



Airways epithelial exposure to *Streptococcus pneumoniae* in the presence of the alarmin IL-33 induces a novel subset of pro-inflammatory ILC2s promoting a mixed inflammatory response

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Abstract

Background We have previously shown that asthma-like airways inflammation may be induced by topical exposure to respiratory tract pathogens such as *S. pneumoniae* (*SP*) in concert with epithelial alarmins such as IL-33. Details of the pathogenesis of this murine surrogate remain however unexplored.

Methods Airways inflammation was induced by repeated, intranasal exposure of *Il-4*^{-/-}, *Rag1*^{-/-} and *Rag2*^{-/-}*Il2rg*^{-/-} mice (in which B lymphocyte IgE switching, adaptive and innate immunity are respectively ablated) as well as wild type mice to inactivated *SP*, IL-33 or both. Airways pathological changes were analysed, and the subsets and functions of locally accumulated ILC2s investigated by single cell RNA sequencing and flow cytometry.

Results In the presence of IL-33, repeated exposure of the airways to inactivated *SP* caused marked eosinophil- and neutrophil-rich inflammation and local accumulation of ILC2s, which was retained in the *Il-4*^{-/-} and *Rag1*^{-/-} deficient mice but abolished in the *Rag2*^{-/-}*Il2rg*^{-/-} mice, an effect partly reversed by adoptive transfer of ILC2s. Single cell sequencing analysis of ILC2s recruited following *SP* and IL-33 exposure revealed a *Klrg1*⁺*Ly6a*⁺ subset, expressing particularly elevated quantities of the pro-inflammatory cytokine IL-6, type 2 cytokines (IL-5 and IL-13) and MHC class II molecules, promoting type 2 inflammation as well as involved in neutrophil-mediated inflammatory responses.

Conclusion Local accumulation of *KLRG1*⁺*Ly6a*⁺ ILC2s in the lung tissue is a critical aspect of the pathogenesis of airways eosinophilic and neutrophil-rich inflammation induced by repeated exposure to *SP* in the presence of the epithelial alarmin IL-33.

Keywords Airways · IL-33 · *S. pneumoniae* bacteria · ILC2s · IL-6

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Introduction

Asthma is a heterogeneous airways disease characterised by airways obstruction caused by spasm of hyperresponsive airways smooth muscle and often by additional narrowing of the airways caused by inflammation and swelling of the airways mucosa, and sometimes additional, irreversible remodelling changes such as goblet cell metaplasia. It currently afflicts more than 300 million people worldwide, while its overall prevalence in Chinese adults is 4.2% [1, 2]. The inflammatory phenotypes of asthma include eosinophilic, neutrophilic, mixed and paucigranulocytic [3]. In recent years, the classification of asthma phenotypes has evolved into asthma endotypes such as type 2 (T2)-high and T2-low [4, 5]. T2-high eosinophilic asthma is the most common endotype of asthma, characterized by elevated expression of IgE and a type 2 immune response driven by Th2 cells (adaptive immunity) and group 2 ILCs (ILC2, innate immunity) [4, 5]. T2-low asthma is more complex, and is often characterized by lack of type 2 biomarkers, the presence of neutrophilia, obesity, and unresponsiveness to corticosteroids [4, 5]. In this scenario, Th17 cells seem to play a pivotal pathogenic role. The mixed endotype is often driven by trans-signalling pathways driven by IL-6, IL-17 and IL-22 and is often associated with severe disease [4–6].

In susceptible individuals, local alarmin cytokines released from epithelial cells in response to environmental insults stimulates local ILC2s, which are key orchestrators of innate immunity, to produce Th2-type cytokines. In addition, ILC2s also have the potential to shape and amplify acquired immune mechanisms, notably by acting as antigen-presenting cells expressing MHC class II and OX40-ligand, the critical molecular switch for the development of CD4⁺ T lymphocytes into Th2-type cells [7]. Recent studies have suggested that ILC2s exhibit a dynamic and plastic role in the promotion of airways inflammation, producing non-Th2 type cytokines such as IL-17 and IL-10 [8, 9]. The mechanism of genesis of this plasticity and its distinct role in vivo remain to be clarified.

Respiratory infections can significantly impact upon the development, progression, and exacerbation of asthma. Recent investigations have shown significant associations of the composition of the bronchial microbiota with asthma endotypes [10]. Durack et al. have shown that T2-high asthma is associated with a low bronchial bacterial burden [11]. Moreover, upregulated local IL-33 production in the airways in response to rhinovirus, influenza A virus and respiratory syncytial virus infections plays a key role both in increasing vulnerability to respiratory viral infection in asthmatic patients and in amplifying the ensuing local, T2 inflammation of the airways [12–14].

ILCs play important role in lung inflammation and respiratory infections. It has been shown that ILC1 can produce antiviral mediators to enhance viral clearance during respiratory viral infection [15]. It has been also reported that in viral and bacterial pneumonia, ILC3s are able to promote microbial clearance as well as tissue repair through secreting the cytokines IL-17 and IL-22 [16]. Interestingly, virus infection can induce ILC2s to produce type 2 cytokines resulting in airway mucus and wheezing, which are associated with increased disease severity [17]. However, more studies are needed to explore the role of ILC2s during respiratory infection.

Streptococcus pneumoniae (*SP*) is a gram-positive bacterium which is the principal cause of community-acquired pneumonia worldwide. Interestingly, early exposure of children to *SP* impacts upon the risk of subsequent development of asthma [18, 19], while the presence of asthma may in turn compromise the patient's anti-microbial defences. Our previous studies using experimental animals have shown that repeated, intranasal, concomitant exposure of the airways to a low concentration of IL-33 and inactivated *SP* can induce asthma-like airways inflammation [20]. The precise pathogenesis of this process, and in particular whether it involves phenotypic changes in the ILC2s remains to be clarified. In the present study, we further investigated these mechanisms using IL-4^{-/-}, *Rag1*^{-/-} and *Rag2*^{-/-}*Il2rg*^{-/-} mice, which respectively lack the ability to produce an IgE response and to mount competent adaptive and innate immune responses. Additionally, using single-cell RNA (scRNA-seq) analysis of ILC2s isolated from the inflamed lung tissue, we examined the molecular signatures of individual ILC2s and aligned these with their potential role in the genesis of airways inflammation following repeated challenge with *SP* in the presence of IL-33.

Materials and methods

Animals and experimental protocol

Specific pathogen-free female BALB/c mice (8–10 weeks old) and C57BL/6 mice (8–10 weeks old) were purchased from Vital River Laboratories (Beijing, China). IL-4-deficient (*Il4*^{-/-}) BALB/c mice and *Rag1* knockout (*Rag1*^{-/-}) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). *Rag2*/*Il2rg* double knockout (*Rag2*^{-/-}*Il2rg*^{-/-}) C57BL/6 mice were purchased from Taconic Biosciences (Germantown, New York). All mice were bred in the Department of Laboratory Animal Sciences, Capital Medical University, Beijing, China. All experimental procedures were approved by the animal ethics Committee of the Capital Medical University, Beijing, China (AEEI-2022-207).

To determine whether exposure of the airways to inactivated *SP* induced lung inflammation in the presence of

the cytokine IL-33, female BALB/c mice were randomly assigned to 4 groups challenged pernasally with normal saline control (NS, 50 μ L saline), IL-33 (mIL-33, R&D Systems, 100 ng in 50 μ L saline), inactivated, whole *SP* (5×10^7 CFU in 50 μ L saline) and a combination of both IL-33 (100 ng) and inactivated *SP* (5×10^7 CFU in 50 μ L saline) on days 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16 and 18 (Fig. 1A), as previously described [20]. *SP* bacteria were obtained from the American Type Culture Collection (ATCC 49,619) and propagated. The bacteria were inactivated by treating with 0.2% formaldehyde at 37 °C for 12 h with shaking.

To investigate the contribution of IL-4 to the pathogenesis of lung inflammation induced by exposure to IL-33 + *SP*, *Il4*^{-/-} and wild-type (WT) female BALB/c mice were randomly assigned to 4 groups challenged pernasally with diluent control, IL-33 alone, inactivated *SP* alone and a combination of both.

To investigate the contribution of adaptive and innate immune cells to the lung inflammatory response induced by exposure to IL-33 and *SP*, groups of wild-type (WT), *Rag1*^{-/-} gene-deleted and *Rag2*^{-/-}*Il2rg*^{-/-} gene-deleted female C57BL/6 mice were randomly assigned to pernasal challenge with IL-33 + *SP* or diluent control as shown in the schedules in Figs. 3A and 4A.

Bronchoalveolar lavage fluid (BALF) collection and differential cell counting

Following euthanasia, BALF was collected from the mice and total cells enumerated as previously described [21]. Cellular smears were then prepared and stained with haematoxylin & eosin (H&E) for differential cellular counts [20].

Lung histology

The left lungs were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Lung Sect. (4 μ m) were stained with HE (Beijing Solarbio Technology Co Ltd.) and then examined under a Leica microscope [20].

Measurement of cytokine concentrations, total and *SP*-specific IgE and IgG₁ in lung homogenates and serum

100 mg of right lung tissue was weighed then homogenised with 5 \times volume PBS containing 1% Triton X-100 and protease inhibitor cocktail (Roche Diagnostics GmbH). After centrifugation to remove debris, the supernatants were collected for measurement of cytokines. Commercial ELISA kits (Invitrogen) were used to measure the concentrations of IL-4, IL-5, IL-6, IL-13, total IgE and IgG₁ in the supernatants of the right lung homogenates and sera.

Concentrations of *SP*-specific IgE and IgG₁ in the sera were measured as described previously [20] with a slight modification. Briefly, the inactivated *SP* homogenized with PBS containing 1% Triton X-100 and protease inhibitor cocktail by ultrasonic crusher. After centrifugation to remove debris, the supernatants were collected as *SP* lysates [20]. Then 96-well microtiter plates were coated with 100 μ L volume of 10 μ g/mL of a whole protein extract of the *SP* lysates [20] overnight. Serum samples were diluted 1:10 (for IgE) or 1:1000 (for IgG₁), and antibody detected using goat anti-mouse IgE (HRP) (1:5000, Invitrogen) or goat anti-mouse IgG₁ (HRP) (1:5000, Abcam) at 37°C for 1 h.

Preparation of single-cell suspensions and flow cytometric analysis

Spleens and lymph nodes were mechanically disrupted using a 70- μ m cell strainer. Lung tissues were cut into pieces and digested in DPBS medium containing DNase I (Sigma-Aldrich) (50U/mL) and collagenase VIII (Sigma-Aldrich) (250 U/mL) at 37°C for 30 min. Single-cell suspensions were obtained by passing the lung tissue digest through a 70- μ m cell strainer. ILC2s were stained with APC-conjugated anti-lineage cocktail (BD Pharmingen), APC-Cy7-conjugated anti-CD45 (30-F11, BD Pharmingen), BB515-conjugated anti-ICOS (7E.17G9, BD Pharmingen), PerCP-eFluor-710-conjugated anti-ST2 (RMST2-33, eBioscience) and BUUV395-conjugated anti-GATA3 (L50-823, BD Pharmingen) on ice for 30 min. ILC2s were identified as lineage⁻CD45⁺ICOS⁺ST2⁺, as previously described [21–23]. For cytokine staining, cells were stimulated with cell stimulation cocktail (500x, eBioscience) for 4 h at 37 °C. At the end of this incubation, the cells were fixed and permeabilised, and then stained with anti-IL-6 (MP5-20F3, BD Pharmingen), anti-IL-5 (TRFK5, BD Pharmingen) and anti-IL-13 (eBio13A, eBioscience) antibody. Isotype and single-stain controls were included. Acquisition was performed on a Cytex Aurora (Cytex) or BD LSRFortessa X20 (BD Biosciences) cytometer, and analysed using FlowJo software (Tree Star Inc.).

ILC2 isolation and adoptive transfer

ILC2s were isolated from the lung tissues of the group of 10 mice euthanised 24 h after the final, pernasal challenge with IL-33 + *SP* (Fig. 1A). Cells were stained and sorted as Lineage⁻CD45⁺ICOS⁺ST2⁺ cells using a FACSAria III (BD Biosciences) system. Sorted ILC2s were cultured for 3 days in RPMI 1640 containing 10% FBS, 1% penicillin/streptomycin, 0.1% β -mercaptoethanol, 10 ng/mL IL-2 (R&D Systems), and 10 ng/mL IL-7 (R&D Systems) [21], then adoptively transferred to *Rag2*^{-/-}*Il2rg*^{-/-} mice (1×10^6 cells per mouse via tail vein injection) before intranasal

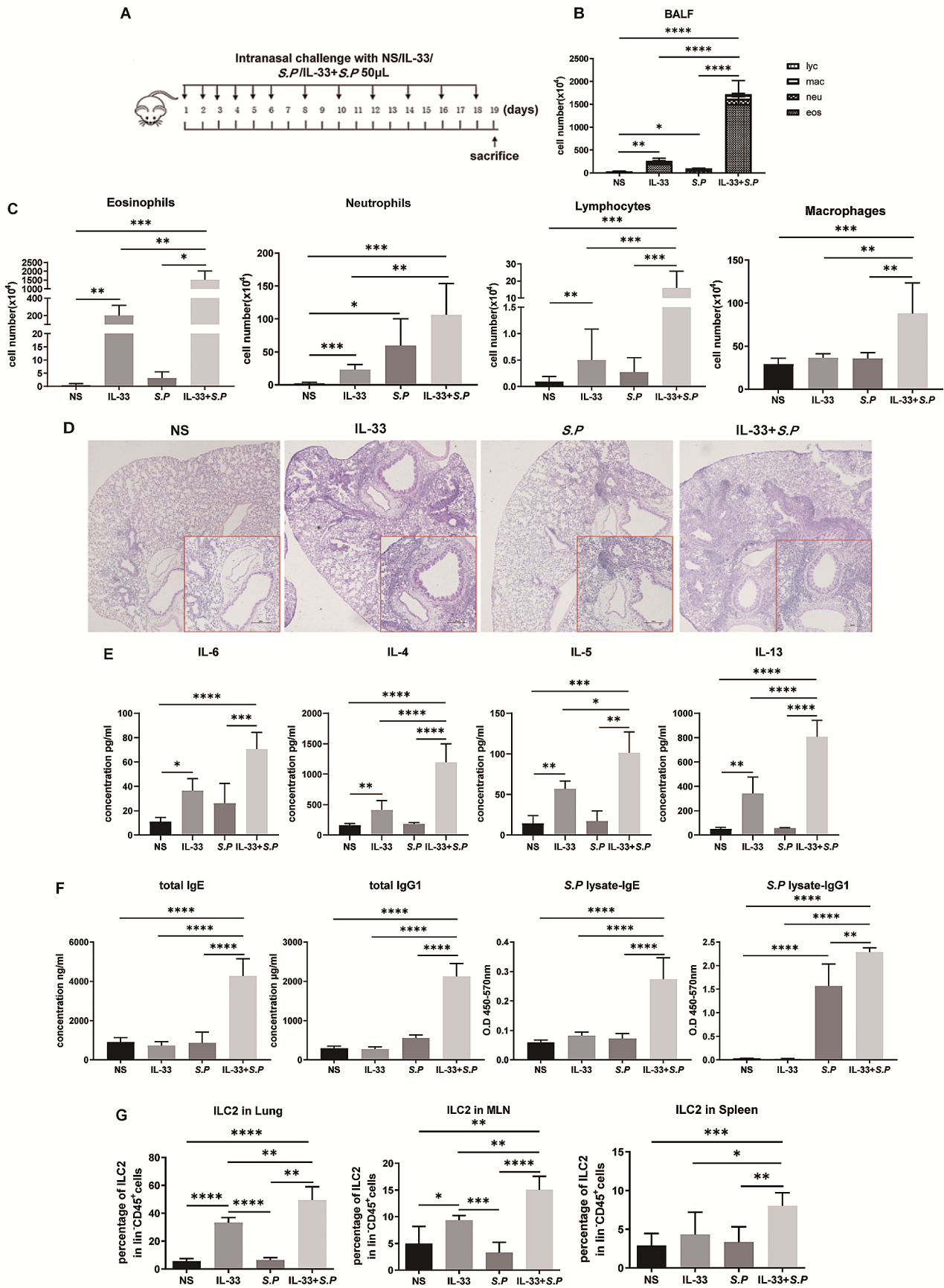


Fig. 1 Combined exposure to IL-33 + *S. pneumoniae* (*SP*) increases type 2 airways inflammation. **A**, Schedule of murine challenge (19 days). **B & C**, Cell counts (total and differential) in the bronchoalveolar lavage fluid (BALF) of the experimental mice at day 19. **D**, Representative photomicrographs of haematoxylin & eosin sections of lung tissues. **E**, Concentrations of IL-6, IL-4, IL-5 and IL-13 in homogenates of the lung tissues of the experimental mice as measured by commercial ELISA. **F**, Concentrations of total serum IgG₁, IgE (left hand panels) and IgG₁ and IgE binding specifically to *SP* (right hand panels) determined by ELISA. Data are presented as the mean \pm SEM ($n=5$ in each group). **G**, Percentages of ILC2s in the lungs, MLNs and spleens of the experimental mice. Bars show the mean \pm SD ($n=5-6$ in each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

administration of IL-33 + *SP* 2 h on days 1, 3, 5 and 7, as previously described [21, 24]. (Fig. 4A).

Single-cell RNA-seq analysis

Lung ILC2s sorted from lung tissues of WT mice and were loaded into Chromium microfluidic chips with 3' v3 chemistry and barcoded with 10 x Chromium Controller (10X Genomics). RNA from the barcoded cells was subsequently reverse-transcribed and sequencing libraries were constructed with reagents from a Chromium Single Cell 3' v3 reagent kit (10X Genomics) according to the manufacturer's instructions. Sequencing was performed using the Illumina HiSeq PE150 platform at Novogene Co. Ltd (Beijing, China). After raw base call files were de-multiplexed into FASTQ files, we used the Cell Ranger Suite (10X Genomics) to perform alignment, filtering, barcode counting processing and single-cell gene unique molecular identifier counting based on the reference genome. Data were imported into R (version 4.0.5) using the Seurat package (version 4.3.0). Integrated analysis was used to combine the samples, while t-distributed stochastic neighbour embedding (t-SNE) was used to visualise the cells, which formed 11 separate clusters based on the Louvain algorithm. We removed contaminating cells and kept ILC2s based on high expression of *Gata3*, *Rora* and also refer to R package Single R (v 2.0.0). Re-clustering of the cells provided 5 separate clusters. Cells were analysed for gene expression. The R package Monocle2 (v 2.22.0) was used to determine gene expression in pseudotime.

Statistical analysis

The software package GraphPad Prism 7.01 (GraphPad, San Diego, CA) was used for all data analysis and preparation of graphs. In general, between-group statistical comparisons were performed using the unpaired *t*-test, while Mann-Whitney test was used to compare the difference between two groups for data that did not conform to normal distribution. Data are expressed as the mean \pm SD. For all tests, $p < 0.05$ was considered significant.

Results

Repeated, topical exposure of the airways to whole, inactivated *SP* induces florid local inflammation and specific antibody production in the presence of IL-33

To investigate the outcome of repeated exposure of the airways to inactivated *SP* in the presence of IL-33, we devised a protocol in which naïve mice were repeatedly challenged pernasally with inactivated bacteria, IL-33, both or vehicle control over an 18 day period prior to euthanasia on day 19, as shown in the schedule in Fig. 1A. Mice challenged with IL-33 alone significantly increased mean numbers of total cells, eosinophils, neutrophils and lymphocytes in BALF as compared with saline control. And the mean numbers of total cells, eosinophils, neutrophils, lymphocytes and macrophages in BALF were significantly higher in mice challenged simultaneously with IL-33 and inactivated *SP* than those of mice challenged with IL-33, inactivated *SP* and saline alone (Fig. 1B, C), as well as florid inflammation of the peribronchial and perivascular regions of their lung tissues, as revealed by H&E (Fig. 1D).

ELISA analysis revealed that IL-33 challenge alone caused modestly elevated expression of the type 2 cytokines IL-4, IL-5 and IL-13 and the pro-inflammatory cytokine IL-6 in lung homogenates from the experimental animals (Fig. 1E). Simultaneous challenge of the airways with IL-33 and inactivated *SP* induced marked, significantly elevated expression of IL-4, IL-5, IL-13, and IL-6, as well as the mean serum concentrations of total and *SP*-specific IgE and IgG₁ compared with those challenges with IL-33, inactivated *SP* alone or saline control (Fig. 1E, F).

Repeated topical exposure of the airways to *SP* in the presence of IL-33 induces local and systemic elevation of the numbers of ILC2s

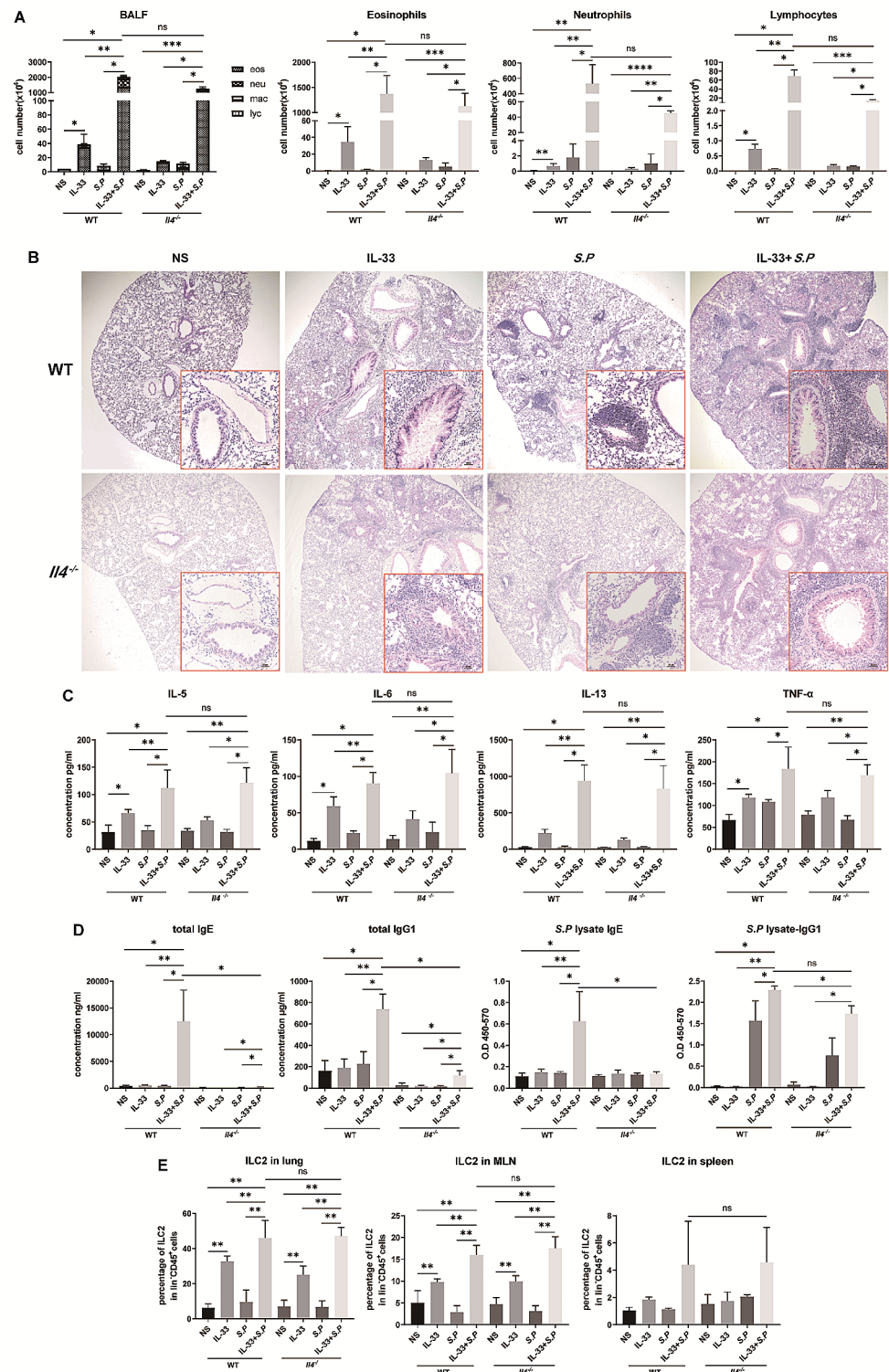
Flow cytometric analysis of single cell suspensions obtained from the lung tissues, spleens and mediastinal lymph nodes of the experimental animals revealed that repeated challenge of the airways with IL-33 and *SP* induced significant elevation of the mean percentages of ILC2s in CD45⁺lin⁻ cells (defined as lineage⁻CD45⁺ICOS⁺ST2⁺ILC2s expressing the transcription factor *GATA-3*) in the lung tissues compared with either stimulus alone or vehicle control (exposure to IL-33 alone also resulted in significant elevation of the mean percentages of ILC2s) (Fig. 1G and Fig. S1). A similar outcome was observed in the spleens and MLNs of the animals, although the percentages of these cells in these more distal organs were considerably smaller (Fig. 1G, and Fig. S1).

Airways inflammation of mice repeatedly, topically exposed to SP in the presence of IL-33 is independent of IL-4 and IgE production

To clarify the possible roles of IL-4 and IgE in the pathogenesis of lung mixed inflammation caused by repeated,

topical exposure to IL-33+SP, we compared the pathological changes in groups of syngeneic, *Il4*^{-/-} and WT mice challenged using the protocol shown in Fig. 1A. Data analysis revealed that there were no significant differences in the mean total numbers of inflammatory cells, eosinophils, neutrophils and lymphocytes in the BALF (Fig. 2A),

Fig. 2 IL-4 gene deletion results in decreased IgE production but no changes in airways eosinophil infiltration in mice exposed to repeated, combined challenge with IL-33 + *S. pneumoniae* (SP). **A**, Total and differential inflammatory cell counts in the bronchoalveolar lavage fluid (BALF) of WT and *Il4*^{-/-} mice. **B**, Representative photomicrographs of lung sections stained with haematoxylin & eosin. **C**, Concentrations of IL-6, TNF- α , IL-5 and IL-13 in lung tissue homogenates of the experimental mice as determined by ELISA. **D**, Serum concentrations of total and SP lysate-specific IgE and IgG1 as determined by ELISA. **E**, Percentages of ILC2s in *lin*⁻CD45⁺ cells from the lungs, MLN and spleens of the experimental mice. Bars show the mean \pm SD ($n=3-5$ in each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$



inflammatory cell influx into the lung tissue (histological examination of H&E-stained sections) (Fig. 2B), cytokine production including IL-6, TNF- α , IL-5, IL-13 (Fig. 2C) as well as the mean percentages of ILC2s in the lung and MLNs (Fig. 2E) between the *Il4*^{-/-} and WT mice (full gating strategies given in Figure S2). In contrast, the significant increases in the mean serum concentrations of total and *SP*-specific IgE and total IgG₁ following repeated challenge with IL-33 + *SP* compared with diluent control were respectively ablated and significantly attenuated in the *Il4*^{-/-} compared with the WT mice, although the mean serum concentration of *SP*-specific IgG₁ was not significantly altered (Fig. 2D).

Cells of the adaptive immune system contribute only marginally to airways inflammation and ILC2 accumulation in the airways of mice repeatedly, topically exposed to *SP* in the presence of IL-33

To investigate the possible role of mature T and B lymphocytes in the pathogenesis of airways inflammation induced by repeated, topical exposure to *SP* in the presence of IL-33, we set up an 8 days, repeated intranasal challenge protocol with both agents or diluent control in *Rag1*^{-/-} mice, which lack mature T and B cells, and WT mice, as shown in Fig. 3A. The significantly elevated mean numbers of total cells, eosinophils and neutrophils infiltrating the BALF

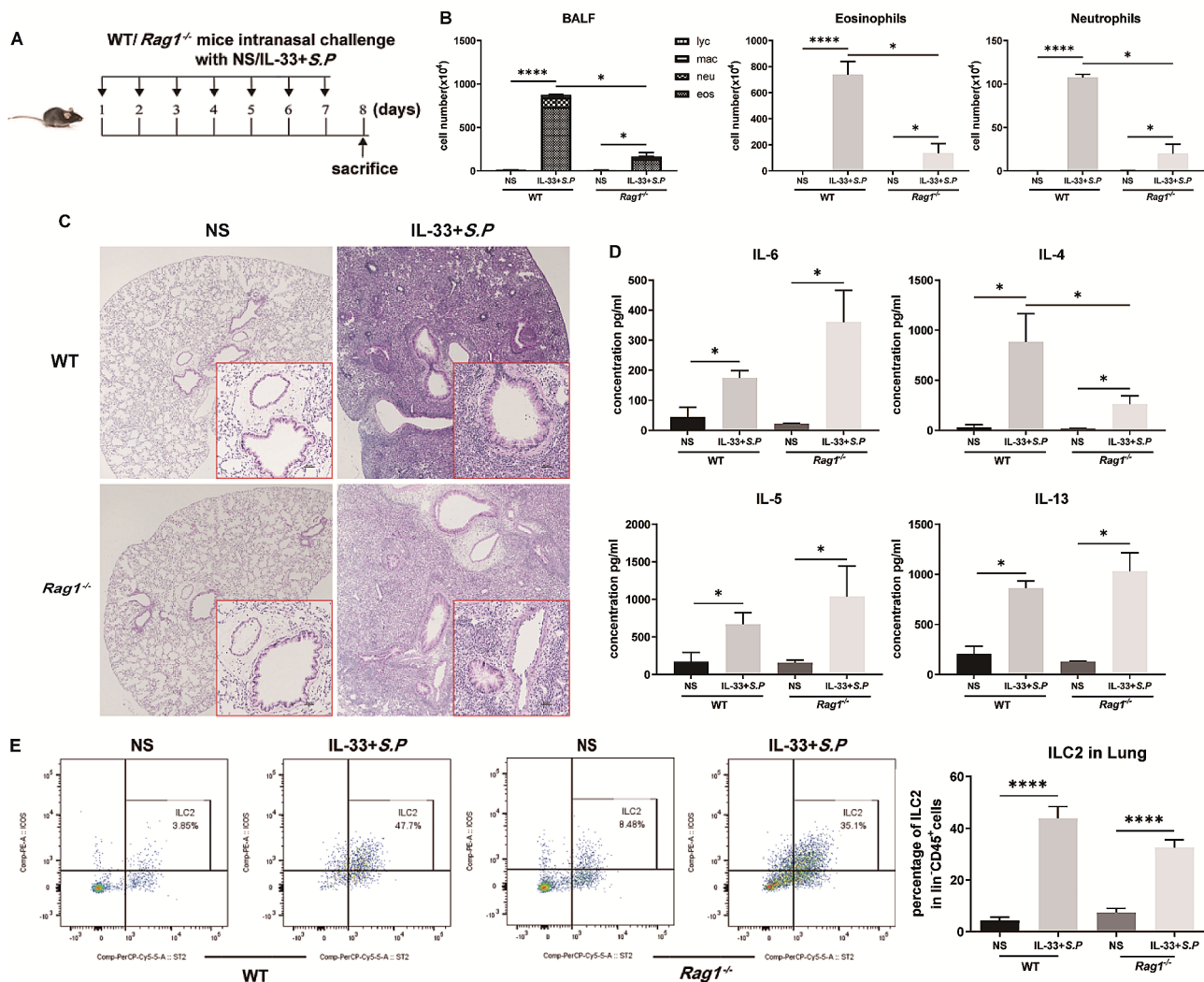


Fig. 3 The adaptive immune system marginally contributes to airways inflammation induced by repeated exposure to inactivated *S. pneumoniae* (*SP*) in the presence of IL-33. **A**, Schedule of murine challenge. **B**, Cell counts (total and differential) in the bronchoalveolar lavage fluid (BALF) of WT and *Rag1*^{-/-} mice. **C**, Representative photomicrographs of haematoxylin & eosin sections of lung tissue. **D**, Concentrations of IL-6, IL-4, IL-5 and IL-13 in homogenates of the

lung tissues of the experimental mice as determined by commercial ELISA. **E**, Representative flow cytometric plots of ILC2s gated as the ICOS⁺ST2⁺ sub-population of CD45⁺lin⁻ lung cells from the WT and *Rag1*^{-/-} mice, and the frequency of ILC2s in lin⁻CD45⁺ cells are shown. Bars show the mean \pm SD ($n=4-5$ in each group). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

following repeated challenge of the WT animals with inactivated *SP* in the presence of IL-33 compared with diluent control were significantly attenuated in the *Rag1*^{-/-} mice (Fig. 3B). In contrast, as revealed by H&E (Fig. 3C) staining, the lung tissues of the *Rag1*^{-/-} mice exhibited prominent inflammatory cellular infiltration, and resembling that observed in the WT mice. Furthermore, the mean concentrations of IL-6, IL-5 and IL-13 in the lung tissue homogenates (Fig. 3D) as well as the mean percentages of ILC2s in the lung tissues (Fig. 3E) were equivalent in the *Rag1*^{-/-} and WT mice.

Adoptive transfer of ILC2s restores the inflammatory response in the airways of *Rag2*^{-/-}*Il2rg*^{-/-} mice to repeated, topical exposure to *SP* in the presence of IL-33

To confirm the role of ILC2s in the pathogenesis of airways inflammation induced by repeated, topical exposure to *SP* in the presence of IL-33, *Rag2*^{-/-}*Il2rg*^{-/-} mice and WT controls were challenged intranasally for 8 days, the *Rag2*^{-/-}*Il2rg*^{-/-} mice having received an intravenous injection of 10⁶ ILC2s or culture medium control immediately prior to challenge on days 1, 3, 5 and 7 via the tail vein

(Fig. 4A). As seen in Fig. 4B–D, adoptive transfer of ILC2s to the *Rag2*^{-/-}*Il2rg*^{-/-} mice partly but significantly restored the inflammatory response seen in the WT mice to repeated, pernasal challenge with *SP* in the presence of IL-33 in the airways lumen and the elevated expression of IL-4, IL-5, IL-13 and IL-6 in the lung tissue homogenates. Notably, adoptive transfer of ILC2s to the *Rag2*^{-/-}*Il2rg*^{-/-} mice also significantly increased mean numbers of neutrophils as compared with those WT animals challenged with inactivated *SP* in the presence of IL-33.

scRNA-seq identifies ILC2 heterogeneity

To further characterise the molecular features of the ILC2s generated following repeated challenge with *SP* in the presence of IL-33, scRNA-seq analyses were performed in the sorted, ILC2-enriched immune cell populations prepared from the experimental mice as shown in Fig. 1A. Using t-SNE analysis in the R package Seurat, five distinct clusters of cells based on their transcriptomes were detected (Fig. 5A). Clusters 0–3 were identified as ILC2s based on their high expression of ILC2 signature genes, such as *Rora*, *Gata3*, *Il1rl1* (encodes ST2) and *Il7r* (Fig. 5D). Resting *Cxcl2*⁺ natural ILC2

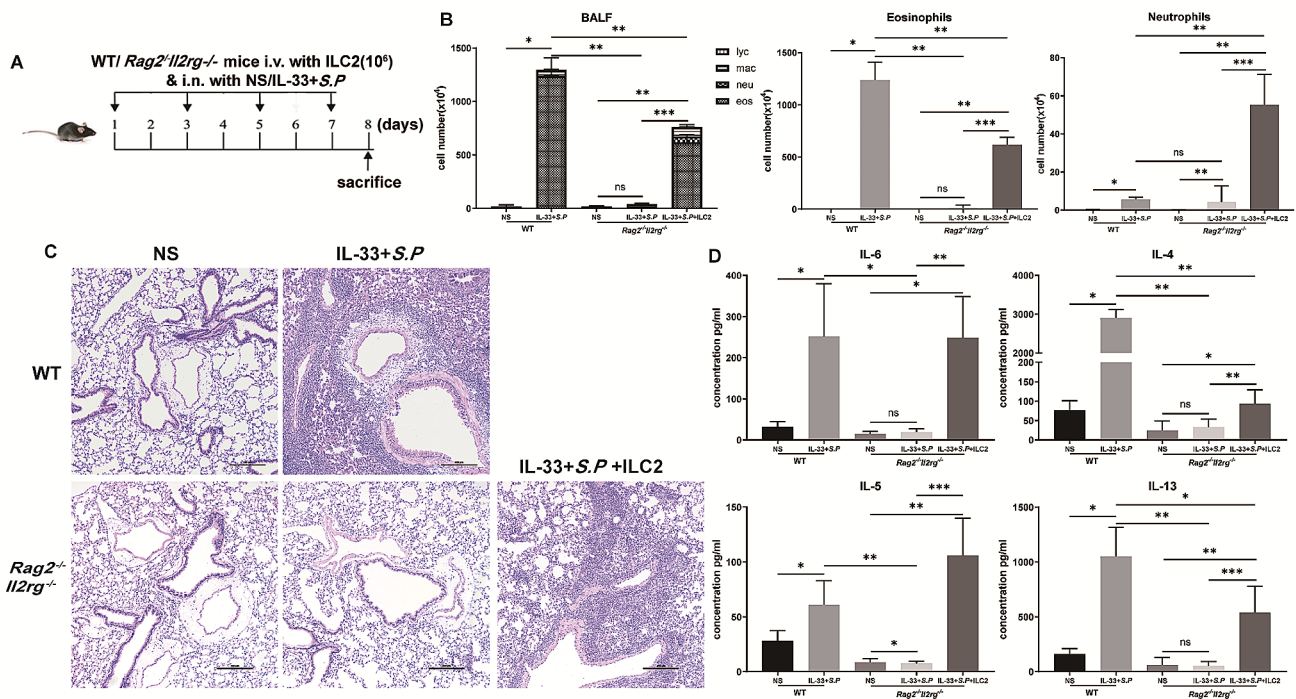


Fig. 4 The innate immune system, ILC2s, play a significant role in IL-33 and *S. pneumoniae* (*SP*)-induced airways eosinophilic inflammation. **A**, Schedule of murine challenge. Cultured ILC2s were adoptively transferred (intravenously) into *Rag2*^{-/-}*Il2rg*^{-/-} mice on days 1, 3, 5 and 7, then the mice challenged pernasally with IL-33 and *SP* or NS on the same days. On day 8, the mice were euthanised. **B**, Cell counts (total and differential) in the bronchoalveolar lavage fluid

(BALF) of WT and *Rag2*^{-/-}*Il2rg*^{-/-} mice. **C**, Representative photomicrographs of lung tissue sections stained with haematoxylin & eosin. **D**, Concentrations of IL-6, IL-4, IL-5 and IL-13 in homogenates of the lung tissues of the WT and *Rag2*^{-/-}*Il2rg*^{-/-} mice as determined by commercial ELISA. Bars show the mean \pm SD ($n = 4-5$ in each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

cells (nILC2) (Cluster 0) [23–25] were defined as *Il1rl1* (ST2)⁺*Cxcl2*⁺*Klrg1*⁻*Il5*⁻*Il13*⁻ (Fig. 5E). Compared with cluster 0, clusters 1, 2 and 3 expressed an enrichment of genes related to ILC2 activation and function, including *Il5* and *Il13* (Fig. 5D), which were further subdivided into *Klrg1*⁺*Ly6a*⁺ILC2 (cluster 1), defined as *Klrg1*⁺*Il17rb*⁺*MHCII*⁺*Ly6a*⁺*Il5*⁺*Il13*⁺, *Klrg1*⁺*Ly6a*⁻ILC2 (cluster 2), defined as *Klrg1*⁺*Ly6a*⁻*Il17rb*⁺*Il5*⁺, as well as *Klrg1*⁻ILC2 (cluster 3) defined as *Klrg1*⁻*Il17rb*⁻*Il13*⁺^{20–22}. Comparison of the percentages of the different populations revealed that nILC2s were enriched in the *SP* and saline control challenged animals (Fig. 5B, C), while *Klrg1*⁺*Ly6a*⁻ILC2s and *Klrg1*⁻ILC2s were abundant in the IL-33 challenged animals. In addition, *Klrg1*⁺*Ly6a*⁺ILC2s were enriched in the IL-33 + *SP* challenged animals (Fig. 5B, C). Analysis of the top 10 differentially expressed genes (DEG) revealed that *Klrg1*⁺*Ly6a*⁺ILC2s highly expressed genes associated with cell differentiation (*Tox2*, *Irf3*), adhesion and antigen presentation (*H2-K1*, *Itgae*), as well as survival and proliferation (*Tff1*, *Ttac39c* and *Plac8*) (Fig. 5E and Table S1). As expected, gene set enrichment analysis (GSEA) indicated that the genes expressed by the *Klrg1*⁺*Ly6a*⁺ILC2s were enriched in those implicated in inflammatory response and antigen processing and presentation (Fig. 5F). Taken together, these data suggest that repeated challenge with *SP* in the presence of IL-33 results in the generation of distinct ILC2 subsets that preferentially upregulate genes associated with activation, proliferation, adhesion and antigen presentation by these cells.

Developmental trajectory analysis defines distinct states of ILC2s associated with repeated challenge with *SP* in the presence of IL-33

To further discern how changes in ILC2 subtype-specific gene expression in each of the ILC2 groups proceeds temporally in response to the IL-33/*SP* stimulus, we applied Monocle2 to reconstruct an inferred, pseudo temporal trajectory of development of all acquired ILC2s (Fig. 5G–I). This analysis defined eight developmental hierarchies (states 1–8), with the state 1 cells (mainly enriched nILC2s) located at the starting point of the cellular evolution on this map (Fig. 5G–I), which suggested multiple pathways of ILC2 development, including into *Klrg1*⁺*Ly6a*⁺, *Klrg1*⁺*Ly6a*⁻ and *Klrg1*⁻ cells. As expected, the state 1 cells were more prevalent in the animals challenged with *SP* alone and saline control, and exhibited elevated expression of *Cxcl2* and *Fos*, but lower expression of *Klrg1*, *Il5* and *Il13* (Fig. 5D, H, I). It seems reasonable to infer that these state 1 cells were lung resident, resting ILC2s. Following repeated challenge with IL-33,

particularly in combination with *SP*, the native population declined in prevalence, while the ILC2s exhibited progressive evidence of activation, with a predominance of *Klrg1*⁺*Ly6a*⁺ cells (Fig. 5G–I). In the course of this developmental trajectory, cells expressing *Arg1*, *H2-D1*, *Il13* and *Cxcr6* were more abundant, while those expressing *Cxcl2*, *csf2* and *Fos* were reduced (Fig. 5J). Collectively, these clustering data are consistent with the hypothesis that repeated challenge of the airways with *SP* in the presence of IL-33 results in the development of distinct subsets of ILC2s, including *Klrg1*⁺*Ly6a*⁺ cells which likely contribute to antigen presentation and inflammatory responses.

Klrg1⁺*Ly6a*⁺ILC2s promote a mixed inflammatory response by producing large amounts of IL-6 and type 2 cytokines

To further characterise the molecular features and functions of distinct subsets of ILC2s generated following repeated challenge with *SP* in the presence of IL-33, flow cytometric analysis of lung cells were performed from the experimental mice as shown in Fig. 1A. The results showed that repeated challenge of the airways with the combination of IL-33 and *SP* induced significant elevation of the mean percentage of *KLRG1*⁺*Ly6a*⁺ILC2s in the lung tissues compared with IL-33 alone, accompanied by a significant decrease in the mean percentage of *KLRG1*⁺*Ly6a*⁻ILC2s (Fig. 6A, B and Fig. S3). As expected, *KLRG1*⁺*Ly6a*⁺ILC2s can express higher levels of pro-inflammatory cytokine IL-6, type 2 cytokines (IL-5 and IL-13) and MHC class II molecules than *KLRG1*⁺*Ly6a*⁻ and *KLRG1*⁻ ILC2s, suggesting that *KLRG1*⁺*Ly6a*⁺ILC2s not only promote type 2 inflammation, but are also involved in neutrophil-mediated inflammatory responses (Fig. 6C).

Discussion

In the present study, we demonstrate for the first time that repeated exposure of the airways to an external, environmental pathogenic and commensal organism, *SP*, even if non-viable, may in the presence of the alarmin cytokine IL-33 induce phenotypic changes in local ILC2s which promote airways inflammation. This in turn induces a systemic antibody response to the organism, including the production of specific IgE, although in our IL-4 gene-deleted animals, in which IgE production was ablated, airways inflammation was not significantly altered. These data suggest that, at least in this particular scenario, IgE production is epiphenomenal. This conclusion is further

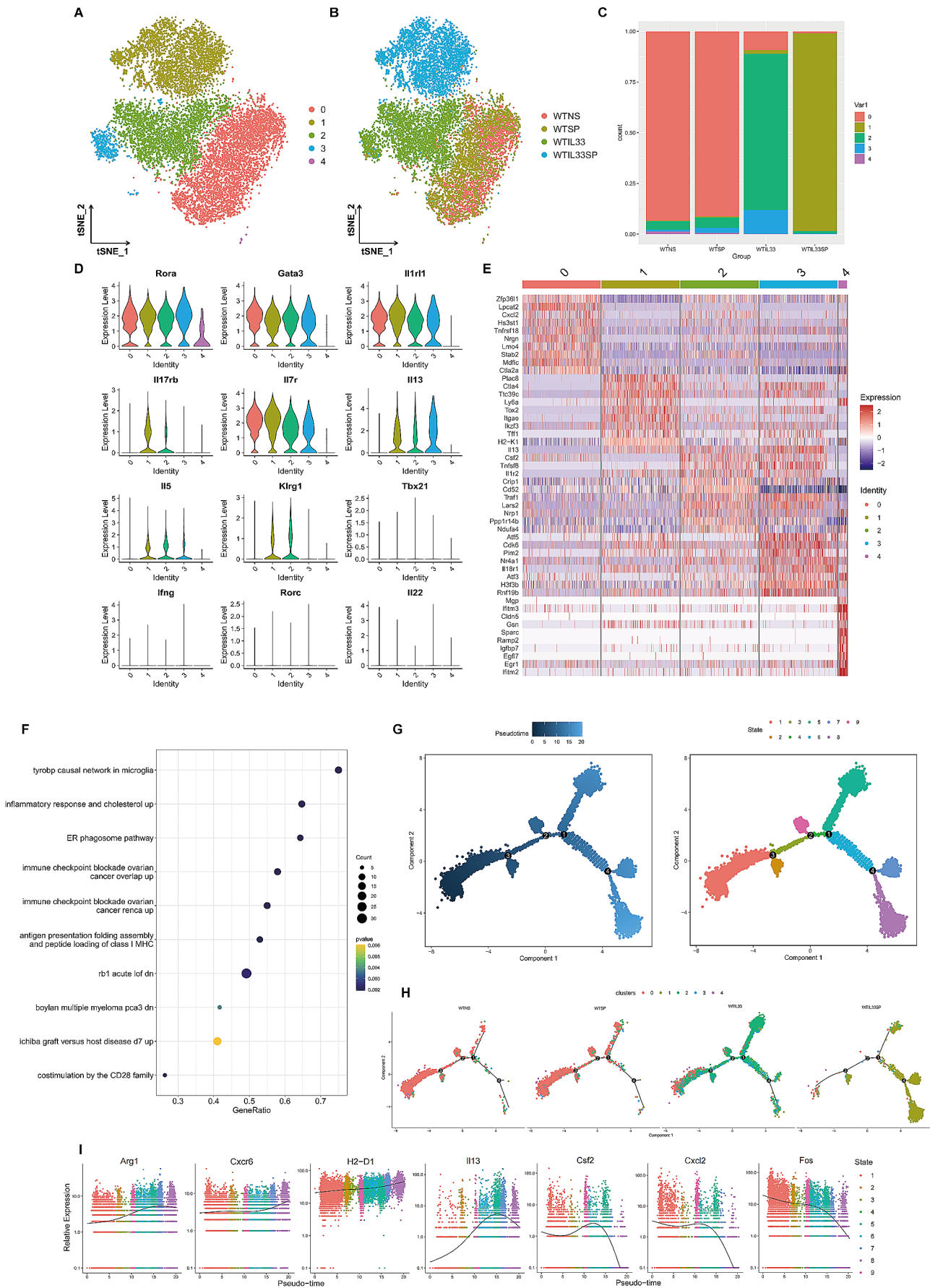


Fig. 5 Single cell RNA seq analysis reveals ILC2 heterogeneity and developmental trajectory. **A & B**, The scRNA-seq data ($n=13,338$ single ILC2s) across all four groups of ILC2s are shown as nonlinear representations of the top 50 principal components: the cells are coloured according to t-distributed stochastic neighbour embedding (t-SNE)-based clusters (**A**) or according to treatment (**B**). Sub-clustering of ILC2s was performed with a resolution of 0.3. **C**, Proportions of the clusters within each group as defined by treatment condition. **D**, Distinct patterns of key differentially expressed genes across clusters. Violin plots show the expression levels (y axis) of the indicated genes across the cells in each cluster (x axis). **E**, Heat map of top 10 highly significantly upregulated, differentially expressed genes in each cluster. **F**, Selected list of inflammatory response Gene Ontology pathways enriched in ILC2s of the animals challenged with IL-33 + *S. pneumoniae* (*SP*). **G & H**, Putative differentiation trajectory of ILC2s in all samples, with each point coloured pseudo temporally (**G**) and by clusters (**H**). **I**, Pseudo temporal plot showing representative gene expression levels of different marker genes along the temporal axis. The size of each dot reflects relative expression

supported by our demonstration that alarmin-associated airways inflammatory responses to environmental antigens may proceed largely independently of T and B lymphocytes of the adaptive immune system, but depend critically on the involvement, and phenotypic transformation of ILC2s.

Using scRNA-seq, we were able to define three subsets of pulmonary activated ILC2s expressing genes encoding Th2-type cytokines including *Il5* and *Il13* to a high degree. Previous scRNA-seq data have shown that the vast majority of pulmonary ILC2s are tissue resident cells under steady state conditions [25–29]. Consistent with these studies, our present data indicate that nILC2s express genes such as *Gata3*, *Il1r1*, *Cd69* and *Cxcl2* to a relatively high degree, but others such as *Klrg1* to a relatively low degree. Data also suggest that nILC2s may be derived locally from the tissues rather than recruited from the circulation. Both our study and previous studies have shown that exposure to the alarmin IL-33 induces distinct, active subsets of ILC2s such as *Klrg1*⁺*Ly6a*⁻ and *Klrg1*⁻ cells^{25–27}. Notably, our present data define a further unique subset of *Klrg1*⁺*Ly6a*⁺ ILC2s expressing the *Il5*, *Il13*, *Il1r1l*, *Klrg1*, *Il17rb*, *Cxcr6*, *Arg1*, *H2-D1*, and *H2-K1* genes to an elevated degree which is distinct from previous reports of an ILC inflammatory subset expressing *Klrg1* and *Il1r1l*^{25,26}. Interestingly, *Klrg1*⁺*Ly6a*⁺ ILC2s develop in the lung tissues of mice following repeated challenge with *SP* in the presence of IL-33 and appear to exhibit, at least under our experimental conditions, elevated production of the pro-inflammatory cytokine IL-6, the type 2 cytokines IL-5 and IL-13 and MHC class II molecules compared with *Klrg1*⁺*Ly6a*⁻ and *Klrg1*⁻ ILC2s. Wallrapp and colleagues [27] have also described a population of respiratory tract ILC2s activated by the local neuropeptide neuromedin U combined with the alarmin IL-25 which amplified Th2-type inflammation and co-expressed

antigen-presentation genes, including *H2-Ab1* and *Cd74*. In addition, Nakatani-Kusakabe and colleagues [30] have shown that IL-33 elicits ILC2-dependent skin inflammation mediated by two distinct functional clusters of ILC2s, one of which expressed H2-related MHC class II genes and was able to track to local lymph nodes. These findings are also in line with the present study suggesting that ILC2s express the antigen-presentation MHC class II molecules, which facilitate dialogue with T cells to initiate systemic, acquired immune responses.

In the present study, adoptive transfer of ILC2s to the *Rag2*^{-/-}*Il2rg*^{-/-} mice partly but significantly restored the inflammatory response observed in the WT mice challenged with *SP*+IL-33 and the elevated expression of IL-4, IL-5, IL-13 and IL-6 in the lung tissue homogenates. Notably, adoptive transfer of ILC2s to the *Rag2*^{-/-}*Il2rg*^{-/-} mice also significantly increased mean numbers of neutrophils as compared with those WT animals challenged with inactivated *SP*+IL-33. These data suggest that ILC2s and products such as IL-6 may also contribute to neutrophil-mediated inflammatory responses. Furthermore, we demonstrated that *KLRG1*⁺*Ly6a*⁺ ILC2s can express elevated quantities of the pro-inflammatory cytokine IL-6 and type 2 cytokines, suggesting that this subset of ILC2s not only promotes eosinophilic inflammation but is also able to induce neutrophil-mediated inflammatory responses, even partially. Hardman and colleagues [31] have shown that heat-killed, skin-resident bacteria may provoke an IL-6 profile in ILC2 in vitro, suggesting that ILC2 might be capable of sensing the cutaneous microbiota including *S. aureus*, *S. epidermidis* and *Pseudomonas aeruginosa*. In addition, our previous data [32] have shown that ILC2s have the capacity to uptake, phagocytose and degrade bacteria as well as generate extracellular traps, indicating that ILC2 may also serve as the first line against foreign microbial invasion. Thus, ILC2s might not only serve as a cellular source of type 2 cytokines, but also potentially participate in the antibacterial process and promote neutrophil-mediated inflammatory responses.

It seems likely that the development of human asthma phenotypes, which may be T2-high, T2-low and of “mixed” phenotype, depends critically on the impact of environmental factors [4]. In the present study, mice challenged with IL-33 developed T2-high eosinophilic inflammation, but those exposed to IL-33+*SP* developed significantly elevated numbers of both eosinophils and neutrophils, suggesting a process of evolution of the immune-inflammatory status, commencing with a canonical T2 signature and progressing to a more complex, mixed profile. Furthermore, consistent with previous studies showing that the IL-6 transsignalling pathway is involved in mixed phenotypes [6], *KLRG1*⁺*Ly6a*⁺ ILC2s produce abundant IL-6, suggesting

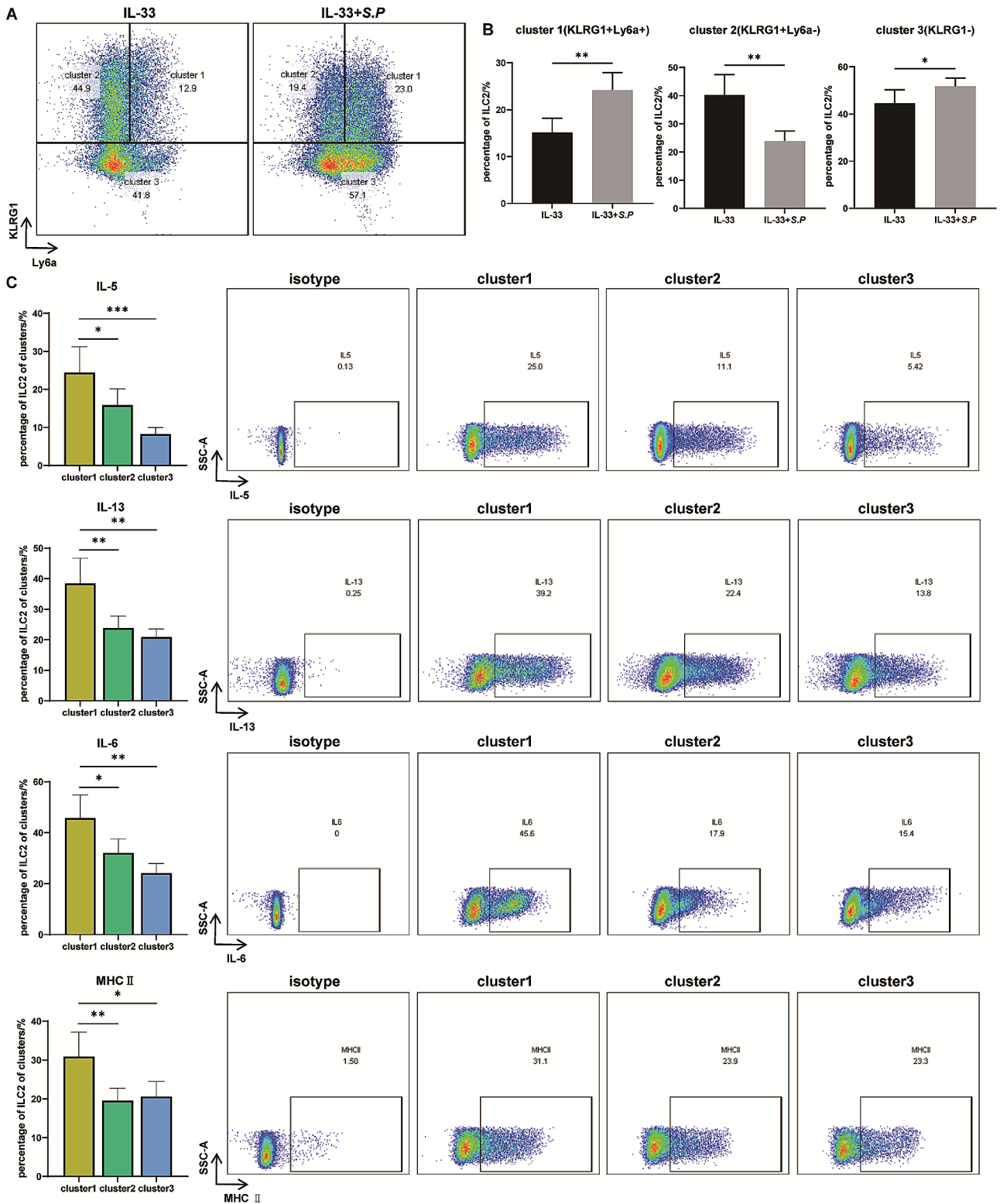


Fig. 6 Repeated challenge of the airways with IL-33 and SP increases the frequency of KLRG1⁺Ly6a⁺ILC2s. **A&B**, The frequencies and representative flow cytometric analysis of KLRG1⁺Ly6a⁺, KLRG1⁺Ly6a⁻ and KLRG1⁻ ILC2s. **C**, The frequencies and represen-

tative flow cytometric analysis of IL-5, IL-13, IL-6 and MHC class II molecules in each subset of ILC2s. Bars show the mean ± SD (n=4–5 in each group). * p < 0.05, ** p < 0.01

that this subset of ILC2s may play an important role in phenotypic overlap.

Conclusion

In conclusion, we present compelling evidence that repeated, commensal exposure of the airways to environmental pathogens such as *SP* predisposes, in susceptible individuals, to IL-33- and KLRG1⁺Ly6a⁺ILC2-dependent, T cell-independent mixed inflammation causing airways obstruction in animal models. These findings will hopefully be extendable in future to the clinic, shedding light on individual environmental stimuli which may be exacerbating symptoms in patients with obstructive airways disease and informing management and, hopefully, prophylaxis.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00011-024-01896-3>.

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Author contributions XD, YL and YX conducted all experiments and drafted the manuscript with input from all authors. YY, CL, YC and ZL analyzed flow cytometry results. CJC, DZ and LZ helped with design mouse experiments. SY and WW supervised the project.

Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate All animal experimental procedures were approved by the animal ethics Committee of the Capital Medical University, Beijing, China (AEEI-2022-207).

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

References

- Huang K, Yang T, Xu J, et al. Prevalence, risk factors, and management of asthma in China: a national cross-sectional study. *Lancet*. 2019;394:407–18.
- Papi A, Brightling C, Pedersen SE, et al. Asthma *Lancet*. 2018;391:783–800.
- Pelaia C, Crimi C, Vatrella A, et al. Molecular targets for biological therapies of severe asthma. *Front Immunol*. 2020;11:603312.
- Ricciardolo FLM, Guida G, Bertolini F, et al. Phenotype overlap in the natural history of asthma. *Eur Respir Rev*. 2023;32:220201.
- Hammad H, Lambrecht BN. The basic immunology of asthma. *Cell*. 2021;184(6):1469–85.
- Chung KF. Type-2-low severe asthma endotypes for new treatments: the new asthma frontier. *Curr Opin Allergy Clin Immunol*. 2023;23:199–204.
- Humbert M, Bousquet J, Bachert C, et al. IgE-mediated multimorbidities in allergic asthma and the potential for omalizumab therapy. *J Allergy Clin Immunol Pract*. 2019;7:1418–29.
- Cai T, Qiu J, Ji Y, et al. IL-17-producing ST2⁺ group 2 innate lymphoid cells play a pathogenic role in lung inflammation. *J Allergy Clin Immunol*. 2019;143:229–44.
- Seehus CR, Kadavallore A, de la Torre B, et al. Alternative activation generates IL-10 producing type 2 innate lymphoid cells. *Nat Commun*. 2017;8:1900.
- Sharma A, Laxman B, Naureckas ET, et al. Associations between fungal and bacterial microbiota of airways and asthma endotypes. *J Allergy Clin Immunol*. 2019;144:1214–27.
- Durack J, Lynch SV, Nariya S, et al. Features of the bronchial bacterial microbiome associated with atopy, asthma, and responsiveness to inhaled corticosteroid treatment. *J Allergy Clin Immunol*. 2017;140:63–75.
- Nikonova A, Shilovskiy I, Galitskaya M, et al. Respiratory syncytial virus upregulates IL-33 expression in mouse model of virus-induced inflammation exacerbation in OVA-sensitized mice and in asthmatic subjects. *Cytokine*. 2021;138:155349.
- Ravanetti L, Dijkhuis A, Dekker T, et al. IL-33 drives influenza-induced asthma exacerbations by halting innate and adaptive antiviral immunity. *J Allergy Clin Immunol*. 2019;143:1355–70.
- Jackson DJ, Makrinioti H, Rana BM, et al. IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo. *Am J Respir Crit Care Med*. 2014;190:1373–82.
- Stier MT, Goleniewska K, Cephus JY, et al. STAT1 represses cytokine-producing Group 2 and Group 3 innate lymphoid cells during viral infection. *J Immunol*. 2017;199:510–9.
- Hoffmann JP, Kolls JK, McCombs JE. Regulation and function of ILC3s in Pulmonary infections. *Front Immunol*. 2021;12:672523.
- Norlander AE, Peebles RS Jr. Innate type 2 responses to respiratory syncytial virus infection. *Viruses*. 2020;12:521.
- Teo SM, Tang H, Mok D, et al. Airway Microbiota dynamics uncover a critical window for interplay of pathogenic bacteria and allergy in childhood respiratory disease. *Cell Host Microbe*. 2018;24:341–52.
- Martinez FD. Childhood asthma inception and progression: role of microbial exposures, susceptibility to viruses and early allergic sensitization. *Immunol Allergy Clin North Am*. 2019;39:141–50.
- Li C, Du X, Huang Q, et al. Repeated exposure to inactivated *Streptococcus pneumoniae* induces asthma-like pathological changes in mice in the presence of IL-33. *Cell Immunol*. 2021;369:104438.
- Li Y, Chen S, Chi Y, et al. Kinetics of the accumulation of group 2 innate lymphoid cells in IL-33-induced and IL-25-induced murine models of asthma: a potential role for the chemokine CXCL16. *Cell Mol Immunol*. 2019;16:75–86.
- Helou DG, Shafiei-Jahani P, Lo R, et al. PD-1 pathway regulates ILC2 metabolism and PD-1 agonist treatment ameliorates airway hyperreactivity. *Nat Commun*. 2020;11:3998.
- Wu YH, Lai AC, Chi PY, et al. Pulmonary IL-33 orchestrates innate immune cells to mediate respiratory syncytial virus-evoked airway hyperreactivity and eosinophilia. *Allergy*. 2020;75:818–30.
- Helou DG, Shafiei-Jahani P, Hurrell BP, et al. LAIR-1 acts as an immune checkpoint on activated ILC2s and regulates the induction of airway hyperreactivity. *J Allergy Clin Immunol*. 2022;149:223–36.

25. Nagashima H, Mahlaköiv T, Shih HY, et al. Neuropeptide CGRP limits group 2 innate lymphoid cell responses and constrains type 2 inflammation. *Immunity*. 2019;51:682–95.
26. Flamar AL, Klose C, Moeller JB, et al. Interleukin-33 induces the enzyme tryptophan hydroxylase 1 to promote inflammatory group 2 innate lymphoid cell-mediated immunity. *Immunity*. 2020;52:606–19.
27. Wallrapp A, Riesenfeld SJ, Burkett PR, et al. The neuropeptide NMU amplifies ILC2-driven allergic lung inflammation. *Nature*. 2017;549:351–6.
28. Wu X, Kasmani MY, Zheng S, et al. BATF promotes group 2 innate lymphoid cell-mediated lung tissue protection during acute respiratory virus infection. *Sci Immunol*. 2022;7:c9934.
29. Xu H, Ding J, Porter C, et al. Transcriptional atlas of intestinal immune cells reveals that neuropeptide α -CGRP modulates group 2 innate lymphoid cell responses. *Immunity*. 2019;51:696–708.
30. Nakatani-Kusakabe M, Yasuda K, Tomura M, et al. Monitoring cellular movement with photo convertible fluorescent protein and single-cell RNA sequencing reveals cutaneous group 2 innate lymphoid cell subtypes, circulating ILC2 and skin-resident ILC2. *JID Innov*. 2021;1:100035.
31. Hardman CS, Chen YL, Salimi M, et al. IL-6 effector function of group 2 innate lymphoid cells (ILC2) is NOD2 dependent. *Sci Immunol*. 2021;6:eabe5084.
32. Yang Y, Li Y, Xu Y, et al. Group 2 innate lymphoid cells can engulf and destroy bacteria. *Cell Mol Immunol*. 2021;18:2569–71.

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