

MicroRNA-155 is a critical regulator of type 2 innate lymphoid cells and IL-33 signaling in experimental models of allergic airway inflammation

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Background: Allergic airway inflammation is triggered by allergen exposure through several steps including release of IL-33, which promotes cytokine (IL-5, IL-13) production by type 2 innate lymphoid cells (ILC2s). MicroRNA (miR)-155 has recently been described to regulate adaptive responses in allergic inflammation. However, the role of miR-155 in the regulation of ILC2s remains unexplored.

Objective: We sought to elucidate the contribution of miR-155 in ILC2 expansion using experimental murine models of allergic airway inflammation.

Methods: To determine the role of miR-155 in the regulation of ILC2s in allergic airway inflammation, miR-155 deficient (miR-155^{-/-}) and wild-type (WT) mice were subjected to acute or chronic allergen-induced inflammation or treated with recombinant IL-33.

Results: miR-155 was 10-fold upregulated in WT-derived ILC2s in response to IL-33. Furthermore, miR-155^{-/-} mice demonstrated impaired lung IL-33 levels in response to allergen challenge and the number of ILC2s was significantly reduced in allergen-challenged miR-155^{-/-} mice compared with WT mice. Exogenous IL-33 treatment revealed that miR-155 is needed for IL-33-induced ILC2 expansion and eosinophilic airway inflammation. Indeed, ILC2s from IL-33-challenged miR-155^{-/-} lungs exhibited impaired proliferation, GATA-3 expression, and IL-13 production as compared with IL-33-challenged WT ILC2s. **Conclusions:** Our findings for the first time demonstrate that ILC2s and IL-33 signaling are regulated by miR-155 in allergic airway inflammation. (J Allergy Clin Immunol 2016;■■■■:■■■■-■■■■.)

Key words: miR-155, ILC2, IL-33, IL-13, allergy, asthma, eosinophils

Allergic airway inflammation is triggered by allergen exposure through several steps, including release of epithelial-

Abbreviations used

ILC2: Type 2 innate lymphoid cell
miR: MicroRNA
OVA: Ovalbumin
WT: Wild-type

derived alarmin cytokines such as IL-33 and IL-25.¹ Allergic inflammation has been shown to be critically dependent on IL-33,^{2,3} where several studies have identified IL-33-responsive type 2 innate lymphoid cells (ILC2s) as a potent source of type 2 cytokines (IL-5 and IL-13),⁴⁻⁷ and recent reports have indicated that ILC2s are not only important for initiation of type 2 immune responses but also support the generation of T_H2 cells via IL-13.^{8,9}

MicroRNAs (miRs) belong to a growing family of small noncoding RNAs that by their ability to inhibit mRNA translation or degradation of mRNA targets alter gene expression, which ultimately results in a regulatory function of cellular responses.^{10,11} We and others have recently demonstrated that miR-155 plays an essential role in the regulation of allergen-induced airway inflammation *in vivo*.¹²⁻¹⁵ These studies have focused on the involvement of adaptive T_H2 and innate dendritic cell responses using experimental models of acute allergic inflammation; thus, the importance of miR-155 in chronic allergic airway inflammation remains unknown. Furthermore, innate ILC2 responses are often studied under short-term acute allergic conditions and few studies have focused on the involvement of ILC2s in chronic allergen-induced inflammatory settings.

In the current study, we demonstrate that miR-155 is critical for allergen-induced ILC2 expansion and maintenance in a model of allergen-induced chronic inflammation. miR-155 was found to be upregulated in stimulated ILC2s, and by using miR-155-deficient (miR-155^{-/-}) mice we further demonstrate that miR-155 is required for allergic induction of IL-33 in the lung. Importantly, exogenous IL-33 administration was unable to induce ILC2 expansion in the miR-155-deficient mouse lung. Furthermore, miR-155^{-/-}-derived ILC2s revealed impaired proliferation, GATA-3 expression, and IL-13 production in response to intranasal IL-33 challenge.

To date, few inhibitory signals for ILC2s have been described. Thus, our finding that ILC2s rely on miR-155 for proper function provides insight into the mechanisms of induction of allergic asthma as well as the maintenance of chronic allergic inflammation, which could ultimately be used in new therapeutic approaches that target ILC2s.

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METHODS

Animals

Mice lacking the function/reporter allele bic/miR-155 (B6.Cg-MiR155^{tm1.1Rsky/J}) were purchased from Jackson Laboratories (Bar Harbor, Me) and maintained for in-house breeding. Control C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany). Age- and sex-matched mice were used in all experiments. All mice were used between age 7 and 12 weeks, housed in pathogen-free conditions, and given food and water *ad libitum*. All procedures were conducted with permission from the Animal Ethics Committee at the University of Gothenburg, Gothenburg, Sweden (permit no. 126/14).

Induction of airway inflammation

Mice were sensitized by intraperitoneal ovalbumin (OVA) injection. Acute inflammation was induced by intranasal OVA challenge on 5 consecutive days, and samples were collected 24 hours after the final challenge. Chronic inflammation was induced by 3 intranasal OVA challenges per week for 12 weeks, and samples were collected 72 hours after the final challenge. Control mice were OVA sensitized and challenged with PBS. Intranasal challenges with recombinant murine IL-33 or PBS control were performed on day 1, 3, and 5, and samples were collected 24 hours after the final challenge. In some experiments, mice were kept naive. Additional information about the experimental models can be found in this article's Online Repository at www.jacionline.org.

Flow cytometry

Lung ILC2s were identified as mononuclear (FSC^{lo}, SSC^{lo}), lineage-negative (CD3[−], B220[−], CD19[−], CD11b[−], TER-119[−], Ly-G6/Gr1[−], NK1.1[−], CD11c[−], FcεRI[−]), CD45⁺, CD127⁺, CD25⁺ cells. Flow cytometric analysis (300,000–800,000 events per sample) was performed using a BD FACS Aria Flow Cytometer running BD FACS Diva version 6.0 Software or BD FACSVerse Flow Cytometer running BD FACS Suite Software (BD Biosciences, San Jose, Calif) and analyzed with FlowJo Software (Tree Star Inc, Ashland, Ore). See additional information in the Online Repository (gating strategy in Fig E2, A, in this article's Online Repository at www.jacionline.org).

In vitro stimulation and cell sorting

Lineage-negative lung cells from IL-33-challenged wild-type (WT) mice were enriched by magnetic depletion. Lineage-negative cells were seeded in cell culture media containing IL-2 and IL-7 (50 ng/mL of each). Cells were cultured for 7 days, and for the final 18 to 20 hours of culture, cells were stimulated with IL-33 (100 ng/mL) or left unstimulated for medium control. ILC2s (Lin[−]CD45⁺ICOS⁺CD25⁺ST2⁺) were sorted from harvested cultures on a BD FACS Aria Flow Cytometer running BD FACS Diva version 6.0 Software and analyzed with FlowJo Software. Sorted cells were collected in RNeasy Protect Cell Reagent (Qiagen, Hilden, Germany). See this article's Online Repository at www.jacionline.org for more details.

Cytokine analysis

IL-33 and IL-25 were measured in supernatants of lung tissue homogenate using ELISA DuoSets (R&D Systems, Minneapolis, Minn) according to the manufacturer's protocol. Absorbance values were analyzed on a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, Calif).

Lung histology

Lungs of chronic OVA-challenged mice and PBS controls were embedded in optimum cutting temperature (OCT) compound followed by immediate snap-freezing and stored at −80°C until sectioning using a Leica CM1900 UV-cryostat (Leica Microsystems Nussloch GmbH, Solms, Germany). Hematoxylin/eosin staining was performed according to standard protocols.

Masson's trichrome stain kit (Connective Tissue Stain; Abcam, Cambridge, United Kingdom) was used according to the manufacturer's protocol. Collagen accumulation was analyzed in trichrome-stained lung sections obtained from chronic OVA-challenged mice and PBS controls. See additional information in this article's Online Repository at www.jacionline.org.

RNA isolation and miR expression

RNA was isolated from lungs (chronic OVA model) and sorted ILC2s using miRNeasy Mini kit or miRNeasy Micro kit (Qiagen), respectively. miR-155 expression was measured using LNA primer sets for miR-155 (Exiqon A/S, Vedbaek, Denmark). See additional information in this article's Online Repository at www.jacionline.org.

Statistical analysis

All data were analyzed with Graph Pad Prism 6 Software (GraphPad Software Inc, La Jolla, Calif) and are expressed as mean ± SEM. Statistical analysis was performed using nonparametric Mann-Whitney *U* test or unpaired *t* test, and significance was defined as **P* < .05, ***P* < .01, and ****P* < .001.

RESULTS

miR-155 deficiency protects against chronic allergen-induced eosinophilic inflammation

To investigate whether miR-155 is required for the development of chronic allergic airway inflammation, we adopted 2 experimental models of allergen-induced inflammation. WT and miR-155^{−/−} mice were OVA sensitized and challenged on 5 consecutive days to induce acute allergic inflammation (Fig 1, A), or 3 times per week during 12 weeks to induce chronic allergic inflammation (Fig 1, B). Impaired eosinophil numbers were detected in the airways of acute or chronic allergen-challenged miR-155^{−/−} mice as compared with WT mice (Fig 1, C and D, including PBS controls in Fig E1, A and B, in this article's Online Repository at www.jacionline.org). Nevertheless, significantly increased eosinophil numbers were found in miR-155^{−/−} mice upon acute allergen challenge as compared with PBS-treated control mice (Fig E1, A). However, eosinophils were not found to increase in miR-155^{−/−} mice following chronic allergen challenge (Fig E1, B). In addition, lymphocytes and neutrophils were also found at significantly lower numbers in the airways of acute or chronic allergen-challenged miR-155^{−/−} mice; macrophages were only significantly reduced in miR-155^{−/−} mice following chronic allergen challenge. Overall, the levels of infiltrating immune cells were lower in response to chronic allergen challenge as compared with acute allergen challenge, suggesting that different inflammatory phases were at play.

To examine the chronic condition further, we performed histologic examination of lungs from chronic allergen-challenged mice to assess collagen accumulation, a hallmark feature of chronic pulmonary inflammation. Allergen-challenged WT and miR-155^{−/−} mice demonstrated basement membrane thickening in airway lining areas (representative lung sections in Fig 1, E, collagen stained blue). WT lungs in addition displayed larger areas of connective tissue that were stained positive for collagen, which was reduced in lungs of miR-155^{−/−} mice (Fig 1, G). Furthermore, examination of hematoxylin and eosin-stained lung tissues confirmed marked infiltration of inflammatory cells in allergen-challenged WT

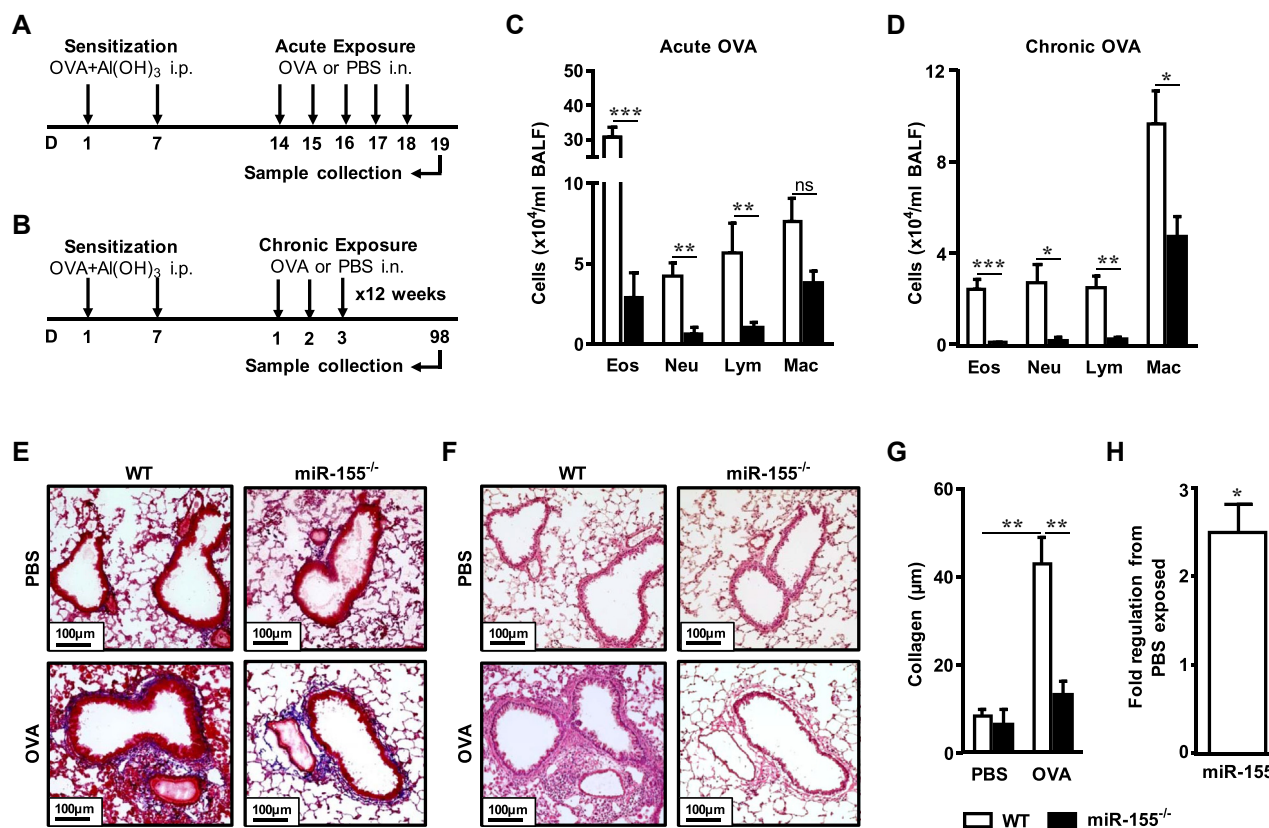


FIG 1. miR-155^{-/-} mice exhibit reduced eosinophilic inflammation and subepithelial collagen deposition in response to chronic allergen challenge. Experimental models of acute (A) or chronic (B) OVA-induced allergic airway inflammation. C and D, Total cells in BALF. Lung sections stained with Masson's trichrome stain (E) and hematoxylin and eosin (F). G, Collagen thickness around airway epithelium. H, miR-155 expression in lungs of OVA-challenged mice compared with PBS-treated mice. Fig 1, E-H, refer to chronic OVA model. BALF, Bronchoalveolar lavage fluid; Eos, eosinophil; i.n., intranasal; i.p., intraperitoneal; Lym, lymphocyte; Mac, macrophage; Neu, neutrophil; ns, nonsignificant. Data are shown as means ± SEM (n = 4-8 per group). *P < .05, **P < .01, and ***P < .001.

mice; miR-155^{-/-} mice demonstrated no increase in cells in the lung tissue as compared with PBS-treated controls (representative lung sections in Fig 1, F). In fact, when evaluating miR-155 expression in the lungs of chronic allergen-challenged WT mice, we found the levels to be significantly upregulated after allergen challenge as compared with those after PBS challenge (Fig 1, H). Taken together, these observations suggest that miR-155 is required to establish chronic airway inflammation by allergen challenge.

miR-155^{-/-} mice display impaired numbers of allergen-induced ILC2s

ILC2s are important players in type 2 immune responses, including allergic inflammation.¹⁶ Thus, we next quantified the number of ILC2s (Lin⁻CD45⁺CD127⁺CD25⁺, gating strategy in Fig E2, A) in single-cell suspensions of lungs by flow cytometric analysis. Given that we previously have demonstrated that miR-155 expression is highly upregulated in lungs of acute allergen-challenged mice,¹² we analyzed ILC2s during steady state and in response to allergen challenge. Lungs of naive miR-155^{-/-} mice demonstrated lower numbers of ILC2s compared with naive WT mice (Fig E2, B and C); however, no significant differences were found either in the percentage

CD127⁺ (IL7Rα) cells (Fig E2, D) or in CD25 (IL2Rα), ICOS, and ST2 (IL33R) receptor expression (Fig E2, E and F). Upon acute allergen challenge, significantly increased numbers of ILC2s were detected in lungs of both WT and miR-155^{-/-} mice as compared with PBS-treated controls (Fig 2, A). However, the total number of ILC2s was significantly lower in allergen-challenged miR-155^{-/-} mice compared with WT mice (Fig 2, A). It is important to note that the fold increase in ILC2 cell numbers from PBS-treated control mice was not different between the 2 strains upon acute allergen challenge (data not shown) due to differences in ILC2 numbers at baseline (Fig E2, B and C). The increased numbers of ILC2s were found to correlate with increasing numbers of airway eosinophils in acute allergen-challenged WT and miR-155^{-/-} mice (see Fig E3, A, in this article's Online Repository at www.jacionline.org). However, the induction of ILC2s in miR-155^{-/-} mice following acute allergen challenge declined on chronic allergen challenge, suggesting that miR-155 is required to maintain ILC2 numbers during a prolonged inflammatory response. Indeed, at this time point, increased numbers of ILC2s (Fig 2, E) were found to correlate with increasing airway eosinophils in WT mice only (Fig E3, B). Furthermore, it is important to note that we found no significant differences in the number of CD4⁺ T cells or activated CD4⁺ T cells (CD25⁺CD4⁺ cells) in lungs of chronic

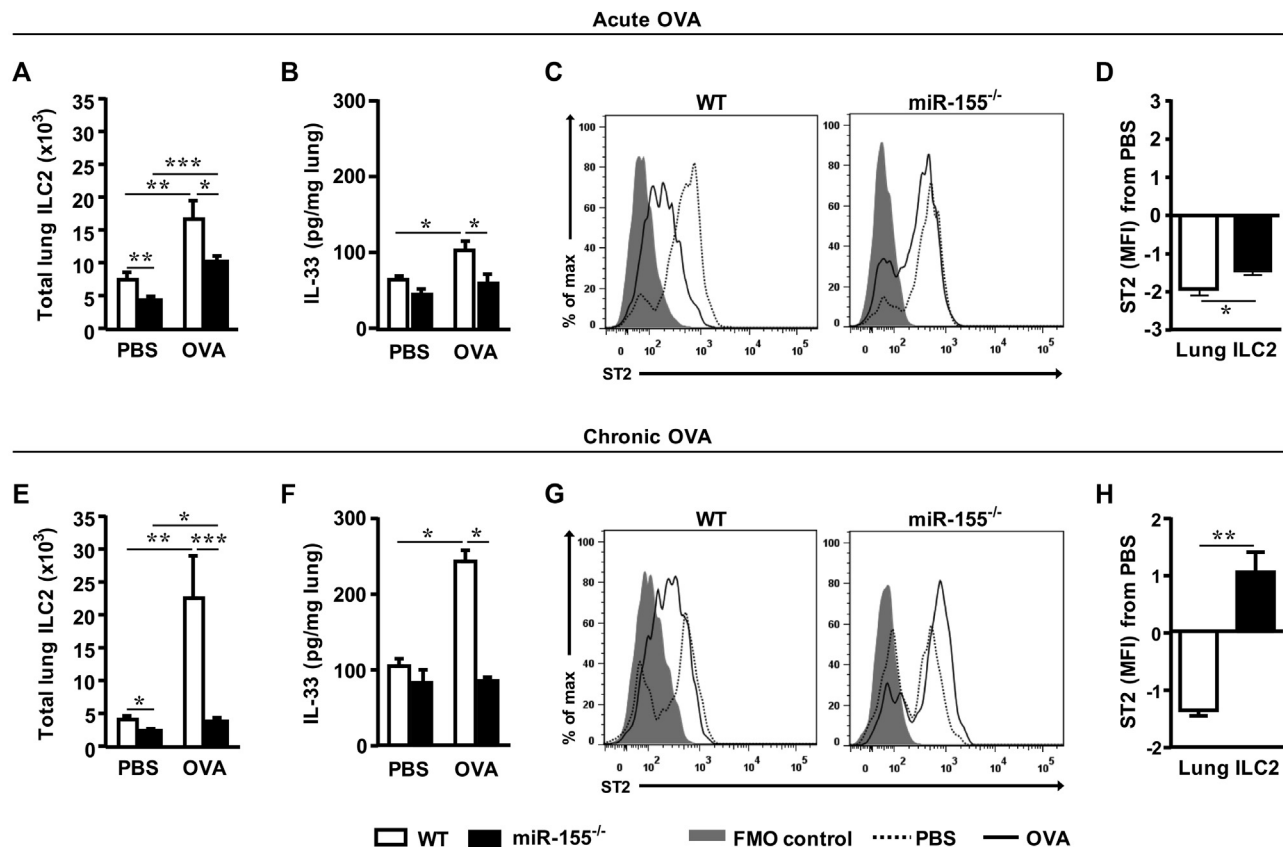


FIG 2. Lack of miR-155 alters ST2/IL33R expression on ILC2s and impairs lung IL-33 production in response to acute or chronic allergen challenge. **A** and **E**, Total number of lung ILC2s. **B** and **F**, IL-33 protein levels in lungs. **C** and **G**, ST2 expression on lung ILC2s (Lin⁻CD45⁺CD127⁺CD25⁺). **D** and **H**, Fold regulation of ST2 MFI on ILC2s from OVA-challenged mice compared with PBS-treated mice. Fig 2, A-D, refer to the acute OVA model and E-H refer to the chronic OVA model. FMO, Fluorescence minus one; MFI, mean fluorescent intensity. Data are shown as means $\pm$ SEM (n = 5-8 per group). * P < .05, ** P < .01, and *** P < .001.

allergen-challenged miR-155^{-/-} and WT mice (see Fig E4, B and C, in this article's Online Repository at www.jacionline.org).

Lack of miR-155 affects IL-33 signaling in response to acute or chronic allergen challenge

It is known that the epithelial-derived cytokine IL-33 is crucial for the induction and activation of subsets of ILC2s in the context of allergic inflammation,^{4,5,7,16} and thus we next measured the levels of lung IL-33. Significantly increased levels of IL-33 were detected in lung tissues from acute or chronic allergen-challenged WT mice (Fig 2, B and F), which correlated with increasing airway eosinophils (see Fig E5, A and B, in this article's Online Repository at www.jacionline.org). In contrast, IL-33 levels in lungs from allergen-challenged miR-155^{-/-} mice remained at the level of PBS controls. To investigate the consequences of the impaired IL-33 production in miR-155^{-/-} mice further, we continued by analyzing the expression of the IL-33 receptor (ST2) on ILC2s. The increasing amounts of IL-33 in WT lungs following acute or chronic allergen challenge correlated with decreased expression of ST2 on ILC2s (see Fig E6, A and B, in this article's Online Repository at www.jacionline.org). In fact, acute allergen challenge resulted in downregulation of ST2 on both WT and miR-155^{-/-} ILC2s, although to a significantly lesser extent in miR-155^{-/-} mice

(Fig 2, C and D). Chronic allergen challenge caused downregulation of ST2 on WT ILC2s but a significant upregulation on miR-155^{-/-} ILC2s (Fig 2, G and H).

miR-155 is required for IL-33-induced eosinophilic inflammation and ILC2 expansion

To examine whether the impaired IL-33 production in miR-155^{-/-} mice was responsible for the lower numbers of ILC2s as well as dysregulation of ST2 expression, we treated WT and miR-155^{-/-} mice with recombinant murine IL-33. Naive mice were given IL-33 (1 μ g) on 3 days (day 1, 3, 5) by intranasal administration and samples were collected 24 hours after the final administration (Fig 3, A). A significant induction of airway eosinophils and lymphocytes was detected in WT mice in response to intranasal IL-33 challenge; however, eosinophilia was absent in IL-33-challenged miR-155^{-/-} mice (Fig 3, B, including PBS controls in Fig E1, C, representative May Grünwald-Geimsa-stained cells in Fig 3, C). Furthermore, the total number of ILC2s increased significantly in WT lungs, which correlated with increasing airway eosinophils (Fig E3, C). Interestingly, no increase in ILC2s was detected in IL-33-challenged miR-155^{-/-} mice, suggesting that miR-155 is required for IL-33-induced ILC2 expansion in the airways (Fig 3, D). The ST2 expression on ILC2s remained unchanged

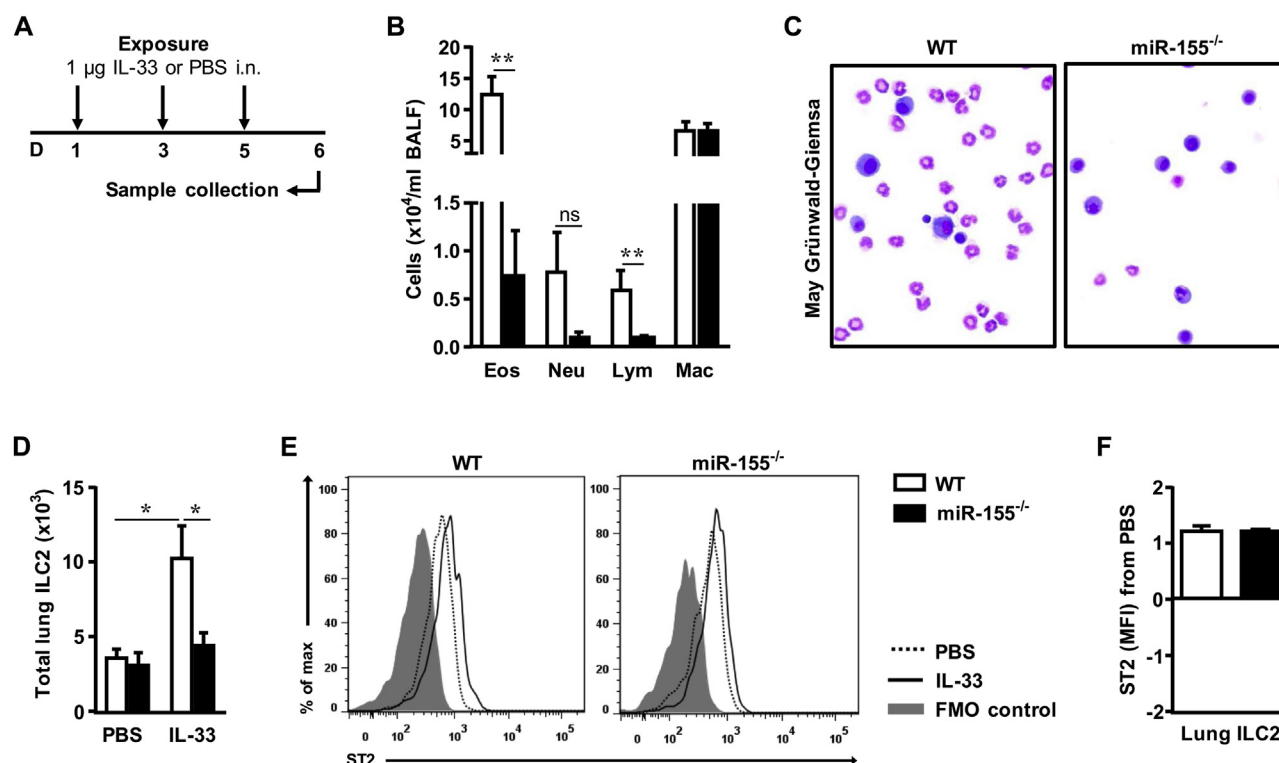


FIG 3. miR-155 is crucial for IL-33-induced eosinophilic inflammation and ILC2 expansion. **A**, Experimental model of IL-33-induced airway inflammation. **B**, Total cells in BALF. **C**, May Grünwald-Giemsa-stained BALF cells from IL-33-challenged mice. **D**, Total number of lung ILC2s. **E**, ST2 expression on lung ILC2s (Lin⁻CD45⁺CD127⁺CD25⁺). **F**, Fold regulation of ST2 MFI on ILC2s from IL-33-challenged mice compared with PBS-treated mice. BALF, Bronchoalveolar lavage fluid; FMO, Fluorescence minus one; MFI, mean fluorescent intensity; ns, nonsignificant. Data are shown as means $\pm$ SEM (n = 4-6 per group). *P < .05 and **P < .01.

in IL-33-challenged WT and miR-155^{-/-} mice as compared with PBS-challenged controls (Fig 3, E and F).

miR-155^{-/-} ILC2s exhibit impaired proliferation, and expression of GATA-3 and ICOS in response to IL-33

To examine factors that potentially play a role in the observed impairment of ILC2 induction in miR-155^{-/-} mice, we investigated cell proliferation in response to IL-33 treatment. Lung cells from IL-33-challenged miR-155^{-/-} mice displayed decreased proliferation in the lineage-negative proportion of cells as compared with WT mice (Fig 4, A and B). Most proliferating cells expressed GATA-3 and ST2 (Fig 4, C). However, the overall number of GATA-3-expressing cells was dramatically reduced in IL-33-challenged miR-155^{-/-} lung (Fig 4, D and E). The costimulatory receptor ICOS has previously been reported to play important roles for efficient function of human and murine ILC2s.^{17,18} Significantly decreased numbers of ICOS-expressing proliferating ILC2s were detected in miR-155^{-/-} mice in response to IL-33 challenge (Fig 4, C).

miR-155^{-/-} ILC2s exhibit impaired IL-13 production

A marked reduction in ST2-expressing lineage-negative ILC2 cells was found in lungs of IL-33-challenged miR-155^{-/-} mice as compared with WT mice (Fig 5, A and B). To examine

possible functional implications of miR-155 deficiency further, we continued by performing intracellular cytokine staining and flow cytometric analysis. miR-155^{-/-} ILC2s exhibited significantly decreased IL-13 production (Fig 5, C and E) but not IL-5 production (Fig 5, D and E) in response to IL-33, indicating that miR-155 has a direct regulatory role in ILC2 function.

IL-33 stimulation upregulate miR-155 expression in ILC2s

We next assessed the expression of miR-155 in ILC2s. Cell cultures of lineage-negative lung cells enriched from IL-33-challenged WT mice were stimulated with IL-33 and ILC2s were sorted using fluorescence-activated cell sorting (sorting strategy in Fig 6, A). *In vitro* stimulation with IL-33 significantly increased ILC2 receptor expression (ICOS/CD25/ST2) (Fig 6, C), which was accompanied by a dramatic upregulation of miR-155 expression (Fig 6, D), suggesting that miR-155 is required for IL-33-mediated activation of ILC2s.

DISCUSSION

ILC2s have been studied extensively in recent years, which has generated evidence demonstrating their contribution to airway inflammation by providing a potent source of immune effector cytokines.^{4,6,7,16,19} miRs constitute a growing group of regulatory

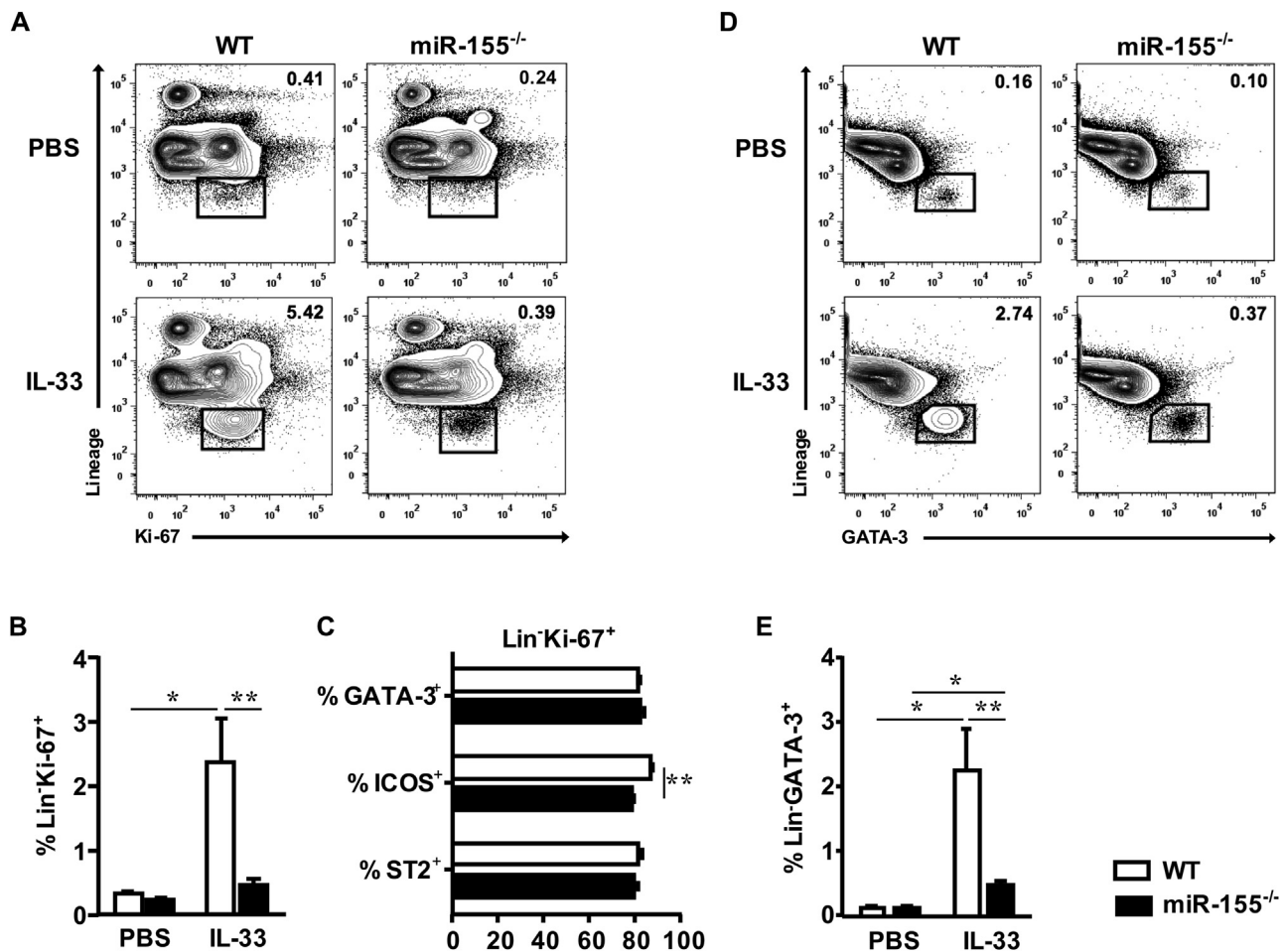


FIG 4. miR-155^{-/-} ILC2s exhibit impaired proliferation and GATA-3 and ICOS expression in response to intranasal IL-33 challenge. **A**, Ki-67⁺ Lin⁻ lung cells. **B**, Percentage proliferating (Ki-67⁺) Lin⁻ cells of CD45⁺ mononuclear lung cells. **C**, Percentage GATA-3⁺, ICOS⁺, and ST2⁺ proliferating Lin⁻ cells of IL-33-challenged mice. **D**, GATA-3⁺ Lin⁻ lung cells. **E**, Percentage GATA-3⁺ Lin⁻ cells of CD45⁺ mononuclear lung cells. Data are shown as means $\pm$ SEM (n = 3-7 per group). **P* < .05 and ***P* < .01.

molecules with important roles in cellular fate.¹⁰ Thus, miRs are of considerable interest for understanding immune regulation in inflammatory diseases such as allergic asthma.^{20,21} In the current study, we demonstrate for the first time that miR-155 is critical for ILC2 regulation and function in experimental models of allergen- and IL-33-induced airway inflammation.

We and others have previously shown that miR-155 is essential for the development of acute inflammation in response to short-term allergen challenge.¹²⁻¹⁵ However, in the current study, we demonstrate that miR-155 deficiency in addition protects from chronic allergic inflammation including reduced collagen deposition in peribronchial airways of miR-155^{-/-} mice compared with WT mice. However, it is important to note that miR-155^{-/-} mice have previously been reported to spontaneously develop pulmonary fibrosis with increasing age.²² In that study, the investigators performed histologic examinations of lungs from naive miR-155^{-/-} mice of around 1 year of age and age-matched WT controls. The discrepancy between their study and ours might be that the mice used in our study of chronic airway inflammation were half a year old at sample collection and had been allergen sensitized and treated with sterile PBS to serve as nonallergic controls. Importantly though, in the current study, nonallergic

miR-155^{-/-} control mice displayed no signs of remodeling of lung airways where no difference could be detected with respect to subepithelial collagen deposition or lung infiltrating cell as compared with age-matched WT mice.

We speculate that ILC2s play an important role in maintaining the chronic inflammatory response of the airways. Indeed, previous studies in mice have suggested that ILC2s and ILC2-inducing cytokines (IL-33, IL25) are important not only for initiation of type 2 immune responses but also contribute to chronic pathological features such as fibrosis.²³⁻²⁶ Recent human studies have also implied the involvement of ILC2s in chronic airway inflammation where airway ILC2s were found to be elevated in sputum²⁷ and bronchoalveolar lavage²⁸ from individuals with asthma compared with disease controls. In our study, miR-155^{-/-} mice revealed capability to induce significant but impaired ILC2 expansion following acute allergic inflammation compared with WT mice. Importantly, this capability was lost in the chronic inflammatory response, demonstrating for the first time miR-155 as a critical regulator not only during acute inflammation but also during a prolonged chronic inflammatory response, possibly through the involvement of ILC2 in maintaining chronic inflammation. Furthermore, a

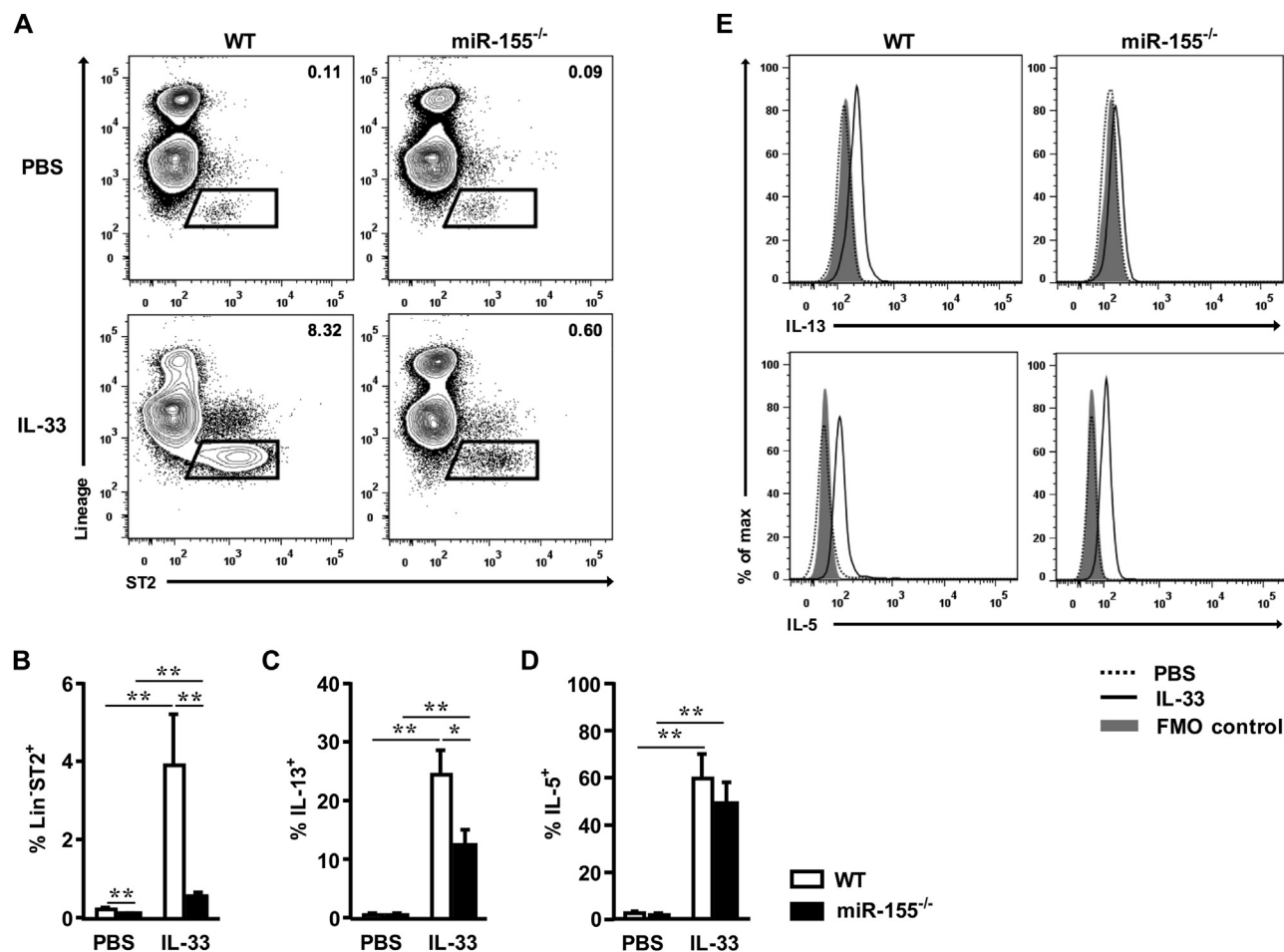


FIG 5. miR-155^{-/-} ILC2s exhibit impaired IL-13 production but not IL-5 in response to intranasal IL-33 challenge. **A**, Lin⁻ST2⁺ lung cells. **B**, Percentage Lin⁻ST2⁺ cells of CD45⁺ mononuclear cells. Percentage IL-13⁺ (**C**) and IL-5⁺ (**D**) cells of Lin⁻ST2⁺ lung cells. **E**, IL-13 or IL-5 expression in lung ILC2s (Lin⁻CD45⁺ST2⁺). FMO, Fluorescence minus one. Data are shown as means $\pm$ SEM (n = 5-7 per group). *P < .05 and **P < .01.

recent report demonstrated that ILC2s but not antigen-specific T cells were essential for the persistence of experimental asthma,²⁸ which is in agreement with our data since we could not detect any differences in CD25⁺ CD4⁺ T cells in the airways between the 2 mouse strains after chronic allergen challenge.

IL-33 is a potent activator of lung ILC2s and the genes encoding IL-33 and its receptor ST2 have been identified as major susceptibility loci for human asthma in several genomewide association studies.²⁹⁻³³ Importantly, we made the novel observation that miR-155^{-/-} mice were unable to induce IL-33 in the lung in response to acute or chronic allergen challenge. Interestingly, naive miR-155^{-/-} mice demonstrated decreased numbers of ILC2s in the lung compared with naive WT mice, although without differences in ST2 expression, which might suggest a role for miR-155 in ILC2 development and/or homeostasis. Upon allergen challenge, the impaired IL-33 production in miR-155^{-/-} mice was reflected on the expression of ST2 on ILC2s. The importance of ST2 in allergic inflammation has previously been reported where investigators demonstrated that ST2 deficiency lead to inhibited T-cell polarization, type 2 cytokine production, and eosinophilia in models of allergic asthma.^{34,35} Furthermore, a study comparing the contribution of IL-33, IL-25, and thymic stromal lymphopoietin in allergic

disease found that only IL-33 signaling was necessary for an intact allergic immune response, including expansion of ILCs *in vivo*.³⁶ Consistent with this, our observations of miR-155 deficiency causing dysregulation of ST2 implies that the necessary dynamics of receptor expression is lost and thus fails to develop a proper type 2 response via IL-33. Further supporting this were increasing ILC2s in lungs of acute allergen-challenged miR-155^{-/-} mice found to correlate with increased airway eosinophils; however, no correlation was found between ILC2 numbers or eosinophil numbers and the levels of IL-33 in the lung. This might suggest that the induction of ILC2s upon acute allergen challenge in miR-155^{-/-} is operating in an IL-33-independent manner. To examine this possibility, we investigated the levels of IL-25, which has been shown to activate subsets of ILC2s,³⁷ in the lung tissue of allergen- or IL-33-challenged WT and miR-155^{-/-} mice. However, IL-25 was undetectable in the lungs of both allergen- or IL-33-challenged WT and miR-155^{-/-} mice (data not shown), suggesting that IL-25 is not a critical player in these models at the investigated time points.

To address the impaired IL-33 levels in miR-155^{-/-} mice, we treated naive WT and miR-155^{-/-} mice with IL-33. Repeated intranasal IL-33 challenges resulted in induction of airway

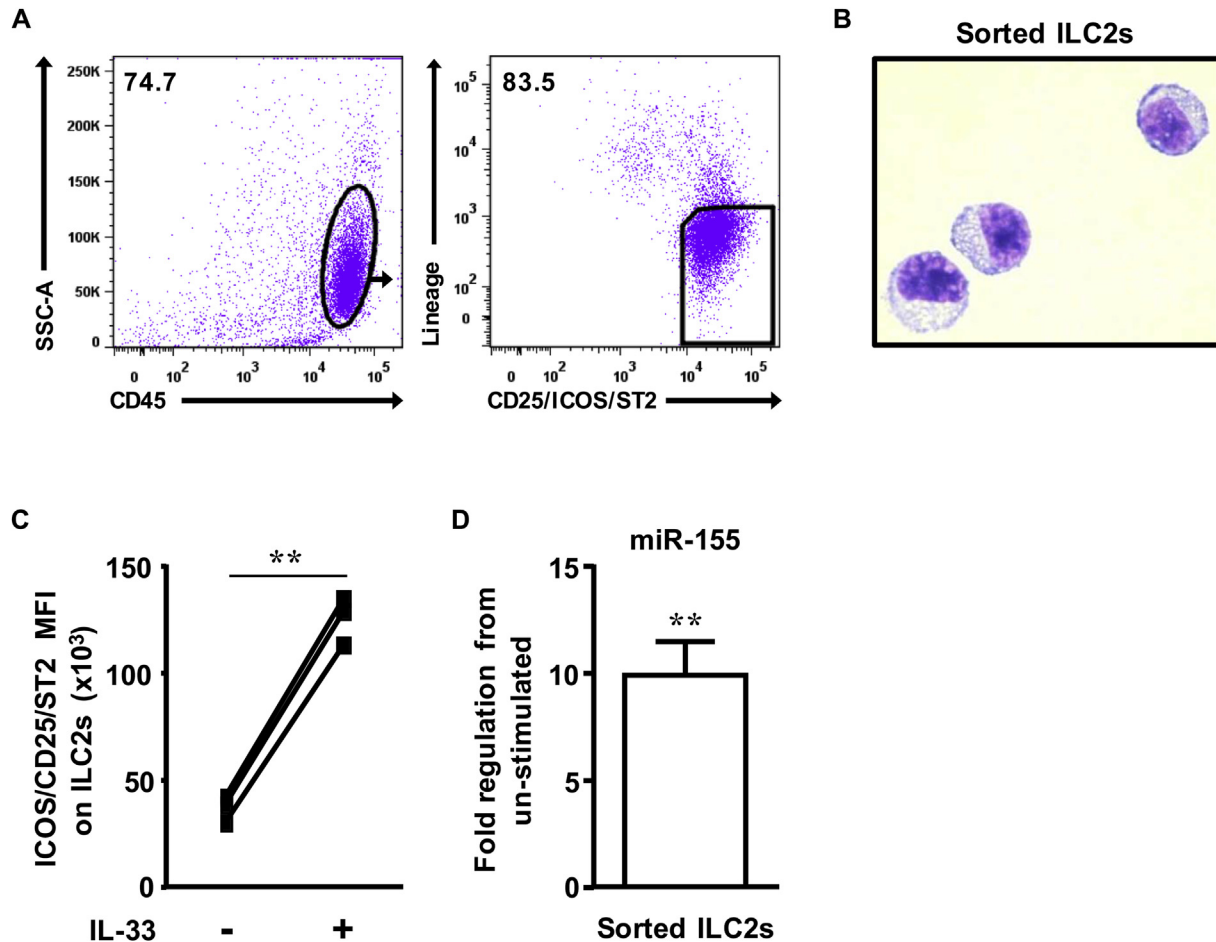


FIG 6. ILC2s upregulate miR-155 expression in response to IL-33 stimulation *in vitro*. **A**, Sorting strategy of lung ILC2s (Lin⁻ CD45⁺ CD25⁺ ICOS⁺ ST2⁺). **B**, Sorted ILC2s stained with Hemacolor rapid stain. **C**, Receptor MFI (ICOS/CD25/ST2) on *in vitro* unstimulated or IL-33-stimulated ILC2s. **D**, miR-155 expression in *in vitro* IL-33-stimulated ILC2s compared with unstimulated ILC2s. Data are shown as means $\pm$ SEM (n = 3-5 per group). MFI, Mean fluorescent intensity. **P < .01.

eosinophilia in WT mice. However, interestingly, not previously shown miR-155^{-/-} mice were unable to develop eosinophilic inflammation in response to intranasal IL-33 treatment, suggesting that in addition to possible epithelial defects in IL-33 production or release, miR-155 might have a direct regulatory role in ILC2 expansion. Indeed, WT and miR-155^{-/-} IL-33-induced ILC2s expressed ST2 to the same extent, indicating that WT and miR-155^{-/-} mice-derived ILC2s are available for potent IL-33 stimuli. However, ILC2 expansion could not be found in lungs of miR-155^{-/-} mice, possibly suggesting that miR-155^{-/-} ILC2s exhibit defect responses downstream IL-33. Importantly, we found that WT ILC2s upregulated miR-155 expression 10-fold in response to IL-33 stimulation, which possibly demonstrates the dependence of miR-155 in IL-33-induced ILC2 activation. Furthermore, although the numbers of ST2-expressing ILC2s were lower in miR-155^{-/-} lung following IL-33 treatment, the ST2-positive cells expressed GATA-3, which has been reported to drive the expression of ST2 in T_H2 cells and ILC2s.^{38,39} Furthermore, a recent study has identified IL-13 as both an inducer of epithelial-derived IL-33 and a factor important for ST2 expression.²⁸ Thus, IL-13 was found to provide a feed-forward loop on IL-33 autoinduction. These results

suggested that ILC2-derived IL-13 is important for the persistence of asthma through its effect on sustained IL-33 production by the epithelium. In agreement with this, the dysregulated ST2 expression on allergen-challenged miR-155^{-/-}-derived ILC2s is further supported by the fact that miR-155^{-/-} ILC2s demonstrated impaired IL-13 production *ex vivo* in response to IL-33. Interestingly, a comparable proportion of WT and miR-155^{-/-} ILC2s was found to produce IL-5 in response to IL-33 challenge *ex vivo*. However, the notably lower numbers of ILC2s in the IL-33-challenged miR-155^{-/-} lung explains the inability to induce lung eosinophilia.

The transcription factor GATA-3 in addition to ST2 is important for the expression of ICOS^{38,40} and is known to play a crucial role in the development, maintenance, and functions of ILC2s.^{39,41-43} We found a significantly decreased expression of ICOS on proliferating ILC2s in miR-155^{-/-} mice, suggesting a defect in ILC2 proliferation or possible survival pathways, functions that recently have been reported to be dependent on ICOS interactions in ILC2s.¹⁷ Nevertheless, our data demonstrated that proliferating ILC2s were GATA-3-expressing cells, consistent with previous reports of GATA-3 being important for ILC2 function and development. However, a marked

reduction in GATA-3-expressing lineage-negative cells was found in miR-155^{-/-} mice compared with WT mice. The impaired expression of GATA-3 in miR-155^{-/-} ILC2s might be a contributing factor to the dramatically impaired type 2 response in miR-155^{-/-} mice in response to allergen or IL-33 challenge.

We have previously shown defects in T_H2 cells in allergen-challenged miR-155^{-/-} mice, including lower levels of GATA-3-expressing CD4⁺ cells and reduced IL-5, IL-13, and IL-4 production *in vitro*.¹² GATA-3 expression impairment was suggested to be due to competition of DNA binding of the miR-155 target PU.1, which was shown to be upregulated in peribronchial lymph nodes of allergen-challenged miR-155^{-/-} mice compared with WT mice. Thus, in the current study, we analyzed PU.1 expression in ILC2s. However, PU.1 was not expressed in either WT or miR-155^{-/-}-derived ILC2s (data not shown), suggesting that other factors impair the GATA-3 expression in ILC2s.

In this study, we have for the first time used a chronic allergen-induced model to determine the role of miR-155 in chronic inflammation. Thus, our results indicate that miR-155 plays a central role not only during acute inflammation but also in the chronic inflammatory response. We clearly show that miR-155 has a previously unknown direct regulatory role in the ILC2 subset that affects IL-33 receptor expression, IL-33 responsiveness, and IL-13 production as well as proliferation capability, possibly due to defects in GATA-3 function. Importantly, our study demonstrates that disruption of the IL-33 pathway, which leads to ILC2 induction, is of critical importance to restrain type 2 responses in allergic airway inflammation. These results open new avenues to increase the understanding of the basic mechanisms involving gene regulatory RNAs in the onset and maintenance of chronic inflammation in asthma.

Key messages

- miR-155 is upregulated in ILC2s in response to IL-33 stimulation *in vitro*.
- miR-155 is critical for allergen-induced ILC2 expansion and IL-33 induction.
- miR-155^{-/-} ILC2s exhibit impaired IL-13 production in response to IL-33 *ex vivo*.
- miR-155 deficiency protects against chronic allergic airway inflammation.

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METHODS

Animal models

Mice were sensitized on day 1 and 6 by intraperitoneal injection of 0.25 mL of 8 μ g of chicken OVA (Sigma-Aldrich, St Louis, Mo) bound to 4 mg of aluminum hydroxide (Sigma-Aldrich) in sterile PBS. Acute inflammation was induced by 5 intranasal challenges on days 15 to 19 consisting of 100 μ g of OVA in 25 μ L sterile PBS as previously described.^{E1} Chronic inflammation was induced from day 15 by 3 intranasal challenges per week for 12 weeks with 100 μ g of OVA in 25 μ L sterile PBS as described in reference E2. Control mice were OVA sensitized as described above but received 25 μ L sterile PBS only. IL-33 stimulation *in vivo* was performed as previously described,^{E3} by intranasal challenges with 1 μ g recombinant murine IL-33 (Peprotech, Rocky Hill, Conn) in 50 μ L sterile PBS on day 1, 3, and 5. Control mice received 50 μ L sterile PBS. The mice were briefly anesthetized with isoflurane (Baxter, Deerfield, Ill) before intranasal challenges.

Sample collection

Bronchoalveolar lavage fluid (BALF) and lung tissue were collected 24 hours (acute OVA model and IL-33 model) or 72 hours (chronic OVA model) after the final challenge. The animals were deeply anesthetized with a mixture of xylazine (130 mg/kg; Rompun, Bayer, Leverkusen, Germany) and ketamine (670 mg/kg; Ketalar, Apoteket AB, Motala, Sweden). Mice were sacrificed by heart puncture and the blood was removed. The mice were then tracheotomized and bronchoalveolar lavage was performed by instilling 0.25 mL (acute OVA model and IL-33 model) or 0.50 mL (chronic OVA model) of sterile PBS through the tracheal cannula, followed by gentle aspiration. The procedure was repeated once and the recovered BALF was pooled and placed on ice until separation of the sample into cell-free BALF for cytokine measurement and BALF cells for cell counting and cytopins. In some experiments, the left lung lobe was inflated with 1 mL of OCT compound (Tissue-Tek; Sakura, Finetek Europe B.V., Zoeterwoude, the Netherlands) supplemented with sterile PBS containing 20% sucrose (Sigma-Aldrich), and immediately frozen in liquid nitrogen for later histological examination. The 4 remaining lung lobes without any connective tissue were placed in Hanks balanced salt solution (HBSS+; Gibco, Paisley, United Kingdom) and kept on ice until preparation of lung single-cell suspensions. In some experiments, left and right lung lobes were placed in HBSS+ for preparation of lung single-cell suspensions. For intracellular cytokine analysis by flow cytometry, left lungs were inflated with HBSS+ supplemented with monensin, a protein transport inhibitor (BD GolgiStop; BD Biosciences) according to manufacturer's instructions (4 μ L/6 mL medium), and placed in HBSS+ containing GolgiStop on ice until preparation of single-cell suspensions. In some experiments, the left lung was collected in plain RPMI-1640 and immediately placed on ice and stored at -80°C until preparation of lung tissue homogenate supernatants for cytokine measurement. For RNA isolation, lung tissue was immediately placed in RNeasy lysis buffer (Qiagen, Crawley, UK) and incubated at 4°C for 24 hours followed by storage at -80°C .

Cytopins

A total of 50,000 or less BALF cells in 100 μ L PBS were used for cytopins. Cells were stained with May-Grünwald-Giemsa (HistoLab Products AB, Gothenburg, Sweden) or Hemacolor Rapid stain (Merck Chemicals, Darmstadt, Germany) according to the manufacturer's protocol. Samples were assessed using a Leica Axioplan 2 Microscope (Carl Zeiss) at a magnification of $\times 100$. Immune cells (eosinophils, neutrophils, macrophages, lymphocytes) were identified on the basis of morphologic criteria and quantified by counting 300 to 400 cells per slide.

Lung histology

The left lung lobe was embedded in Tissue-Tek OCT, snap-frozen, and stored at -80°C until being sliced into 5- μ m sections using Leica CM1900 UV-cryostat (Leica Microsystems Nussloch GmbH, Solms, Germany). Hematoxylin and eosin staining was performed as previously described.^{E1}

Briefly, sections were immediately placed in 4% formaldehyde (HistoLab Products AB) for 15 minutes or more, rinsed in dH_2O , and placed in Mayer's hematoxylin (Sigma-Aldrich) for 5 minutes. The sections were placed in slow running tap water for 10 minutes and then placed in eosin solution consisting of 100 μ g eosin (Merck Chemicals), 50 mL 95% ethanol, and 2 drops of acetic acid for 15 seconds. Excess eosin was removed by washing in absolute alcohol, and slides were mounted in Neo-Mount (Merck). Trichrome stain kit (Connective Tissue Stain; Abcam) was used according to the manufacturer's protocol. Collagen accumulation was analyzed using a light microscope (EVOS, ThermoFisher Scientific) in trichrome-stained lung sections (chronic OVA model). Results are expressed as basement membrane thickness (μ m collagen) lining airways (100–350 μ m in diameter). Three to 8 airways were assessed in each section.

Preparation of lung homogenates

Lungs were thawed on ice, weighed, and placed in 1 mL sterile PBS supplemented with protease inhibitor cocktail (1 tablet Complete-Mini/10 mL PBS; Roche, Mannheim, Germany) in gentleMACS C-tubes (Miltenyi Biotec, Bergisch Gladbach, Germany). The lung tissue was disrupted mechanically using a gentleMACS Dissociator (Miltenyi Biotec), and cell-free supernatants were isolated following centrifugation and kept at -80°C until cytokine analysis.

Preparation of lung single-cell suspensions

Preparation of lung single-cell suspensions was performed as previously described.^{E4} In brief, lungs were weighed and rinsed in a petri dish before being transferred to a gentleMACS C Tube containing 5 mL freshly prepared lung digestion medium consisting of Dulbecco modified Eagle medium high glucose (HyClone, Logan, Utah), 10% heat-inactivated FBS (Sigma-Aldrich), 1% penicillin/streptomycin (HyClone), 50 μ M 2-mercaptoethanol (Sigma-Aldrich), 50 U/mL DNase I (Roche, Mannheim, Germany), and 250 U/mL Collagenase type IV (Gibco). Lungs were dissociated using a gentleMACS Dissociator (Miltenyi Biotec) according to the manufacturer's instructions (including 30-minute incubation at 37°C). The digested tissue was filtered through a 70- μ m mesh cell strainer and washed in serum-free RPMI-1640 (HyClone), and supernatants were removed after centrifugation. The cell pellet was resuspended in 5 mL 40% Percoll (GE Healthcare, Uppsala, Sweden) in RPMI-1640 followed by centrifugation with low brake. After removing debris, cells were resuspended in 5 mL red blood cell lysis solution consisting of 0.1 mM EDTA (Sigma-Aldrich) in distilled water supplemented with 0.8% NH_4Cl (Merck) and incubated on ice for 10 minutes. Following centrifugation, cells were resuspended in PBS supplemented with 2% FBS for cell counting. The total number of cells was determined by standard hematologic procedures. For intracellular cytokine analysis by flow cytometry, preparation of lung single-cell suspensions was performed as described above but in all liquid steps GolgiStop was added.

Flow cytometry

Antibodies used for flow cytometric analysis are listed in Table E1. Antibodies for surface antigens were incubated for 30 minutes followed by 4% paraformaldehyde solution (Sigma-Aldrich) fixation and washed in PBS supplemented with 1% FCS. Cells were analyzed immediately or subjected to further intracellular staining. Staining of transcription factors was performed according to manufacturer's instructions using Foxp3 staining buffer set (eBioscience, San Diego, Calif). All steps in the staining procedure of intracellular cytokines were performed using solutions supplemented with GolgiStop up until fixation of cells. Following fixation, cells were washed with PBS supplemented with 1% FCS followed by permeabilization with 0.1% saponin (Sigma-Aldrich) in HBSS and 40-minute incubation of antibodies for intracellular antigens.

ILC2 stimulation and FACS sorting

Lungs from IL-33-challenged WT mice were sampled and processed to generate single-cell suspensions. Lineage-negative lung cells were enriched

by using magnetic separation according to the manufacturer's protocol (Lineage Cell Depletion Kit, MACS; Miltenyi Biotec GmbH). A total of 500,000 lineage-negative cells were seeded in 1 mL complete cell culture media (RPMI-1640 medium, 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 55 µM 2-mercaptoethanol, 50 ng/mL recombinant mouse [rm]IL-2, 50 ng/mL rmIL-7). rmIL-2 and rmIL-7 were purchased from Peprotech. Cells were cultured for 7 days (fresh culture medium was exchanged once). At 18 to 20 hours before harvest, rmIL-33 (Peprotech) was added to stimulated wells (100 ng/mL). Cells were harvested and antibodies for surface antigens were incubated for 30 minutes followed by 1 wash in PBS supplemented with 1% FCS, and immediately subjected to FACS sorting. Sorted ILC2s (Lin⁻CD45⁺ICOS⁺CD25⁺ST2⁺) were collected in RNeasy Protect Cell Reagent (Qiagen) and stored at -80°C until RNA isolation.

RNA isolation

Lungs from chronic OVA-challenged mice and PBS controls were stored in RNAlater for RNA isolation. The tissue was homogenized and lysed using QIAzol Lysis Reagent (Qiagen) in gentleMACS M Tubes using a gentleMACS Dissociator (Miltenyi Biotec GmbH), followed by QIAshredder spin columns (Qiagen). Total RNA (including small RNA) was isolated using miRNeasy Mini Kit (Qiagen). Total RNA from sorted ILC2s (stored in RNeasy Protect) was isolated using miRNeasy micro kit in addition to QIAshredder spin columns (Qiagen). Contaminating DNA was removed using Turbo DNA-free kit (Ambion, Life Technologies) according to the manufacturer's protocol. RNA yield and integrity were analyzed using Agilent RNA 6000 Nano kit (lung tissue) or Agilent RNA 6000 Pico kit (cells) on Agilent 2100 Bioanalyzer running 2100 Expert Software (Agilent Technologies, Waldbronn, Germany).

miR-155 expression

Reverse transcription of miRNA was performed (replicate samples) using 20 ng total RNA (lung tissue) or 0.2 to 2 ng total RNA (cells) per cDNA reaction with the Universal cDNA Synthesis kit (Exiqon A/S) according to the manufacturer's protocol. Quantitative real-time PCR was carried out on CFX-96 Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, Calif) using diluted reverse transcription product, LNA Primer sets for mmu-miR-155-5p, U6 snRNA and hsa-miR-103a-3p, and SYBR Green Master Mix Kit according to the manufacturer's recommendations (Exiqon). The expression of miR-155 was normalized to U6 snRNA and hsa-miR-103a-3p. Fold regulation in lungs of chronic OVA-challenged WT mice was calculated from PBS-exposed WT controls, and fold regulation in IL-33-stimulated ILC2s was calculated from unstimulated medium control ILC2s using CFX Manager 3.0 Software (Bio-Rad Laboratories).

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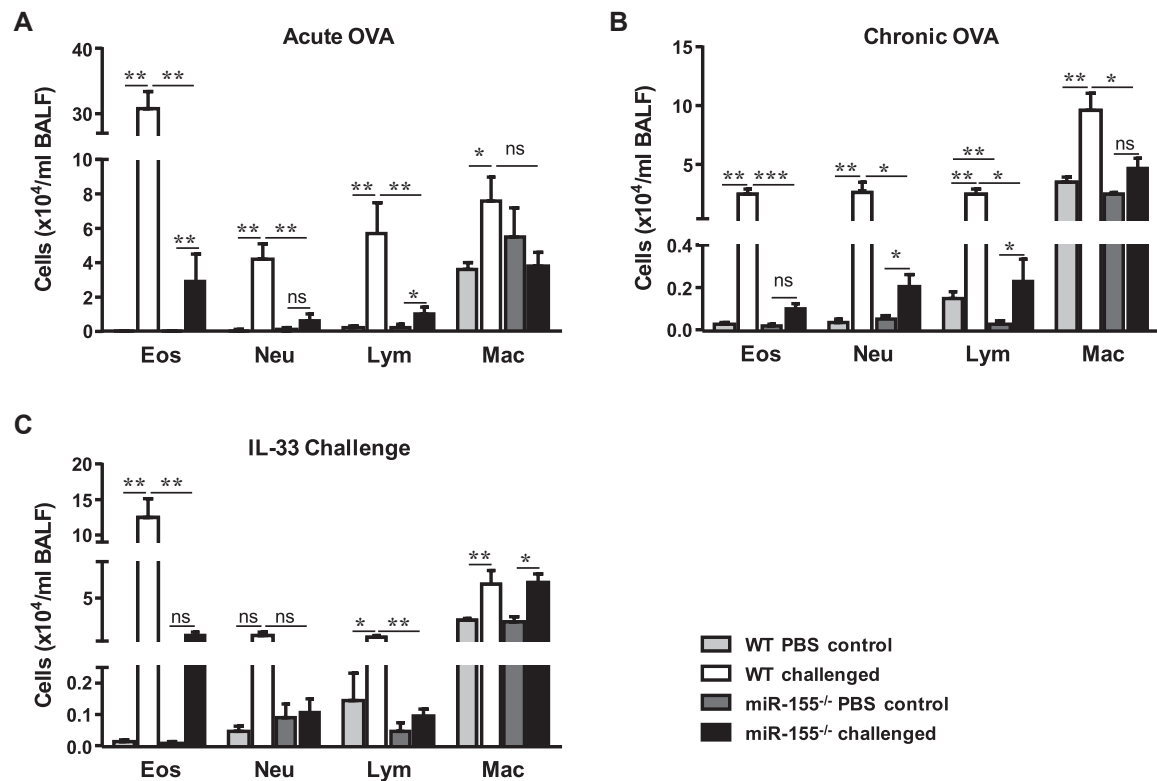


FIG E1. Total cells in BALF from acute OVA (A), chronic OVA (B), or IL-33-challenged (C) WT and miR-155^{-/-} mice and PBS control mice. *Eos*, Eosinophil; *Neu*, neutrophil; *Lym*, lymphocyte; *Mac*, macrophage; *ns*, nonsignificant. Data are shown as means $\pm$ SEM (*n* = 4-8 per group). **P* < .05, ***P* < .01, and ****P* < .001.

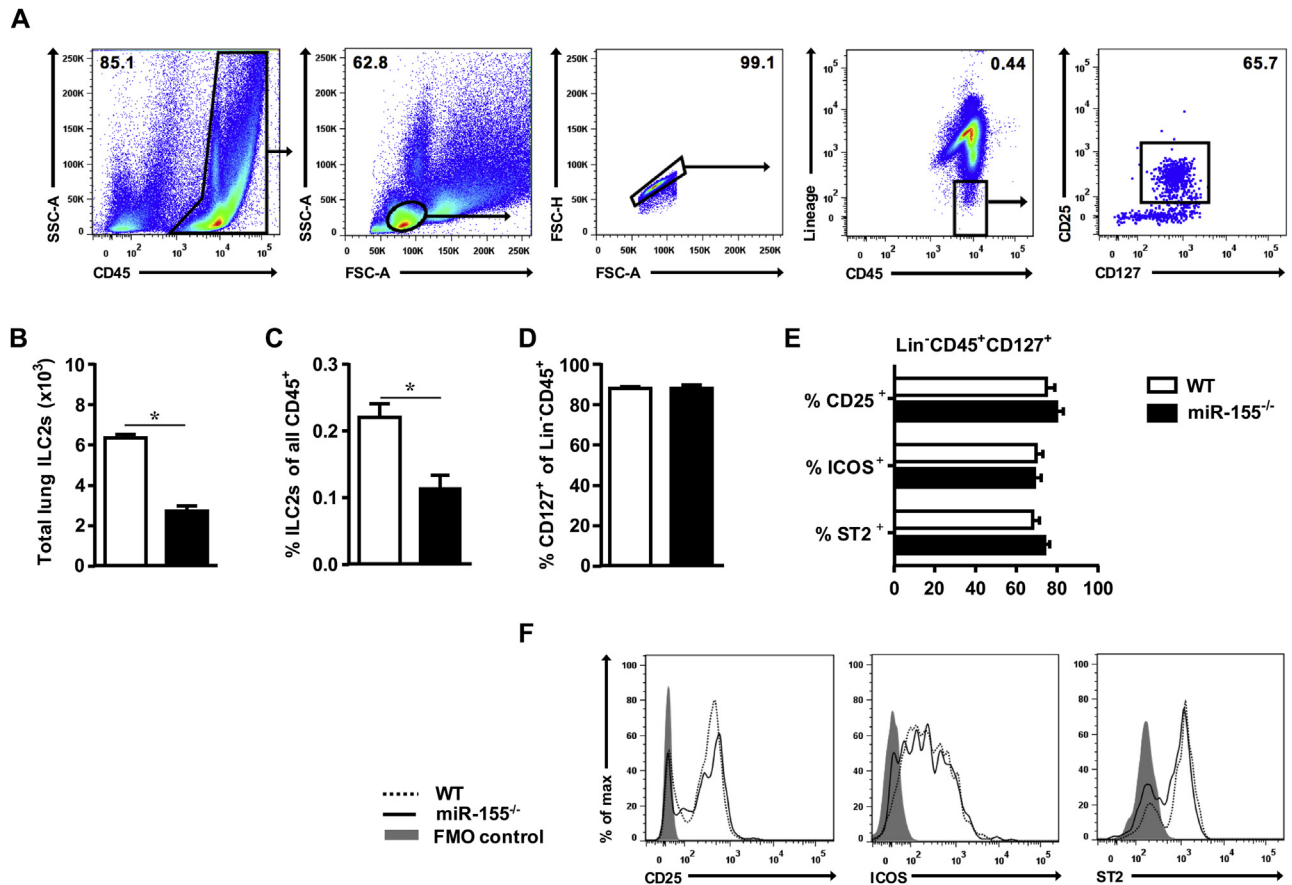


FIG E2. **A**, Gating strategy of lung ILC2s (Lin⁻CD45⁺CD127⁺CD25⁺). **B**, Total number of lung ILC2s. **C**, Percentage ILC2s of all leukocytes (CD45⁺). **D**, Percentage CD127⁺ (IL7R α) cells of Lin⁻CD45⁺ mononuclear lung cells. **E**, Percentage CD25⁺ (IL2R α), ICOS⁺, and ST2⁺ (IL33R) cells of Lin⁻CD45⁺CD127⁺ cells. **F**, CD25, ICOS, and ST2 expression on Lin⁻CD45⁺CD127⁺ cells. Lungs from naive WT and miR-155^{-/-} mice are analyzed in Fig E2, B-F. *FMO*, Fluorescence minus one; *FSC-A*, forward-scatter area; *FSC-H*, forward-scatter height; *SSC-A*, side-scatter area. Data are shown as means $\pm$ SEM ($n = 4$ per group).

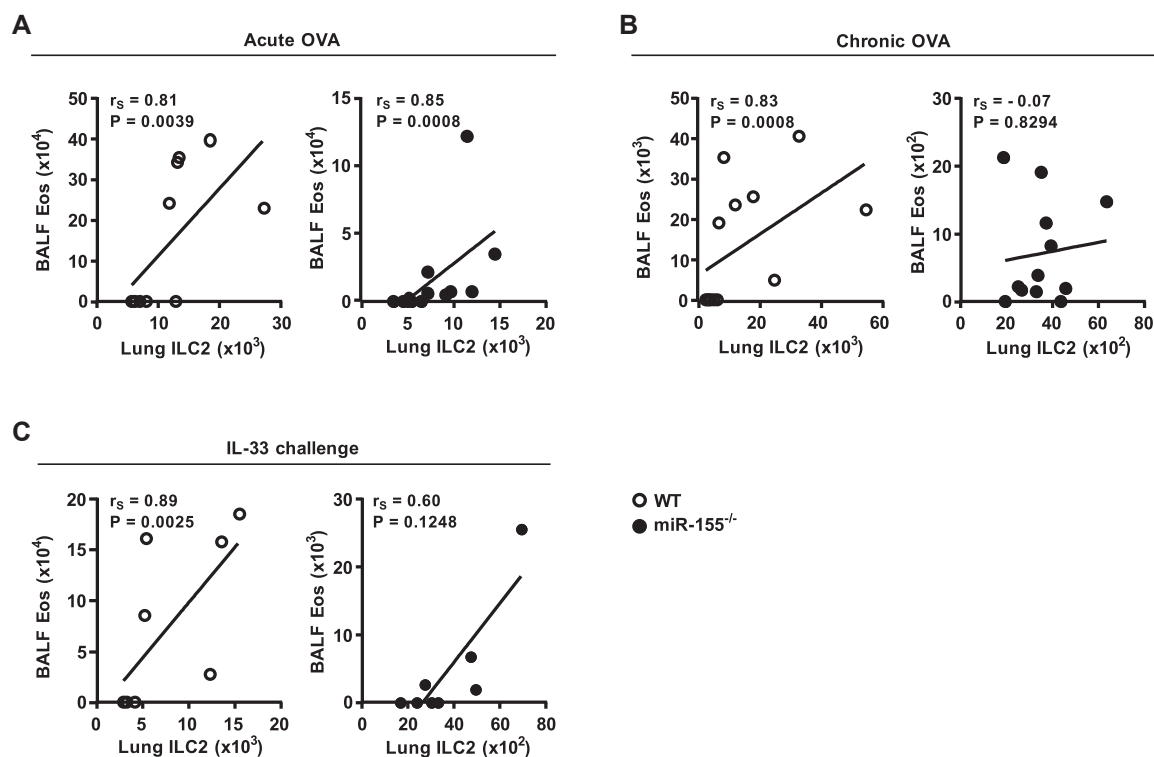


FIG E3. Scatter graph demonstrating correlation analysis between BALF eosinophils (Eos; mL^{-1} BALF) and total lung ILC2s of acute OVA (**A**), chronic OVA (**B**), or IL-33-challenged (**C**) WT and miR-155^{-/-} mice and PBS control mice. Eos, Eosinophils; r_s , Spearman correlation coefficient. Data are shown as means $\pm$ SEM ($n = 4-8$ per group).

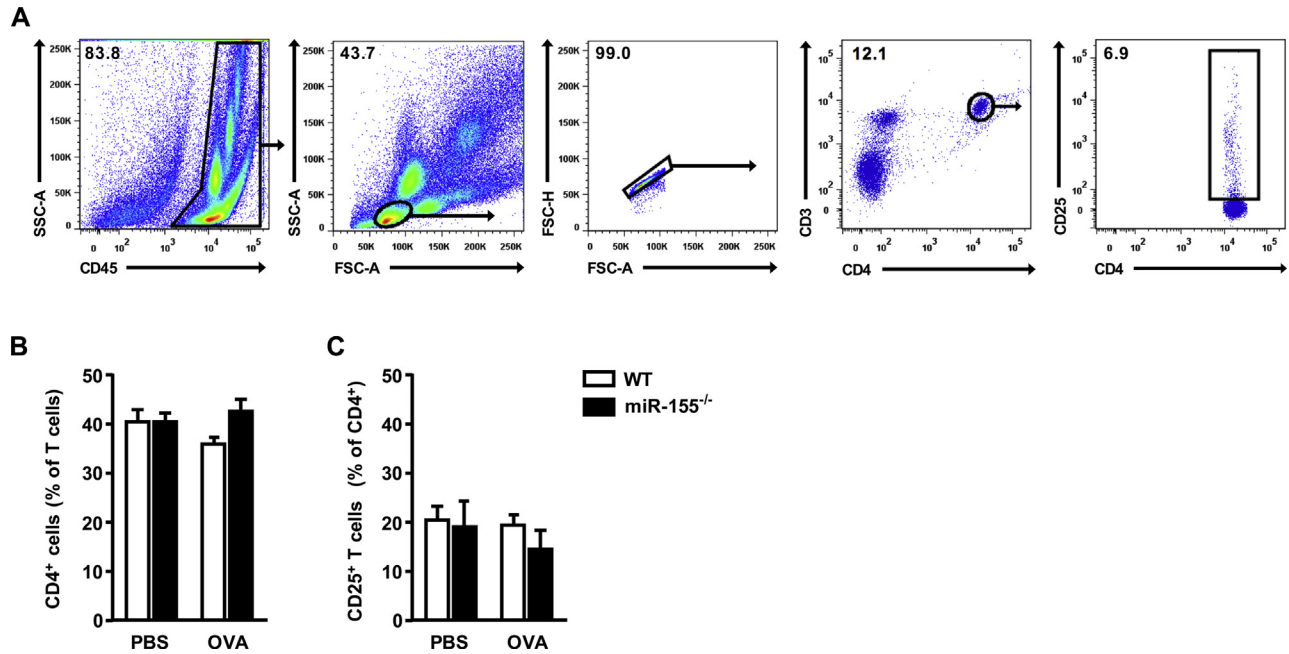


FIG E4. A, Gating strategy of lung T helper cells ($CD45^{+}CD3^{+}CD4^{+}$). Percent $CD4^{+}$ T helper cells of all T cells (**B**) ($CD45^{+}CD3^{+}$) and $CD25^{+}CD4^{+}$ T helper cells (**C**) in lungs from chronic allergen-challenged WT and $miR-155^{-/-}$ mice and PBS control mice. *FSC-A*, Forward-scatter area; *FSC-H*, forward-scatter height; *SSC-A*, side-scatter area. Data are shown as means $\pm$ SEM ($n = 5-8$ per group).

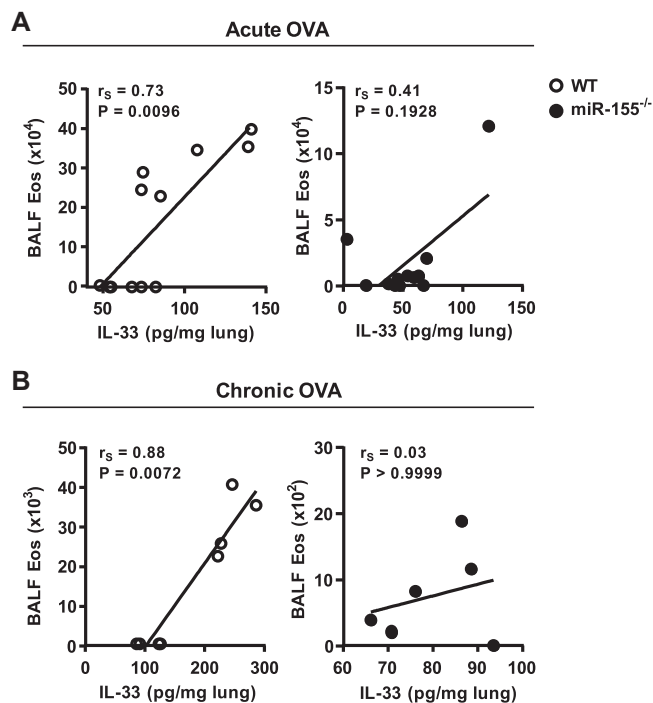


FIG E5. Scatter graph demonstrating correlation analysis between BALF eosinophils (Eos; mL^{-1} BALF) and lung IL-33 (pg/mg lung) of acute (**A**) and chronic (**B**) OVA-challenged WT and miR-155^{-/-} mice and PBS control mice. Eos, Eosinophils; r_s , Spearman correlation coefficient. Data are shown as means $\pm$ SEM ($n = 5-8$ per group).

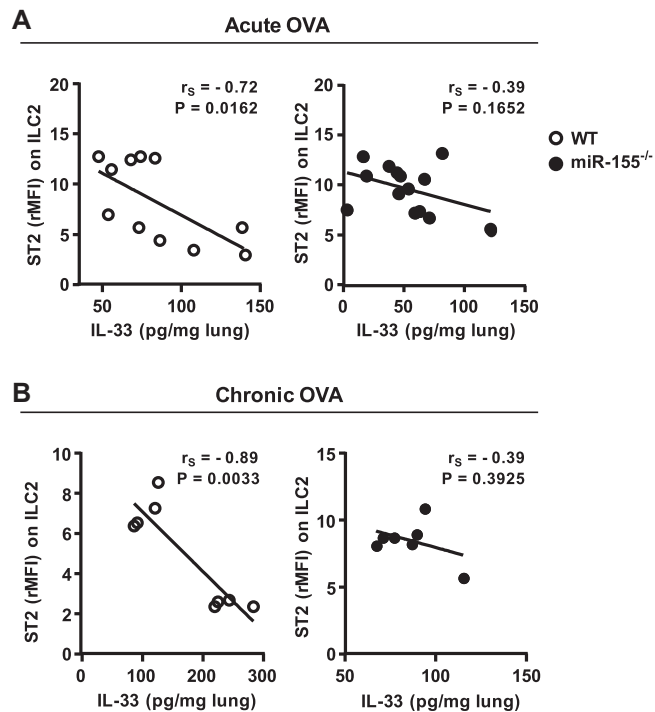


FIG E6. Scatter graph demonstrating correlation analysis between ST2 (relative mean fluorescent index; rMFI) on lung ILC2s and IL-33 (pg/mg lung) of acute (**A**) and chronic (**B**) OVA-challenged WT and miR-155^{-/-} mice and PBS control mice. r_s , Spearman correlation coefficient. Data are shown as means $\pm$ SEM ($n = 5-8$ per group).

TABLE E1. Antibodies for flow cytometric analysis

Antigen (clone)	Format	Manufacturer
Hematopoietic Lineage cocktail: CD3 (17A2), CD45R/B220 (RA3-6B2), CD11b (M1/70), TER-119 (TER-119), Ly-G6/Gr-1 (RB6-8C5)	FITC	eBioscience
CD11c (N418)	FITC	eBioscience
CD19 (1D3)	FITC	BD Bioscience
NK-1.1 (PK136)	FITC	BD Bioscience
FceR1 (MAR-1)	FITC	eBioscience
CD3 (145-2C11)	FITC	BD Bioscience
CD127/IL-7Ra (A7R34)	PE	eBioscience
GATA-3 (TWAJ)	PE	eBioscience
Isotype control GATA-3/Rat IgG2b, κ (eB149/10H5)	PE	eBioscience
IL-13 (eBio13A)	PE	eBioscience
Isotype control GATA-3/Rat IgG1, κ (eBRG1)	PE	eBioscience
CD4 (H129.19)	PE	BD Bioscience
CD45 (30-F11)	PerCP	BD Bioscience
ICOS/CD278 (C398.4A)	PE-Cy7	BioLegend
ICOS/CD278 (C398.4A)	APC	eBioscience
ST2/IL-33R (RMST2-2)	APC	eBioscience
CD25 (PC61)	APC	BD Bioscience
PU.1 (9G7)	AF647	Cell Signaling Technology
CD4 (GK.1.5)	APC-H7	BD Bioscience
CD25/IL-2RA (PC61)	BV421	BD Bioscience
Ki-67 (16A8)	BV421	BioLegend
Isotype control Ki-67/Rat IgG2a, κ (RTK2758)	BV421	BioLegend
IL-5 (TRFK5)	BV421	BioLegend
Isotype control IL-5/Rat IgG1, κ (RTK2071)	BV421	BioLegend
CD127/IL-7Ra (SB/199)	BV510	BD Bioscience
CD4 (RM4-5)	BV510	BD Bioscience

AF647, Alexa fluor 647; APC, allophycocyanin; APC-H7, H7 conjugate of Allophycocyanin; BV421, Brilliant violet 421; BV510, Brilliant violet 510; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PE-Cy7, C7 conjugate of phycoerythrin; PerCP, peridinin chlorophyll protein.