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IL-33-experienced group 2 innate lymphoid cells in the lung are poised to enhance type 2 inflammation selectively in adult female mice

Haya Aldossary[1], Rami Karkout[1], Katalina Couto[1], Lydia Labrie[1] and Elizabeth D. Fixman[1*]

Abstract

While Th2 adaptive immunity has long been considered to orchestrate type 2 inflammation in the allergic lung, group 2 innate lymphoid cells (ILC2s), with the ability to produce a similar profile of type 2 cytokines, likely participate in lung inflammation in allergic asthma. ILC2s are also implicated in sex disparities in asthma, supported by data from murine models showing they are inhibited by male sex hormones. Moreover, larger numbers of ILC2s are present in the lungs of female mice and are correlated with greater type 2 inflammation. Lung ILC2s exhibit intriguing memory-like responses, though whether these differ in males and females does not appear to have been addressed. We have examined type 2 lung inflammation in adult male and female Balb/c mice following delivery of IL-33 to the lung. While the number of ILC2s was elevated equally in males and females four weeks after exposure to IL-33, ILC2s from female mice expressed higher levels of ST2, the IL-33 cognate receptor subunit, and a larger proportion of ILC2s from females expressed the IL-25 receptor (IL-25R), which has previously been linked to memory-like ILC2 responses in mice. Our data show that the subset of ILC2s expressing IL-25R, upon activation, was more likely to produce IL-5 and IL-13. Moreover, STAT6 was absolutely required for enhanced responsiveness in this model system. Altogether, our data show that enhanced type 2 inflammation in females is linked to durable changes in ILC2 subsets with the ability to respond more robustly, in a STAT6-dependent manner, upon secondary activation by innate epithelial-derived cytokines.

Keywords IL-33, IL-25, ILC2, Trained immunity, Th2 adaptive immunity, Lung inflammation

Introduction

Asthma is a chronic condition characterized by airway inflammation, bronchial hyperreactivity, goblet cell hyperplasia and smooth muscle hypertrophy [1]. Abundant data support a role for type 2 inflammation in allergic asthma - the most common phenotype of asthma. Allergic airway inflammation is associated with increases in type 2 $CD4^+$ T helper cells (Th2), IgE-secreting B cells, eosinophils, mast cells, and group 2 innate lymphoid cells (ILC2s). Th2 cells and ILC2s produce type 2 cytokines, including IL-4, IL-5, and IL-13, which orchestrate the inflammatory responses in the lung, including eosinophil recruitment and activation, goblet cell hyperplasia, and airway hyperreactivity (AHR).

In murine models of allergic airways disease, ILC2s contribute significantly to the overall population of cells producing IL-5 and IL-13 [2–4]. Lung ILC2s are considered important targets of epithelial cell derived cytokines, including IL-25, thymic stromal lymphopoietin (TSLP), and IL-33, though a wide assortment of mediators

*Correspondence:
Elizabeth D. Fixman
elizabeth.fixman@mcgill.ca
[1]Meakins-Christie Laboratories, Research Institute of the McGill University Health Centre, Montreal, QC, Canada

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modulates activation or inhibition of ILC2s [5]. IL-33 is generally considered the most potent of the alarmins, though ILC2s have the ability to respond to each of these cytokines. IL-33 and TSLP promote their expression in a reciprocal manner [6]; and activation of ILC2s by TLSP is associated with steroid resistance in both humans and mice [7, 8].

More males than females are diagnosed with asthma before puberty, with a shift in adults, where prevalence is greater in females who, in addition, often experience more severe forms of the disease [9, 10]. Abundant data from both human studies and murine models implicate sex hormones in these differences, with androgens inhibiting and estrogens promoting disease [11–14]. Recently, modulation of ILC2s by sex hormones has been defined in a number of studies. Female mice have larger numbers of ILC2s, primarily due to the presence of a population of ILC2s that do not express the killer-cell lectin like receptor G1 (KLRG1) [13, 15]. KLRG1 association with E-cadherin, present on lung epithelial cells, inhibits ILC2s in vitro [16]; thus, it has been proposed that KLRG1– ILC2s may have greater activity. While androgens negatively regulate lung ILC2s [12–14] and estrogens are largely without effect [11], progesterone may positively regulate ILC2s [17]. Nevertheless, in mice estrogens increase levels of IL-33 in the lung, thereby enhancing allergen-induced type 2 inflammation [11]. Consistent with findings from mice, adult females with moderate to severe asthma have larger numbers of circulating ILC2s compared to adult males [11].

The complex relationship between ILC2s and other immune cells through cytokine production and/or cell: cell interactions has led to an increasing interest in the fate of activated ILC2s even after the resolution of airway inflammation [18]. Data from murine studies show that, upon restimulation, ILC2s, previously activated in vivo (by IL-33, *Alternaria alternata*, papain, or the serine protease from *Aspergillus oryzae*), respond more dramatically compared to their naïve counterparts, through enhanced proliferation and cytokine production [19, 20]. These memory-like ILC2s have a gene expression profile similar to that of $CD8^+$ memory T cells; they also express higher levels of the IL-17RB subunit of the IL-25 receptor (IL-25R) and respond to intranasal administration of IL-25, a response lacking in ILC2s from naïve mice [19]. More recently, gene expression programs have been linked to enhanced responsiveness and memory development in ILC2s, one of which - the so-called 'preparedness' program - includes the STAT6 transcription factor [20], a well-known inducer of type 2 inflammation [21].

Here, we have investigated ILC2 responses in models of allergic airways disease and type 2 inflammation in which male and female mice were sensitized through lung delivery of ovalbumin (OVA) in the presence of IL-33. Mice were then challenged one month later by delivery of OVA or alarmin cytokines to the lung. Our data show that females had larger numbers of eosinophils and ILC2s in the lung following secondary challenge with either OVA or alarmins and that even without challenge, IL-33-experienced lung ILC2s persisted at greater levels for at least one month in both males and females. While the number of ILC2s did not differ, their phenotype did. ILC2s from female mice expressed greater levels of ST2; and a larger number of ILC2s in females expressed the IL25R, which was associated with a greater propensity for cytokine production upon delivery of either IL-25 or IL-33. Moreover, enhanced responsiveness to IL-25 was absent in ILC2s from STAT6 knockout (KO) mice. Together, our data suggest that upon previous activation by IL-33, lung ILC2s in female mice are poised to exert stronger effector functions following secondary challenge with disparate stimuli.

Materials and methods

Mice

Male and female, wild type and STAT6 knockout (KO) Balb/c mice, originally purchased from The Jackson Laboratory (Bar Harbor, ME) were bred in house under pathogen free conditions in the Animal Resource Division at the Research Institute of McGill University Health Centre and used at ages 5–6 weeks. Mice were kept in cages supplemented with water and irradiated food at all times. All animal studies were approved by the McGill University Animal Care Committee and performed following guidelines of the Canadian Council on Animal Care.

In vivo stimulation

Mice were briefly anesthetized with isoflurane, followed by intranasal treatment with IL-33 (0.05 μg), and/or OVA (50 μg) in a volume of 30 μl on days 1 and 2. OVA alone was used as the negative control. IL-33 was purchased from Thermo Fisher Scientific (Carlsbad, CA) and OVA was purchased from Worthington Biochemical Corp (Lakewood, NJ). Mice were allowed to rest for 4 weeks and then, in some experiments, either sacrificed directly or challenged with OVA (10 μg) daily for each of 4 days and euthanized 48 h later for lung collection and processing. In other experiments, mice were sensitized as above and 4 weeks later, treated with a single intranasal injection of IL-25 (0.25 μg) or IL-33 (0.25 μg) or saline and euthanized 48 h later. In experiments to examine whether the absence of STAT6 activity affected memory-like ILC2 responses, female wild-type and STAT6 KO mice were treated with IL-33 prior to the 4-week rest and in vivo delivery, as above, with saline or IL-25.

Lung digestion

Whole lungs were collected in RPMI medium. Then, lung tissue was minced and digested for 35 min at 37 °C in 1mL of enzymatic solution comprised of RPMI with DNase I (200 µg/ml; Sigma-Aldrich, Oakville, ON), Liberase™ (100 µg/ml; Roche, Indianapolis, IN), hyaluronidase 1a (1 mg/ml; Life Technologies, Carlsbad, CA), and collagenase XI (250 µg/ml; Life Technologies). Enzymatic digestion was stopped by the addition of 1mL RPMI+5% FBS [22, 23]+1% penicillin/streptomycin. Red blood cells were lysed by adding 3 ml of ammonium-chloride-potassium (ACK) lysis buffer, followed by 2 washes with RPMI+5% FBS+1% penicillin/streptomycin after which the cells were filtered, counted and then diluted using RPMI+5% FBS to obtain 2×10^6, and 1×10^6 cells for ILC2 and eosinophil staining, respectively.

Flow cytometry staining

All cells for flow cytometry analysis were first washed with phosphate-buffered saline (PBS) before being stained. Then, cells were plated in low-adherent round-bottom 96 wells plates. ILC2s were incubated with 0.665 µl/mL of Golgi Stop (BD Biosciences, Franklin Lakes, NJ) in RPMI containing 10% FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 10 mM HEPES and 55 µM 2-mercaptoethanol for 4 h at 37 °C and 5% CO_2. Then, cells were washed with PBS and incubated in the dark at 4 °C with eFlour789 viability dye (eBioscience, San Diego, CA) for 20 min. Next, cells were washed and incubated for 5 min with anti-mouse CD16/CD32 (Fc block) (BD Biosciences, San Jose, CA). ILC2s were stained using BUV395-CD45.2, EF-450-Thy1.2, PECy7-CD127, PerCP-eF710-ST2, BV605-KLRG1, BV510-MHCII, BV786-IL-17RB and a combination of PE-conjugated antibodies against CD3e, CD11c, CD11b, CD49b, CD45R, TCRyD, Ly6G, and FceR1a. Eosinophils were stained with BUV395-CD45.2, Alexa Fluor 700-Ly6G, APC-F4/80, Alexa Fluor 488-CD11c, PeC7-CD11b, PE-Siglec F. After 30 min, cells were washed twice with PBS and fixed overnight with intracellular fixation buffer (eBioscience).

The next day, cells for ILC2 flow cytometry were washed and permeabilized with Perm/Wash buffer (BD Biosciences, Franklin Lakes, NJ) and stained intracellularly with AF488-IL-13 and APC-IL-5. After 45 min, cells were washed twice with Perm/Wash buffer and one time with PBS. Finally, cells were resuspended in 100 uL of PBS for acquisition. In all flow panels, fluorescence minus one (FMO) controls were used to define and gate positive populations. More information about the antibodies can be found in Supplemental Tables 1 and 2. Cells were acquired using BD LSRFortessa or BD LSRFortessa X-20 (Immunophenotyping Core Facility, RI-MUHC) flow cytometers. Analysis was completed with FlowJo V10 (FlowJo LLC, Ashland OR).

Statistical analysis

Data analyses and graphs were generated using Prism (Graphpad Software, San Diego, CA). Data were analyzed using one-way or two-way ANOVA followed by Tukey's multiple comparisons test as indicated in the figure legends. A p-value less than 0.05 was considered significant. Outcomes are presented as mean ± standard error of the mean (SEM). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Grubb's test with $\alpha=0.05$ or ROUT test with Q=1% were employed to remove outliers.

Results

ILC2s persist in the lungs of male and female Balb/c mice for up to 4 weeks post OVA/IL-33 delivery

To better understand the influence of sex on the fate of IL-33-treated lung ILC2s in type 2 inflammation and allergic airways disease, we first examined ILC2 responses in a murine asthma model. Adult mice were treated with OVA alone (as the negative control) or OVA/IL-33 delivered to the lungs, after which mice were rested for 4 weeks and then either sacrificed directly or challenged with OVA to reactivate OVA-specific T cells prior to sacrifice (Fig. 1A). This model is similar to those of the Croft lab, showing that addition of lipopolysaccharide or a Nod2 agonist (muramyl dipeptide) to OVA at the time of antigen priming through the lung reduces induction of OVA-induced tolerance and, at the same time, promotes Th2 differentiation and type 2 inflammation following OVA challenge [24, 25]. Consistent with this, four weeks after initial priming, eosinophils were not elevated in mice sensitized with OVA alone, whether challenged with OVA or not. However, following OVA challenge of mice that had been sensitized with OVA/IL-33, eosinophils in both males and females were increased, to levels that were significantly greater in females (Fig. 1B) (exhibiting sex differences similar to those in house dust mite models of allergic airways disease) [14, 26].

Mice treated with OVA alone had negligible differences in total ILC2s, whether they were from males or females, and whether they were examined before or after OVA challenge (Fig. 1C). However, even without OVA challenge, ILC2s in mice sensitized with OVA/IL-33 were significantly increased, to levels that were similar in males and females (Fig. 1C). Following OVA challenge of OVA/IL-33 sensitized mice, ILC2s increased about 3-fold in both males and females, and similar to eosinophils, there were significantly more ILC2s in females compared to males (Fig. 1C). These data suggest that ILC2s persist in the lungs of both male and female mice for up to 4 weeks post activation.

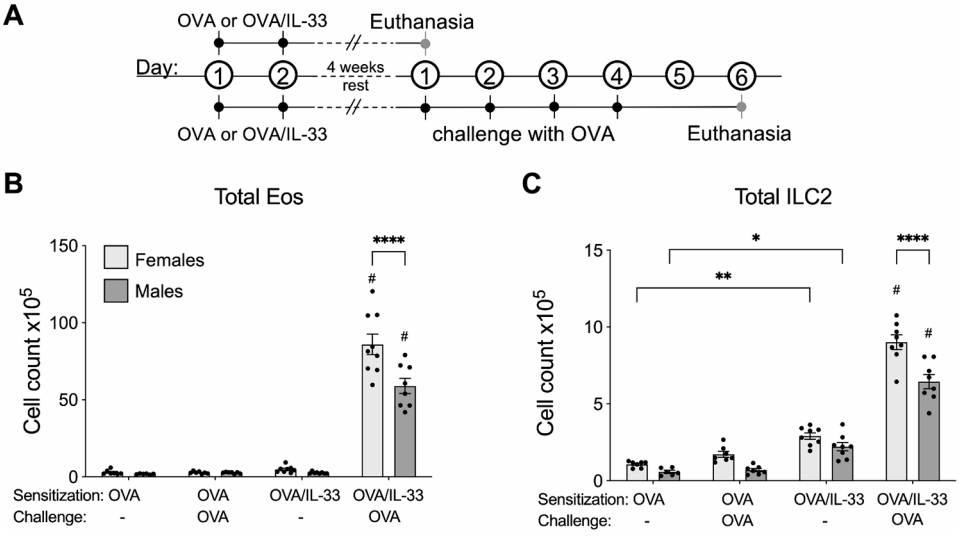

Fig. 1 ILC2s persist in the lungs of male and female Balb/c mice for up to 4 weeks post OVA/IL-33 delivery. **(A)** Male and female Balb/c mice were sensitized with OVA or OVA/IL-33 once per day for 2 days. Mice were rested for 4 weeks and then either sacrificed directly or challenged with OVA once per day for each of 4 days and euthanized 48 h later, after which lungs were harvested for flow cytometry analysis. Absolute count of **(B)** eosinophils and **(C)** ILC2s in the lung. Data are the combination of 2 independent experiments, presented as mean ± SEM, with 6–8 mice/group. Outcomes were assessed by two-way ANOVA, followed by Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. #: significantly different than all other groups within the same sex

IL-33-experienced ILC2s exhibit phenotypic differences in male and female mice

Sex-specific phenotypic differences in ILC2s in murine models have been noted both before and after activation. One notable difference is that in adult female mice there are larger numbers of ILC2s lacking expression of KLRG1 [13], findings we have confirmed [15]. Thus, we examined ILC2 populations expressing KLRG1 (or not) 4 weeks following exposure to OVA or OVA/IL-33. No sex differences were observed in the numbers of KLRG1$^+$ ILC2s in any group, though the KLRG1$^+$ population remained significantly elevated 4 weeks post OVA/IL-33 treatment in both males and females and increased further, and to the same extent, in mice of each sex upon OVA challenge (Fig. 2A). Moreover, compared to males, KLRG1$^-$ ILC2s were elevated in females, though a statistically significant sex difference was present only after OVA challenge of OVA/IL-33 sensitized mice. In addition, unlike ILC2s expressing KLRG1, the KLRG1$^-$ ILC2 population was not significantly elevated 4 weeks after OVA/IL-33 exposure (in mice of either sex) (Fig. 2B).

Because upregulation of IL-25R has previously been associated with memory-like responses in ILC2s [19], we examined whether levels of this receptor were increased in IL-33-experienced ILC2s before or after OVA challenge, using the gating strategy presented in Fig S1. Following OVA challenge of OVA/IL-33-sensitized mice, the total number of IL-25R-positive ILC2s, expressing KLRG1 or not, was significantly increased only in female mice (Fig. 2C and D). Interestingly, there was also a clear trend for these cells to increase 4 weeks after OVA/IL-33 administration and prior to OVA challenge, again selectively in females. No change in the median fluorescence intensity (MFI) of IL-25R was detected (data not shown). Finally, while we defined ILC2s based on their ST2 expression (Fig S1), we also found that, 4 weeks after OVA/IL-33 exposure, ILC2s in female mice expressed higher levels of ST2; an increase that was present in both KLRG1$^+$ and KLRG1$^-$ ILC2s (Fig. 2E and F), but absent in their male counterparts. This increase in ST2 expression in ILC2s from female mice was no longer apparent following OVA challenge. These data suggest that, one month after initial activation, IL-33-experienced ILC2s in female mice are poised to respond in a more robust manner to secondary challenge with IL-33 or IL-25.

Sex differences in cytokine producing ILC2s are due to KLRG1$^-$ ILC2s in female mice

We also quantified cytokine producing ILC2s in mice exposed to OVA or OVA/IL-33, with and without OVA challenge. Cytokine producing ILC2s were not significantly elevated 4 weeks after OVA/IL-33 administration in either males or females; however, OVA challenge induced a significant increase in the number of IL-13$^+$ KLRG1$^+$ ILC2s in both males and females (Fig. 3A), but a similar increase in IL-13$^+$ KLRG1$^-$ ILC2s was present only in females (Fig. 3C). IL-5$^+$ KLRG1$^+$ ILC2s tended to increase 4 weeks after OVA/IL-33 delivery, but OVA challenge did not further increase their number above that present in OVA/IL-33 treated mice (with no OVA challenge) (Fig. 3B). Females had significantly greater numbers of IL-5$^+$ KLRG1$^-$ ILC2s compared to males

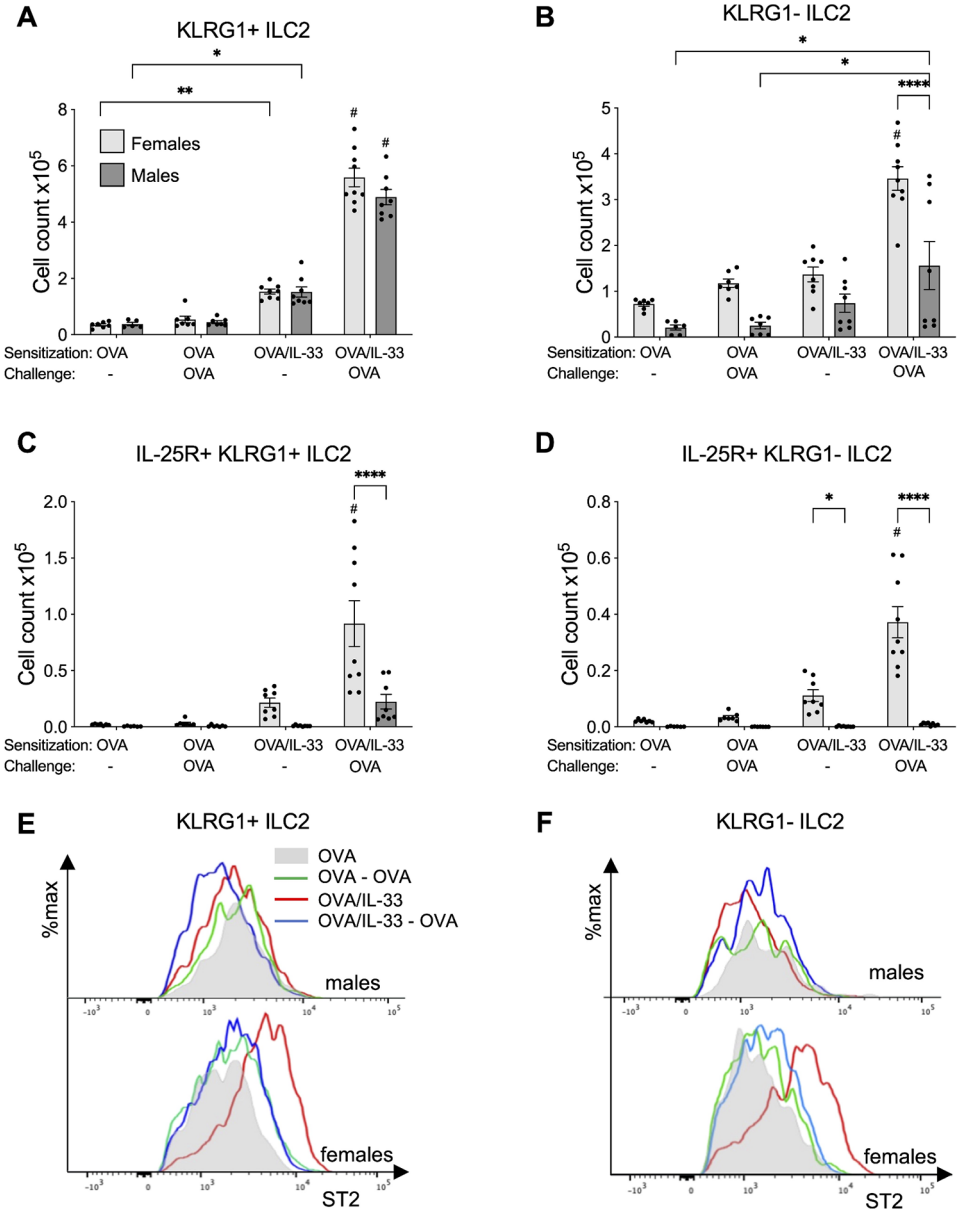

Fig. 2 IL-33-experienced ILC2s exhibit phenotypic differences in male and female mice. Mice were treated as described in Fig. 1A, after which lungs were harvested for flow cytometry analysis. Absolute count of **(A)** KLRG1$^+$ ILC2s **(B)** KLRG1$^-$ ILC2s **(C)** IL25R$^+$KLRG1$^+$ ILC2s **(D)** IL-25R$^+$KLRG1$^-$ ILC2s in the lung. MFI of ST2 on **(E)** KLRG1$^+$ ILC2s **(F)** KLRG1$^-$ ILC2s. Data are the combination of 2 independent experiments, presented as mean ± SEM, with 6–8 mice/group for A-D and representative of 2 independent experiments with 6–8 mice/group in E and F. Outcomes were assessed by two-way ANOVA, followed by Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. #: significantly different than all other groups within the same sex

in mice sensitized and challenged with OVA, as well as in mice exposed to OVA/IL-33, both before and after OVA challenge (Fig. 3D). While there was no significant increase in IL13$^+$IL-5$^+$KLRG1$^+$ 4 weeks post OVA/IL-33 exposure in males or females, OVA challenge increased their number significantly in both sexes (Fig S2A). In addition, IL13$^+$IL-5$^+$KLRG1$^-$ ILC2s were elevated four weeks post OVA/IL-33 treatment in female mice only (Fig S2B) and these numbers increased significantly upon OVA challenge only in females (Fig S2B). Together, these data suggest that production of IL-13 and IL-5 is differentially regulated in ILC2s, with IL-13-producing ILC2s being much more responsive to OVA challenge in this model of Th2 adaptive immunity in the lung. Nevertheless, the larger number of IL-5$^+$ KLRG1$^-$ ILC2s in females prior to OVA challenge was not sufficient to increase eosinophil numbers, as reflected in the small number of eosinophils present in all mice prior to OVA challenge (Fig. 1B). The larger number of cytokine-producing KLRG1$^-$ ILC2s in females likely contributes to the greater

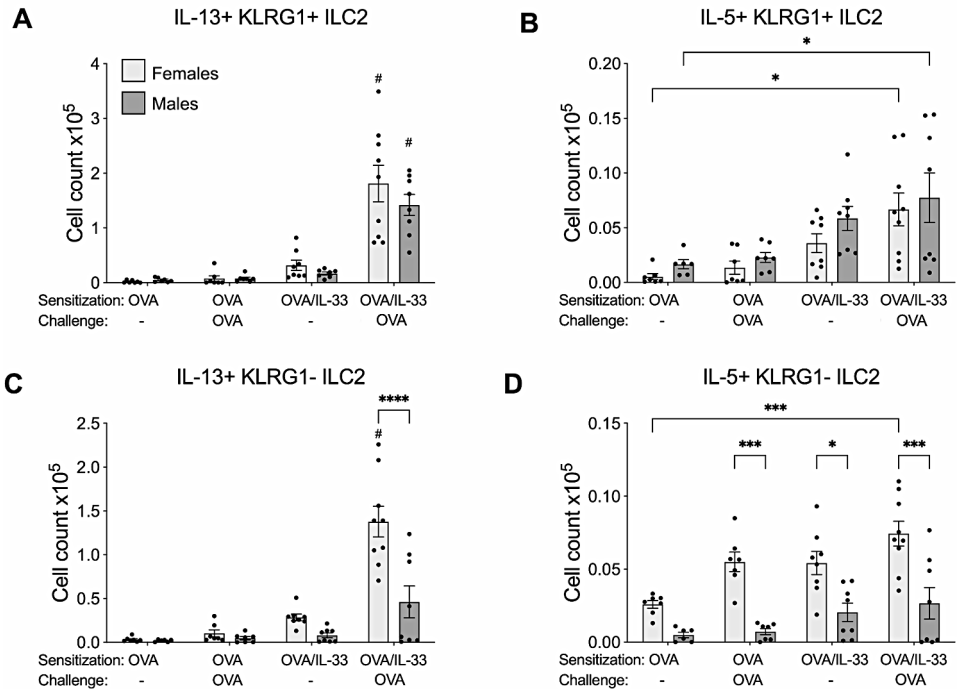

Fig. 3 Cytokine producing KLRG1$^-$ ILC2s are greater in female mice. Mice were treated as described in Fig. 1A, after which lungs were harvested for flow cytometry analysis. Absolute count of **(A)** IL-13$^+$KLRG1$^+$ ILC2s **(B)** IL-5$^+$KLRG1$^+$ ILC2s **(C)** IL-13$^+$KLRG1$^-$ ILC2s **(D)** IL-5$^+$KLRG1$^-$ ILC2s in the lung. Data are the combination of 2 independent experiments, presented as mean ± SEM, with 6–8 mice/group. Outcomes were assessed by two-way ANOVA, followed by Tukey's post hoc test. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. #: significantly different than all other groups within the same sex

inflammatory responses in females compared to males, post OVA challenge.

IL-33-experienced ILC2s are more responsive to IL-25 in female mice

We next examined the functional significance of IL-25R expression on IL-33-experienced ILC2s. Mice were treated with OVA or OVA/IL-33 as above. Four weeks later, OVA-sensitized mice were challenged with a single low dose of IL-25 and OVA/IL-33 sensitized mice were challenged with IL-25 or control saline as shown in Fig. 4A. These treatment groups are designated OVA→IL-25, OVA/IL-33→saline, and OVA/IL-33→IL-25, respectively. Compared to OVA→IL-25 treated mice, both eosinophils and ILC2s were significantly increased in OVA/IL-33→IL-25 treated male and female mice (Fig. 4B, C). In addition, in OVA/IL-33→saline exposed mice, eosinophil numbers were not elevated (Fig. 4B), even while ILC2s were increased (though the increase was not statistically significant) (Fig. 4C), similar to our findings presented in Fig. 2A. Eosinophils and ILC2s were both significantly increased, selectively in female mice, treated with OVA/IL-33→IL-25 (Fig. 4B, C), whereas these cell populations did not increase in male mice. Similar changes were noted in the KLRG1$^+$ ILC2 population (Fig. 4D) as well as the KLRG1$^-$ ILC2 population (Fig. 4E), though, as expected, larger numbers of KLRG1$^-$ ILC2s were present in females compared to males. Taken together, these data demonstrate that IL-33-experienced ILC2s in female mice are markedly responsive to a single challenge with IL-25, whereas their male counterparts were largely unaffected.

We also examined whether IL-25 promoted cytokine production by OVA/IL-33-experienced ILC2s. No significant differences in numbers of cytokine producing ILC2s (whether they expressed KLRG1 or not) were present in OVA/IL-33→saline exposed mice (Fig. 5A-D). IL-13$^+$KLRG1$^+$ and IL-13$^+$KLRG1$^-$ ILC2s were significantly increased in OVA/IL-33→IL-25 exposed females (Fig. 5A, C), whereas similar changes were absent in male mice. Interestingly, no significant differences were observed between males and females in the numbers of IL-5$^+$KLRG1$^+$ ILC2s in OVA/IL-33→IL-25 exposed mice, where increases were noted in both males and females (Fig. 5B). In addition, IL5$^+$KLRG1$^-$ ILC2s were significantly increased only in OVA/IL-33→IL-25 treated female mice (Fig. 5D). Similar responses in ILC2 cytokine production were present in mice challenged with IL-33 (Fig S3A-D), providing evidence that enhanced responsiveness in females was not limited to IL-25.

Our data presented in Fig. 2C showed a trend for an increase in the number of IL-25R$^+$ ILC2s selectively in females 4 weeks post OVA/IL-33 administration. Thus,

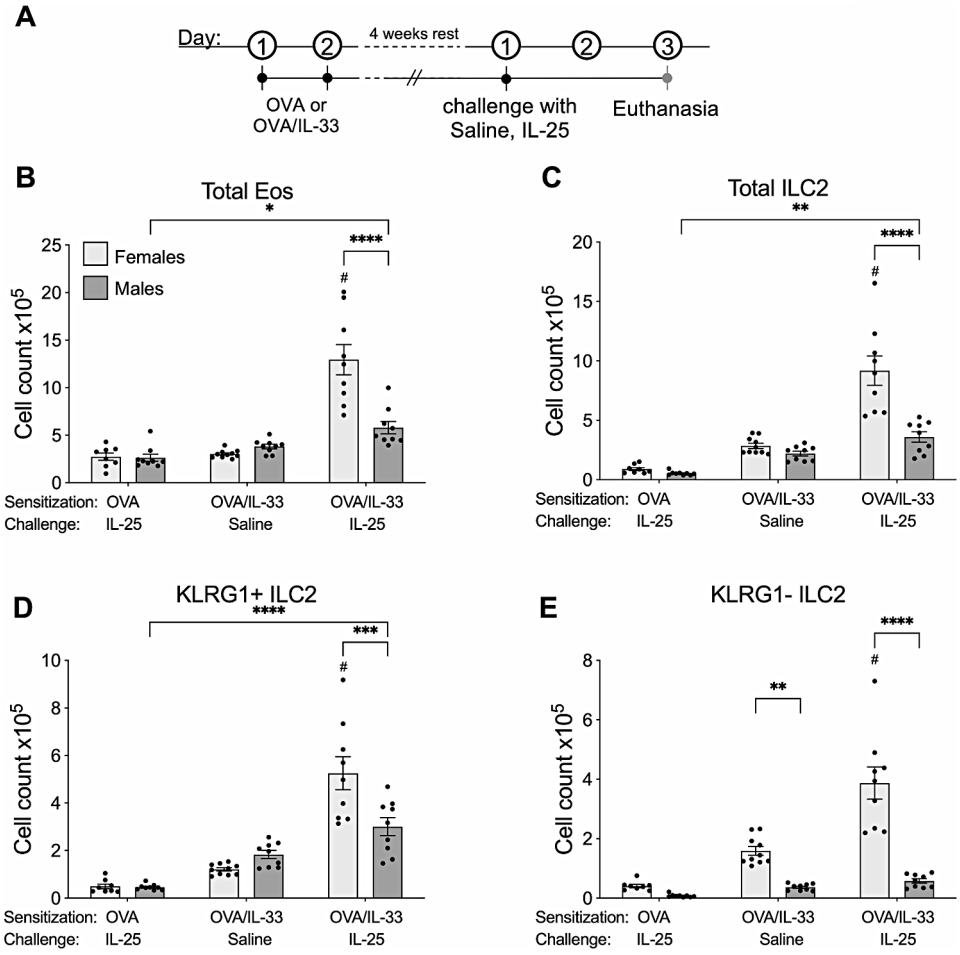

Fig. 4 IL-33-experienced ILC2s in female mice are more responsive to IL-25. **(A)** Male and female Balb/c mice were sensitized with OVA or OVA/IL-33 once per day for 2 days. Mice were rested for 4 weeks, and then OVA-treated mice were challenged once with IL-25, while OVA/IL33-treated mice were challenged once with saline or IL-25 before being euthanized 48 h later and harvesting lungs for flow cytometry analysis. Absolute count of **(B)** eosinophils **(C)** ILC2s **(D)** KLRG1$^+$ ILC2s **(E)** KLRG1$^-$ ILC2s in the lung. Data are the combination of 2 independent experiments, presented as mean ± SEM, with 8–10 mice/group. Outcomes were assessed by two-way ANOVA, followed by Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. #: significantly different than all other groups within the same sex

we hypothesized that IL-25 challenge would induce the expansion of IL-25R$^+$ ILC2s in females. Our data show that a single challenge with IL-25 significantly increased the numbers of IL-25R$^+$KLRG1$^+$ and IL-25R$^+$KLRG$^-$ ILC2s, as expected, only in female mice (Fig. 5E, F). IL25R$^+$ ILC2s were also increased in response to secondary challenge with IL-33 (Fig S3E, F). Altogether, these data provide further evidence that IL-33-experienced ILC2s in female mice are much more responsive to secondary activation compared to ILC2s in males.

The expression of IL-25R on IL-33-experienced ILC2s is associated with greater propensity to produce type 2 cytokines

We also examined how expression of IL-25R impacted responsiveness to secondary exposure to IL-25 or IL-33. While larger numbers of ILC2s lacking expression of IL-25R were present in both males and females (data not shown), ILC2s expressing IL-25R had a greater propensity to produce IL-13 and/or IL-5: compare black portion of circles in Fig. 6A and B for males and Fig. 6C and D for females. Moreover, the proportion of cytokine producing ILC2s (whether IL-25R$^+$ or not) was greater in females compared to males: compare Fig. 6C to Fig. 6A and Fig. 6D to Fig. 6B. Similar results were observed in mice challenged with IL-33 (data not shown). Together, our data provide evidence that expression of IL-25R marks ILC2s that are poised to respond in an enhanced manner upon secondary activation, whether by IL-25 or IL-33.

Enhanced responsiveness of IL-33-experienced ILC2s to IL-25 is dependent upon STAT6

STAT6 has recently been linked to memory responses in ILC2s [20]. Thus, we also examined whether the absence of STAT6 had an impact on the enhanced responsiveness to IL-25 of ILC2s previously exposed to IL-33. Wild-type

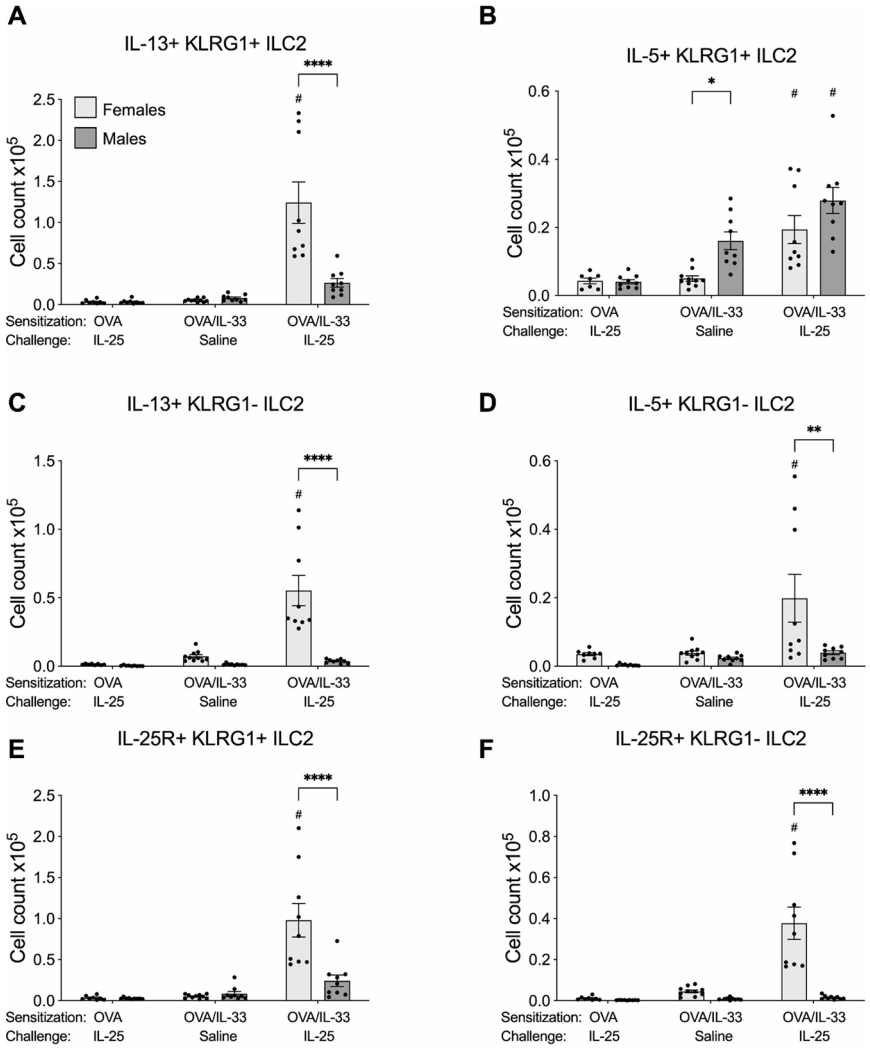

Fig. 5 IL-25 increases cytokine production and number of IL-25R expressing ILC2s selectively in female mice. Mice were treated as described in Fig. 4A, after which lungs were harvested for flow cytometry analysis. Absolute count of **(A)** IL-13$^+$KLRG1$^+$ ILC2s **(B)** IL-5$^+$KLRG1$^+$ ILC2s **(C)** IL-13$^+$KLRG1$^-$ ILC2s **(D)** IL-5$^+$KLRG1$^-$ ILC2s **(E)** IL-25R$^+$KLRG1$^+$ ILC2s (**F**) IL-25R$^+$KLRG1$^-$ ILC2s in the lung. Data are the combination of 2 independent experiments, presented as mean ± SEM, with 8–10 mice/group. Outcomes were assessed by two-way ANOVA, followed by Tukey's post hoc test. $^{**}p < 0.01$, $^{****}p < 0.0001$. #: significantly different than all other groups within the same sex

and STAT6 KO female mice were treated with IL-33 and challenged, one month later, as above with control saline or IL-25. Similar to our findings presented in Figs. 4 and 5, our data show that IL-33-experienced KLRG1$^+$ ILC2s from wild-type mice were significantly elevated 4 weeks after IL-33 exposure and further increased upon secondary activation with IL-25 (Fig. 7A, C, E; **left panels**). Increases were present in total ILC2s (Fig. 7A) as well as those producing IL-13 (Fig. 7C) or IL-5 (Fig. 7E). Similar increases were noted for KLRG1$^-$ ILC2s (Fig. 7B, D, F; **left panels**). In STAT6 KO mice, KLRG1$^+$ ILC2s producing IL-13 or IL-5 were significantly increased 4 weeks after IL-33 exposure and a similar trend was observed for total KLRG1$^+$ ILC2s (Fig. 7A, C, E; **right panels**), providing evidence that ILC2s from STAT6 KO mice retain the ability to respond to IL-33. However, delivery of IL-25 did not further enhance total or cytokine-producing KLRG1$^+$ ILC2s in STAT6 KO mice (Fig. 7A, C, E; **right panels**). KLRG1$^-$ ILC2s from STAT6 KO mice were less responsive than their counterparts expressing KLRG1 (Fig. 7B, D, F; **right panels**). Altogether, these data suggest that the absence of STAT6 does not limit the ability of ILC2s to respond to IL-33, though it is required for enhanced responsiveness to IL-25, a response associated with memory like activity in IL-33-experienced ILC2s.

Discussion

In the current study, we examined ILC2 responses in models of allergic airways disease and type 2 inflammation in male and female Balb/c mice. When examined 4 weeks after OVA/IL-33 exposure, ILC2s were present at similar levels in the lungs of all mice; however, these cells

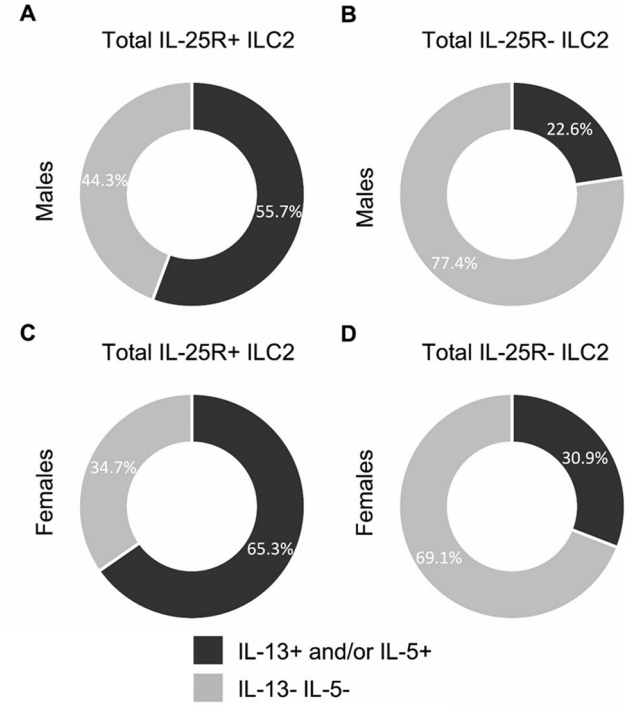

Fig. 6 The expression of IL-25R on IL-33-experienced ILC2 is associated with a greater propensity for cytokine production in response to IL-25 challenge. Mice were sensitized with OVA/IL-33 and challenged with IL-25 as described in Fig. 4A, after which lungs were harvested for flow cytometry analysis. The frequency of ILC2s producing IL-13 and/or IL-5 (black portion of rings) or no cytokines (grey portion of rings) in **(A)** IL-25R$^+$ ILC2s in male mice **(B)** IL-25R$^-$ ILC2s in male mice **(C)** IL-25R$^+$ ILC2s in female mice **(D)** IL-25R$^-$ ILC2s in female mice. Data are the combination of 2 independent experiments, with 8–10 mice/group

exhibited sex-specific phenotypic differences, which were associated with enhanced responsiveness in female mice challenged with OVA or alarmin cytokines. Our data show that IL-33-experienced ILC2s from female mice were more responsive to a single challenge with IL-25, in agreement with the larger number of ILC2s from female mice expressing the receptor for this cytokine. Expression of IL-25R was also correlated with greater propensity to produce type 2 cytokines, whether cells were re-activated by IL-25 or IL-33. Interestingly, the ability of ILC2s to establish memory-like responses in female mice was almost entirely dependent upon STAT6. Whether this is due to ILC2-specific STAT6 activity and/or that in other cells that influence ILC2 activation and/or memory responses is not yet known.

Takei and colleagues showed enhanced responsiveness to IL-25 in ILC2s previously activated by IL-33 [19], though the sex of mice used in their study was not defined. Their more recent data show that lung ILC2s in females are more metabolically active than their male counterparts [27], responding more vigorously to IL-33, in agreement with our findings. Takei and colleagues also reported that activation of lung ILC2s by IL-33 or papain has long-lasting effects on their function [19]. The initial exposure to IL-33 results in a rapid expansion of the lung ILC2 population, followed by a contraction phase. Administration of bromodeoxyuridine at the time of initial activation showed that some of the activated ILC2s survive for several months and that in vivo activation with IL-33 maintains higher numbers of ILC2s for a longer period of time compared to papain, suggesting that the stronger the initial activation, the longer ILC2s persist in the lung. While their experiments were conducted in C57Bl/6 mice treated with a cumulative dose of 0.75ug IL-33, we found similar responses in Balb/c mice. Our data indicate that ILC2 numbers were elevated in the lungs of both male and female mice for at least 4 weeks post OVA/IL-33 sensitization, although we used a cumulative dose of 0.1 µg IL-33 to generate IL-33-experienced ILC2s.

Following OVA challenge of mice previously sensitized to OVA/IL-33, larger numbers of ILC2s were present in female mice compared to males. KLRG1 is commonly used to identify mature ILC2s and ex vivo binding of KLRG1 to E-cadherin inhibits ILC2 function [13, 16]. Kadel et al. have shown that at 3 weeks of age, ILC2s lacking expression of KLRG1 are a minor subset in the lungs of both male and female mice but that these cells increase after reproductive age selectively in females, as elevated androgen levels in males reduce the number of KLRG1$^-$ ILC2s, the result of which is larger numbers of ILC2s as well as severity of type 2 inflammation induced upon allergen exposure in females. Our data confirmed no sex difference in the numbers of ILC2s expressing KLRG1 in OVA/IL-33 treated mice both before and after OVA challenge, while OVA challenge increased the number of KLRG1$^-$ ILC2s to a greater extent in female mice, providing evidence that the larger number of ILC2s in females post OVA challenge is due to the larger number of the KLRG1$^-$ subset, consistent with our previous work in an acute type 2 innate immunity model [15].

We did not observe sex differences in cytokine production by KLRG1$^+$ ILC2s before or after OVA challenge. However, in male mice, KLRG1$^-$ ILC2s had reduced capacity to produce these cytokines. Of particular interest, IL-13 producing KRLG1$^+$ ILC2s increased dramatically post OVA challenge (compared to IL-5 producing KLRG1$^+$ ILC2s) in both males and females. Similar responses following OVA challenge were confined to the KLRG1$^-$ ILC2 subset producing IL-13 in females. These data provide evidence of differential regulation of ILC2 populations producing IL-5 or IL-13, linked in part to their expression of KLRG1, with secondary activation having a more profound effect on IL-13 producing ILC2s.

Our data suggest that the development of memory-like responses in ILC2s is more pronounced in females. Four weeks after initial activation with OVA/IL-33, ILC2s

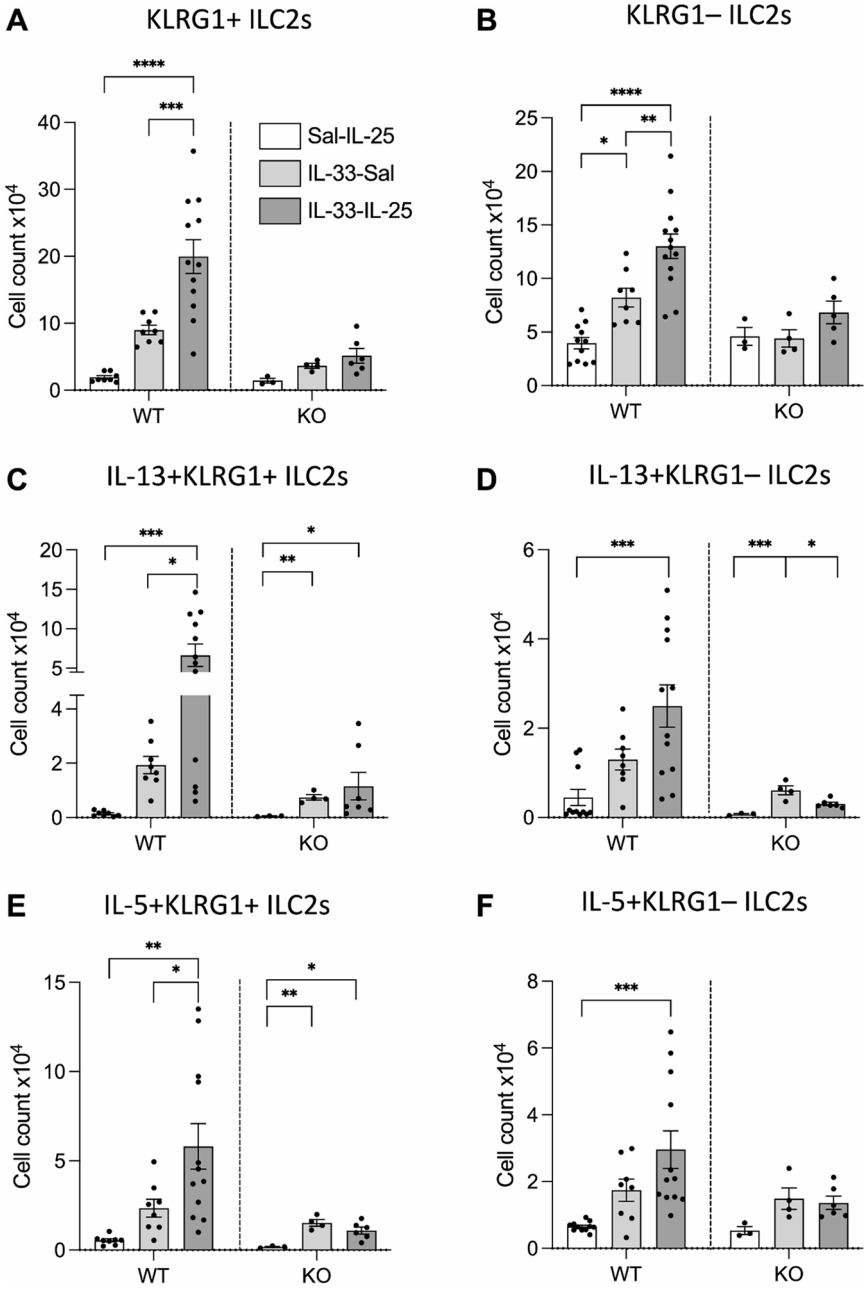

Fig. 7 Enhanced responsiveness of IL-33-experienced ILC2s to IL-25 is dependent upon STAT6. Mice were treated as described in Fig. 4A, replacing OVA with saline as described in methods, after which lungs were harvested for flow cytometry analysis. Absolute count of **(A)** KLRG1$^+$ ILC2s **(B)** KLRG1$^-$ ILC2s **(C)** IL-13$^+$KLRG1$^+$ ILC2s **(D)** IL-13$^+$KLRG1$^-$ ILC2s **(E)** IL-5$^+$KLRG1$^+$ ILC2s (**F**) IL-5$^+$KLRG1$^-$ ILC2s in the lung. In each graph wild-type mice are on the left, and STAT6 KO mice are on the right. Data are the combination of 3 independent experiments in wild-type mice and, in STAT6 KO mice, 1 experiment for Sal-IL-25 group and IL-33-Sal group and the combination of 2 independent experiments for the IL-33-IL-25 group. Data are presented as mean ± SEM with 3–13 mice/group. Outcomes were assessed by one-way ANOVA, followed by Tukey's post hoc test for mice of each genotype. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

in female mice responded more robustly to a number of different stimuli, including OVA, IL-25, or IL-33. While the precise mechanisms that promote ILC2 memory-like properties are poorly understood, several proteins have been implicated [19, 20, 28]. Our data support a role for IL-25R in the sex disparity of ILC2 memory responses. We also sought to determine the advantage of the expression of IL-25R by long lived ILC2s. Our data show that, in female mice only, a single exposure to IL-25 increased lung eosinophils and ILC2s in mice that had been sensitized with OVA/IL-33 4 weeks earlier. A similar pattern was observed when mice were challenged with IL-33.

While IL-25R-expressing ILC2s remained a small proportion of the overall ILC2-responding population, these cells had a greater propensity for cytokine production in response to a secondary challenge, whether this was with IL-25 or IL-33. Moreover, this enhanced cytokine production was more pronounced in ILC2s from females compared to males. While the exact mechanism by which IL-25R confers a greater likelihood for cytokine production is not well understood, this receptor can, nevertheless, serve as a marker for ILC2s more likely to respond upon secondary activation, as shown by our work as well as that of others [29].

Our data also support a role for STAT6 in memory-like ILC2 responses as we show that enhanced responsiveness to IL-25 was lost in mice lacking STAT6. Whether STAT6 in ILC2s is directly required for enhanced responsiveness is as yet unclear. Of note, the impact of IL-4 and IL-13, STAT6-activating cytokines, on ILC2 responses has been shown in a number of studies [30–32]. Interestingly, ILC2s and $CD4^+$ Th2 cells also interact, both directly and indirectly, to promote type 2 inflammation. ILC2-specific expression of MHCII [31] and OX40L [33] can activate $CD4^+$ T cells. In turn, T cell-derived IL-2, IL-4, and IL-13 have each been implicated in enhanced responses of ILC2s [31, 32]. Moreover, ILC2 specific production of IL-13 indirectly promotes Th2 differentiation, through activation of DCs [34], a response that is enhanced by IL-33-experienced ILC2s [19]. Thus, in addition to promoting T cell independent type 2 innate inflammatory responses, long-lived ILC2s likely also promote Th2 adaptive immunity through their ability to promote Th2 differentiation. While we did not directly examine $CD4^+$ T cell responses in this study, these cells likely contributed to the large increase in eosinophils in mice sensitized to OVA/IL-33 and challenged with OVA, in which both T cells and ILC2s were likely activated. Defining mechanisms by which these cells interact to promote type 2 inflammation in this model awaits further investigation.

Although the development and innate functions of ILC2 have been well documented, the role of the memory-like ILC2s in more chronic allergic lung inflammation remains largely unexplored. While it is known that asthmatic females have elevated levels of circulating ILC2s compared to their male counterparts [12], examining their responsiveness ex vivo and how that compares to ILC2s from healthy controls could shed light on how ILC2s might contribute to enhanced responses in human disease. Finally, understanding the molecular mechanism(s) underlying the longevity of ILC2s and how they impact inflammatory responses upon secondary activation will likely shed light on how these intriguing cells participate in asthma pathogenesis, including exacerbations in affected individuals, and provide information to target these cells therapeutically to improve the health of all asthmatics, whether male or female.

Supplementary Information

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Supplementary Material 1

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Author contributions
HA, RK, and EDF were responsible for writing the manuscript. HA, RK, KC, and EDF designed the experiments. HA, RK, KC, and LL participated in animal handling, tissue collection and data acquisition.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Animal studies were approved by the McGill University Animal Care Committee and performed following guidelines of the Canadian Council on Animal Care.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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