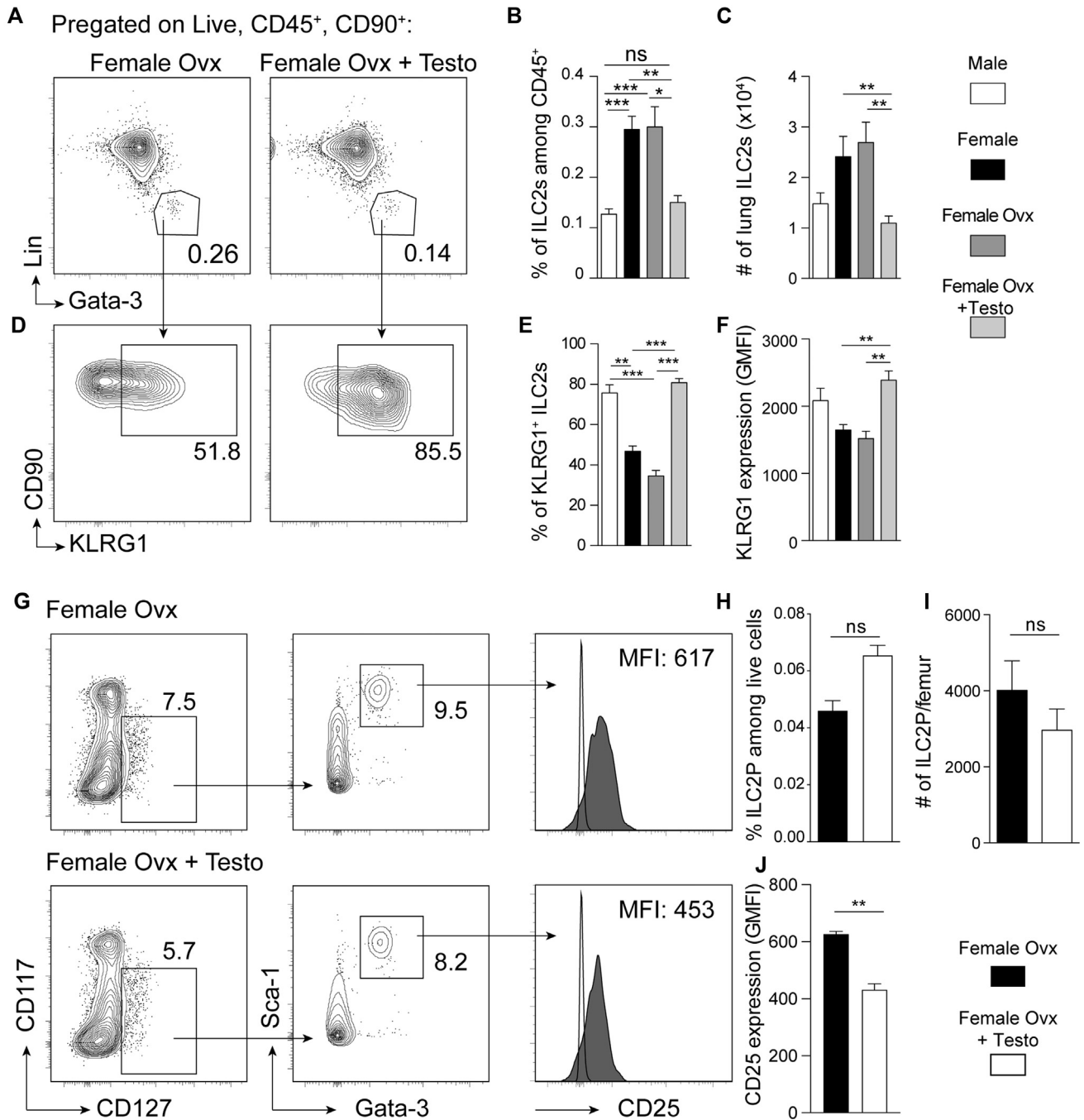


FIG 1. Testosterone administration diminishes lung ILC2 number and enhances KLRG1 expression on ILC2s from female mice. **A-F**, ILC2 analysis from the lungs of male, female, and Ovx female mice treated for 10 days with placebo or testosterone pellets. **A**, Representative cytometry analysis of ILC2s. ILC2s were defined as SingletLiveCD45⁺CD90⁺Lineage⁻Gata-3⁺ cells. Frequency adjusted to total CD45⁺ cells. (Lineage: CD3, CD4, CD8, TCRαβ, TCRγδ, CD19, B220, CD11b, CD11c, Gr1, and DX5) (Detailed gating strategy in Fig E1, A). Frequency (Fig 1, B) and number of ILC2s (Fig 1, C) among hematopoietic cells (SingletLiveCD45⁺ cells). **D**, KLRG1 expression on lung ILC2s and associated frequency (Fig 1, E). **F**, GMFI of KLRG1 on ILC2s. **G-J**, ILC2P analysis in the bone marrow. **G**, Representative flow cytometry analysis of ILC2P in the bone marrow of Ovx female mice treated with testosterone or placebo. ILC2P were defined as SingletLiveLin⁻CD117^{dim}CD127⁺Sca-1⁺Gata-3⁺ cells (Detailed gating strategy in Fig E1, E). Frequency (Fig 1, H) and number (Fig 1, I) of ILC2P per femur. **J**, GMFI of CD25 on ILC2P. FMO of CD25 is shown (unfilled). FMO, Fluorescence minus one; GMFI, geometric mean of fluorescence intensity; MFI, mean of fluorescence intensity; ns, not significant; Testo, testosterone. Data are representative of 2 independent experiments (5-7 mice per group). **P* < .05; ***P* < .01; ****P* < .001.

Repository at www.jacionline.org). ILC2s develop from the ILC2 progenitor (ILC2P) in the bone marrow,²⁸ numbers of which are also reduced in male mice.¹⁴ Unlike lung ILC2, percentage (Fig 1, G and H; Fig E1, E) and total numbers (Fig 1, I) of ILC2P were not affected by testosterone treatment. However, the CD25 expression levels of ILC2P were significantly and homogeneously decreased in androgen-treated mice (Fig 1, G and J), in agreement with our previous observation in male ILC2P.¹⁴

We next examined whether exogenous androgen therapy could be protective in ILC2-driven lung inflammation in female mice. In agreement with our previous work,¹⁴ intact and Ovx female mice developed stronger type-2 lung inflammation as compared with male mice, with higher numbers of cells in the BAL fluids (Fig 2, A-C). Interestingly, females treated with testosterone or DHT exhibited a marked reduction in BAL total cell numbers, to levels similar to those of males (Fig 2, C; Fig E2, F). Likewise, lung histological score was significantly reduced in androgen-supplemented females, as compared with Ovx females, which exhibited diffuse infiltration of inflammatory cells around bronchia and blood vessels (Fig 2, A and B; Fig E2, E). This reduction in lung inflammation in androgen-treated mice was correlated with a reduction in the frequency (Fig 2, D and E; Fig E2, C) and total number (Fig 2, F) of ILC2s in the lungs. Although frequencies of KLRG1⁺ ILC2s and KLRG1 expression levels were similar between intact and Ovx females, both parameters were significantly upregulated in intact male and Ovx female supplemented with androgen (Fig 2, G and H; Fig E2, D). Together, these data show that ILC2s are amenable to pharmacological manipulation with AR agonist ligands, and that treatment of female mice with testosterone during 10 days is sufficient to reduce lung ILC2 numbers and ILC2-driven lung inflammation to levels similar to male mice.

Sex bias in ILC2s is independent of adaptive immune system and endogenous IL-33

We next investigated whether sex bias in lung ILC2s was maintained in the absence of the adaptive immune system. We used Rag2-deficient mice (Rag2^{-/-}), which were injected with IL-2/anti-IL-2 antibody complexes to promote *in vivo* ILC2 expansion, without the confounding factor associated with the presence of T cells, especially regulatory T cells, highly expressing CD25.²⁹ At steady state, sex bias in lung ILC2 relative frequency and KLRG1 expression was maintained in Rag2^{-/-} mice (Fig 3, A and B). Administration of IL-2/anti-IL-2 antibody complexes induced lung ILC2 proliferation, as assessed by Ki67 staining in both male and female mice (not shown). Pulmonary ILC2 frequencies and numbers were significantly higher in female than in male mice (Fig 3, C-E). As expected, castration of males abolished the sex differences in lung ILC2s (Fig 3, C-F). Together, these results showed that the sex bias in lung ILC2s was maintained in absence of T and B cells both at steady state and on ILC2 activation induced through IL-2R signaling.

In Rag2-deficient mice, we also investigated the protective effect of androgens on lung inflammation triggered by intranasal challenge with papain, a protease known to be allergenic both in human and in mouse. Rag2^{-/-} female mice treated with testosterone showed decreased total cell number in BAL and fewer eosinophils in lungs (see Fig E3, A-C, in this article's Online

Repository at www.jacionline.org). Again, testosterone treatment in female Rag2^{-/-} mice was associated with a diminution in the frequency and in the total number of ILC2s (Fig E3, D-F) together with increased expression of KLRG1 on ILC2s (Fig E3, G-I). Thus, the presence of an adaptive immune system was dispensable for testosterone-mediated protection of female mice from allergen-induced lung inflammation.

Because sex differences in alarmin production in the lung tissue have been reported,^{15,30} we next investigated whether IL-33 was responsible for the sex differences in pulmonary ILC2 numbers. Tissue-resident cells were extracted from the lung tissue of IL-33-deficient male and female mice. Similarly to WT or Rag2^{-/-} mice, the frequency and total number of lung ILC2s were higher in female compared with male mice (Fig 4, A-C). Likewise, sex differences in KLRG1 expression (Fig 4, D), Ki67⁺ ILC2 frequency (Fig 4, E), and ST2 expression (Fig 4, F) were conserved in the constitutive absence of IL-33.

ILC2s are amenable to pharmacological downregulation through cell-intrinsic AR-dependent activation

Next-generation sequencing analyses have reported high-level expression of *Ar* gene in bone marrow ILC2P¹⁴ as well as tissue-resident ILC2s, including lung ILC2s.^{31,32} Here, we now show that AR protein is readily detectable in *in vitro* expanded lung ILC2s from WT->WT but not AR^{KO}-> WT chimeric mice (Fig 5, A). To determine whether endogenous AR can be activated in mature ILC2s, we used transgenic reporter mice in which luciferase gene expression is driven by endogenous AR-binding to androgen response elements (ARE-Luc).³³ We purified ILC2s from the lungs of WT or ARE-Luc bone marrow B6 chimeras previously treated with IL-33 as shown in Fig 2. Strong luciferase activity was detected in ILC2s expressing the highly specific androgen reporter luciferase construct (Fig 5, B), demonstrating that ILC2s express a functionally active endogenous AR. Moreover, *in vitro*-expanded ILC2s from male or female mice expressed comparable amount of AR protein, which can be further increased by adding DHT to the culture (Fig 5, C). These results show that ILC2s from male and female mice express a functionally active full-length AR protein, which can be stabilized by high-affinity androgen binding in both sexes in accordance with the literature.^{34,35}

We next evaluated whether ILC2 regulation by *in vivo* androgen treatment occurred through cell-intrinsic AR activation. Mixed WT(CD45.1)/AR^{KO}(CD45.2)-BM chimeras were generated in male and female CD45.2 recipient mice. A group of female mice was treated with testosterone (Fig 5, D). All groups were injected with IL-33 during the last week before lung ILC2 analysis. The chimerism was checked in IL-33-injected CD45.2 female reconstituted with CD45.1 bone marrow cells, and we found that most (>95%) lung ILC2s were derived from the transplanted bone marrow (see Fig E4 in this article's Online Repository at www.jacionline.org). In the mixed AR^{KO}(CD45.2)/WT(CD45.1)->CD45.2WT chimeras, we calculated the ratio of CD45.2/CD45.1 cells in various populations isolated from the lung of IL-33-treated mice (see Fig E5 in this article's Online Repository at www.jacionline.org). In male and in androgen-treated female mice, we observed a significant reduction in WT ILC2s

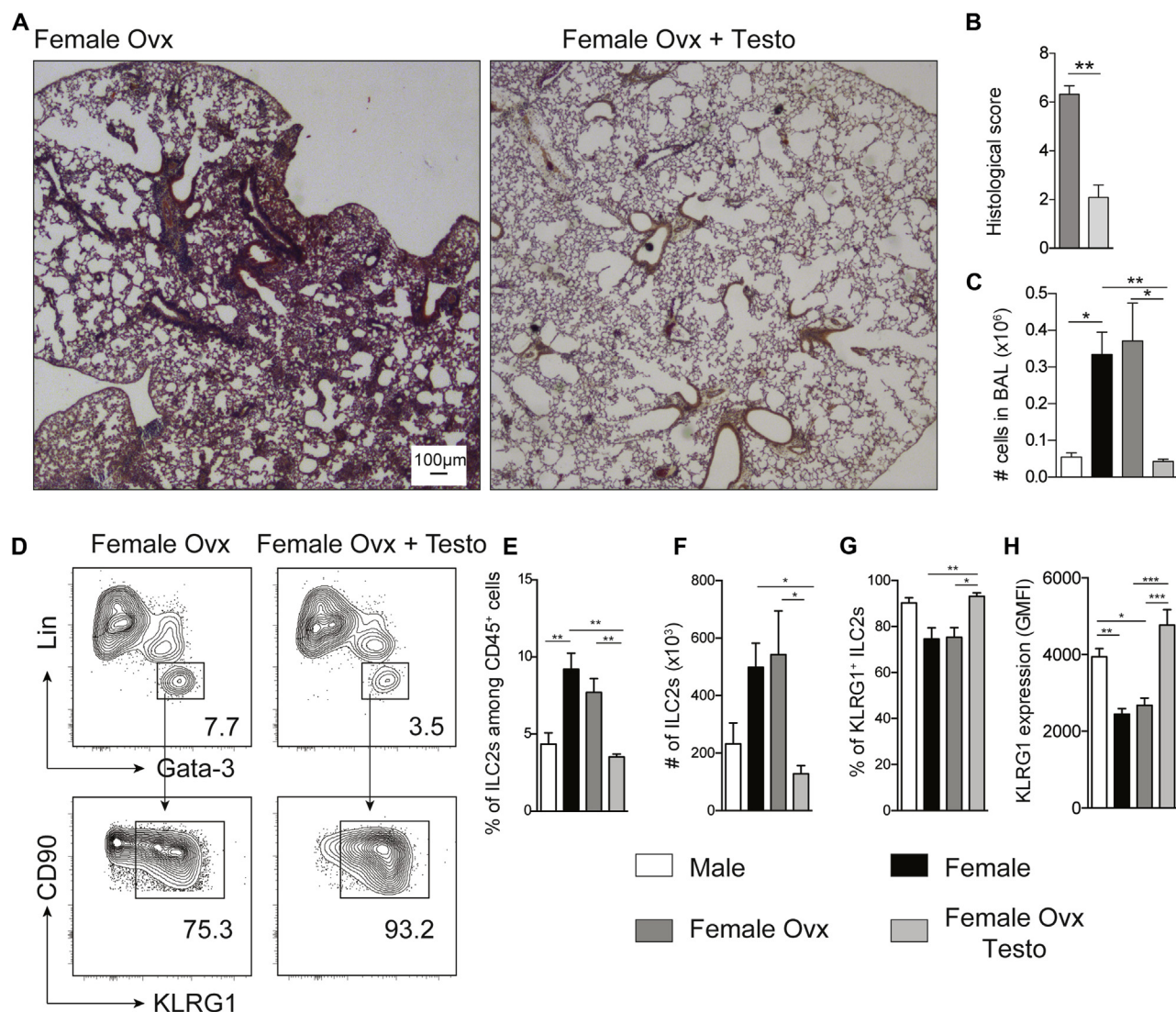


FIG 2. Testosterone treatment limits IL-33-mediated lung inflammation. Male, female, and Ovx female mice treated for 10 days with placebo or testosterone pellets were intraperitoneally injected with huIL-33 (4 μ g/mouse/d) on day 0, 1, 4, 5, and 6, and further analyzed on day 7. **A**, Representative hematoxylin and eosin-stained lung sections from Ovx female mice treated with testosterone or placebo. **B**, Quantification of lung inflammation. **C**, Total number of cells in BAL fluid. **D**, Representative flow cytometry staining of lung ILC2s from Ovx female mice treated with placebo or testosterone (pregated on singlet, live, CD45⁺, CD90⁺). Frequency adjusted to total CD45⁺ cells. **E**, Frequency of ILC2s in lungs. **F**, Number of ILC2s in lungs. **G**, Frequency of lung ILC2s expressing KLRG1. **H**, GMFI of KLRG1 on lung ILC2s. *GMFI*, Geometric mean of fluorescence intensity; *ns*, not significant; *Testo*, testosterone. Data are representative of 2 independent experiments (5-7 mice per group). * $P < .05$; ** $P < .01$; *** $P < .001$.

compared with AR^{KO} ILC2s, resulting in higher AR^{KO}/WT ratio compared with lung-infiltrating natural killer (NK) cells or eosinophils, which do not express AR (Fig 5, E). In intact females, the AR^{KO}/WT ratios were similar between ILC2s and the other cell populations examined, showing that AR activation within WT ILC2 is responsible for the inhibition of ILC2 expansion *in vivo* through a cell-intrinsic mechanism (Fig 5, E). A trend toward higher frequencies of KLRG1⁺ cells in AR-sufficient ILC2s was observed in male and female mice (Fig 5, F). This trend was markedly enhanced on androgen supplementation, when WT ILC2s homogeneously expressed high levels of KLRG1 as compared with AR-deficient ILC2s from the same individual

mice (Fig 5, F). Together, these results suggest that testosterone activates AR signaling within ILC2s to promote an inhibitory pathway, limiting ILC2 proliferation and/or maintenance in lung tissues.

Generation and characterization of KLRG1-deficient mice

KLRG1 expression on lung ILC2s appears tightly regulated by androgens^{14,16} and our present results (Fig 1, D-F, and Fig 5). KLRG1 has been shown to negatively regulate *in vitro* cytokine production and proliferation of human ILC2s²⁰; however,

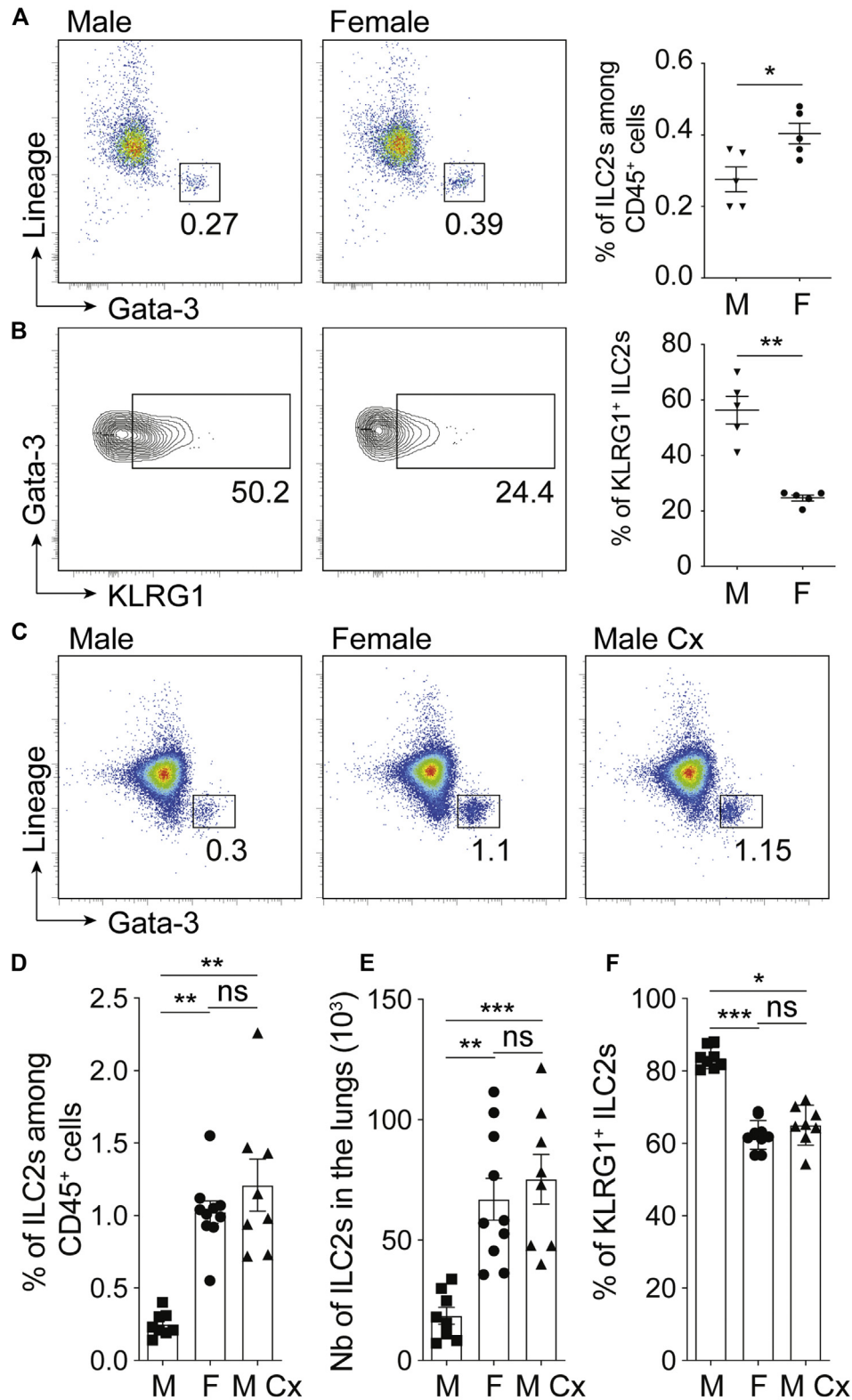


FIG 3. Sex bias in lung ILC2s is maintained in absence of adaptive immune system. **A** and **B**, ILC2 analysis from the lungs of male and female Rag2^{-/-} mice. **A**, Representative cytometry analysis and frequency of ILC2s in the lungs. ILC2s were defined as SingletLiveCD45⁺CD90⁺Lineage⁻Gata-3⁺ cells. Frequency adjusted to total CD45⁺ cells. (Detailed gating strategy in Fig E 1, A). **B**, KLRG1 expression and frequency on lung ILC2s. **C-F**, Male, female, and castrated male were intraperitoneally injected with IL-2-anti-IL-2 complex (1 μ g/mouse/d of IL-2 and 5 μ g/mouse/d of anti-IL-2) on day 0, 2, 4, and 6 and further analyzed on day 7. **C**, Representative flow cytometry staining of lung ILC2s (pregated on singlet, live, CD45⁺, CD90⁺). **D**, Frequency of ILC2s in lungs. **E**, Number of ILC2s in lungs. **F**, Frequency of lung ILC2s expressing KLRG1. **F**, Female; **M**, male; **M Cx**, castrated male; *ns*, not significant. Data are representative of 2 independent experiments (5-10 mice per group). * $P < .05$; ** $P < .01$; *** $P < .001$.

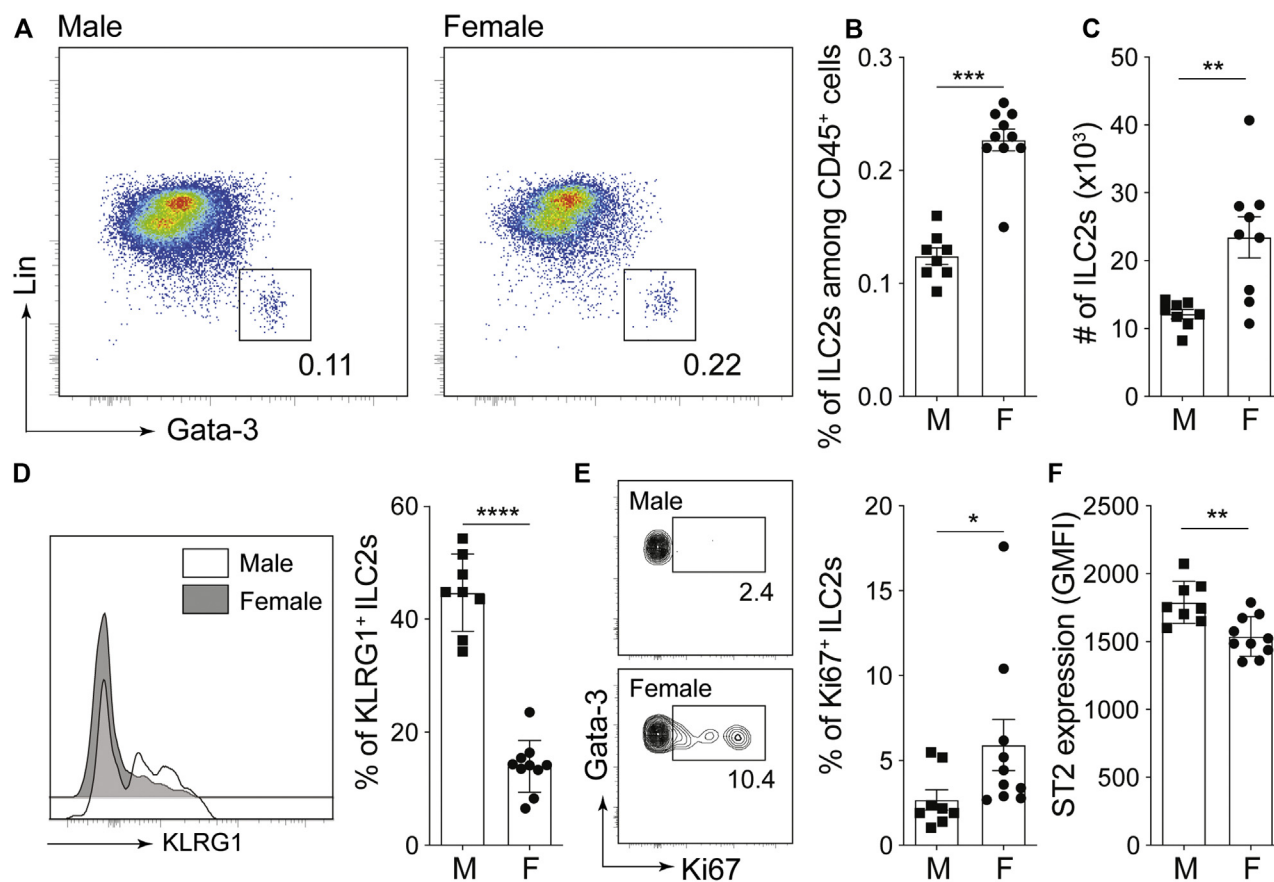


FIG 4. IL-33 deficiency does not affect the sex bias in lung ILC2s. **A**, Representative flow cytometry of ILC2s in the lungs of male and female IL-33^{-/-} mice. Percentages are adjusted to CD45⁺ cells. **B**, Percentage of ILC2s in the lungs. **C**, Number of ILC2s in the lungs. **D**, KLRG1 expression on ILC2s. **E**, Percentage of ILC2s expressing Ki67. **F**, GMFI of ST2 on lung ILC2s (8-10 mice per group). *F*, Female; *GMFI*, geometric mean of fluorescence intensity; *M*, male; *ns*, not significant. Comparison between groups was done using Mann-Whitney test. Bar graphs indicate the mean \pm SEM. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

whether KLRG1 expression controls steady-state ILC2 numbers and ILC2-driven inflammation *in vivo* is currently unknown. To address this issue, we generated KLRG1-deficient mice by targeting exon 1 of the *Klrk1* gene using CRISPR-Cas9 technology (see Fig E6, A and B, in this article's Online Repository at www.jacionline.org). Two KLRG1-deficient mouse lines that gave similar results were generated (Fig E6, C-K). KLRG1 deficiency was not associated with developmental defect, overt pathology, or changes in immune cell population frequencies in lymphoid tissues (data not shown and Fig E6), in agreement with previous work.³⁶ KLRG1-deficient mice were born at expected Mendelian ratio and lacked expression of KLRG1 on NK cells (Fig E6, C and F) and lung ILC2s (Fig 6, A and D). We observed a trend toward a reduction in KLRG1⁺ cell frequencies and KLRG1 expression levels (geometric mean of fluorescence intensity) on circulating NK (Fig E6, I and J) and lung ILC2s (Fig 6, A and D) from heterozygous KLRG1^{+/-} mice.

Sex-based differences in the percentage (Fig 6, B) and total numbers (Fig 6, C) of steady-state lung ILC2s were maintained whatever the mouse genotype (KLRG1^{+/-} or KLRG1^{-/-}). However, KLRG1 deficiency abrogated sex differences in ST2 expression and Ki67 staining in lung ILC2s (Fig 6, E and F). Because sex bias in the frequency of proliferating (Ki67⁺) cells was abolished in absence of KLRG1, we next examined the effect of

KLRG1/E-cadherin interactions on ILC2 proliferation and functional properties.

KLRG1 limits lung ILC2 proliferation and competitive fitness in response to IL-33

E-cadherin expressed on epithelial cells is the only known ligand of KLRG1,^{18,19} and *in vitro* incubation of human ILC2s on recombinant E-cadherin inhibits their proliferation and the production of type-2 cytokines (IL-5 and IL-13).²⁰ To test the role of KLRG1 expression on mouse ILC2 proliferation, we differentiated ILC2s from bone marrow ILC2P in the presence of DHT. In agreement with our previous work,¹⁴ culture of ILC2P in the presence of DHT upregulated the expression of KLRG1 in more than 50% of mature ILC2s after 1 week of culture on stromal OP9-Delta1 cells (Fig 7, A-C). This *in vitro* effect of DHT was dose-dependent and blocked by flutamide, a pure AR antagonist (Fig 7, B and C). Using this culture model, ILC2s were generated from KLRG1^{-/-} and KLRG1^{+/+} littermate controls in the presence of DHT (Fig 7, D) and then cultured for an additional 3 days in the presence of IL-33 and IL-7 on feeder cell line expressing or not E-cadherin (Fig 7, E). KLRG1-sufficient ILC2s exhibited a reduced capacity to proliferate as compared with KLRG1^{-/-} ILC2s but only when cultured in presence of CMT-93 cells, which

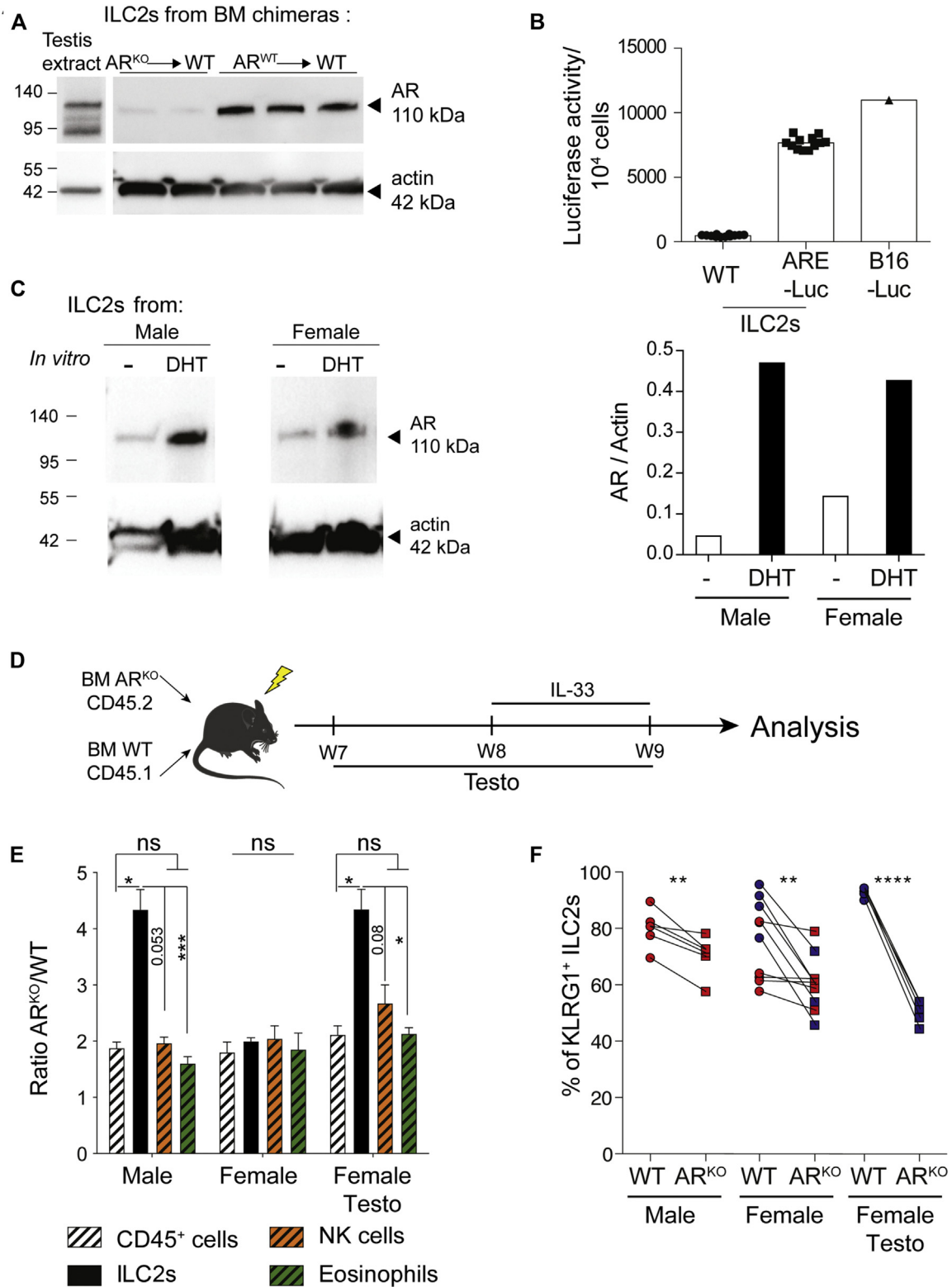


FIG 5. Testosterone-mediated cell-intrinsic activation of AR inhibits ILC2s. **A**, Western blot of AR in sorted lung ILC2s from lethally irradiated female reconstituted with WT (AR^{WT}) or AR-deficient (AR^{KO}) cells. Eight weeks later, female mice were treated with hull-33. Testis extract was used as a positive control. **B**, Lethally irradiated female B6 mice were reconstituted with ARE-Luc reporter or control WT bone marrow cells. Eight weeks posttransplant, mice were treated with hull-33 for 7 days. Bioluminescence activity was then measured in *in vitro* expanded ILC2s (n = 9 replicates). B16 cells stably transfected with luciferase were used as positive control. **C**, Western blot and associated quantification of AR expression in ILC2s purified from lungs of female and male Rag2^{-/-} mice previously treated with hull-33. ILC2s were further expanded

express E-cadherin (Fig 7, F). Unlike results from human ILC2s, production of IL-5 or IL-13 was not affected by KLRG1/E-cadherin interactions (see Fig E7, A and B, in this article's Online Repository at www.jacionline.org).

KLRG1 expression on lung ILC2s could act in a cell-intrinsic manner to downregulate their expansion on IL-33 injection. To test this hypothesis, competitive bone marrow chimeras were generated by reconstituting lethally irradiated male and female B6 mice with an equal mixture of bone marrow cells from CD45.1 WT and CD45.2 KLRG1^{-/-} mice. On 6 weeks of reconstitution, female mice were supplemented with testosterone or left untreated as shown in Fig 7, G. All mice were then injected with IL-33, and the ratios between KLRG1^{-/-} (CD45.2⁺) and WT (CD45.1⁺) lung ILC2s, NK cells, eosinophils, and CD45⁺ cells were calculated 1 week later (see Fig E8 in this article's Online Repository at www.jacionline.org; Fig 7, H). We observed a significant deficiency in the proportion of WT ILC2s in the 3 groups of mice, irrespective of the presence of androgens (Fig 7, H). For comparison, the KLRG1^{-/-}/WT ratio was similar for total CD45⁺ lung-infiltrating cells, lung NK cells, and lung eosinophils in the 3 experimental groups (Fig 7, H). Together, our results show that KLRG1 expression on ILC2s, by interacting with E-cadherin, negatively controls ILC2 activation threshold and proliferative potential in competitive mixed bone marrow chimeras.

KLRG1 expression is dispensable to maintain the sex bias in pulmonary ILC2 numbers

Although we observed that KLRG1^{-/-} ILC2s were at a competitive advantage in a mixed bone marrow chimera model, our results also indicated that KLRG1 was dispensable for the androgen-mediated downregulation of lung ILC2s in male at steady state. Because KLRG1 is strongly upregulated on ILC2s in both males and females on IL-33-mediated lung inflammation, we next investigated whether KLRG1 may serve as a negative regulator of ILC2 expansion during lung inflammation induced by IL-33 injection. As expected, WT B6 male had reduced eosinophils and total cell numbers in BAL compared with female mice (see Fig E9, A-C, in this article's Online Repository at www.jacionline.org). In lungs, the frequency of inflammatory eosinophils defined as in Fig E1, C, was also higher in females compared with males (Fig E9, D). Similar results were observed when comparing KLRG1^{-/-} males and females (Fig E9, A-D). Of note, the nature and the number of inflammatory cells in BAL and lung were similar between WT and KLRG1-deficient mice whatever the sex (Fig E9, A-D). Pulmonary ILC2 numbers were upregulated in female compared with male mice, and no differences were seen between WT and KLRG1^{-/-} mice (Fig E9, E and F). Together, KLRG1 deficiency had no measurable effect on ILC2-mediated lung inflammation in mice whatever the sex.

KLRG1 is dispensable for the sex-specific differences in lung ILC2 numbers and function during allergen challenge

We next examined whether KLRG1 could play a role in pulmonary ILC2 regulation in response to protease allergen challenge. We chose a model of mild inflammation, dependent on ILC2, induced by 3 intranasal instillations of papain.³⁷ In this model, lung inflammation was significantly higher in females than in males, as shown by the enhanced frequencies of eosinophils in female lungs compared with male lungs (Fig 8, A and B). Number and percentage of eosinophils in BAL were 3 to 4 times higher in females than in males. Similar results were observed in KLRG1-deficient mice (Fig 8, A-D). The severe inflammation in females was correlated with an increase in frequency and total number of lung ILC2s (Fig 8, E-G) and a reduced expression of KLRG1 on lung ILC2s (see Fig E10 in this article's Online Repository at www.jacionline.org). Interestingly, the frequency of ILC2s producing IL-5 and IL-13 after *ex vivo* stimulation with IL-2/IL-33 was significantly higher in females than in males (Fig 8, H and I). In contrast, no sex differences in the frequencies of IL-5⁺/IL-13⁺ ILC2s were observed on stimulation with phorbol 12-myristate 13-acetate/ionomycin stimulation (Fig 8, J). This sex bias in lung ILC2 function in response to *ex vivo* IL-33 stimulation was maintained in the absence of KLRG1 (Fig 8, H and I). Together, our data show that KLRG1 expression has no significant consequence on pulmonary ILC2 function and expansion *in vivo* in response to allergen challenge. In conclusion, upregulation of KLRG1 by androgens appears dispensable for the downregulation of lung ILC2 numbers and functional properties in males.

DISCUSSION

Here, we show that short-term androgen therapy limits pulmonary ILC2 numbers at steady state and subsequently ILC2-dependent lung inflammation in female mice. Lung ILC2s express a functionally active AR able to promote reporter gene expression through direct binding to androgen response element. KLRG1 is an androgen-regulated gene in lung ILC2s at steady state and is upregulated during lung inflammation on ILC2s in both sexes.¹⁴ Using a newly generated KLRG1-deficient mouse model, we now show that KLRG1 deficiency was not sufficient to restore pulmonary ILC2 numbers and functions in male mice to levels similar to those found in females. Thus, despite its strong association with AR signaling, KLRG1 was dispensable for androgen-mediated regulation of pulmonary ILC2s.

In our previous work¹⁴ and in the present study, we performed a careful examination of ILC2s in mouse tissues based on the expression of CD45, CD90, and GATA-3 and the lack of lineage-specific markers. We showed that males exhibit reduced numbers of ILC2s in various tissues, including the lung, which was associated with reduced susceptibility to allergic airway inflammation in response to the environmental allergen house

for 1 week with IL-2 (10 ng/mL) in presence or not of AR agonist (DHT, 10⁻⁸ mol). D-F, Lethally irradiated B6 mice were intravenously reconstituted with an equal mixture of CD45.1⁺ (WT) and CD45.2⁺ (AR^{KO}) BM cells. Eight weeks postreconstitution, mice were intraperitoneally injected with hIL-33 and (Fig 5, D) the relative frequency of WT and AR^{KO} hematopoietic cells (CD45⁺), ILC2s, NK cells, and eosinophils was assessed (gating strategy in Fig E5). E, Percentage of ILC2s expressing KLRG1. BM, Bone marrow; ns, not significant; Testosterone. Data are the pool of 2 independent experiments (5-10 mice per group). *P < .05; **P < .01; ****P < .0001.

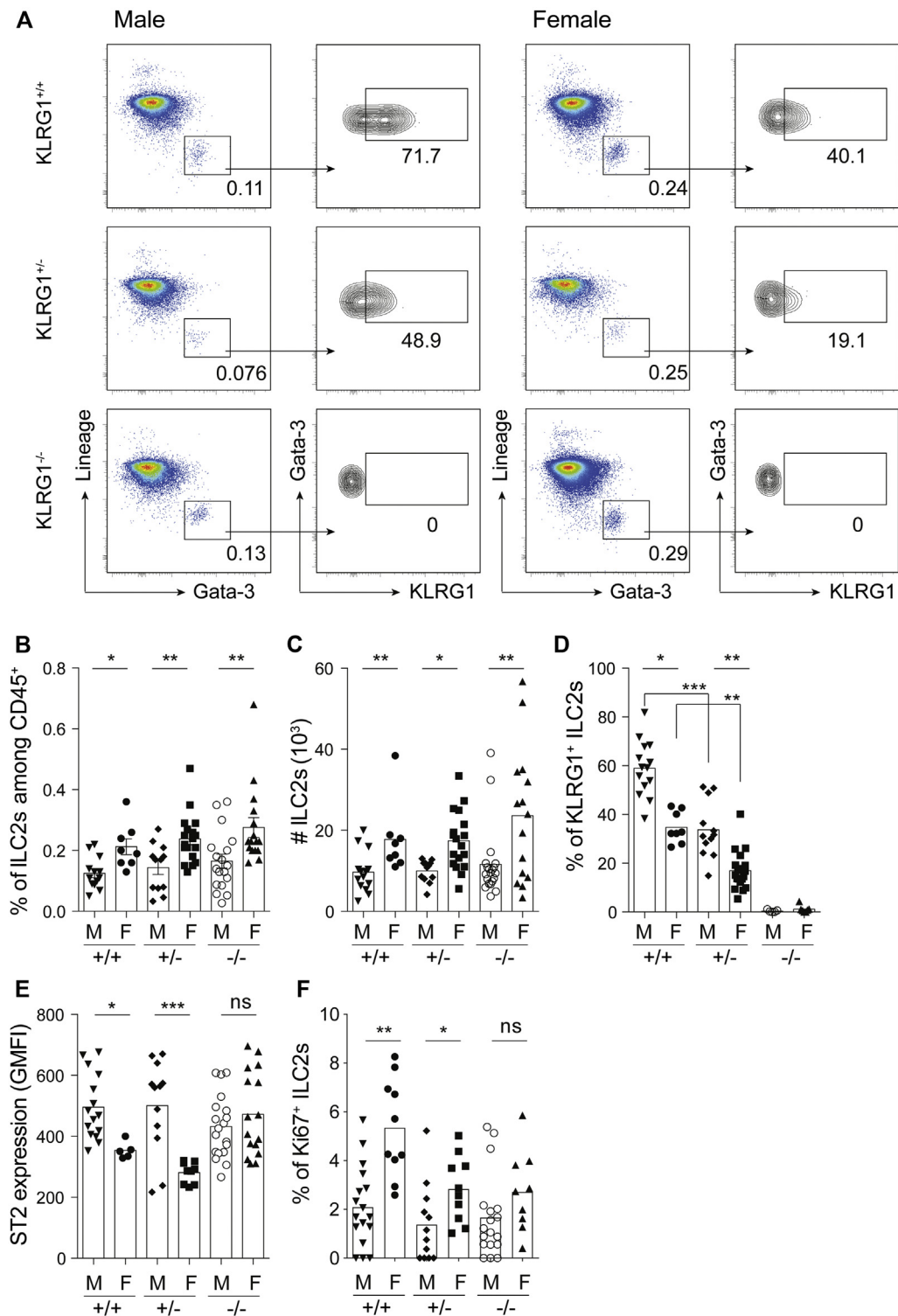


FIG 6. KLRG1 deficiency does not affect lung ILC2s at steady state in either male or female mice. ILC2 number and phenotype were analyzed in the lungs of 8-week-old KLRG1-deficient (KLRG1^{-/-}) male and female mice and their WT (KLRG1^{+/+}) and heterozygote (KLRG1^{+/-}) littermate controls. **A**, Representative lung ILC2 flow cytometry staining and their KLRG1 expression. **B**, Frequency of lung ILC2s. **C**, Number of lung ILC2s. **D**, Percentage of ILC2s expressing KLRG1. **E**, GMFI of ST2 on lung ILC2s. **F**, Frequency of Ki67-expressing ILC2s. **F**, Female; **GMFI**, geometric mean fluorescence intensity; **M**, male; **ns**, not significant. Data pooled of 2 different experiments (5-12 mice per group). **P* < .05; ***P* < .01; ****P* < .001.

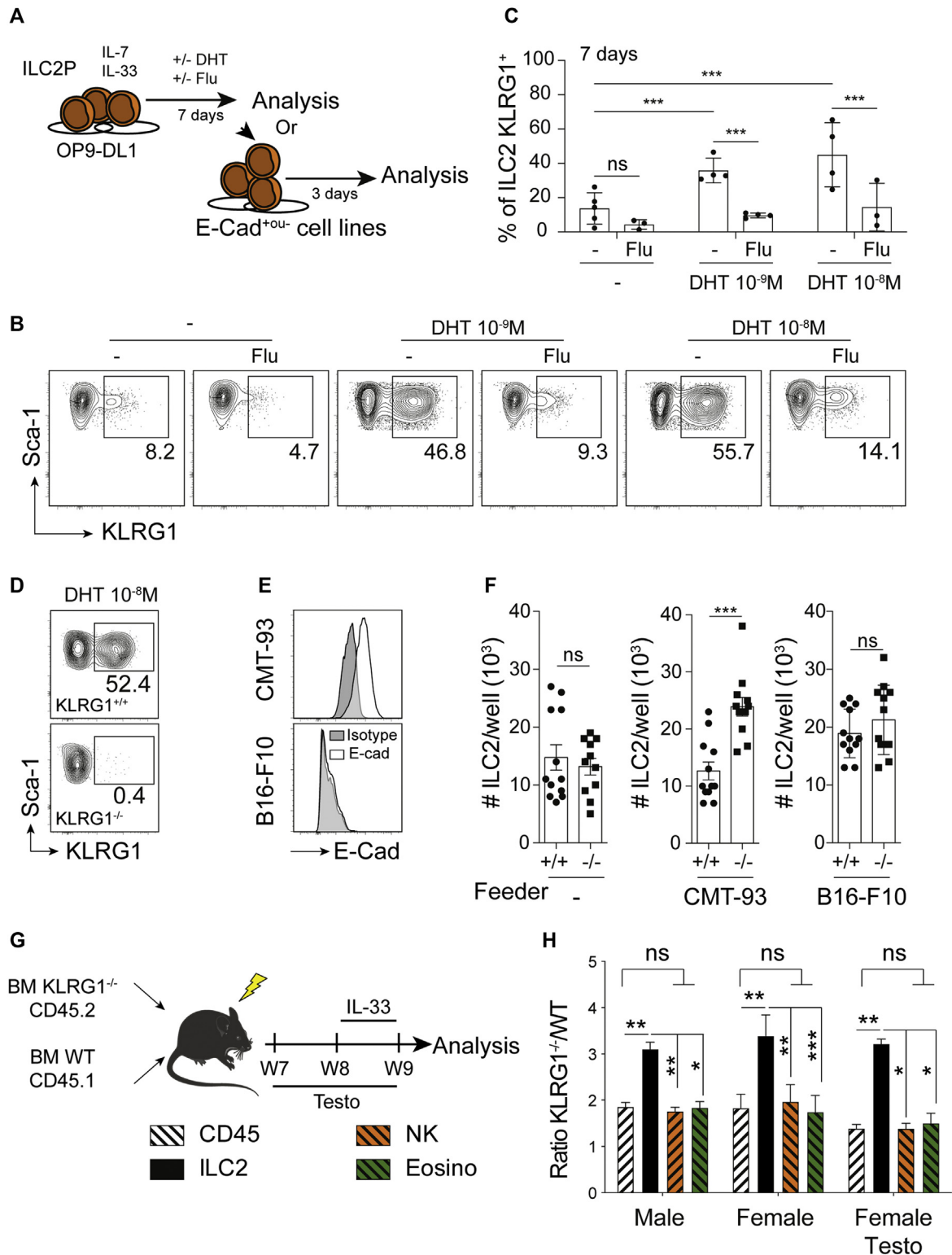


FIG 7. KLRG1 expression on ILC2s is upregulated by AR signaling and limit ILC2 proliferation and competitive fitness *in vivo*. **A-C**, *In vitro* differentiation of female ILC2P on OP9-DL1 feeder in culture medium supplemented with IL-7 and IL-33 in the presence of DHT or flutamide (Flu). **A**, Experimental design. **B**, KLRG1 expression on ILC2s on day 7. **C**, Associated percentages. Each dot represents the mean of 3 replicates. Between 3 and 5 independent experiments per condition were performed. **D-F**, Seven days after cell sorting, ILC2P were transferred on cell lines expressing E-cadherin (CMT93) or not (B16-F10) for 3 days in medium supplemented with IL-7 and IL-33. **D**, KLRG1 expression on ILC2s. **E**, E-cadherin expression on cell lines. **F**, ILC2 number at the end of the 10-day culture. **G** and **H**, Lethally irradiated B6 mice were intravenously reconstituted with an equal mixture of CD45.1⁺ WT and CD45.2⁺ KLRG1^{-/-} bone marrow cells. Eight weeks post-reconstitution, mice were intraperitoneally injected with hull-33. **H**, Ratio of WT to KLRG1^{-/-} CD45⁺, ILC2, NK, and eosinophils was assessed. *E-Cad*, E-cadherin; *Eosino*, eosinophils; *ns*, not significant; *Testo*, testosterone. Data are a pool of 2 independent experiments (5-10 mice per group). ***P* < .01; ****P* < .001.

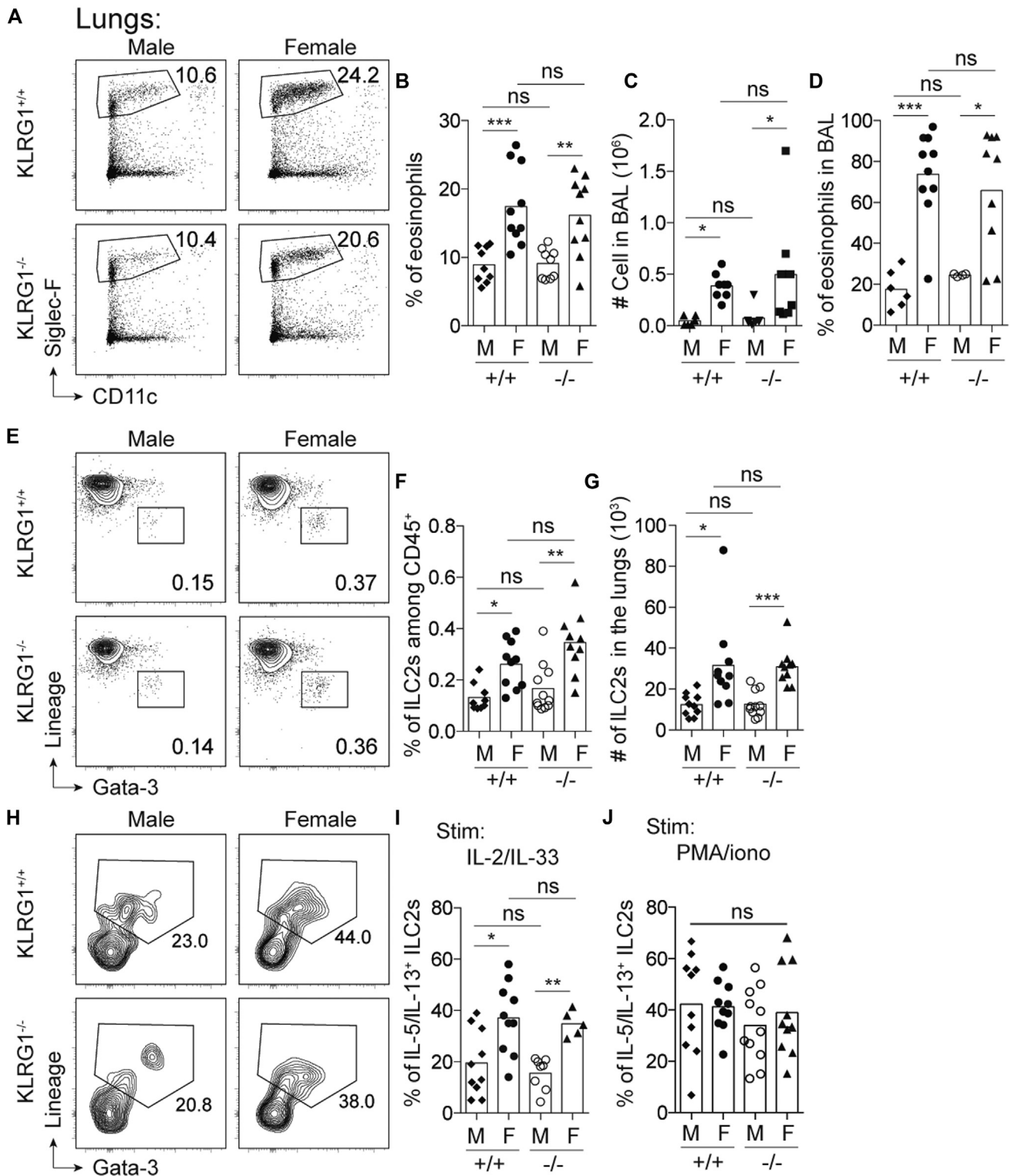


FIG 8. KLRG1 expression does not affect papain-induced lung inflammation. KLRG1^{+/+} and KLRG1^{-/-} male and female mice were intranasally challenged with papain for 3 days and analyzed on day 4. **A**, Representative flow cytometry staining of lung eosinophils. **B**, Percentage of lung eosinophils. **C**, Number of total cells in BAL. **D**, Frequency of eosinophils in BAL. **E**, Representative flow cytometry staining of lung ILC2s. **F**, Percentage of lung ILC2s. **G**, Number of lung ILC2s. **H**, Representative flow cytometry of IL-5- and IL-13-producing ILC2s stimulated overnight with IL-33 and IL-2 and **I**) associated percentage. **J**, Percentage of IL-5- and IL-13-producing ILC2s stimulated with PMA/ionomycin for 3 hours (brefeldin A during the last 1.5 hours). *F*, Female; *M*, male; *ns*, not significant; *PMA*, phorbol 12-myristate 13-acetate; *Stim*, stimulated. Data are a pool of 2 independent experiments (9-10 mice per group). **P* < .05; ***P* < .01; ****P* < .001.

dust mite extracts, or IL-33–driven lung inflammation.¹⁴ This robust sex bias in pulmonary ILC2s was also reported on challenge with various allergens, including *Alternaria alternata* extracts¹⁵ and the protease papain (our present work in Fig E3 and Fig 8), which both activate ILC2s through proteolytic maturation of IL-33 in the lung.³⁸ Androgens, through AR signaling, were found to account for most of the sex differences in lung ILC2 numbers.^{14,15} Moreover, exogenous androgens were reported to downregulate ILC2 functions on allergen challenge.¹⁵ However, it was unclear whether this inhibition was mediated through cell-intrinsic AR signaling in ILC2s. Indeed, we now provide evidence that short-term (7–10 days) treatment of adult female mice with androgens is sufficient to reduce lung ILC2 numbers. ILC2Ps in the bone marrow exhibited signs of androgen-mediated regulation as shown by their homogeneous downregulation of CD25 expression in bone marrow ILC2Ps. Although we previously reported that ILC2P express *Ar* and are sensitive to androgen-mediated regulation *in vitro*,¹⁴ the fast reduction in lung-resident ILC2s induced by androgen treatment is unlikely to occur through downregulation of ILC2P egress from the bone marrow. Indeed, experiments in parabiotic mice have shown that pulmonary ILC2s persist in adult as tissue-resident effector cells at steady state with limited input from distant cellular source, such as the bone marrow.^{25–27} The tissue-autonomous maintenance of ILC2s in adult tissue has been recently reported to depend in part on the *in situ* differentiation of ILC2s from resident progenitors.³⁹ Whether androgen signaling imparts ILC2 renewal and phenotypic diversification through its action on such tissue-resident progenitors or mature lung ILC2s will deserve further investigations.

The rapid and dramatic reduction in pulmonary ILC2s in androgen-treated mice was strongly correlated with an upregulated expression of KLRG1, which recognizes E-cadherin as a key component of epithelial intercellular junctions.⁴⁰ Although KLRG1 has been reported to inhibit human skin ILC2s function,²⁰ its role *in vivo* is still unclear. Here, we generated a new model of KLRG1 deficiency, and we showed that KLRG1^{-/-} mice from both sexes displayed normal frequencies of pulmonary ILC2s, not only at steady state but also during IL-33 or allergen challenge. Surprisingly, we showed that KLRG1/E-cadherin interaction blunted mouse ILC2 proliferation *in vitro*. Similarly, in response to IL-33 *in vivo*, KLRG1 expression impaired the competitive fitness of pulmonary ILC2s in a sex- and androgen-independent manner. However, despite the robust effect of KLRG1 in competitive settings, KLRG1 deficiency neither altered ILC2 numbers nor functional properties *in vivo*, nor did it lead to hyperactive ILC2s, in agreement with previous analyses of T-cell responses³⁶ and gut GATA-3⁺ regulatory T cells.⁴¹ Because expression of KLRG1 increases with the maturation states of lung ILC2s at steady state and following helminth infection,³⁹ and is upregulated in IL-33–activated ILC2 *in vivo*, we cannot exclude that KLRG1 might play a role in the tissue maintenance of mature/memory ILC2s on allergen challenge⁴² or in other functions of ILC2s associated with tissue homeostasis or tissue repair function.^{43,44}

Cues from the surrounding environment are critical in shaping ILC2s adaptation and functional properties in tissue.^{39,45,46} In the lung, ILC2 activation is predominantly mediated by IL-33.⁴⁶ In mice, IL-33 is a nuclear protein expressed in epithelial cells, fibroblast-like cells, and myofibroblasts, both at steady state and inflammation.³⁷ IL-33 can influence many aspects of allergic

responses from activation and development of eosinophils⁴⁷ to cytokine secretion by T_H2 cells and ILC2s.³⁷ Age-dependent sex differences in the expression of IL-33, IL-7, and thymic stromal lymphopoietin (TSLP) have been recently reported, with enhanced expression of lung epithelial cytokines in lung homogenates of adult female mice challenged with IL-33 or *A alternata* extracts compared with male mice.^{15,30} However, these differences were unlikely to contribute to the higher responsiveness of female ILC2s as we show here that sex differences in lung ILC2 numbers and phenotypes at steady state were not affected by IL-33 deficiency.

Asthma is more prevalent in boys than in girls during childhood, but in adolescence, asthma becomes more severe and prevalent in girls,^{4,8,48} consistent with the observation that asthma hospitalizations are prominent in young boys and middle-aged women.⁴⁹ The reason why asthma is more severe in young males is not understood. Early in life, males exhibit enhanced T_H2-type immunity and an increased susceptibility to T_H1-dependent infection, such as respiratory virus infection, compared with females.^{50,51} Of note, higher proportions of ILC2s have been reported in male cord bloods, whereas sex differences in circulating ILC2s were not found in adults,⁵² probably as a consequence of androgen upregulation after sexual maturity in males. Outgrowing of severe asthma is observed in longitudinal analysis during puberty, suggesting a protective action of sex steroid hormones.⁵³ Indeed, increased levels of androgens have been associated with asthma protection in many recent studies.^{54–56} Moreover, pharmacological and genetic studies support a protective action of androgens.^{57–59} Supplementation with the androgen dehydroepiandrosterone (DHEA)—which can be metabolized into testosterone, the precursor of DHT, by the dehydrogenase encoded by the *HSD3B1* gene—is beneficial in mild to moderate asthma.^{57,58} Two variants of HSD3B1 with consequences on DHEA metabolism have been described. The HSD3B1 (1245A) variant limits DHEA conversion to androgens, whereas HSD3B1 (1245C) increases DHEA metabolism to more potent androgens.⁶⁰ Patients with severe asthma homozygous for HSD3B1 1245C have better lung functions compared with AA or AC carrier patients, particularly in the context of glucocorticoid therapy.⁵⁹ Although AR expression and function in human ILC2s still remain to be shown, it is likely that androgens may benefit asthma through multiple mechanisms due to the pleotropic expression of AR in various tissues, including, airway smooth muscle,⁶¹ or other lung-resident cells.

Conclusions

Taken together, our results provide compelling evidence that androgen acting through AR expressed in ILC2s is able to exert rapid and efficient negative control of lung ILC2 maintenance and phenotypic diversification in both homeostasis and in settings of pulmonary diseases. Understanding how AR signaling regulates gene expression to promote this robust sex bias in pulmonary ILC2s will deserve further investigation. To our knowledge, beside glucocorticoids, no drug is currently available to reduce ILC2 numbers and function *in vivo*.⁶² Targeting AR signaling in human ILC2s could represent an alternative approach to inhibit ILC2s in patients with asthma, including corticosteroid-resistant asthma, in which ILC2s are suspected to play a prominent role over CD4 T cells.^{63,64} Along these lines, IL-2, IL-7, and TSLP

have been identified as modifiers of ILC2 corticosteroid sensitivity in a signal transducer and activator of transcription 5 (STAT-5)–dependent manner.⁶⁵ Indeed, STAT-5 is a key molecule in TSLP-induced corticosteroid resistance in mouse airway inflammation.⁶³ As we show that ILC2 activation and proliferation was still significantly blunted in male mice compared with female mice on IL-2 treatment, this suggests that the STAT-5–dependent pathway is not refractory to pharmacological inhibition by androgens. Selective AR modulators have been developed to maintain tissue-specific beneficial androgenic activities, while minimizing the masculinizing and virilizing side effects of testosterone.⁶⁶ We speculate that *in vivo* pharmacological manipulation of ILC2s using nonsteroidal selective AR modulator could represent an alternative to glucocorticoid treatment in corticoid-resistant asthma and could be instrumental in improving long-term outcomes of allergic asthma not only in men but also in women.

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Clinical implications: This study supports the proof of concept that targeting AR in ILC2s may be beneficial in allergic asthma by reducing ILC2 numbers and functions both at steady state and during allergen challenge.

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METHODS

Cholesterol-based time-release pellet for androgen delivery and androgen dosage in plasma

Pellets releasing either vehicle (cholesterol), testosterone, or DHT were prepared as described.^{E1} Testosterone (Tocris, Bristol, UK) or DHT (kindly provided by Dr F. Giton, INSERM U955, Créteil, France) was thoroughly mixed in powder form with cholesterol (C3045, cell culture grade purity >99%, Sigma-Aldrich, St Louis, Mo) at a w:w ratio of 10% testosterone or DHT for 90% cholesterol, and compacted to obtain pellets (20 mg total), which were then implanted subcutaneously into female mice as previously described.^{E2} Placebo-treated mice were implanted with 100% cholesterol pellets. Plasma testosterone and DHT levels were assessed by GC/MS, as described previously^{E3} using TQ8050 (Shimadzu, Japan) triple quadrupole mass spectrometer equipped with a chemical ionization source and operating in Q3 single-ion monitoring mode for detection. The linearity of steroid measurement was confirmed by plotting the ratio of the steroid peak response/internal standard peak response to the concentration of testosterone or DHT for each calibration standard. In female mice ($n = 4/\text{group}$) treated with testosterone pellets, plasma androgen concentrations were relatively constant from day 7 to 28, with testosterone ranging from 3.9 ± 1.4 ng/mL to 1.79 ± 0.424 ng/mL and DHT ranging from 0.077 ± 0.019 ng/mL to 0.028 ± 0.016 ng/mL. In placebo-treated mice, plasma androgens were below the detection limits.

AR protein expression by western blot

Cell lysates were prepared in Laemmli sample buffer (Invitrogen, Carlsbad, Calif), sheared through a 31-G needle, and total protein was quantified by a bicinchoninic acid protein assay (Pierce, Rockford, Ill). Protein (20–25 μg per lane) was fractionated by SDS-PAGE on precast 4% to 15% gradient gels (Bio-Rad, Hercules, Calif) and transferred to Amersham Hybond 0.45- μm PVDF membranes (GE Healthcare, Chicago, Ill). Membranes were blocked with 5% skim milk and 0.1% Tween-20 in PBS, and probed overnight with antibodies to β -actin (mouse monoclonal IgG₁, clone AC-15; Millipore Sigma, Chicago, Ill) or with the anti-AR antibody (abcam, clone EP670Y). Finally, membranes were incubated with peroxidase-conjugated secondary antibodies, anti-rabbit IgG (Cell Signaling Technology, 7074; Danvers, Mass). Chemiluminescent detection was carried out with Amersham ECL Select or ECL Prime reagents (GE Healthcare) as necessary, and densitometric analysis was performed with the Image Lab 5.0 software (Bio-Rad).

Generation of CRSPR/Cas9-mediated KLRG1-deficient mice

We designed 2 CRISPR-Cas9 crRNA targeting exon 1 of *Klrg1* using the publicly available design program Chop-Chop (<http://chopchop.cbu.uib.no/search.php>) (Mm.Cas9.KLRG1.1.AB 5'-TTCAACTAGAGCTGCCGG-3' and Mm.Cas9.KLRG1.1.AJ 5'-CTATTCAACTAGAGCTGC-3') (see Fig E6, A). Each crRNA was complexed with tracrRNA to generate gRNA complexes, which were used at a 1/1 molar ratio to form the ribonucleoprotein complex with Cas9 (Integrated DNA Technologies, Leuven, Belgium). C57BL/6 1-cell embryos ($n = 32$) were microinjected with Cas9-ribonucleoprotein complex targeting the 2 overlapping *Klrg1* exon 1 sequences. Mice were first screened by restriction enzyme digestion of genomic DNA using MspI, AluI, and TseI to identify mice carrying insertion/deletion (indel) of some bases generated upstream of the PAM motif, by the site-specific double-strand break followed by NonHomologous End Joining repair. Mice were further characterized by DNA sequencing (primer Fw: 5'-CAGCGGGCCTCTAAAGACA-3', Rev: 5'-ATTCCCTCACCTCTCTCC-3'). Mosaic founder mice containing 7 indels identified were mated with C57BL/6J mice. Two of them (indel#1; indel#4) were selected further because they resulted in earlier appearance of STOP codons. Mice heterozygous for these mutated alleles of KLRG1 were backcrossed at least 2 times on C57BL/6J background, and then intercrossed for generating KLRG1 knockout mice. KLRG1^{-/-} mice expressing either indel#1 or indel#4

gave similar results. Most of the experiments shown were performed with the KLRG1^{-/-} indel#1 line.

KLRG1 allele-specific amplification

Primers for WT (KLRG1⁺ allele), forward, 5'-ACACTA GAGCTGCCGGA-3'; reverse, 5'-GATCGGGATCAATAAAGAC-3', generate DNA segment of 373 bp. Primers for Indel#1 (KLRG1^{Indel#1}), forward, 5'-GTTTACTGCAGCGTCCA-3'; reverse, 5'-AGGTGCCTCACTA GAGCT-3', generate amplicons of 358 bp. Primers for Indel#4 (KLRG1^{Indel#4}), forward, 5'-ATCTATTCAACTAGAGCG-3'; reverse, 5'-ATCTGAGACTCGTACCATAG-3', generate amplicons of 250 bp size (Fig E6, B). PCR products were analyzed on 2% agarose gel electrophoresis.

Flow cytometry analysis

The following antibodies were used for the identification and purification of ILC2s, NK cells, and eosinophils: CD19 (ID3), B220 (RA3-6B2), CD3 (145-2C110), CD4 (GK1.4), CD8 α (53-6.7), CD11b (M1/70), Gr-1 (RB6-8C5), TCR β (H57-597), TCR $\gamma\delta$ (GL3), NKp46 (29A1.4), NK1.1 (PK136), CD49b (DX5), Pan-CD45 (30-FU), CD45.1 (A20), CD45.2 (104), CD117 (2B8), CD127 (A7R34), Sca1 (E13-161.7), KLRG1 (2F1), Thy1.2 (30H12), SiglecF (E50-2440), CD11c (HL3), and ST2 (DJ8 or RMT2-2). Intracellular staining was performed using the Intracellular Fixation & Permeabilization Buffer Set (ebioscience) to analyze transcription factor (Gata3, clone TWAJ) and Ki67 expression, and the BD Cytofix/Cytoperm kit to evaluate IL-13 (eBio13A) and IL-5 (TRFK5) cytokine expression. Intracellular cytokine staining for IL-13 and IL-5 was performed following stimulation for 4 hours with PMA (50 ng/mL) and ionomycin (100 ng/mL), in the presence of brefeldin A (1 $\mu\text{g}/\text{mL}$) or following overnight stimulation with IL-2 and IL-33 (10 ng/mL) and 5 hours of brefeldin A. Cells were acquired on an LSRFortessa (BD Biosciences) and analyzed with FlowJo software (FlowJo, Ashland, Ore). Flow cytometric sorting was performed with a FACSaria (BD Biosciences).

Bioluminescence assay

Lethally irradiated female B6 mice were reconstituted with ARE-Luc reporter bone marrow, as described above. Eight weeks posttransplant, mice were treated with huIL-33 for 7 days, and ILC2s sorted as described previously. Firefly-luciferase activity in cell lysates was assessed with Luciferase Assay System reagents from Promega according to the manufacturer's instructions. Briefly, 10⁴ ILC2s or B16-Luc were lysed using 100 μL passive lyse buffer (Promega). Twenty microliter/well of lysate and 100 μL well of substrate buffer (Promega) were mixed for bioluminescence detection. Chemiluminescence was measured in triplicate over a 10-second period, with a 2-second delay, on a Berthold Centro LB960 microplate luminometer.

Lung histology

Lung tissue was fixed in 10% buffered formalin for 24 hours and then placed in ethanol 70% before embedding in paraffin. Sections (4 μm) were stained with hematoxylin and eosin. Histological disease scores from 0 to 3 were attributed on the basis of severity of peribronchial, perivascular, and interstitial immune cell infiltration, and thickening of peribronchial epithelium, resulting in a maximum score of 12.

ELISA

IL-15 and IL-13 were quantified by 2-site sandwich ELISA (antibodies from BD Pharmingen). Absorbance was measured using a Varioscan microplate reader (Roche).

ILC2 stimulation and differentiation

To induce ILC2 expansion *in vivo*, mice were injected with 4 μg huIL-33 (as described above) and cells isolated from the lungs. Lin⁺ cells were depleted using the Mouse Hematopoietic progenitor cell enrichment kit (StemCell Technologies). Lin⁻ cells were then stained with

anti-CD90-allophycocyanin (APC) and positively selected with anti-APC microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). ILC2s ($\text{Lin}^- \text{CD90}^+$) were then FACS-sorted for bioluminescence assay or further expanded in RPMI medium supplemented with 10% FCS, 1% penicillin-streptomycin, 50 μM β -mercaptoethanol, and 10 ng/mL recombinant mouse IL-2 (10 ng/mL, R&D Systems). ILC2s were used at day 7 for western blot analysis (purity > 95%).

To induce the ILC2 differentiation *in vitro*, bone marrow cells were collected from hips, femurs, and tibias. ILC2P were FACS-sorted from magnetically enriched Sca-1⁺ cells as described (Fig E1, E). Purified ILC2P were cultured for 7 days in α MEM complete medium (10% heat-inactivated FCS, 1% penicillin-streptomycin, 50 μM β -mercaptoethanol) on OP9-DL1 (kindly provided by Dr Zúñiga-Pflücker, University of Toronto), with 2 ng/mL of IL-33, 5 ng/mL of IL-7, and 10 ng/mL of stem cell factor. The AR agonist DHT and the AR antagonist flutamide (Flu) (Sigma-Aldrich) were diluted in methanol at 10^{-2} mol for stock solution and then added at the

indicated concentrations (from 10^{-9} mol to 10^{-7} mol) at day 0 and +4 of culture. CMT93 and B16-F10 were plated 3 hours before ILC2 addition in 96-well plates (50,000 cells/well).

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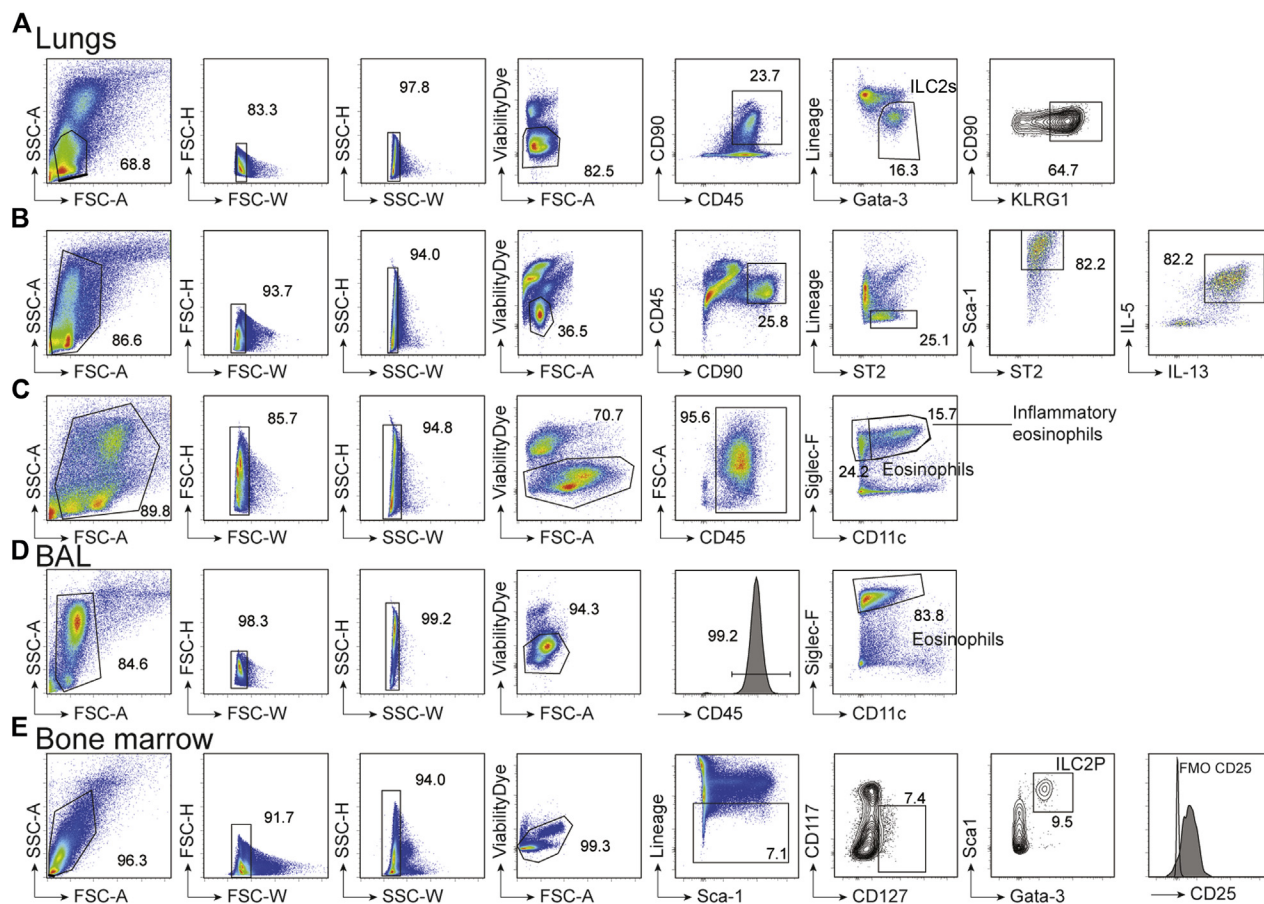


FIG E1. Flow cytometry gating strategies used for the analysis of lung-infiltrating cells. **A-C**, Lung analysis. **A**, ILC2 gating strategy. **B**, IL-5 and IL-13 production by ILC2s. **C**, Total eosinophils and inflammatory eosinophils. **D**, BAL cells analysis. **E**, ILC2P in the bone marrow. *FSC-A*, Forward scatter-area; *FSC-H*, forward scatter-height; *FSC-W*, forward scatter-width; *SSC-A*, side scatter-area; *SSC-H*, side scatter-height; *SSC-W*, side scatter-width.

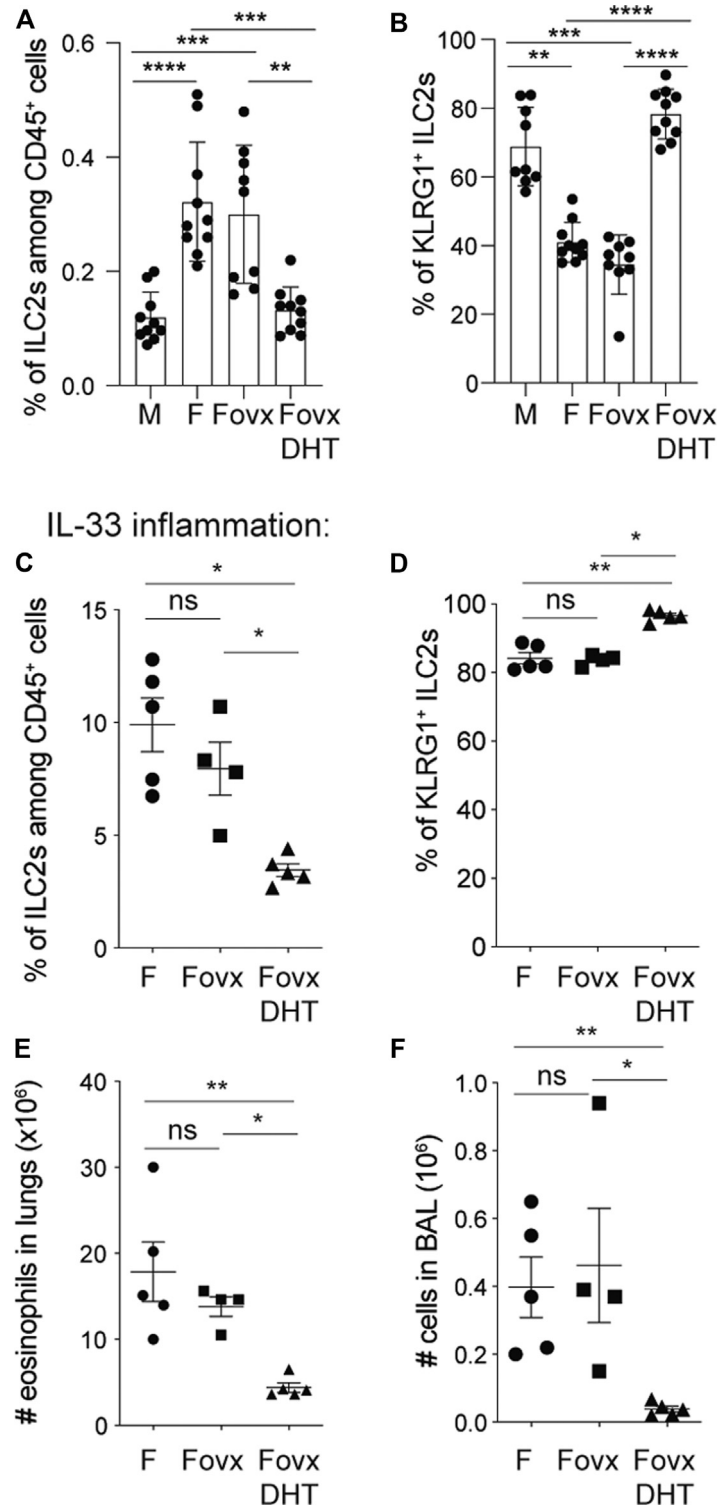


FIG E2. DHT treatment protects females from IL-33–driven lung inflammation. **A** and **B**, ILC2 analysis from the lungs of male (M), female (F), and OvX female (Fovx) treated with placebo or DHT pellets for 10 days. **A**, Frequency of ILC2s adjusted to total CD45⁺ cells. **B**, KLRG1 expression on lung ILC2s. **C–F**, OvX mice were implanted subcutaneously with placebo pellets (Fovx) or DHT-containing pellets (Fovx DHT) 10 days before intraperitoneal injection with hIL-33 (4 μg/mouse/d) on day 0, 1, 4, 5, and 6 and further analyzed on day 7. **C**, Frequency of ILC2s in lungs. **D**, Frequency of lung ILC2s expressing KLRG1. **E**, Number of eosinophils. **F**, Number of cells in BAL fluid. **F**, Female; **M**, male; *ns*, not significant. Comparison between groups was done using Kruskal-Wallis test. Bar graphs indicate the mean ± SEM. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

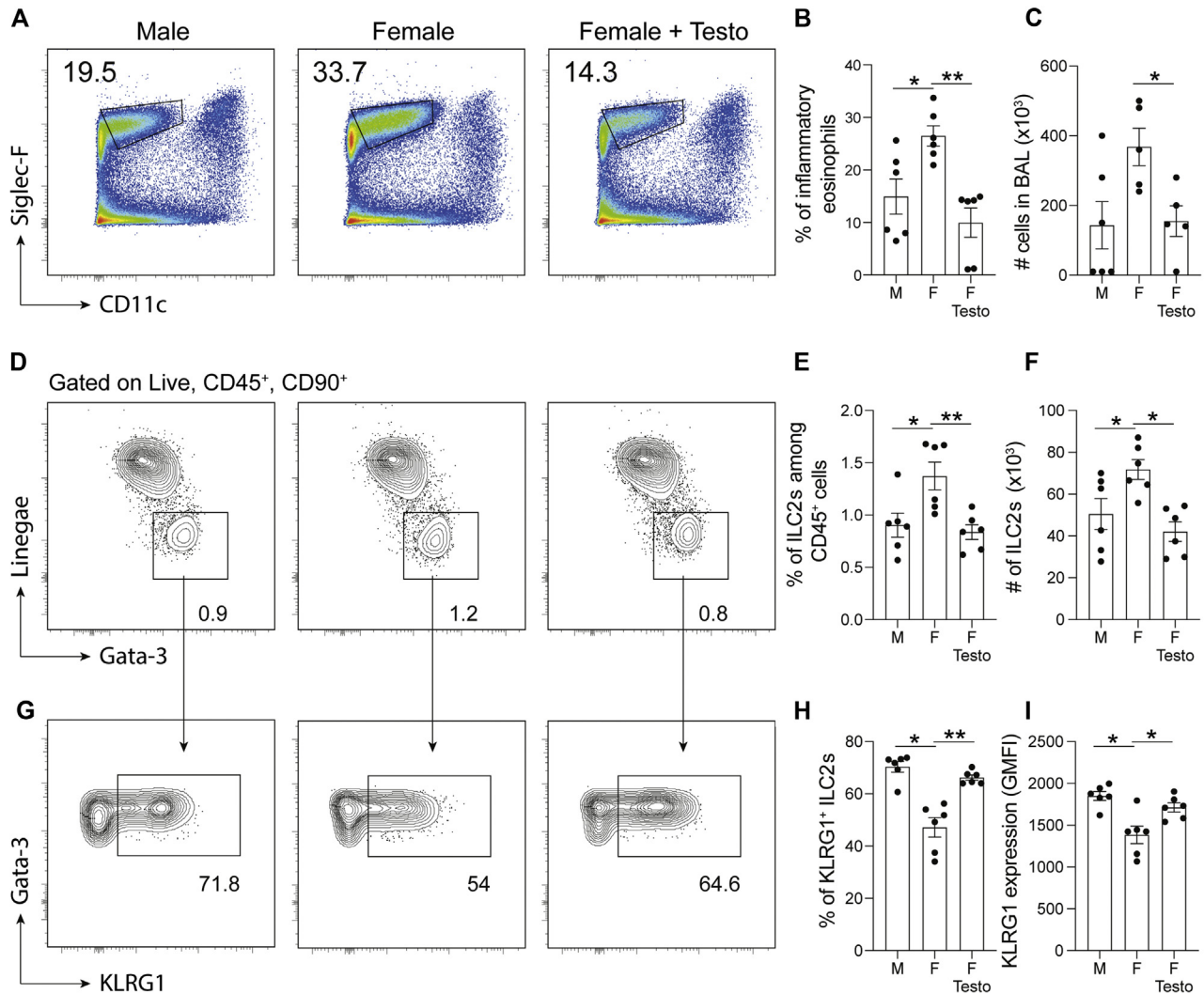
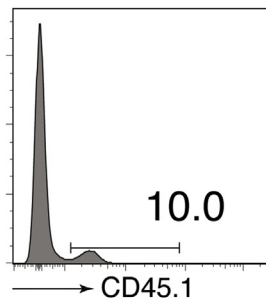
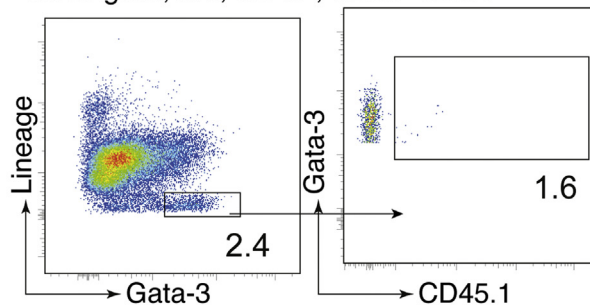


FIG E3. Testosterone treatment limits lung inflammation induced by the protease allergen papain in Rag2-deficient mice. Rag2^{-/-} male (M), placebo- (F), or testosterone-treated female (F Testo) mice were intranasally challenged with papain for 3 days and analyzed on day 4. **A**, Representative flow cytometry staining of lung inflammatory eosinophils. **B**, Percentage of lung inflammatory eosinophils. **C**, Number of total cells in BAL. **D**, Representative flow cytometry staining of lung ILC2s. **E**, Percentage of lung ILC2s. **F**, Number of lung ILC2s. **G**, Representative flow cytometry of KLRG1⁺ ILC2s. **H**, Associated percentage of fluorescence intensity. **I**, GMFI (6 mice per group). F, Female; GMFI, geometric mean of fluorescence intensity; M, male; ns, not significant. * $P < .05$; ** $P < .01$.

A On singlets, live, CD45⁺ cells :



B On singlets, live, CD45⁺, CD90⁺ cells :



C

- CD45.1 (Host)
- CD45.2 (Graft)

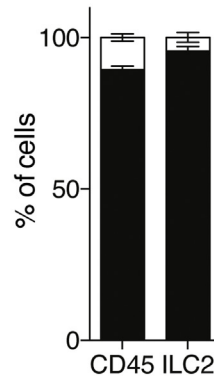


FIG E4. Lung ILC2s largely originate from the transplanted bone marrow. Lethally irradiated CD45.1 mice (host) were reconstituted with CD45.2 bone marrow cells (graft). Six weeks posttransplant, the relative ratio of CD45.1 and CD45.2 cells was analyzed in the lungs. Flow cytometry analysis of (A) total lung CD45 cells and (B) ILC2s. C, Proportion of CD45.1 among CD45.2 cells in the lungs.

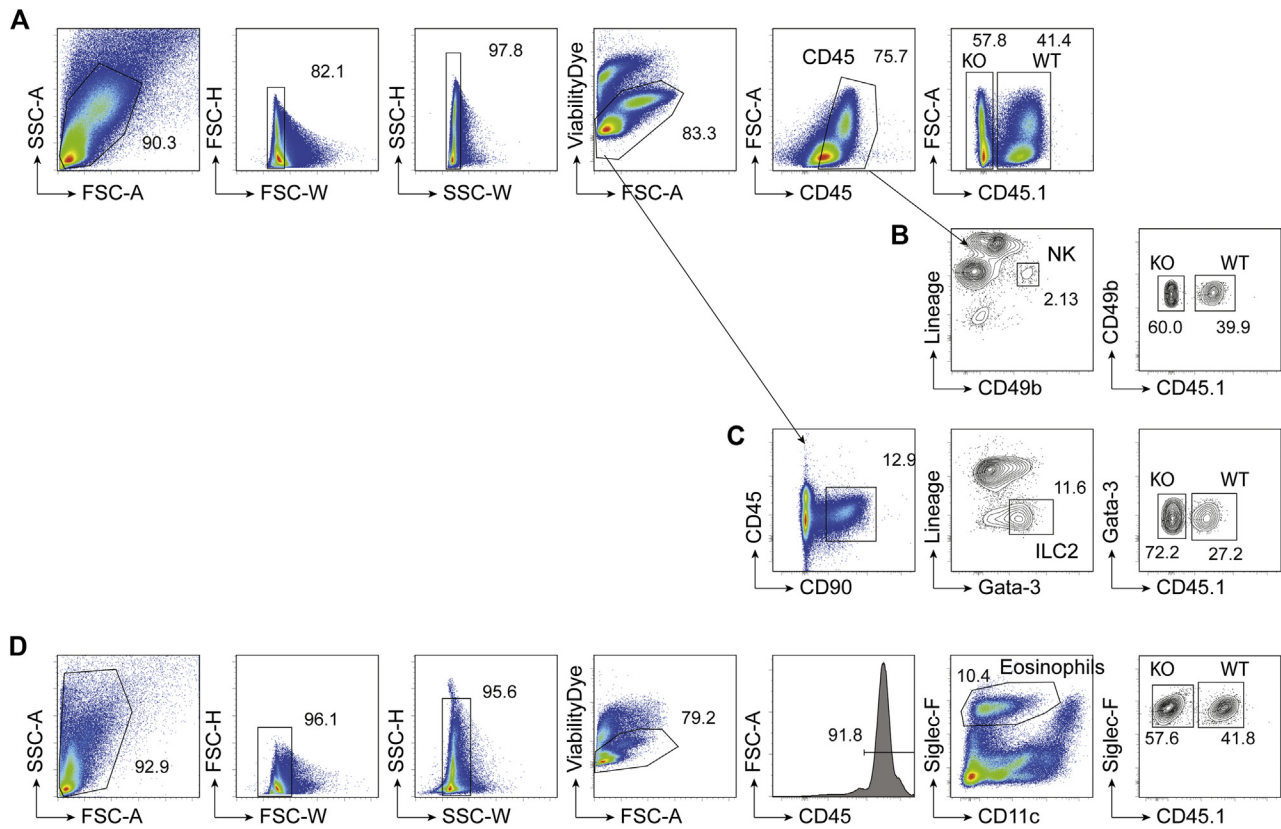


FIG E5. Flow cytometry gating strategy of lung-infiltrating cells in mixed bone marrow chimeras. Bone marrow chimeras were generated as described in Fig E4 by transplanting a mixture (1:1) of bone marrow cells from CD45.1 (WT) and CD45.2 (AR^{KO}) mice. **A**, CD45 cells analysis. **B**, NK cells gating strategy. **C**, ILC2 gating strategy. **D**, Eosinophils proportion in the lungs. *FSC-A*, Forward scatter-area; *FSC-H*, forward scatter-height; *FSC-W*, forward scatter-width; *KO*, knockout; *SSC-A*, side scatter-area; *SSC-H*, side scatter-height; *SSC-W*, side scatter-width.

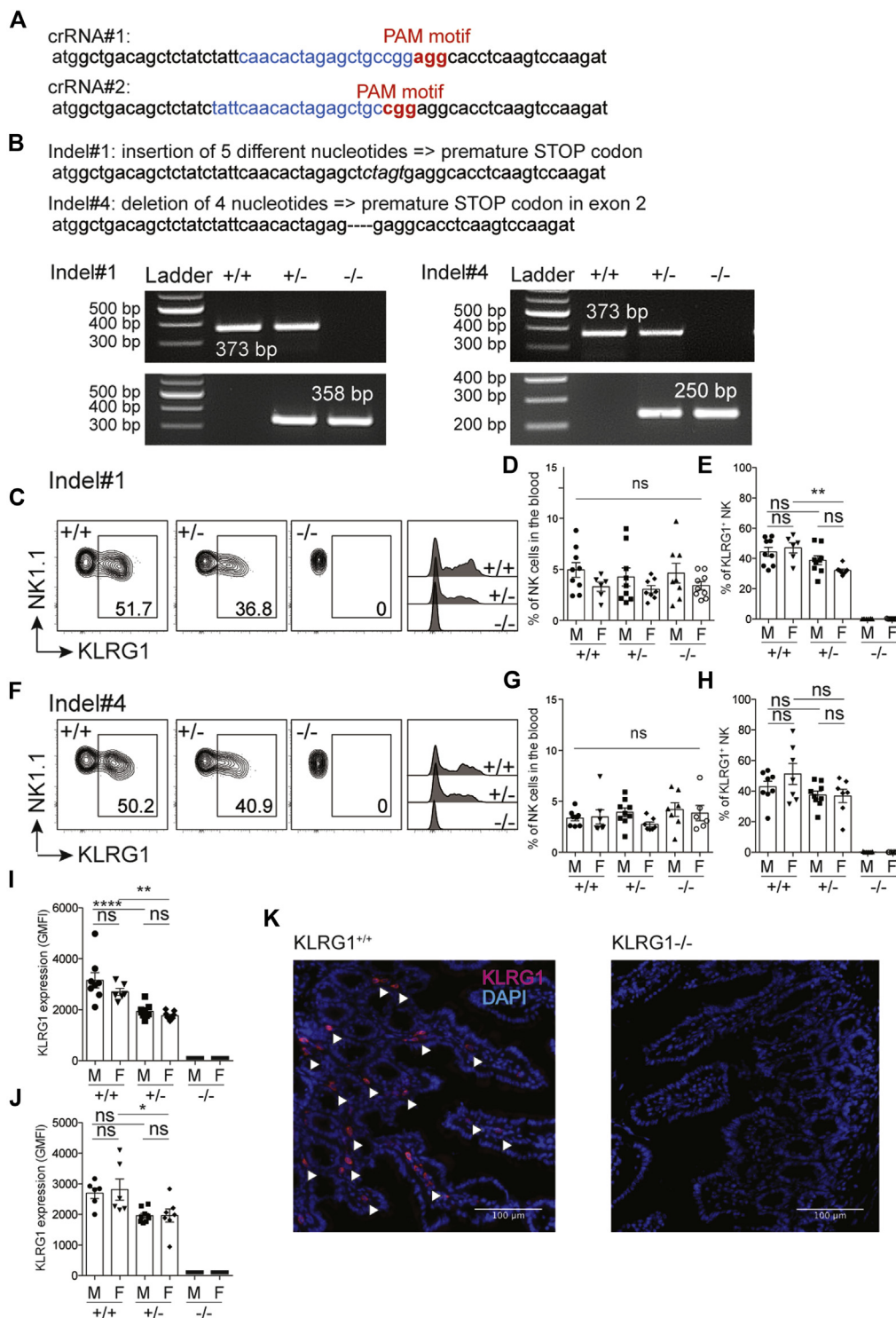


FIG E6. Generation of $KLRG1^{-/-}$ mice. Mutations (Indel#1 and Indel#4) were generated in $KLRG1$ locus using CRISPR-Cas9 method to induce the complete loss of the protein expression. **A**, Sequences of the 2 RNA guides used for the CRISPR-Cas9 are underlined with PAM motif in bold. **B**, Sequence of *Klr1g* gene in the 2 mutations. PCR amplification of $KLRG1^{+/+}$, $KLRG1^{+/-}$, and $KLRG1^{-/-}$ in Indel#1 and Indel#4 mice. **C**) Flow cytometry analysis of NK cells ($CD3^{-}$, $CD19^{-}$, $NK1.1^{+}$) in the peripheral blood of 4-week-old Indel#1 mice and littermate control and associated **(D)** percentage. **E**, Percentage of NK cells expressing KLRG1. **F**) Flow cytometry analysis of NK cells in the peripheral blood of 4-week-old Indel#4 mice and littermate control and associated **(G)** percentage. **H**, Percentage of NK cells expressing KLRG1. **I** and **J**, KLRG1 GMFI on NK cells in Indel#1 (Fig E6, I) and Indel#4 (Fig E6, J) mutant mice. **K**, KLRG1 staining on gut cryostat sections from $KLRG1^{+/+}$ and $KLRG1^{-/-}$ mice treated with hUL-33 as in Fig 2. *DAPI*, 4'-6-Diamidino-2-phenylindole, dihydrochloride; *F*, female; *GMFI*, geometric mean of fluorescence intensity; *M*, male; *ns*, not significant. Comparison between groups was done using Kruskal-Wallis test. Error bars indicate the mean \pm SEM. * $P < .05$; ** $P < .01$; **** $P < .0001$.

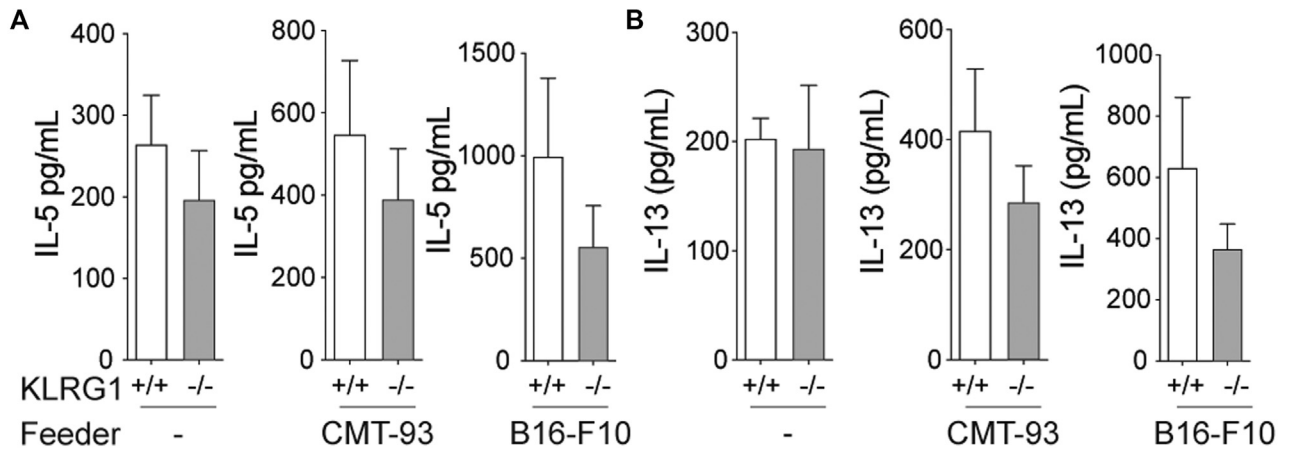


FIG E7. E-cadherin/KLRG1 interaction does not affect type 2 cytokine production by ILC2. *In vitro* differentiation of bone marrow ILC2P on OP9-DL1 feeder supplemented with DHT for 7 days and further transferred on cell lines expressing E-cadherin (CMT93) or not (B16-F10) for 3 days. **(A)** IL-5 and **(B)** IL-13 concentration in supernatants of the last 3 days of culture. Data were expressed as cytokine concentrations from individual wells normalized to 1000 ILC2s per well.

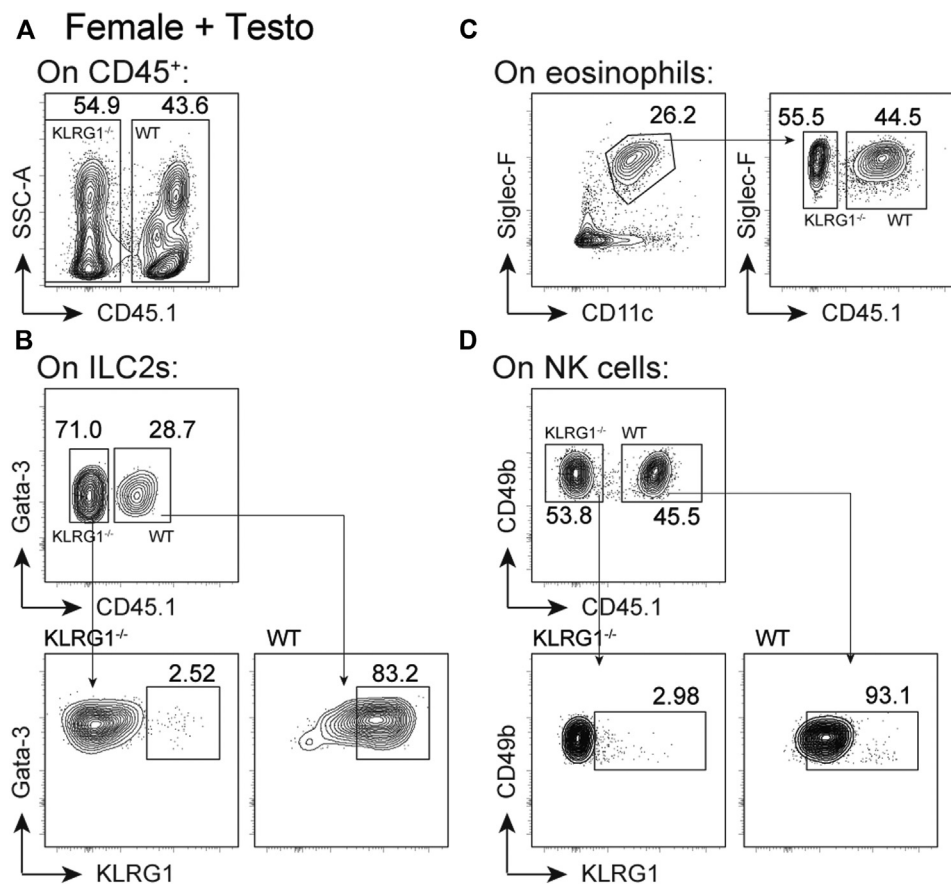


FIG E8. Flow cytometry gating strategy of lung-infiltrating cells in WT/KLRG1^{-/-} mixed bone marrow chimeras. Lethally irradiated B6 (CD45.2) mice were intravenously reconstituted with an equal mixture of CD45.1⁺ (WT) and CD45.2⁺ (KLRG1^{-/-}) bone marrow cells as described in Fig 7, G. Representative flow cytometry of female chimeric mice treated with testosterone. Analysis of CD45.1⁻ (KLRG1^{-/-}) and CD45.1⁺ (WT) cells on (A) total hematopoietic cells, (B) ILC2s, (C) eosinophils, and (D) NK cells. KLRG1 expression is shown for ILC2 and NK cells. SSC-A, Side scatter-area; *Testo*, testosterone.

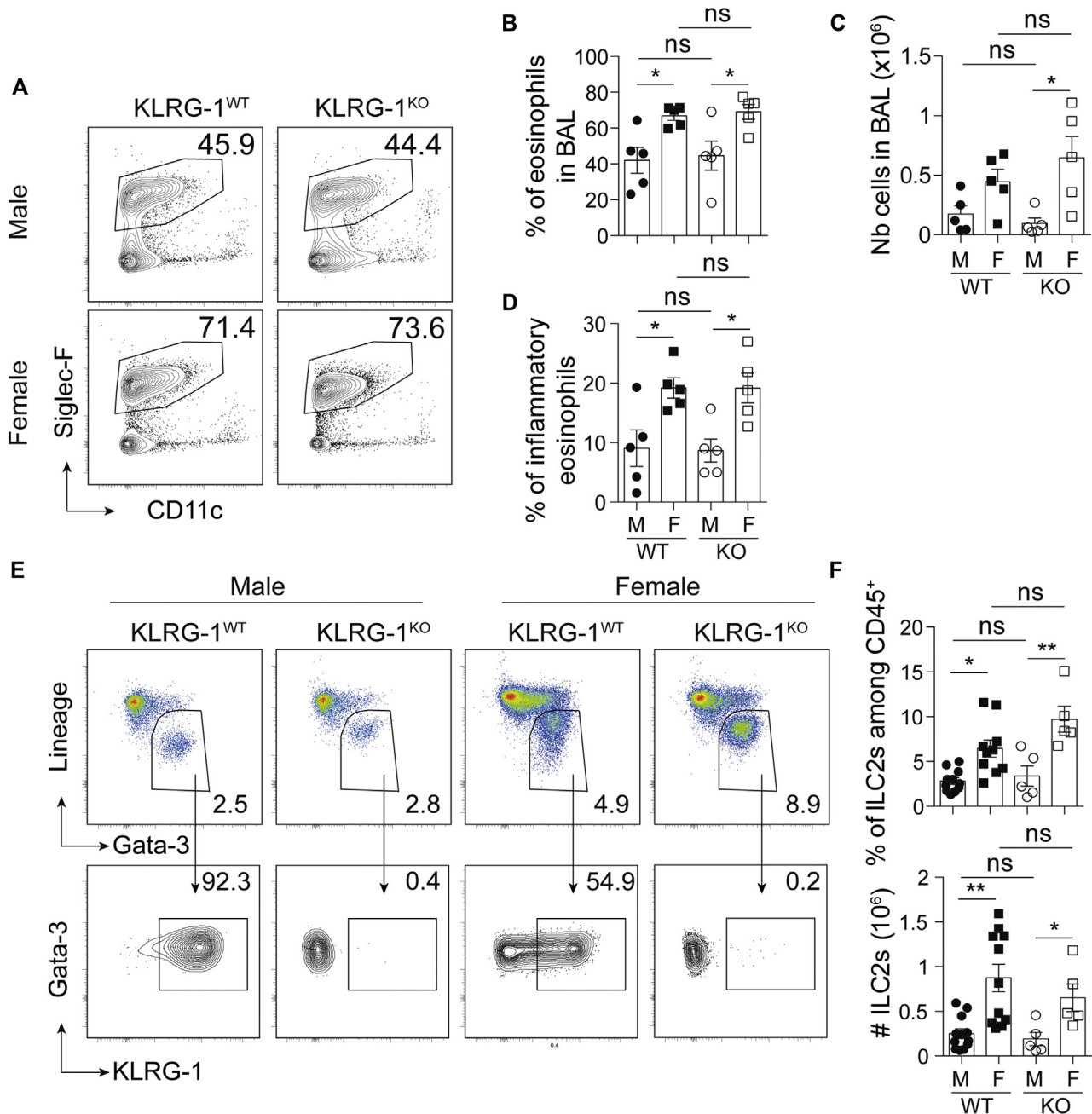


FIG E9. KLRG1 expression is dispensable to maintain the sex bias in ILC2-dependent lung inflammation induced by IL-33 administration. KLRG1^{+/+} and KLRG1^{-/-} male and female mice were intraperitoneally injected with huIL-33 (4 μ g/mouse/d) on day 0, 1, 4, 5, and 6 and analyzed on day 7. **(A)** Representative flow cytometry staining of eosinophils in BAL and **(B)** percentage associated. **(C)**, Number of cells in BAL. **(D)**, Percentage of inflammatory eosinophils in the lungs. **(E)**, Representative flow cytometry staining of ILC2s and their KLRG1 expression. **(F)**, Percentage and number of lung ILC2s (5-10 mice per groups). F, Female; KO, knockout; M, male; ns, not significant. Comparison between groups was done using Kruskal-Wallis test. Bar graphs indicate the mean \pm SEM. * $P < .05$; ** $P < .01$.

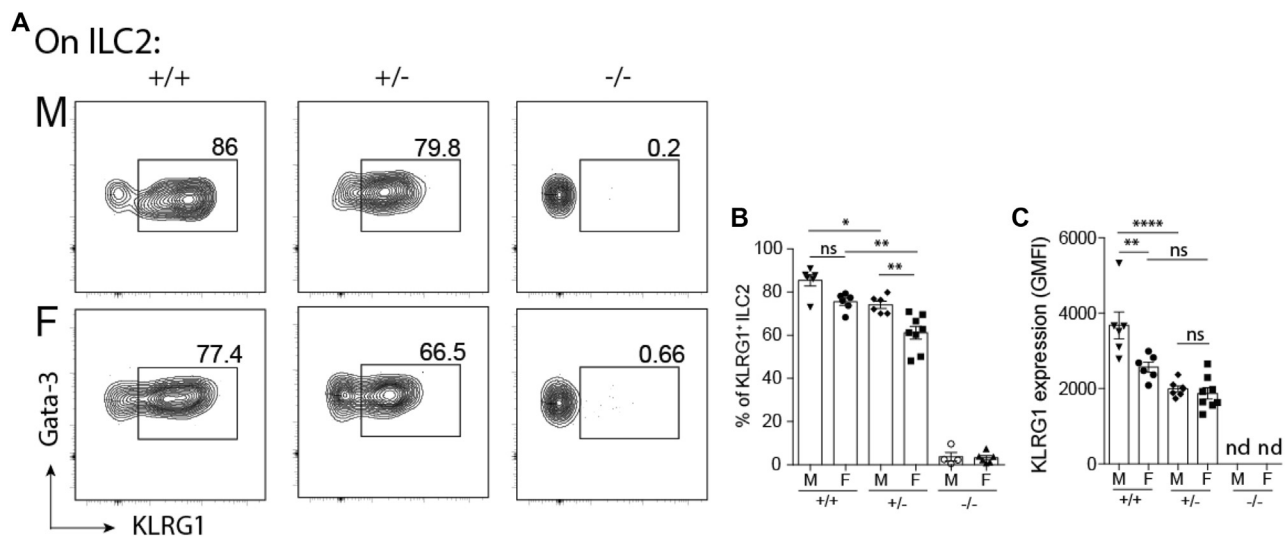


FIG E10. KLRG1 expression on pulmonary ILC2s during papain inflammation in male and female mice. KLRG1^{+/+}, KLRG1^{+/-}, and KLRG1^{-/-} male (M) and female (F) mice were intranasally treated with papain for 3 days and analyzed on day 4. **(A)** Representative flow cytometry staining of KLRG1 on ILC2s with **(B)** percentage and **(C)** GMFI associated. *F*, Female; *GMFI*, geometric mean of fluorescence intensity; *M*, male; *ns*, not significant. Comparison between groups was done using Kruskal-Wallis test. Bar graphs indicate the mean \pm SEM. * $P < .05$; ** $P < .01$; *** $P < .001$.