PULMONARY INNATE LYMPHOID CELLS - GATEKEEPERS OF RESPIRATORY HEALTH

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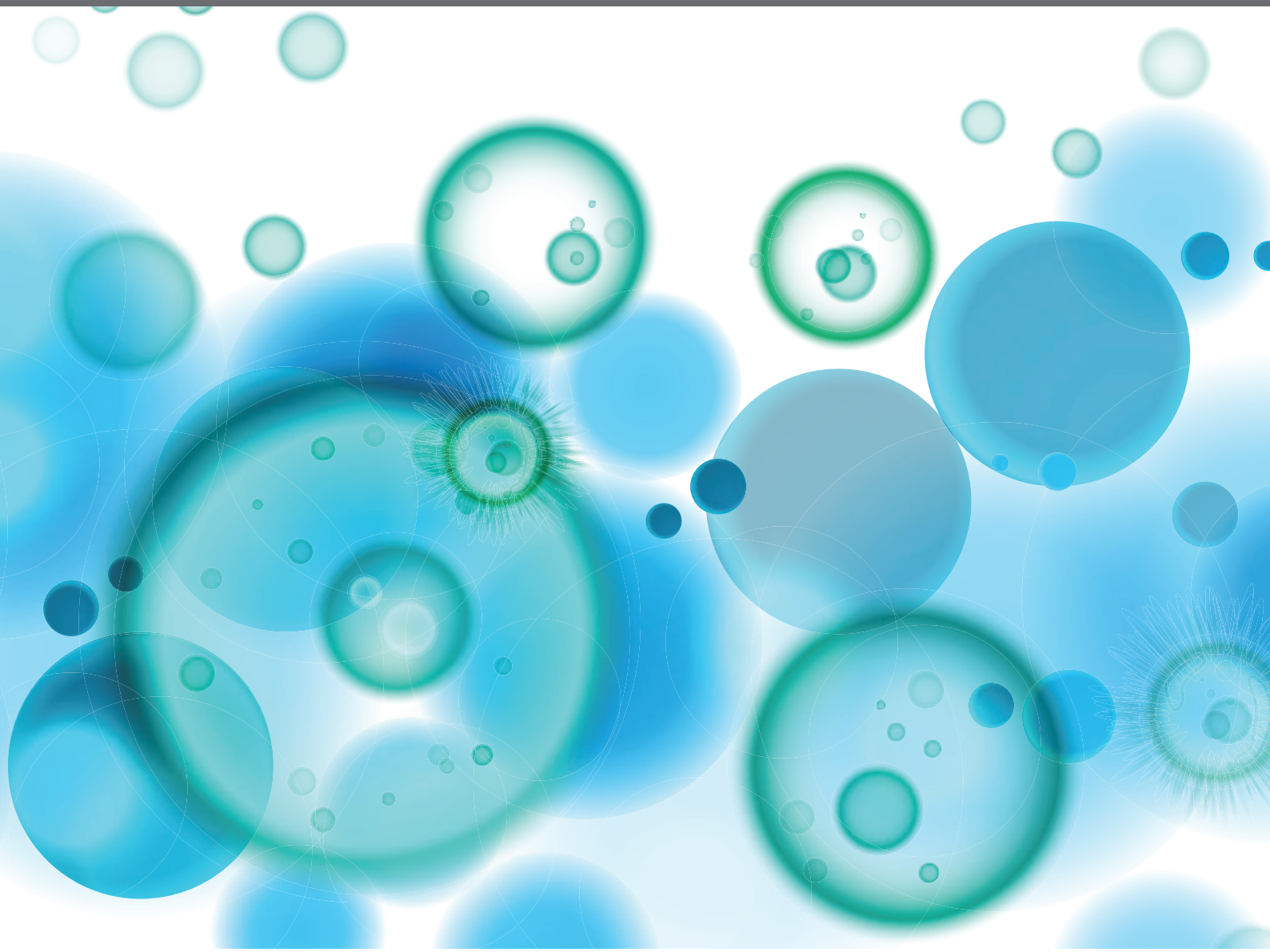

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PULMONARY INNATE LYMPHOID CELLS - GATEKEEPERS OF RESPIRATORY HEALTH

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Editorial: Pulmonary Innate Lymphoid Cells - Gatekeepers of Respiratory Health

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Keywords: innate lymphoid cells, ILC, lung, airway, respiratory, pulmonary, infection, disease

Editorial on the Research Topic

Pulmonary Innate Lymphoid Cells - Gatekeepers of Respiratory Health

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The Research Topic "*Pulmonary Innate Lymphoid – Gatekeepers of Respiratory Health*", provides a series of up-to-date reviews including a fresh look at innate lymphoid cell (ILC) development (Shin et al.); the fate of activated ILC2 (Wirtz et al.); the role of ILC2 and ILC3 in pulmonary infections (Fonseca et al. and Hoffmann et al.) and an update on ILCs in chronic respiratory diseases (Hsu et al. and Rao et al.). These reviews provide the ideal framework to enable this editorial to focus on the six original research articles in the collection that bring new knowledge to the field of pulmonary ILC biology.

c-Rel IS REQUIRED FOR ILC2 ACTIVATION AND PULMONARY INFLAMMATION

ILC2 activation is governed by a network of transcriptional regulators including nuclear factor (NF)-kB family transcription factors. While it is known that activating interleukin (IL)-33 receptor signaling results in downstream NF-kB activation, the underlying molecular mechanisms remain elusive (Mindt et al.).

In this Research Topic, back-to-back papers demonstrate that the NF-kB subunit c-Rel is required to mount effective pulmonary type 2 immune responses (Mindt et al. and Zaini et al.). IL-33-mediated activation of ILC2 *in vitro* as well as *in vivo* induced c-Rel mRNA expression and increased c-Rel protein levels (Mindt et al.). Furthermore, IL-33-mediated activation of pulmonary ILC2 caused nuclear translocation of c-Rel (Mindt et al.). Although c-Rel was found to be a critical mediator of pulmonary type 2 immune responses, ILC2-intrinsic deficiency of c-Rel did not influence the developmental capacity of ILC2 nor affected homeostatic numbers of lung-resident ILC2 at steady state (Mindt et al. and Zaini et al.). Moreover, ILC2-intrinsic deficiency of c-Rel alters

the capacity of ILC2 to upregulate the expression of the key stimulatory receptors ICOSL and OX40L, and the expression of the signature type 2 cytokines IL-5, IL-9, IL-13, and granulocyte-macrophage colony-stimulating factor (Mindt et al.). c-Rel-deficient mice displayed significantly reduced lung inflammation in response to pulmonary challenge with either papain or recombinant IL-33 (Mindt et al. and Zaini et al.).

Collectively, c-Rel promotes ILC2-driven allergic airway inflammation and suggest that c-Rel may contribute to the pathophysiology of ILC2-mediated allergic airway disease. c-Rel thereby represents a promising future target for the treatment of allergic asthma. (Mindt et al. and Zaini et al.). However, the role of c-Rel in allergen-induced asthma models e.g., following instillation of house dust mite and the impact on functional parameters such as airway hyperresponsiveness and mucus secreting cell metaplasia, remain to be elucidated.

PUTTING THE STING INTO ILC2-ILC1 SHIFTING WITH CYCLIC-DI-GMP

Type 2 inflammation underpins several endotypes of asthma. In asthma, recurrent viral infections, bacterial colonization, and host cell death drive the accumulation of intracellular cyclic-di-nucleotides including cyclic-di-GMP (CDG). However, the impact of CDG on allergic airway inflammation is unknown. To explore this, Cavagnero et al. intranasally administered CDG, which induced early airway type 1 interferon (IFN) production and suppressed IL-7R$^+$ ST2$^+$ ILC2 and type 2 lung inflammation, following pulmonary challenge with either Alternaria or recombinant IL-33 (Cavagnero et al.). An IL-7R$^-$ST2$^-$CD90.2$^+$ lung ILC subset, that had a transcriptional signature that was consistent with ILC1, were expanded by administration of CDG when it was delivered in combination with either the fungal allergen, Alternaria, or recombinant IL-33. CDG-mediated suppression of pulmonary type 2 inflammation occurred independently of IL-18R, IL-12, and STAT6, but required the stimulator of interferon genes (STING) and type 1 IFN signaling (Cavagnero et al.).

Collectively, this study demonstrates that CDG drives STING-dependent IFN production, ILC1 activation and accumulation, as well as ILC2 suppression and abrogation of innate type 2 innate airway inflammation. This study adds to our understanding of how the pathogenesis of allergic airway disease may be impacted by lung insults due to cellular stress, bacterial and viral infections (Cavagnero et al.).

THE α7nAChR AGONIST PNU-282987 INHIBITS ILC2 FUNCTION AND ALLERGIC AIRWAY INFLAMMATION

The cholinergic anti-inflammatory pathway controls inflammation through the release of the neurotransmitter acetylcholine. Acetylcholine can also stimulate the a7 nicotinic acetylcholine receptor (a7nAChR) that is highly expressed on ILC2. The a7nAChR agonist, GTS-21, is known to attenuate ILC2-dependent airway hyperreactivity in mice. In this Research Topic, Yuan et al. explore the ability of an alternate a7nAChR agonist, PNU-282987, to suppress ILC2-mediated allergic airway inflammation (Yuan et al.). Both PNU-282987 and GTS-21 significantly reduced airway mucus secreting cell hyperplasia, eosinophil infiltration into the airways, and ILC2 numbers in bronchoalveolar lavage fluid, following respiratory challenge with recombinant IL-33 or Alternaria (Yuan et al.). In summary, PNU-282987 inhibited ILC2-associated airway inflammation, comparable to that of GTS-21.

RHINOVIRUS C INFECTION INDUCES ILC2 EXPANSION AND AIRWAY INFLAMMATION

Rhinovirus C (RV-C) infection is associated with severe asthma exacerbations. Rajput et al. hypothesized that RV-C infection, in contrast to RV-A, would preferentially stimulate type 2 inflammation, leading to exacerbated eosinophilic airway inflammation. To test this hypothesis the team developed a novel mouse model of RVC infection. Mice inoculated with RV-C15 showed lung viral titers of 1 x 10^5 TCID50 units 24 h after infection, with levels declining thereafter (Rajput et al.). IFN-α, β, γ and λ2 mRNA expression peaked 24-72 hours post-infection. Compared to RV-A1B, mice infected with RV-C15 demonstrated higher bronchoalveolar eosinophils, mRNA expression of IL-5, IL-13, IL-25, Muc5ac and Gob5, protein production of IL-5, IL-13, IL-25, IL-33 and TSLP, and expansion of ILC2 (Rajput et al.). In contrast to ILC2-sufficient *Rora*$^{fl/fl}$ littermates, RV-C-infected ILC2-deficient *Rora*$^{fl/fl}$ *Il7r*^{cre} mice failed to show eosinophilic inflammation or mRNA expression of *Il13*, *muc5ac* and *muc5b*. It was concluded that, compared to RV-A1B, RV-C15 infection induces ILC2-dependent type 2 airway inflammation, providing new insights into the mechanism of RV-C-induced asthma exacerbations (Rajput et al.).

CHILDREN WITH ASTHMA HAVE INCREASED CIRCULATING ILC2 AND NCR$^-$ ILC3

Asthma is the most frequent cause of hospitalization among children; however, little is known regarding the effects of asthma on immune responses in children. Hosseini et al. aimed to evaluate peripheral blood mononuclear cell composition in children with and without asthma. They found that the frequency of circulating ILC2 and NCR$^-$ ILC3 were significantly higher in asthmatics compared to non-asthmatic controls. There was no change in the frequency of other leukocyte subsets commonly associated with asthma such as eosinophils and CD4$^+$ T helper cells.

CONCLUSION

The collection of original articles and reviews presented in this Research Topic highlight the ability of pulmonary ILC to modulate the severity of allergic airway inflammation,

respiratory infections, and respiratory diseases. Specific advances were made in describing the role for the NF-kB subunit c-Rel in activation and effector function of pulmonary ILC2 in mice; showing that CDG induces a ILC2-ILC1 shift that may reduce type 2 inflammation and promote antimicrobial ILC1 responses *in vivo*; demonstrating that the a7nAChR agonist PNU-282987 attenuates ILC2-associated airway inflammation; evidence that respiratory RV-C infection induces ILC2 and eosinophilic airway inflammation in mice; and that children with asthma have increased circulating ILC2 and NCR^- ILC3.

AUTHOR CONTRIBUTIONS

MS led the research collection and wrote the editorial. HD, NL, and LL edited manuscripts for the Research Topic and the editorial. All authors contributed to the article and approved the submitted version.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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A Selective α7 Nicotinic Acetylcholine Receptor Agonist, PNU-282987, Attenuates ILC2s Activation and *Alternaria*-Induced Airway Inflammation

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Background: The anti-inflammatory effect of an α7nAChR agonist, PNU-282987, has previously been explored in the context of inflammatory disease. However, the effects of PNU-282987 on type 2 innate lymphoid cells (ILC2s)-mediated allergic airway inflammation has not yet been established.

Aims: To determine the effects of PNU-282987 on the function of ILC2s in the context of IL-33– or *Alternaria Alternata* (AA)– induced airway inflammation.

Methods: PNU-282987 was administered to mice that received recombinant IL-33 or AA intranasal challenges. Lung histological analysis and flow cytometry were performed to determine airway inflammation and the infiltration and activation of ILC2s. The previously published α7nAChR agonist GTS-21 was employed as a comparable reagent. ILC2s were isolated from murine lung tissue and cultured *in vitro* in the presence of IL-33, IL-2, and IL-7 with/without either PNU-282987 or GTS-21. The expression of the transcription factors GATA3, IKK, and NF-κB were also determined.

Results: PNU-282987 and GTS-21 significantly reduced goblet cell hyperplasia in the airway, eosinophil infiltration, and ILC2s numbers in BALF, following IL-33 or AA challenge. *In vitro* IL-33 stimulation of isolated lung ILC2s showed a reduction of GATA3 and Ki67 in response to PNU-282987 or GTS-21 treatments. There was a significant reduction in IKK and NF-κB phosphorylation in the PNU-282987–treated group when compared to the GTS-21–treated ILC2s.

Conclusion: PNU-282987 inhibits ILC2-associated airway inflammation, where its effects were comparable to that of GTS-21.

Keywords: type 2 innate lymphoid cells, airway, inflammation, nicotinic acetylcholine receptor, Alternaria

INTRODUCTION

Asthma is an immune disorder of the lungs associated with airway inflammation, mucus secretion, and airway hyperresponsiveness (AHR), and is associated with the type 2 cytokines, interleukin (IL)-4, IL-5, and IL-13 (1, 2). While $CD4^+$ T helper 2 (Th2) cells play an important role in the development of allergic cascades, innate immune cells including macrophages, eosinophils, and type 2 innate lymphoid cells (ILC2s) critically contribute to the pathogenesis of the disease (3). Classically, Th2 cells were regarded as the central cell involved in asthma, as they were believed to be the only cellular source of type 2 cytokines. However, the discovery of the ILC2s highlighted a new class of cells that can induce inflammatory cascades independent of T and B cells in response to epithelial alarmin factors, such as IL-25 and IL-33 (4, 5). Upon exposure to these factors, ILC2s secrete Th2 cytokines including IL-4, IL-5, and IL-13 through GATA3 transcription (6–9). Indeed, $Rag2^{-/-}$ mice that lack T cells and B cells presented with pathological features of allergic airway disease due to the presence of ILC2s (10). Furthermore, mouse models of asthma have revealed that the genetic depletion of IL-33 results in a marked reduction of eosinophilic inflammation and mucus secretion following aerosol allergen exposure, indicating the key role of IL-33 in the pathogenesis of asthma (11). IL-33 initiates inflammation by activating ST2/IL-1RAcP, which in turn recruits IRAKs and TRAF6 for the activation of IKK, NF-κB, and MAPK signaling pathways (10). In $Rag2^{-/-}Il2rg^{-/-}$ mice (lacking T cells, B cells, and ILCs), the levels of eosinophilic inflammation and the secretion of mucus decreased significantly, highlighting the importance of the IL-33/ILC2s axis in the development of asthma (12).

Importantly, Tracey et al. identified a cholinergic anti-inflammatory pathway (CAP) that is mediated by the vagus nerve, a major neurological regulator of organ function throughout the body (12, 13). CAP controls inflammation through the release of the neurotransmitter acetylcholine (12, 13). Acetylcholine can also stimulate the α7 nicotinic acetylcholine receptor (α7nAChR) that is expressed on macrophages, T cells, and B cells (14–16). Animal models of diabetes, sepsis, cystic fibrosis, ulcerative colitis, and arthritis in α7nAChR-deficient mice have revealed that this receptor downregulates the function of macrophages and lymphocytes, and attenuates the development of pathology (17–21). Interestingly, recent observation has found that ILC2s expression of α7nAChR is significantly higher than that on macrophages and other lymphocytes (22).

GTS-21 is recognized as an α7nAChR agonist that also binds to α4β2nAChR with high affinity, where the binding power of the latter receptor is 100 times higher compared to the former. α4β2nAChR is predominantly expressed in the central nervous system and regulates psychological activities including mood, memory, and learning (23). Previously, Lauriane Galle-Treger et al. demonstrated that GTS-21 has a significant inhibitory effect on ILC2s-mediated airway inflammation, suggesting GST-21 could be exploited therapeutically to suppress ILC2s-associated inflammatory disorders (22). However, its action on α4β2nAChR may yield unwanted side-effect as the aberrant activation of this receptor leads to anxiety, downregulation of D3 dopamine receptor and interference of growth hormone release (24–26). Interestingly, the compound PNU-282987 has a higher affinity to α7nAChR with little or no effect on the α1, β1, γδ, and α3β4 variants, and monoamine, muscarinic, glutamate, and GABA receptor (27). Moreover, PNU-282987 has been demonstrated to reduce acute lung injury by altering macrophage proliferation in mice (28). Therefore, we plan to explore the effects of PNU-282987 on ILC2s and to compare the difference between PNU-282987 and GTS-21 on ILC2s using both *in vivo* models and *in vitro* stimulation of isolated ILC2s from mouse lung.

MATERIALS AND METHODS

Mice

6 to 8 week old C57BL/6J female mice were purchased from Beijing Vitonlihua Company [license No. SCXK (Beijing) 2012/0001] and were raised at the SPF animal housing facility, Zhengzhou University. The experiment was approved and permitted by the Animal Ethics Committee of Zhengzhou University (Approval Number: ZZURIB20180120).

Murine Model

Mice were intranasally (i.n.) administered with recombinant mouse IL-33 (0.5 μg/dose, R&D, California, USA) in mice intraperitoneally (i.p.) administered with PNU-282987 (20 mg/kg, Abcam, California, USA) or GTS-21 (20 mg/kg, Abcam, California, USA) over three consecutive days (22). For *Alternaria* experiments, mice were i.n. administered with AA (100 μg/dose, Greer Labs, Lenoir, North Carolina, USA) in the presence or absence of PNU-282987 or GTS-21 on four consecutive days. Mice were sacrificed on the second day after the last challenge.

Collection of Bronchoalveolar Lavage Fluid (BALF) Cells and Lung Histology

Mouse lung was lavaged three times with 0.8 ml of ice-cold PBS for the collection of BALF cells. Supernatants were collected for ELISA. Red blood cells were then removed by using hypotonic red blood cell lysis buffer and BALF was then centrifuged to collect cellular infiltrate. Total cell numbers were quantified using a hemocytometer. In some experiments, lung tissues were digested with Liberase™ and DNase (Roche, Basel, Switzerland) for single-cell suspensions. BALF and lung cells were analyzed *via* flow cytometry.

In additional experiments, lung tissues were stained with hematoxylin and eosin (for histopathology) or periodic acid-Schiff (for mucus-secreting cells). Sections were then stained with chromotrope-hematoxylin or periodic acid-Schiff (PAS). Scorings for histopathology (inflammatory infiltrates) and PAS (mucus-producing cells) were performed according to a set of morphological criteria as previously described (29).

Flow Cytometry

Purified rat anti-mouse CD16/CD32 (553141), PE-conjugated hamster anti-mouse KLRG1 (561621), PerCP-Cy™5.5-conjugated hamster anti-mouse KLRG1 (563595), FITC-conjugated rat anti-mouse Ly-6A/E (557405), PE-conjugated rat anti-mouse Siglec-F (552126) and PE-conjugated rat anti-mouse/

anti-human IL-5 (562019) were purchased from BD Biosciences. PE-conjugated anti-IL-13 monoclonal antibody (eBio 13A) was purchased from Invitrogen (San Diego, California, USA). PE-conjugated anti-mouse phospho-IKKα/β (Ser 176/180) and Alexa Fluor 488-conjugated anti-mouse phospho-NF-κB p65 (Ser 536) (93H1) rabbit mAbs were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). Lung ILC2s was defined as lack of classical lineage markers (CD5, CD45R, Anti-Gr-1, CD11b, 7-4, and Ter119), KLRG1$^{+,}$ and Sca-1^{+} populations. Lineage marker negative cells were enriched by both density gradient centrifugation and magnetic beads isolation and purified by flow cytometry for the collection of ILC2s.

Intracellular staining was performed with BD Fixation and Permeabilization Solution (BD Biosciences, San Jose, California, USA) according to the manufacturer's instructions. Cell stimulants and protein transport inhibitors were added before staining. The analysis of IKK-P and NF-κB p65 was carried out per the manufacturer's instructions. FACSCanto II Flow (BD Biosciences, San Jose, California, USA) and MoFlo XDP cell sorter (Beckman coulter, Brea, California, United States) were employed for flow cytometry and cell sorting. Data were analyzed with software FlowJo version 10.0 (Franklin Lakes, New Jersey, United States). The gating strategy was shown in **Figures S1** and **S2**.

In Vitro Stimulation of ILC2s

ILC2s were isolated and cultured in 96-well plates with a volume of 100 μl per well in RPMI-1640 medium (BD Biosciences, San Jose, CA). The cells were plated at 1.5×10^4 cells per well and stimulated with IL-33 (50 ng/ml, BD Biosciences, San Jose, California, USA), IL-2 (20 ng/mL, BD Biosciences, San Jose, California, USA), and IL-7 (20 ng/mL, BD Biosciences, San Jose, California, USA) in the presence or absence of PNU-282987 (20 μM) or GTS-21 (20 μM) for 24 or 72 h.

Proliferation Assay Method

0.4% trypan blue staining solution was absorbed with a dropper and added to the cell suspension at 1:1. The staining solution was dropped gently from the edge of the counting board. Cell count was performed 1 min after staining under light microscopy. The concentration of cell suspension and the ratio of survival to dead cells were then calculated.

Quantitative PCR

Total RNA was extracted and the levels of IL-5, IL-13, GATA3, and IL-33 transcripts were quantitated at the mRNA level by quantitative real-time RT-PCR with Applied Biosystems QuantStudio™ 5 system (Applied Biosystems, Carlsbad, California, USA) following the manufacturer's protocol. The primers were as follows: IL-5 (forward, 5′-TGAGACGATGAGGCTTCCTG-3′ and reverse, 5′-CCACACTTCTCTTTTTGGCGG-3′) (30), IL-13 (forward, 5′-CCCTCAGCCATGAAATAACT-3′ and reverse, 5′-GCGTAACAGGCCATTCTTCC-3′) (30), GATA3 (forward, 5′-CGAGATGGTACCGGGCACTA-3′ and reverse, 5′-GACAGTTCGCGCAGGATGT-3′) (31), IL-33 (forward, 5′-ACTATGAGTCTCCCTGTCCTG-3′ and reverse, 5′-ACGTCACCCCTTTGAAGC-3′) (32).

ELISA

IL-5 and IL-13 from BALF and *in vitro* culture supernatants were detected according to the manufacturer's instructions (MultiSciences Biotech, Hangzhou, Zhejiang, China). Data detection and analysis were performed on a microplate reader (Molecular Devices, San Jose, California, United States).

Statistical Analysis

SPSS21.0 software was used for statistical analysis. One-way ANOVA was used to compare multiple samples. The results were expressed as the mean ± standard error of the mean (SEM). When $P < 0.05$, the difference between samples is considered statistically significant.

RESULTS

PNU-282987 and GTS-21 Inhibit IL-33–Induced Airway Inflammation

Both IL-33 and ILC2s are critically involved in the early stages of asthma exacerbation (33). Firstly, by using recombinant IL-33 to induce lung inflammation *in vivo*, we explored the effects of PNU-282987 and GTS-21 on IL-33–mediated airway inflammation. PAS and HE staining of lung tissue revealed a significant increase of goblet cells in the airway epithelium of the IL-33–exposed group when compared to the PBS group (**Figure 1A**). Treatments with PNU-282987 and GTS-21 significantly attenuated goblet cell hyperplasia and mucus production in airways, and reduced the histopathological scores following IL-33 i.n. inoculation (**Figure 1A**). We also examined the infiltration of eosinophils and ILC2s in BALF and showed that both compounds drastically decreased the migration of both cells (**Figure 1B**). Furthermore, both agonists showed similar responses in the suppression of IL-33–induced airway inflammation and mucus hypersecretion. The populations of CD4^{+} T cells and CD19^{+} B cells in the three groups were unchanged between the IL-33–treated groups (**Figure 1C**), suggesting that the inhibitory action of PNU-282987 and GTS-21 is through their action on ILC2s.

PNU-282987 and GTS-21 Attenuate the Production of IL-5 and IL-13 Secreted by ILC2s *In Vivo*

Next, we determined whether PNU-282987 and GTS-21 inhibit the secretion of IL-5 and IL-13 by ILC2s. RNA extracted from mouse lung tissue was used to determine the transcription levels of IL-5 and IL-13, and the protein levels of IL-5 and IL-13 in BALF were also examined. Levels of IL-5 and IL-13 transcripts in the IL-33 treatment group were significantly higher than in the control PBS group. Treatments with PNU-282987 or GTS-21 significantly decreased the levels of IL-5 and IL-13 transcripts in lung and protein in BALF following IL-33 treatment (**Figures 2A, B**). Although the levels of IL-13 mRNA in the IL-33/GTS-21 group were lower than that in IL-33/PNU-282987 group, there was no difference in the BALF protein level of IL-13 between the two groups (**Figure 2B**). Furthermore, there was no significant difference between PNU-282987 and GTS-21 in inhibition of IL-5^{+}ILC2s and IL-13^{+}ILC2s (**Figure 2C**).

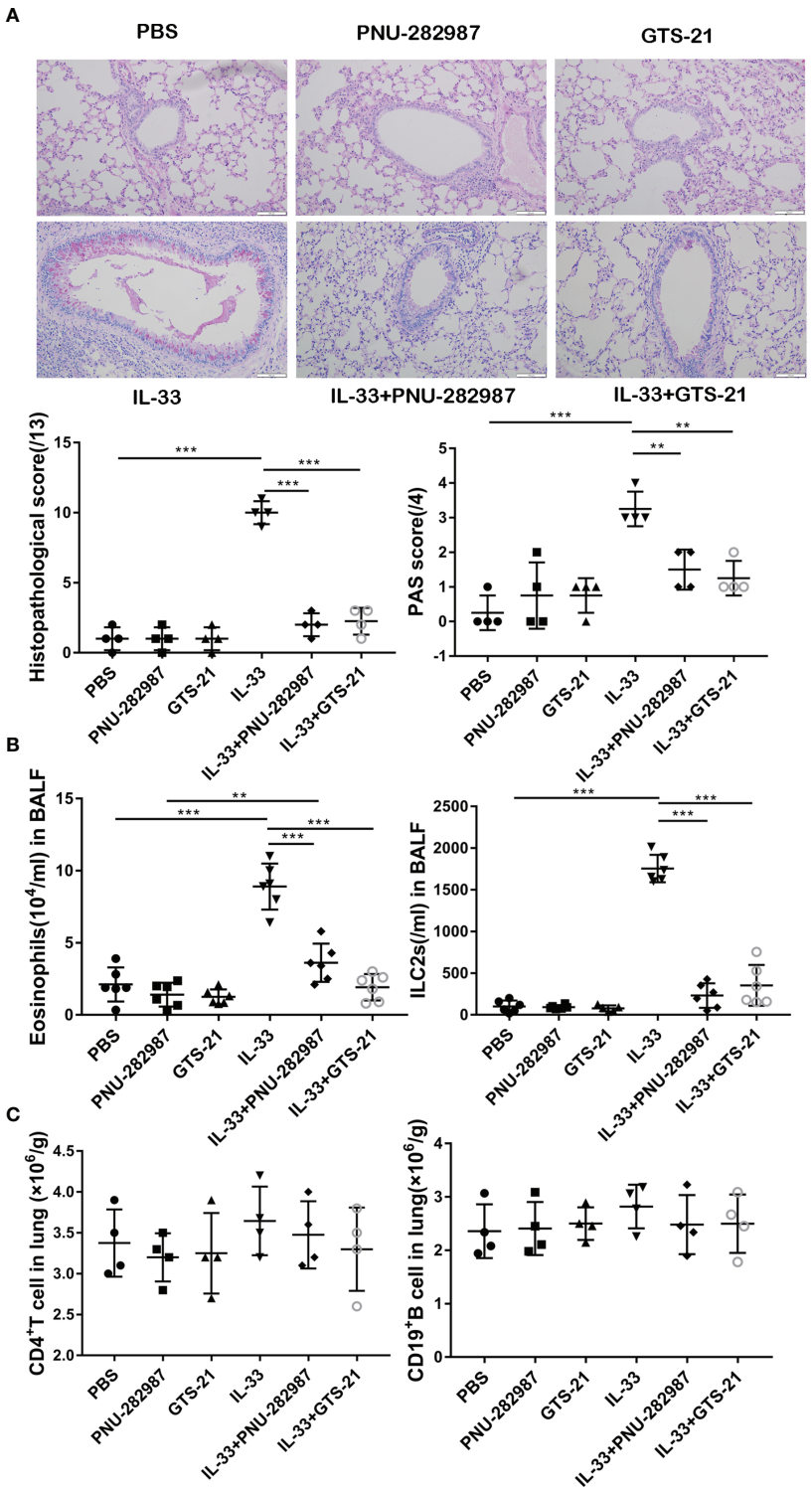

FIGURE 1 | Compared with GTS-21, PNU-282987 has the same inhibitory effect on ILC2-mediated airway inflammation. C57BL/6J mice intranasally challenged with recombinant mouse IL-33 or PBS and also received PNU-282987/GTS-21 or PBS on days 1 to 3. Assessment of lung tissue and analysis of bronchoalveolar lavage fluid (BALF) **(A)** Periodic acid-Schiff reagent lung section (×200, Scale bars at 100 μm). Scorings for histopathology and PAS were assessed. **(B)** Total number of eosinophils and ILC2s in BALF. **(C)** Total number of $CD4^+$ T cells and $CD19^+$ B cells determined by flow cytometry in lung. Data are representative of at least four independent experiments and presented as means ± s.e.m (n = 4 – 6; $^{**}P < 0.01$; $^{***}P < 0.001$).

No IL-5– or IL-13–secreting ILC2s were identified in PBS, PNU-282987, or GTS-21 treatment groups.

PNU-282987 and GTS-21 Abolish AA-Mediated Airway Inflammation

We then determined the anti-inflammatory effects of PNU-282987 on ILC2-regulated airway inflammation and compared its function to that of GTS-21 with AA- challenged animals (22). Our experiments confirmed that the level of IL-33 in the AA treatment group was significantly higher than that in the PBS group (**Figure 3A**). After the administration with PNU-282987 and GTS-21, the levels of RNA in lung and protein in BALF of IL-33 were significantly decreased when compared to the AA alone group. Furthermore, treatments with PNU-282987 or GTS-21 attenuated the AA-induced airway inflammation when we examined the histopathological changes of lung tissue (**Figure 3B**). The levels of eosinophil and ILC2s in the BALF of the AA/PNU-282987 and AA/GTS-21 groups were significantly lower than those of AA only group, and there was no difference between the two former groups (**Figure 3C**). There was no difference in the levels of $CD4^+$ T cells and $CD19^+$ B cells in lung between AA, AA/PNU-282987, and AA/GTS-21 groups (**Figure 3D**). These results suggest that PNU-282987 also inhibits the ILC2-mediated airway inflammation caused by the AA.

PNU-282987 and GTS-21 Inhibit the Production of IL-5 and IL-13 in the Lung of AA-Challenged Mice

Similar to what occurred in the IL-33 model, PNU-282987 and GTS-21 inhibited the production of IL-5 and IL-13 by ILC2s isolated from mice that were exposed to AA, both at transcription and protein levels (**Figures 4A, B**). Furthermore, intracellular staining revealed that the levels of IL-5– and IL-13–producing ILC2s in the AA/PNU-282987 and AA/GTS-21 groups were profoundly reduced compared to the AA alone group (**Figure 4C**). There was no difference between PNU-282987 and GTS-21 in the inhibition of the IL-5– and IL-13–producing function of ILC2s.

PNU-282987 and GTS-21 Inhibited the Proliferation and Functional Activation of ILC2s *In Vitro*

ILC2s from mouse lung tissue were purified by flow cytometry and cultured with or without PNU-282987 or GTS-21 in the presence of IL-2+IL-7 and/or IL-33. After 72 h of culture, the cell numbers were determined for each group, where it was found that that PNU-282987 or GTS-21 administration was associated with a significantly decreased proliferation of ILC2s despite IL-33 exposure (**Figure 5A**). Per the above results, Ki67, a nuclear non-histone that represents cell proliferation, was significantly decreased in the PNU-282987 and GTS-21 groups following IL-33 exposure (**Figure 5B**). The transcription factor GATA3, which is essential for the growth and development of ILC2s, was also inhibited in the PNU-282987 and GTS-21 groups (**Figure 5C**) (34). Data plots for Ki67 and GATA3 of ILC2s were shown in **Figures S3** and **S4**. PNU-282987 and GTS-21 had comparable inhibitory effects on both Ki67 and GATA3.

IKK and NF-κB p65 levels were evaluated to determine the effects of PNU-282987 and GTS-21 on the proliferation and activation of ILC2s (35). Our results showed that both PNU-282987 and GTS-21 could inhibit the phosphorylation of IKK and NF-κB p65, however the inhibitory effect of PNU-282987 on the phosphorylation of IKK and NF-κB p65 was significantly higher than that of GTS-21 24 h after treatments (**Figures 5D, E**). Seventy-two hours after stimulation, there was no detection of the phosphorylation of these two transcriptional regulators among all groups (data not shown). NF-κB pathway critically regulates the expression of IL-6, BCL-6, c-FLIP, and ST2 that play important role in cell proliferation and inflammation (36). Moreover, IL-33 significantly increased ST2 and c-FLIP expression, which were suppressed by the addition of PNU-282987 and GTS-21 (**Figure S5**). Interestingly, both antagonists had no effect on IL-33–induced higher expression of IL-6 and BCL-6 (**Figure S5**). Cell supernatants were collected, and the levels of IL-5 and IL-13 were assayed by ELISA after 72 h of culture. The results showed that PNU-282987 and GTS-21 had the same effect on inhibiting the secretion of IL-5 and IL-13 by ILC2s (**Figures 5F, G**).

DISCUSSION

Fungal allergens are a common household cause of asthma, where fungal proteases are potent initiators of allergic inflammatory cascades (37). Such inflammatory cascades induce the release of alarmin factors (e.g. IL-33), which critically drive the activation of ILC2s and increase secretions of type 2 cytokines. The recent discovery of vagus nerve neuro-regulation of ILC2s through CAP and the α7nAChR has highlighted a novel pathway that could be exploited to attenuate asthma, which has previously been demonstrated with the α7nAChR agonist GTS-21 (22). Here, we demonstrated that the agonist PNU-282987 not only exhibits the same inhibitory effects of GTS-21 but also acts as a potent suppresser of IKK and NF-κB activity in ILC2 cells.

We have firstly employed both recombinant IL-33 and AA to challenge mice for the induction of airway inflammation. Our results showed enhanced infiltration of inflammatory cells and increased levels of proinflammatory cytokines including IL-1β, IL-6, and TNF-α (**Figures S6** and **S7**). Furthermore, we have demonstrated that PNU-282987 reduces eosinophil and ILC2 numbers in BALF and lung tissue and decreases goblet cell hyperplasia in the airway. Although $CD4^+$ Th2 cells are also the major cellular source of IL-5 and IL-13, we did not observe any changes of their infiltration. Our IL-33 and AA models are both short model, therefore it is unlikely that $CD4^+$ antigen specific Th2 cells can differentiate within such narrow time window, highlighting the importance of ILC2s in the disease. In both IL-33- and AA-exposed mice, PNU-282987 and GTS-21 not only abated the proliferation of ILC2s but also inhibited the ability of ILC2s to secrete IL-5 and IL-13 *in vivo*. Furthermore, the inhibition effects of PNU-282987 and GTS-21 on IL-33 protein in BALF are potentially linked to the reduced level of IL-33 protein release from the cells. These data suggest that both PNU-282987 and GTS-21 reduce type 2 cytokines by acting on ILC2s, and that both agonists may directly regulate upstream effect by regulating pre-synthesized IL-33 protein

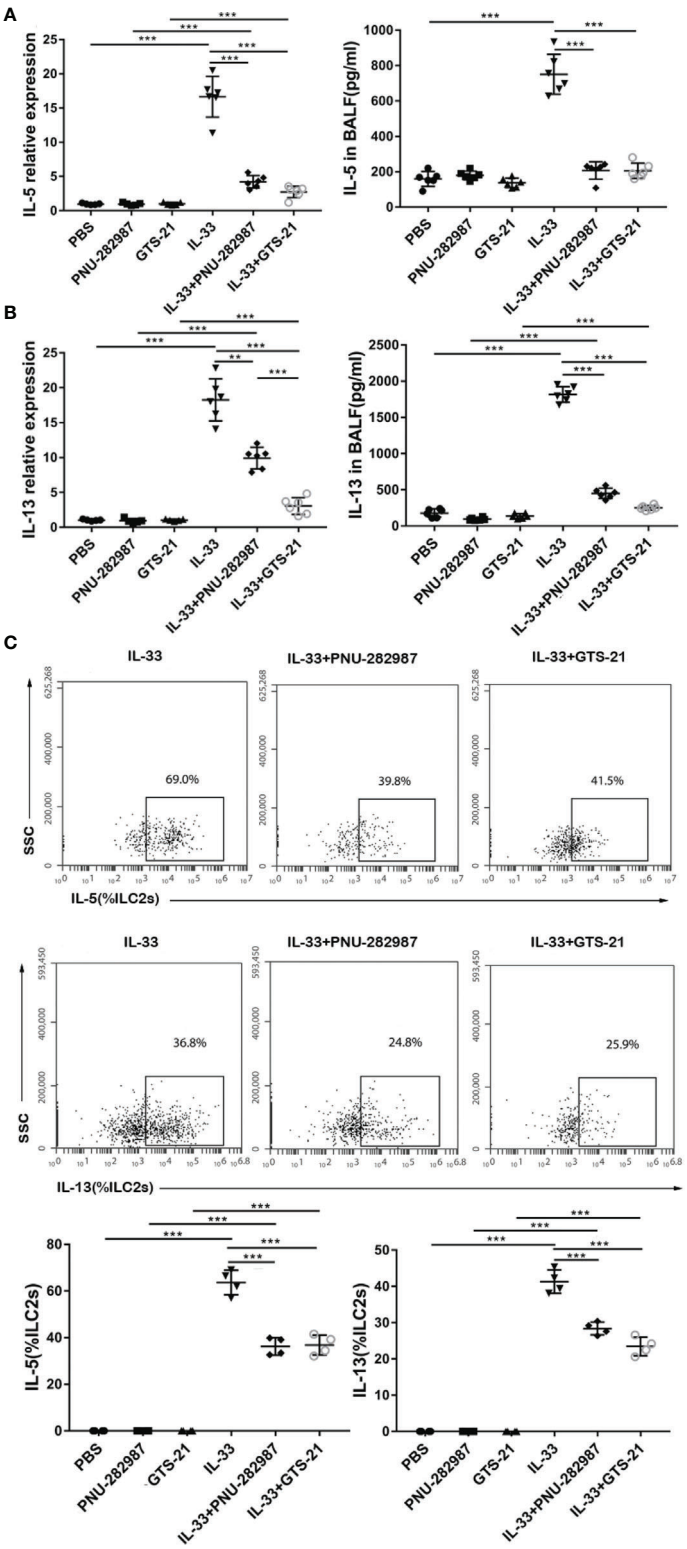

FIGURE 2 | PNU-282987 and GTS-21 attenuate the production of type 2 cytokines secreted by ILC2s *in vivo*. Levels of RNA transcript in lung and protein in BALF of **(A)** IL-5 and **(B)** IL-13 were measured by qPCR and ELISA respectively. **(C)** Percentage of IL-5^{+}ILC2s and IL-13^{+}ILC2s were determined by flow cytometry. Data are representative of at least four independent experiments and presented as means ± s.e.m (n = 4 - 6; $^{**}P < 0.01$; $^{***}P < 0.001$).

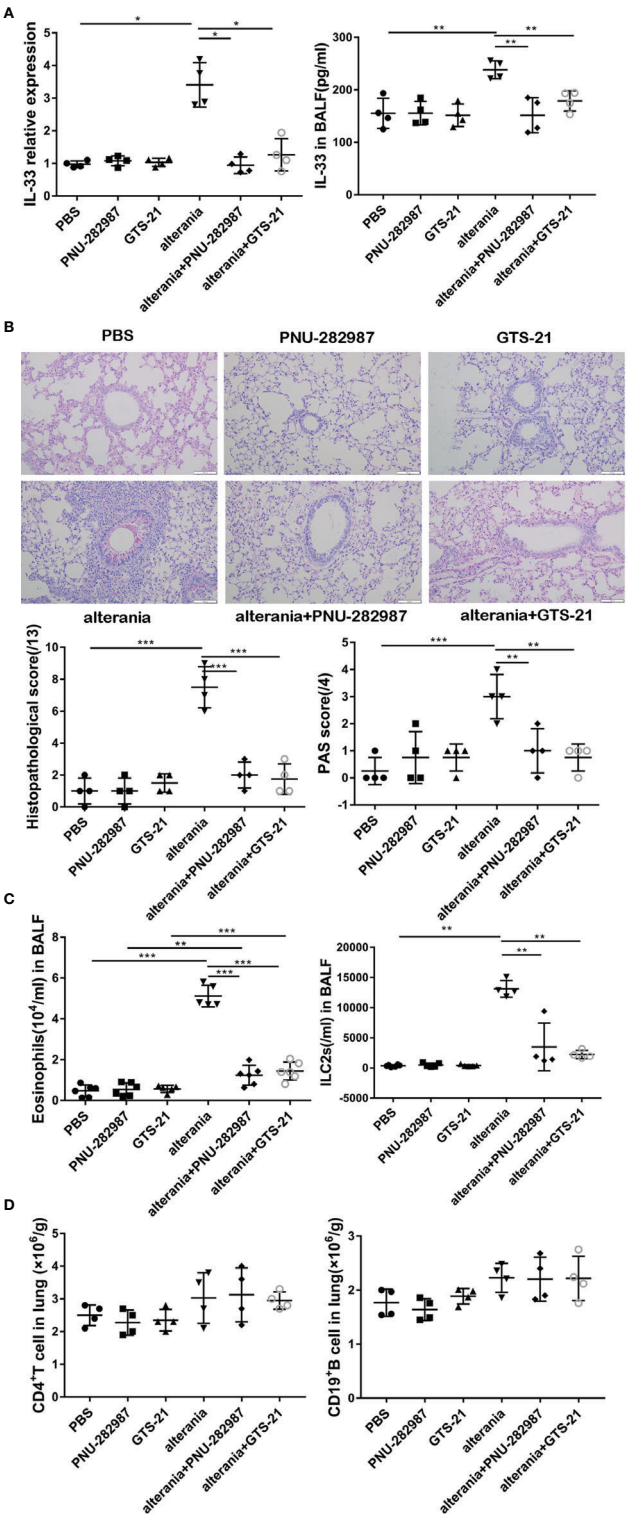

FIGURE 3 | PNU-282987 and GTS-21 inhibit Alternaria-mediated airway inflammation. C57/BL6J mice intranasally received an extract of Alternaria (AA), AA with or without PNU-282987/GTS-21 on days 1 to 4. **(A)** levels of IL-33 RNA in lung and protein in BALF were assessed by qPCR and ELISA respectively. **(B)** Lung tissues were stained by PAS staining. Scoring for histopathology and PAS was assessed by light microscopy (×200, Scale bars at 100 μm). **(C)** Levels of eosinophils and ILC2s in BALF, **(D)** $CD4^+$ T cells and $CD19^+$ B cells in lung were determined by flow cytometry. Data are representative of at least four independent experiments and presented as means ± s.e.m (n = 4 - 5; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

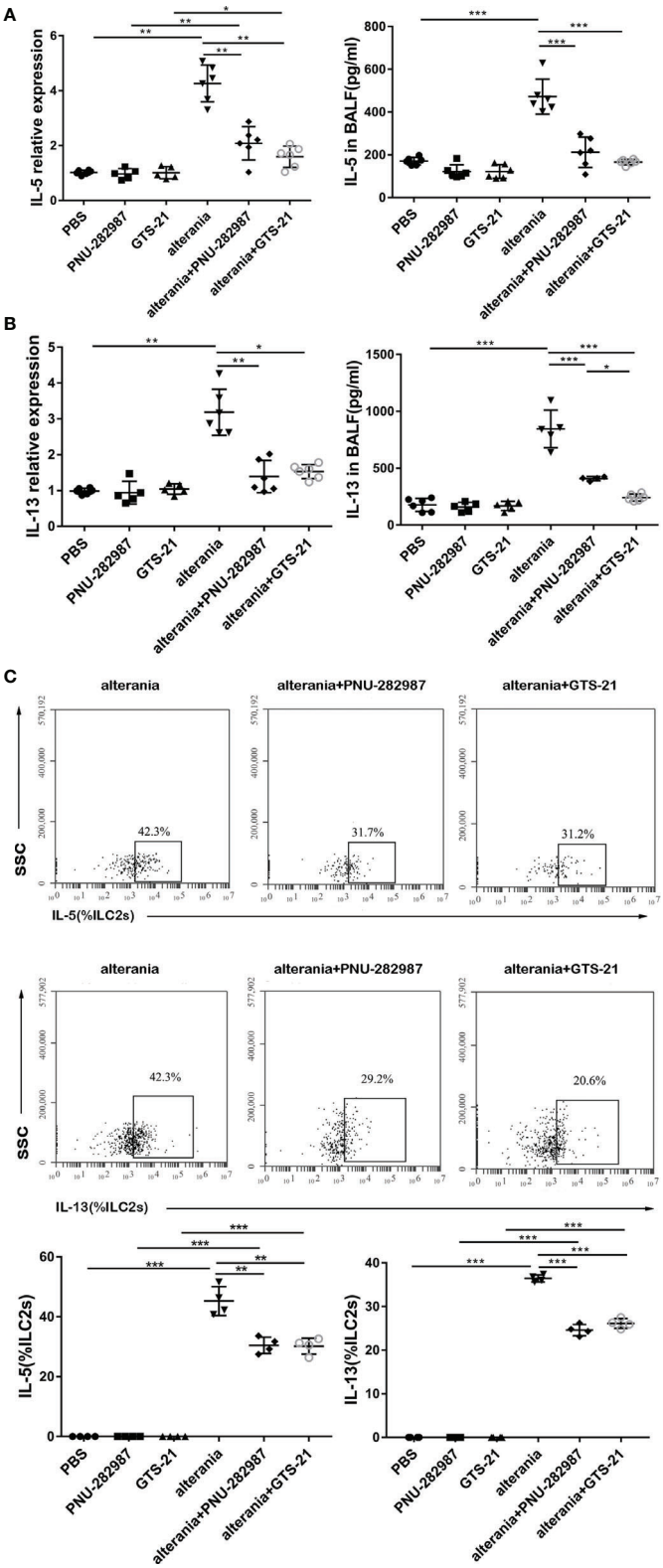

FIGURE 4 | PNU-282987 and GTS-21 inhibit the function of ILC2s in Alternaria-mediated Airway inflammation. Levels of RNA transcript in lung and protein in BALF of **(A)** IL-5 and **(B)** IL-13 were measured by qPCR and ELISA respectively. **(C)** Percentage of IL-5^{+}ILC2s and IL-13^{+}ILC2s was determined by flow cytometry. Data are representative of at least four independent experiments and presented as means ± s.e.m (n = 4 - 6; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

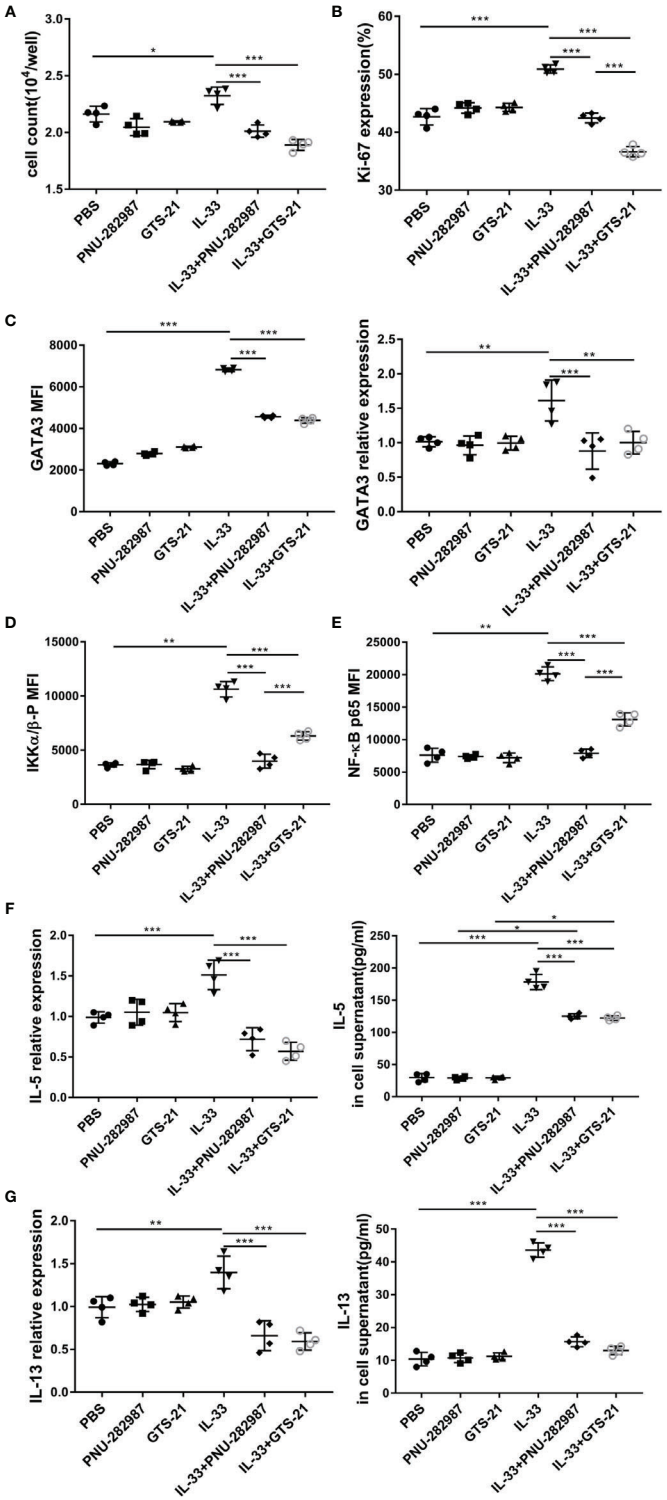

FIGURE 5 | PNU-282987 and GTS-21 inhibit the proliferation and functional activation of ILC2s *in vitro*. Lin^- cells from the lung of C57/BL6J female mice were first separated by magnetic beads, and ILC2s were further isolated by FACS. **(A)** Cell count of ILC2s and **(B)** Percentage of $Ki67^+$cells in isolated lung ILC2s, stimulated with IL-33 in the absence and presence of PNU-282987/GTS-21 for 72 h. **(C)** Mean fluorescence intensity and the RNA levels of GATA3 in isolated lung ILC2s were analyzed by flow cytometry and qPCR. MFI of phosphorylated **(D)** IKKα/β and **(E)** NF-κB p65 with or without PNU-282987/GTS-21 for 24 h. RNA and protein levels of **(F)** IL-5 and **(G)** IL-13 from cultured ILC2s were measured by qPCR and ELISA. Data are representative of at least four independent experiments and presented as means ± s.e.m (n = 4; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

release. Notably, these two cytokines are involved in eosinophil recruitment, AHR, and mucus production (38). While the effectiveness of PNU-282987 and GTS-21 in inhibiting the function of ILC2s was comparable to each other, the former one had a stronger inhibitory effect on IKK and NF-κB, which play a key role in the growth and development of ILC2s (35). This provides stronger evidence for the future study of a unique α7nAChR/IKK/NF-κB focused pathway. Furthermore, the suppression of two NF-κB associated molecules, c-FLIP and ST2, supports the key roles of NF-κB in the differentiation of ILC2s. Through these experiments, we have demonstrated that in addition to GTS-21, PNU-282987 inhibits airway inflammation associated with asthma by interfering with the proliferation and function of ILC2s.

To further explore through what transcription pathways PNU-282987 and GTS-21 enact in ILC2s, flow cytometry was used to assess the expression of the cell proliferation marker, Ki67, and the transcription factors, GATA3, on ILC2s, which are essential for cell proliferation and the production of IL-5 and IL-13. As we expected, the expressions of Ki67 and GATA3 were down-regulated in IL-33/PNU-282987 group when compared to the IL-33 group. This was not surprising as many α7nAChRs-mediated pathways are involved in the process of inhibiting inflammation. This includes the JAK2-STAT3 signaling pathway, α7nAChR/IKK/NF-κB signaling pathway, and α7nAChR/MyD88/IKK/NF-κB pathway (39, 40). STAT3 activated by α7nAChR is a negative regulator of the inflammatory response, and in α7nAChR/IKK/NF-κB signaling axis, α7nAChR further inhibits the nuclear translocation of NF-κB by inhibiting the phosphorylation of upstream signal IKK (41). As Dowling et al. found that nicotine can inhibit the NF- κB signal pathway by binding to α7nAChR, this result supports the notion that the anti-inflammatory mechanism of α7nAChR may be related to IKK and NF- κB transcriptional regulators (42, 43).

We also determined that α7nAChR interacts with I-κB kinase (IKK) and NF-κB as I-κB kinase (IKK) induces NF-κB nuclear activation. After ILC2s were cultured for 24 h *in vitro*, we found that PNU-282987 inhibited the expression of IKK and NF-κB p65 at a significantly higher level when compared with GTS-21. For the different inhibitory effects of PNU-282987 and GTS-21 on IKK and NF-κB, we hypothesized that this observation may be related to the binding ability of GTS-21 to other nicotine receptors, which may interfere with the inhibitory effects of this agonist on α7nAChR activities (44, 45).

Acetylcholine is a major parasympathetic neurotransmitter that has previously been shown to effectively inhibit the release of inflammatory cytokines, such as TNFα from peripheral macrophages stimulated by LPS *in vitro* (13). Stimulation of efferent vagus nerve can inhibit systemic inflammation, as demonstrated in a study that the level of TNFα in serum and liver decreased significantly in Lewis rats with endotoxemia after cervical vagotomy by electric stimulation (13). Furthermore, the release of norepinephrine by the vagus nerve stimulates memory T cells to secrete acetylcholine for the negative regulation of inflammation on α7nAChR-positive cells (46, 47). Our finding that ILC2-driven airway inflammation is negatively regulated by α7nAChR is supported by the observation that ILC2 activity is reduced by memory T-cell release of acetylcholine (48). Interestingly, it is also known that ILC2s express β_2-adrenergic receptors (that respond to norepinephrine), which when activated will agonistically suppress ILC2 proliferation and secretion of type 2 cytokines (49). While this study only focused on exploiting α7nAChR-agonism to regulate ILC2 inflammation, the effects of norepinephrine on the inflammatory cascades explored above should be further investigated to determine if any overlap exists between the two neuro-inhibitory pathways.

Based on our *in vivo* data, the viability of ILC2s in culture was inhibited by PNU-282987. Except for IKK and NF-κB, there was no significant difference in the proliferation of ILC2s and the secretion of IL-5 and IL-13 between PNU-282987 and GTS-21 *in vitro*. Although this mechanism needs to be explored further, it is clear that our results show that PNU-282987 is effective in the treatment of ILC2-mediated airway inflammation induced by IL-33 and AA. The importance of the cholinergic anti-inflammatory pathway and α7nAChR as a pharmacological target for the treatment of inflammatory diseases needs further investigation. More importantly, the inhibitory effect of PNU-282987 on IKK and NF-κB should warrant further attention and it is worthy of future exploration.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Zhengzhou University.

AUTHOR CONTRIBUTIONS

Performed the experiments: FY, LJ, QL, YW, WL, HT. Analyzed and interpreted the data: FY, QL, XL, LZ, FL, MY. Conceived and designed the experiments: FL, MY. Wrote and edited the paper: FY, LS, GZ, FL, MY. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.598165/full#supplementary-material

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Cyclic-di-GMP Induces STING-Dependent ILC2 to ILC1 Shift During Innate Type 2 Lung Inflammation

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Type 2 inflammation is found in most forms of asthma, which may co-exist with recurrent viral infections, bacterial colonization, and host cell death. These processes drive the accumulation of intracellular cyclic-di-nucleotides such as cyclic-di-GMP (CDG). Group 2 innate lymphoid cells (ILC2s) are critical drivers of type 2 lung inflammation during fungal allergen exposure in mice; however, it is unclear how CDG regulates lung ILC responses during lung inflammation. Here, we show that intranasal CDG induced early airway type 1 interferon (IFN) production and dramatically suppressed CD127+ST2+ ILC2s and type 2 lung inflammation during *Alternaria* and IL-33 exposure. Further, CD127–ST2–Thy1.2+ lung ILCs, which showed a transcriptomic signature consistent with ILC1s, were expanded and activated by CDG combined with either *Alternaria* or IL-33. CDG-mediated suppression of type 2 inflammation occurred independent of IL-18R, IL-12, and STAT6 but required the stimulator of interferon genes (STING) and type 1 IFN signaling. Thus, CDG potently suppresses ILC2-driven lung inflammation and promotes ILC1 responses. These results suggest potential therapeutic modulation of STING to suppress type 2 inflammation and/or increase anti-viral responses during respiratory infections.

Keywords: ILC, ILC1, ILC2, asthma, Cyclic-di-GMP, STING, COVID-19, innate lymphoid cell

INTRODUCTION

Group 2 innate lymphoid cells (ILC2s) were identified in 2010 and have since been recognized for their essential role in orchestrating innate type 2 immune responses in murine asthma models (1–3). Airway exposure to the clinically-relevant fungal allergen *Alternaria alternata* (*Alt*) promotes rapid epithelial cell IL-33 secretion and ILC2 production of IL-5 and IL-13, which drive peribronchial eosinophilic inflammation (4). ILC2s belong to a greater family of ILCs that includes ILC1s, which are involved in protective immune responses against intracellular pathogens. While ILC2s express the key transcription factor GATA-3 and produce type 2 cytokines, ILC1s express T-bet and produce interferon-γ (IFNγ).

Insights into asthma pathogenesis have revealed associations with bacterial colonization (5), viral infections (6), as well as inappropriate cell death and mitochondrial stress (7). These processes drive accumulation of cyclic-di-nucleotide danger signals inside of host cells (8). Free intracellular viral and host DNA are converted to cyclic-di-nucleotides by the host cytosolic enzyme cyclic-GMP-AMP-synthase (9). One particular cyclic-di-nucleotide, cyclic-di-GMP (CDG), is synthesized by bacteria ubiquitously as a secondary messenger (10). CDG is not host cell membrane permeable, suggesting CDG accumulation inside of host cells occurs by way of active transport (11, 12). CDG is unique to microbes yet binds numerous mammalian receptors including HCN (13), HpoR (14), Siderocalin (15), and the stimulator of interferon genes (STING) (16). Importantly, CDG has been shown to induce robust type 1 and type 3 interferons (IFN) that are critical for anti-viral responses (16–18).

Viral infections are a common cause of asthma exacerbations, though how type 1 and 3 IFNs contribute to asthma pathogenesis is unclear and may be different in specific asthma endotypes. Recently, the global SARS-CoV2 pandemic has led to rapid, broad interest in anti-viral and immunomodulatory strategies to prevent and treat severe disease. Studies in COVID-19 patients have shown that type 1 IFN responses are delayed and/or exaggerated at later phases of severe COVID-19 (19–22). Thus, strategies to control early airway infections through strong local anti-viral responses may be advantageous through modulation of type 1 and 3 IFN and ILC subsets. The effect of cyclic-di-nucleotides on lung ILC responses is unknown. Here, we investigated the role of CDG in fungal allergen and IL-33 driven asthma models.

RESULTS

CDG Abrogates Alternaria-Induced ILC2-Driven Type 2 Airway Inflammation and Promotes Interferon Production

To elucidate whether CDG regulates innate lung immunity, we utilized a well-described 3-day *Alt*-induced ILC2-driven model of eosinophilic airway inflammation (**Figure 1A**) (4). Intranasal CDG administration nearly abolished *Alt*-induced bronchoalveolar lavage fluid (BAL) and lung eosinophilia (**Figures 1B,C**, **Supplementary Figure 1A**). Concordantly, CDG significantly reduced BAL IL-5 and IL-13 levels (**Figures 1D,E**). Interestingly, CDG synergistically potentiated *Alt*-induced BAL and lung neutrophil accumulation (**Figures 1F,G**, **Supplementary Figure 1B**). In the absence of *Alt,* CDG did not have a significant effect on airway granulocytic infiltration (**Figures 1B,C,F,G**).

Recent studies have shown that ILC2 cytokine production is suppressed by multiple IFNs (23–25). Therefore, we next asked whether CDG drove IFN production during *Alt* exposure. Indeed, BAL IFNγ (type 2 IFN) levels increased over 20-fold on average in mice receiving both *Alt* and CDG compared to *Alt* alone (**Figure 1H**). Further, BAL IFNβ (type 1 IFN) was increased 30-fold 3 h following the first intranasal challenge in mice that received CDG (**Figure 1I**). BAL IFNλ (type 3 IFN) levels were also higher in CDG treated mice at this early timepoint, though the difference was not significant (**Supplementary Figure 1C**).

To determine whether adaptive or innate lymphocytes, or both, were required for CDG-induced attenuation of type 2 inflammation, we performed experiments with *Rag2*-/- mice that possess ILCs (including NK cells) but are deficient in B and T cells as well as *Il7r*-/- mice that lack both ILCs and the majority of T/B cells. Thus, the major difference between these strains is the presence of ILCs (26). No IL-5 and relatively few eosinophils were detectable in the airways of *Il7r*-/- mice (**Figures 1J,K**, **Supplementary Figure 1D**). Following *Alt* challenge, lung eosinophil and BAL IL-5 levels were markedly increased in *Rag2*-/- mice compared to *Il7r*-/- mice. As in wild type mice, CDG nearly abolished *Alt*-induced lung eosinophilia and reduced BAL IL-5 ($p = 0.0518$) in *Rag2*-/- mice. Collectively, these results both demonstrate that CDG combined with *Alt* induces a neutrophilic response associated with increased type 1 and 2 IFNs and suggest that CDG reduces ILC2-driven eosinophilic airway inflammation, independent of adaptive immunity.

CDG Induces a Lung ILC2 to ILC1 Shift

We next investigated the effect of CDG on ILC2 responses in the 3-day *Alt* model. Given the observed increase in BAL IFNγ, we also focused on changes in ILC1 responses after CDG exposure. ILC subsets are heterogeneous and show significant plasticity under different conditions (4, 27). Thus, prior to assessing the effect of *in vivo* CDG challenge on ILC subsets, we used transcriptomic analysis to validate the identity ILC1s and ILC2s following *Alt* administration. We reanalyzed our published RNAseq dataset of *Alt* challenged murine lung ST2+CD127+ and ST2–CD127– ILCs (**Figure 2A**) (4) and found that ST2+CD127+ ILCs highly expressed canonical ILC2 genes (*Klrg1*, *Il7r*, *Areg*, *Il1rl1*, and *Gata3*), whereas ST2–CD127– ILCs highly expressed key ILC1 genes (*Gzma*, *Gzmb*, *Irf8*, *Klrk1*, and *Klrb1*) (**Figure 2B**). Furthermore, gene ontology pathway analysis indicated that one of the most differentially expressed pathways between ST2+CD127+ and ST2–CD127– ILCs was IFNγ production (**Figure 2C**). Thus, ST2+CD127+ and ST2–CD127– populations contain strong ILC2 and ILC1 signatures, respectively.

Intranasal CDG administration dramatically reduced the number of *Alt*-induced IL-5+ and IL-13+ lung ILC2s following *ex vivo* PMA/ionomycin stimulation (**Figure 2D**, **Supplementary Figures 2A,B**). Because of the relatively artificial nature of *ex vivo* stimulation, we next utilized Red5 IL-5 reporter mice and Smart13 IL-13 reporter mice to provide *in vivo* evidence of CDG-elicited ILC2 suppression (28, 29). Concordantly, we found that CDG attenuated *Alt*-induced lung ILC2 IL-5 and IL-13 production *in vivo* (**Figures 2E,F**). Contrary to ILC2 responses, the total number of IFNγ+ ILC1s was significantly increased following CDG challenge and *ex vivo* PMA/ionomycin stimulation (**Figure 2G**, **Supplementary Figure 2C**). Further, BAL IFNγ levels were markedly increased in *Rag2*-/- mice

Abbreviations: ILC, Innate lymphoid cell; ILC2, group 2 innate lymphoid cell; ILC1, group 1 innate lymphoid cell; *Alt*, *Alternaria alternata*; CDN, cyclic-di-nucleotide; CDG, cyclic-di-GMP; STING, stimulator of interferon genes; IFN, interferon; BAL, bronchoalveolar lavage.

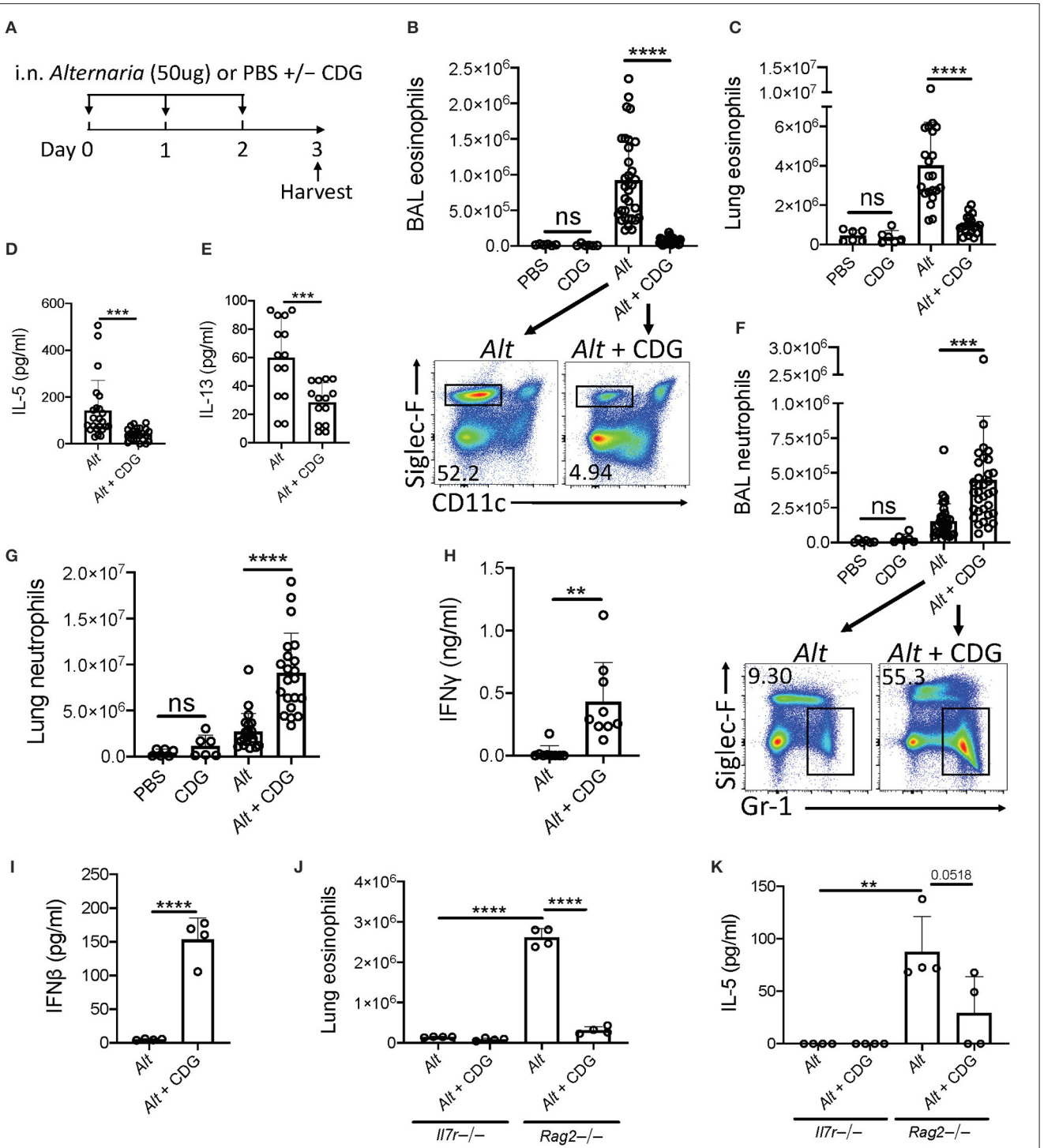

FIGURE 1 | CDG abrogates *Alt*-induced type 2 inflammation and promotes neutrophilia. **(A)** Intranasal challenge mouse model of innate airway inflammation. **(B)** Total BAL eosinophils (top) and representative flow plots (bottom). **(C)** Total lung eosinophils. BAL IL-5 **(D)** and IL-13 **(E)** ELISA. **(F)** Total BAL neutrophils (top) and representative flow plots (bottom). **(G)** Total lug neutrophils. **(H)** BAL IFNγ ELISA. **(I)** BAL IFNβ ELISA 3 h after first challenge. *Il7r*-/- and *Rag2*-/- lung eosinophils **(J)** and BAL IL-5 ELISA **(K)**. Data shown are representative of 2–10 independent experiments with 2–4 mice per group. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, unpaired *t*-test.

compared to *Il7r*-/- mice, suggesting that ILCs (and possibly NK cells) contribute significantly to CDG-induced IFNγ secretion (**Figure 2H**).

In light of the CDG-induced shift from an ILC2 to ILC1 response and recent reports of ILC plasticity (27, 30), we further investigated the impact of CDG on ILC identity, activation,

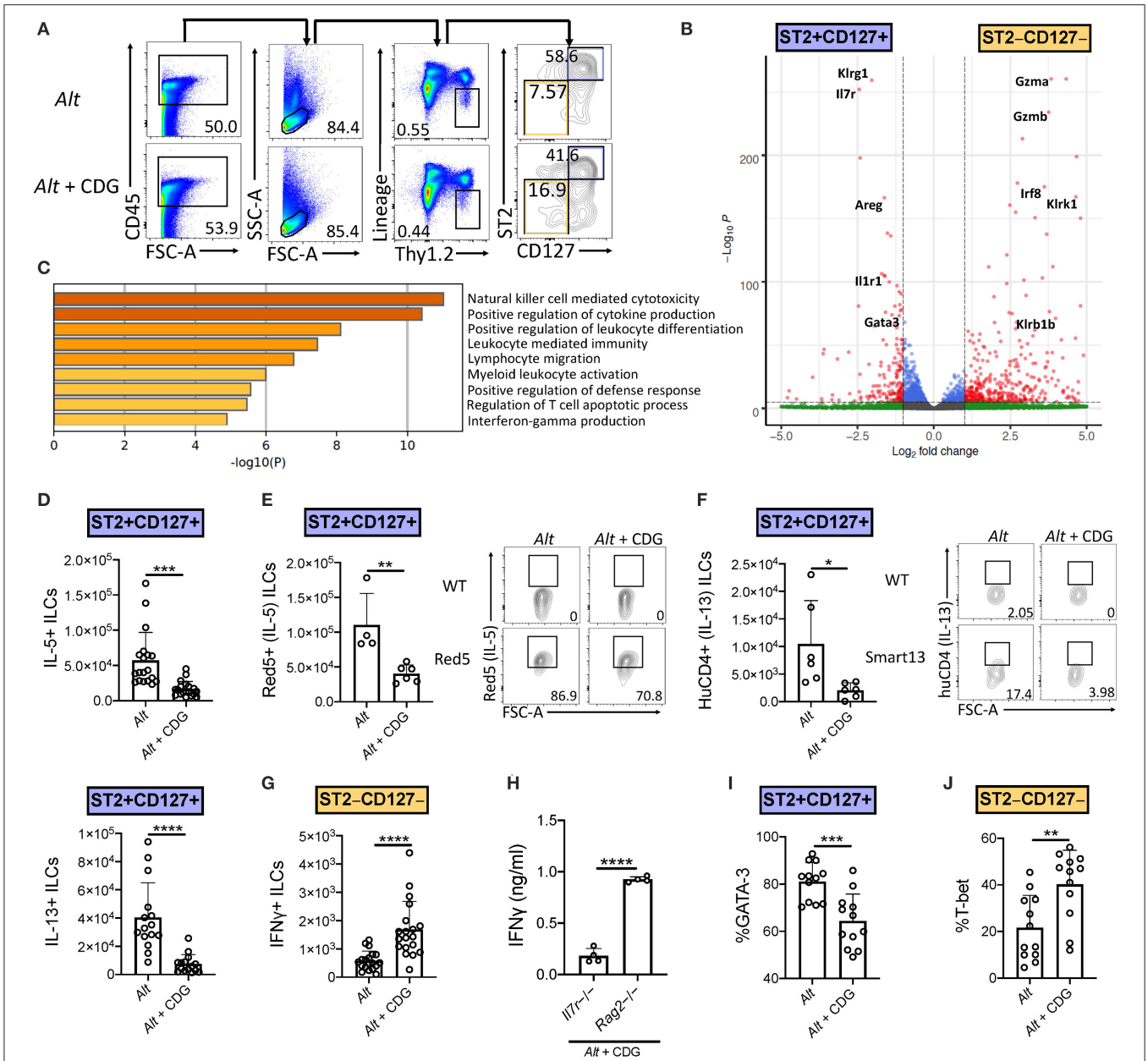

FIGURE 2 | CDG induces ILC2 to ILC1 compartmental changes. Mice were challenged using the same model as in **Figure 1A**. **(A)** Flow cytometric gating scheme for identification of lung ILC2s and ILC1s in *Alt* and *Alt* plus CDG challenged mice. **(B)** RNAseq volcano plot of differentially expressed genes between *Alt* challenged lung ST2+CD127+ ILCs and ST2–CD127– ILCs. **(C)** Differentially expressed gene ontology pathways in ST2+CD127+ and ST2–CD127– ILC subpopulations. **(D)** Total number of IL-5+ (top) and IL-13+ (bottom) lung ILC2s following PMA/ionomycin stimulation *ex vivo*. **(E)** Total number of Red5+ (IL-5+) lung ILC2s (left) and representative flow plots (right). **(F)** Total number of HuCD4+ (IL-13+) lung ILC2s (left) and representative flow plots (right). **(G)** Total number of IFNγ+ lung ILC1s following PMA/ionomycin stimulation *ex vivo*. **(H)** *Il7r*-/- and *Rag2*-/- BAL IFNγ ELISA. Frequency of GATA-3+ lung ILC2s **(I)** and T-bet+ lung ILC1s **(J)**. Data shown are representative of 2–7 independent experiments with 2–4 mice per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, unpaired *t*-test.

and proliferation. CDG decreased lung ILC2 expression of the canonical ILC2 master type 2 cytokine regulator GATA-3 (**Figure 2I**, **Supplementary Figure 2D**) and surface markers KLRG1 and ICOS (**Supplementary Figures 2E,F**). Conversely, CDG increased ILC1 expression of the master type 1 cytokine regulator T-bet by 20% on average (**Figure 2J**, **Supplementary Figure 2G**). Further, CDG attenuated ILC2 proliferation, as evidenced by decreased expression of Ki67 (**Supplementary Figure 2H**), but did not affect ILC2 activation status, as indicated by unchanged CD69 expression levels (**Supplementary Figure 2I**). Conversely, CDG did not affect ILC1 proliferation (**Supplementary Figure 2J**) but did significantly increase ILC1 activation status (**Supplementary Figure 2K**). Taken together, these findings

indicate that CDG drives concomitant ILC2 suppression and ILC1 activation.

As conventional NK cells are also a source of IFNγ, we assessed numbers and activation of lung NK cells in the model. We found a similar increase in NK1.1+CD49b+CD3− lymphocytes in the *Alt*, CDG, and *Alt*+CDG groups of mice compared to PBS treated mice (**Supplementary Figure 3A**). Further, there was no significant difference in NK cell IFNγ expression between groups (**Supplementary Figure 3B**). Thus, IFNγ+ NK cell do not appear to be differentially responsive after CDG+*Alt* exposure as compared with ST2–CD127−ILCs.

CDG Induces ILC2 to ILC1 Shift During IL-33-Driven Type 2 Lung Inflammation

We next asked whether CDG-induced ILC2 inhibition occurred downstream of IL-33 release. To that end, we challenged mice with exogenous IL-33 instead of *Alt*. Consistent with the *Alt* model, CDG severely attenuated IL-33-induced lung eosinophilia (**Figure 3A**) and robustly increased lung neutrophil accumulation (**Figure 3B**). Additionally, CDG administration markedly decreased BAL IL-5 (**Figure 3C**) and increased BAL IFNγ by over 100-fold (**Figure 3D**). As with *Alt*, CDG decreased the total number of IL-33-induced IL-5+ and IL-13+ lung ILC2s (**Figures 3E,F**, **Supplementary Figures 4A,B**) and increased the total number of IFNγ+ ILC1s (**Figure 3G**, **Supplementary Figure 4C**). Thus, CDG had effects on IL-33 driven lung responses comparable to those of the fungal allergen *Alt*, suggesting that CDG inhibition of ILC2 responses is not related to an effect on IL-33 levels during *Alt* exposure.

ILCs Express STING but Are Not Directly Regulated by Extracellular CDG

We next sought to determine whether ILCs are directly regulated by CDG in the presence of IL-33 activation. We first investigated whether ILCs express STING. Expression analysis indicated that along with lung macrophages and dendritic cells (DCs), ILCs highly expressed STING (**Supplementary Figure 5A**). We stimulated sort purified ILCs (**Supplementary Figure 5B**) with IL-33 and CDG for 48 h. As expected, IL-33 increased IL-5 and IL-13 production (**Supplementary Figures 5C,D**). However, extracellular CDG had no effect on ILC type 2 cytokine production showing that ILCs are not directly suppressed by CDG despite expressing STING. CDG has been reported to directly activate phagocytic cells including dendritic, monocyte, and macrophage cell lines, suggesting that phagocytosis is a mechanism by which extracellular CDG accumulates intracellularly and can then activate STING (16). Importantly, these cells could produce IL-12, IL-18, or type 1 IFNs that could suppress ILC2s and promote ILC1 responses.

IL-12 and IL-18R Are Dispensable for CDG-Induced Suppression of Type 2 Response

The ILC2-driven *Alt* asthma model has been described as largely IL-33 dependent, though conversion of ST2+ ILC2s to ILC1s during lung inflammatory responses to influenza has been shown to be promoted by IL-12 and IL-18 (31, 32). Thus, we investigated whether IL-12 and IL-18 signaling were required for the observed granulocytic shift after addition of CDG to *Alt*. Surprisingly, we found that the levels of airway eosinophilia and neutrophilia, and the number of ILC2s and ILC1s, remained constant in mice lacking IL-12 (**Figures 3H,I,L**, **Supplementary Figures 4H,I,M**) and IL-18R (**Figures 3J,K,M**, **Supplementary Figures 4J–L**). Collectively, these results demonstrate that CDG induces ILC2 to ILC1 and eosinophil to neutrophil shifts independent of IL-12 and IL-18 signaling.

CDG Suppression of Type 2 Inflammation and ILC2 Responses Is STING-Dependent

Given the robust airway IFN accumulation induced by CDG, we next investigated whether STING (the *st*imulator of *in*terferon *g*enes) was required for suppression of type 2 lung inflammation and ILC2 responses. Strikingly, through comparison of wild type and STING deficient (*Tmem173*-/-) mice, we found that removal of STING completely rescued CDG-driven abrogation of *Alt*-induced lung eosinophilia and fully abrogated CDG-induced lung neutrophilia (**Figures 4A,B**). Moreover, lung ILC2 function was completely restored in the absence of STING (**Figure 4C**), and CDG-driven ILC1 IFNγ induction was entirely STING-dependent (**Figure 4D**).

STAT6 Is Not Required for Neutrophilic Airway Inflammation Induced by CDG

A recent study demonstrated that STAT6 was required for STING-dependent chemokine production (33). Therefore, we sought to determine whether STAT6 was required for neutrophil accumulation in lung tissue following CDG challenge. Consistent with previous reports, *Alt*-induced eosinophilia was highly dependent upon STAT6 (**Figure 4E**) (26). Notably, we found that residual lung eosinophil levels following CDG administration were further reduced in the absence of STAT6. Unlike eosinophils, neutrophils accumulated in the airway independent of STAT6 (**Figure 4F**). These results demonstrate that while STAT6 regulates innate type 2 lung inflammation, likely through chemokine expression, it has no role in the STING-induced effects on neutrophil accumulation (26, 34).

Type 1 IFN Signaling Is Indispensable for CDG-Induced Suppression of Type 2 Inflammation

Type 1 IFNs are thought to be the major products of *st*imulator of *in*terferon *g*enes (STING) activation. To investigate whether type 1 IFN production was responsible for the observed CDG-induced immunomodulation, we compared type 1 IFN signaling deficient (*Ifnar1*-/-), wild type, and *Tmem173*-/- mice. Removal of type 1 IFN signaling rescued CDG-induced abrogation of BAL and lung eosinophilia (**Figure 5A**) but did not prevent CDG-induced BAL and lung neutrophilia (**Figure 5B**). Moreover, the decrease in total IL-13+ ILCs caused by CDG was dependent on type 1 IFN signaling (**Figure 5C**). Thus, type 1 IFNs are indispensable for CDG-mediated suppression of type 2 inflammation but not neutrophilia. These results are consistent with recent work demonstrating that type 1 IFNs suppress lung ILC2s during viral infection (24).

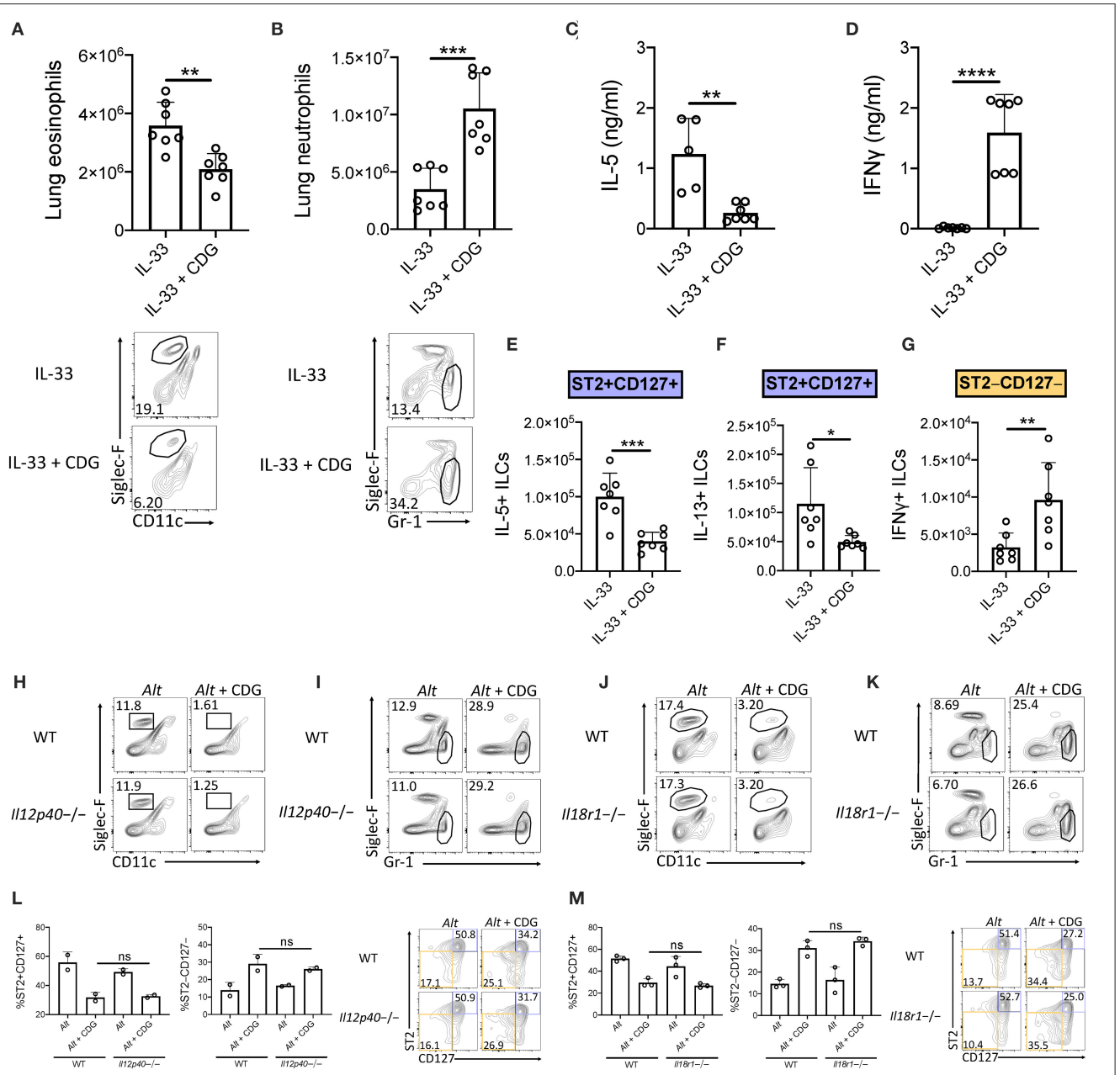

FIGURE 3 | Suppression of type 2 inflammation occurs downstream of IL-33 and is independent of IL-12 and IL-18 signaling. **(A–G)** Mice were challenged using the same model as in **Figure 1A** but with IL-33 instead of *Alt*. **(A)** Total lung eosinophils (top) and representative flow plots (bottom). **(B)** Total lung neutrophils (top) and representative flow plots (bottom). BAL IL-5 **(C)** and IFNγ **(D)** ELISA. Total number of IL-5+ lung ILC2s **(E)**, IL-13+ lung ILC2s **(F)**, and IFNγ+ lung ILC1s **(G)** following PMA/ionomycin stimulation *ex vivo*. **(H–M)** Mice were challenged using the same model as in **Figure 1A**. *Il12p40-/-* lung eosinophil **(H)** and neutrophil **(I)** frequencies and *Il18r1-/-* lung eosinophil **(J)** and neutrophil **(K)** frequencies. Data shown are representative of two independent experiments with 2–4 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, unpaired *t*-test.

IFNγ Is Dispensable for CDG-Induced ILC and Granulocyte Changes

Given that CDG elicited increases in airway IFNγ that may also suppress ILC2 function (23, 25, 35), we next investigated whether IFNγ was required for CDG-induced lung granulocyte or ILC changes. IFNγ monoclonal antibody blockade led to a modest non-significant increase in airway eosinophilia and IL-5+ ILC2s in mice challenged with *Alt* and CDG (**Supplementary Figures 6A,C,E**). Similar to type 1 IFN receptor deficient mice, inhibition of IFNγ signaling had no effect on airway neutrophilia (**Supplementary Figures 6B,D**). These results support that IFNγ is largely dispensable for CDG-induced immunomodulation of ILC2 responses and neutrophilia after exposure to *Alt*+CDG.

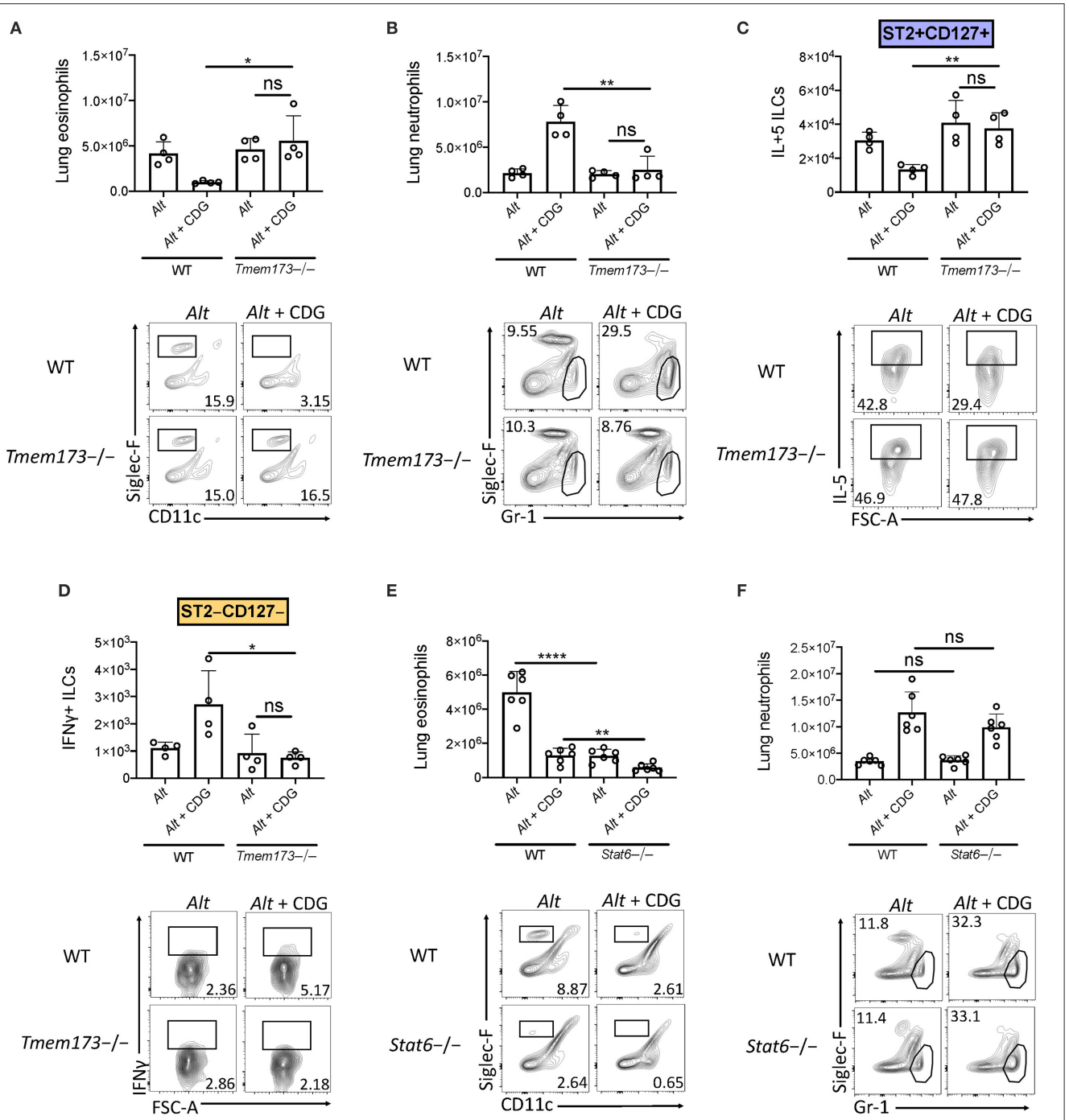

FIGURE 4 | CDG modulation of airway inflammation is STING-dependent and STAT6 independent. **(A–D)** Wild type and *Tmem173-/-* mice were challenged using the same model as in **Figure 1A**. **(A)** Total lung eosinophils (top) and representative flow plots (bottom). **(B)** Total lung neutrophils (top) and representative flow plots (bottom). **(C)** Total lung IL-5+ ILC2s (top) and representative flow plots (bottom). **(D)** Total lung IFNγ+ ILC1s (top) and representative plots (bottom). **(E,F)** Wild type and *Stat6-/-* mice were challenged using the same model as in **Figure 1A**. **(E)** Total lung eosinophils (top) and representative flow plots (bottom). **(F)** Total lung neutrophils (top) and representative flow plots (bottom). Data shown are representative of two independent experiments with 2–3 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, unpaired *t*-test.

DISCUSSION

Asthma is largely a type 2 inflammatory airway disease and is associated with bacterial and viral infections, mitochondrial stress, and host cell death, which all lead to the accumulation of the danger-associated cyclic-di-nucleotides (5–8, 36). ILC2s promote type 2 inflammation in experimental asthma models and likely contribute to airway inflammation and

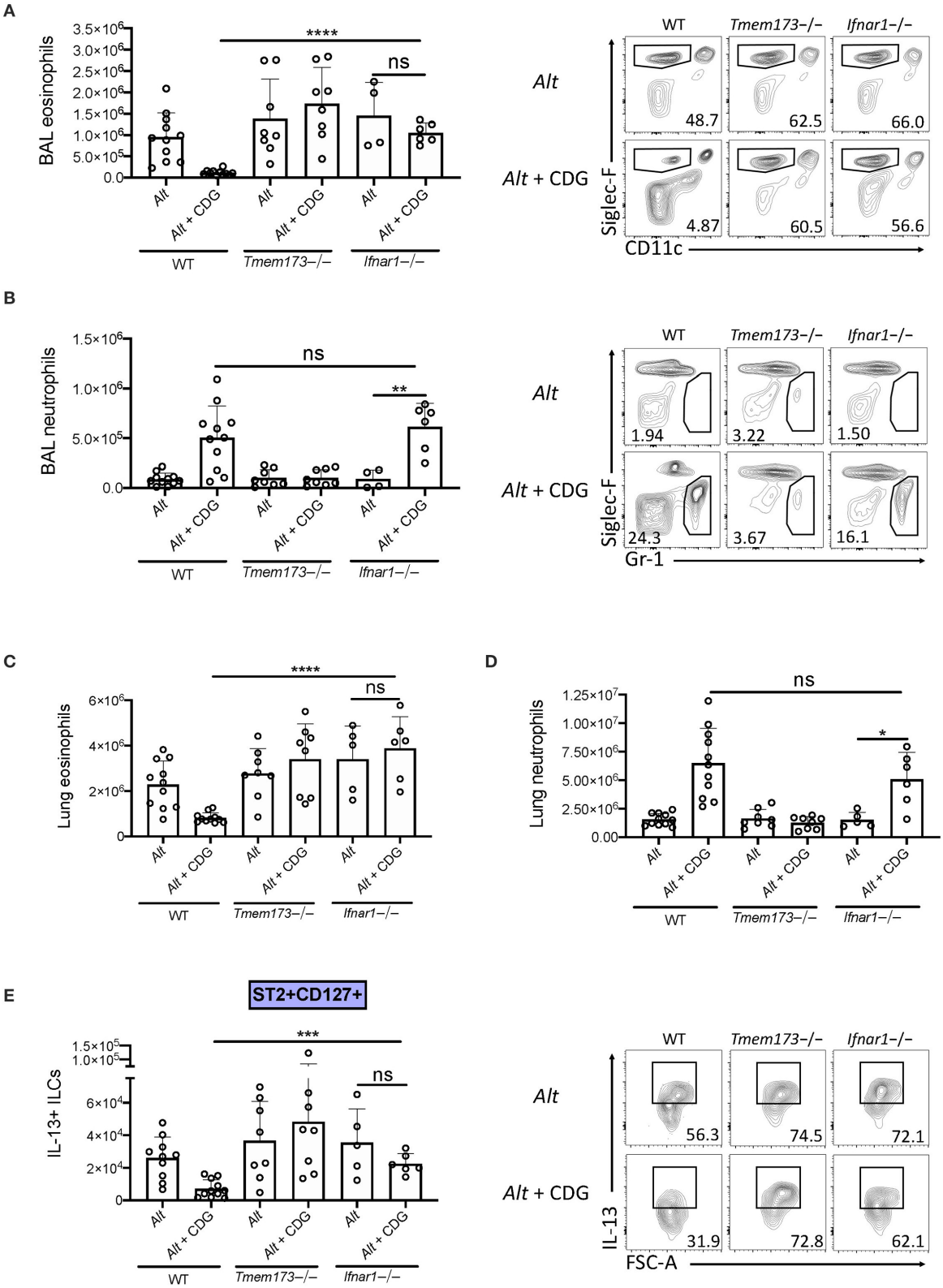

FIGURE 5 | Type 1 IFN signaling is required for CDG induced suppression of ILC2s and type 2 inflammation. Wild type, *Tmem173-/-*, and *Ifnar1-/-* mice were challenged using the same model as in **Figure 1A**. **(A)** Total number of BAL eosinophils (left) and representative plots (right). **(B)** Total number of BAL neutrophils (left) and representative flow plots (right). **(C)** Total number of lung eosinophils. **(D)** Total number of lung neutrophils. **(E)** Total number of IL-13+ST2+CD127+ ILC2s (left) and representative flow plots (right). Data shown are representative of 5 independent experiments with 1–3 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, unpaired *t*-test.

hyperresponsiveness in humans (37). Here, we investigated how *in vivo* administration of the cyclic-di-nucleotide CDG regulates ILC2s and innate type 2 inflammation. Our novel findings show that intranasal CDG challenge nearly abolished *Alt*-induced airway eosinophilia but promoted neutrophil accumulation. Congruently, CDG drove contraction of IL-5+ and IL-13+ lung ILC2s and expansion of IFNγ+ ILC1s. Mechanistically, we found that CDG modulated ILC responses downstream of *Alt*-induced IL-33 release and that the effect of CDG on airway type 2 inflammation was entirely STING- and type 1 IFN-dependent. Interestingly, the effect of CDG on airway neutrophilia was STING-dependent and type 1 IFN-independent. Thus, STING's involvement in pathogen responses, as well as activation by cytosolic mitochondrial DNA (from damaged mitochondria) and autophagy, could represent a critical, common pathway in the development of mixed airway inflammatory responses often found in asthma (8, 38–42).

While type 1 IFNs are the major products of STING activation, STING agonism has also been shown to induce the production of several inflammatory mediators including type 3 IFNs, IL-1β, IL-6, TNFα, CCL2, and CCL20 (43–46). In addition to elevated levels of type 1 IFNs, we observed increases in type 2 and 3 IFN following CDG administration, though the latter failed to reach significance. While type 1, 2, and 3 IFNs have all been shown to inhibit ILC2 function and restrict type lung 2 inflammation (23–25, 47, 48), we found that only type 1 IFN was required for CDG-induced ILC2 suppression. Taken together with recent reports, our findings suggest a model in which intranasal CDG challenge drives STING-dependent early production of type 1 IFN by airway phagocytes, thereby suppressing ILC2-driven airway eosinophilia. Because the effect of CDG on *Alt*-induced neutrophilia we observed was independent of type 1 and 2 IFN signaling, non-IFN mediators downstream of STING likely contribute to CDG-induced neutrophil accumulation.

The effect of CDG on airway eosinophil and neutrophil levels we report here agrees with a previous report in which CDG was administered in an adaptive Th2 cell-driven chronic asthma model, though ILC changes were not assessed (49). Further, the ILC compartmental changes we observed mirror influenza virus induced ILC2 to ILC1 plasticity described in a recent study (32). However, that study demonstrated that IL-12 and IL-18 drove the attenuation of type 2 cytokine production, whereas we found that CDG abrogated type 2 inflammation independent of IL-12 and IL-18 signaling and dependent on STING and type 1 IFN. Collectively, these findings indicate that there are multiple ways to induce an ILC2 to ILC1 shift in the lungs.

The role of STING in ILC responses and lung inflammation we report here are novel and may be tissue specific. A recent investigation into the role of STING in the gastrointestinal mucosa revealed a nearly 2-fold reduction in ILC2 frequency and IL-4 and IL-13 levels, and a 3-fold increase in ILC1 frequency, in STING deficient mice (50). Conversely, we found markedly increased airway ILC2s and type 2 cytokines, and decreased ILC1s and type 1 cytokines, in STING deficient mice following *Alt* and CDG challenge. Such highly divergent findings suggest the presence of distinct, tissue-specific mechanisms by which STING controls ILC responses. These results are consistent with recent work demonstrating other tissue specific ILC responses (51).

The mechanism by which CDG-induced STING activation regulates lung inflammation that we report here is also strikingly different from the mechanism by which a STING gain-of-function mutation was recently reported to regulate lung inflammation (52). The N153S STING gain-of-function mutation induced type 1 IFN-independent and B and T cell-dependent spontaneous lung disease, while we found that CDG induced type 1 IFN-dependent and B/T cell-independent suppression of type 2 inflammation. These contrasting results suggest that the mechanism of STING activation governs the downstream signaling response, of which there are numerous.

The findings we report here might be leveraged for developing treatments to induce early, local control of respiratory viruses, including SARS-CoV-2, in which type 1 and 3 IFN control of virus may be critical for prevention of late complications in severe disease (19, 20). RNA viruses, such as coronaviruses, induce STING downstream of RIG-I and MAVS signaling (53). Importantly, however, coronaviruses have evolved mechanisms for inhibiting STING activation. For example, coronaviruses encode proteases that antagonize STING and prevent downstream IFN production (54, 55). Interestingly, bats, which harbor coronaviruses yet show no signs of immunopathology, constitutively express IFN and were recently shown to possess a distinct STING from that of other mammals (56). When bat STING protein was humanized with a single amino acid substitution, viral tolerance was lost, suggesting a critical role for STING activation and IFN expression in controlling viral replication. Additionally, patients with severe COVID-19 have impaired type 1 IFN responses (20), and early administration of the type 1 interferon IFN-α2b led to reduced in-hospital mortality in COVID-19 patients (21). Collectively, our work, coupled with these reports, suggests that CDG, or other human STING agonists, may be a potential low-cost, local respiratory therapeutic for early treatment of COVID-19 following SARS-CoV2 infection through broad generation of anti-viral type 1, 2, and 3 IFNs.

Our study is limited in that the mechanisms downstream of STING that account for airway neutrophil accumulation remain unclear. Consistent with previous reports, we showed that lung eosinophilia is dependent on STAT6 (26). A prior study showed that STAT6 was required for STING-mediated chemokine production and immune cell recruitment (33); however, in our study, CDG-induced airway neutrophil accumulation occurred independent of STAT6, which suggests the existence of an alternative pathway by which immune cells accumulate in the presence of STING agonism. Further, we cannot exclude additional cell types such as NK cells as important sources of IFNγ during CDG exposure. Future studies investigating these mechanisms are warranted.

In summary, our work demonstrates that CDG drives STING-dependent IFN production, ILC1 activation and accumulation, as well as ILC2 suppression and abrogation of innate type 2 innate airway inflammation. The knowledge set forth in this report is critical for understanding how allergic airway disease pathogenesis may be impacted by lung insults

due to cellular stress, bacterial infection, or viral infections such as SARS-CoV2.

MATERIALS AND METHODS

Mice

6- to 12-week-old female C57BL/6 WT mice were obtained from The Jackson Laboratory (Bar Harbor, Me). *Rag2*-/-, *Il7r*-/-, *Il12p40*-/-, *Ifnar1*-/-, *Tmem173*-/-, and *Stat6*-/- mice were obtained from The Jackson Laboratory and bred in-house. Red5 mice were obtained from Dr. Nunzio Bottini (UCSD), originated from Dr. Richard Locksley (UCSF), and bred in-house (29). Smart13 mice were obtained from The Jackson Laboratory, originated from Dr. Richard Locksley (UCSF), and bred in-house (28). *Il18r1*-/- mice were obtained from Dr. Hal Hoffman (UCSD) and bred in-house. All animal experiments were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

In vivo Alternaria and CDG Models

WT, gene knockout, and cytokine reporter mice were challenged intranasally with PBS, 5 ug CDG, 50 ug of *Alt* extract (Greer, lot number 299382), or 5 ug CDG and 50 ug *Alt* extract in 40 uL every 24 h for 3 days and euthanized 24 h following the third challenge for BAL collection and lung tissue harvest. A subset of experiments was performed with only the latter two experimental groups. Further, in select experiments, *Alt* was replaced with exogenous IL-33 (500 ng). Additionally, a subset of experiments was performed with intraperitoneal IFNγ blocking antibody or isotype (333 ug, BioXCell) injections on D-1, D0, and D1. For experiments in which ILCs were isolated, mice were challenged with *Alt* four times over 10 days to expand the ILC population. For experiments investigating early cytokine release, mice were euthanized 3 h following the first challenge. BAL was performed with 2% BSA in PBS; the first draw was 500 uL and draws 2–5 were 600 uL. BAL was centrifuged at 1,500 rpm for 5 min at 4°C and the supernatant was stored at −20°C for ELISA and cells were counted and phenotyped using flow cytometry. Lungs were digested using the Mouse Lung Dissociation Kit (Miltenyi Biotec) according to the manufacturer's protocol, filtered with a 40 um mesh, and cells were counted and phenotyped using flow cytometry.

Flow Cytometry

Mouse BAL and lung cells were suspended in a solution of 2% FBS and 0.01% sodium azide in PBS and counted on a Novocyte (Acea Biosciences). Cells were incubated with an unconjugated mAb to CD16/CD32 for 10 min at 4°C to block non-specific Fc receptor binding and then incubated for 30 min with fluorochrome conjugated antibodies at 4°C. All antibodies were purchased from BioLegend unless otherwise noted. To identify eosinophils (CD45+Siglec-F+CD11c–) and neutrophils (CD45+GR-1+Siglec-F–), BAL or lung cells, or both, were stained with PerCP-conjugated anti-CD45.2, PE-conjugated anti-Siglec-F (BD), FITC-conjugated anti-CD11c, and APC-conjugated anti-GR-1. For the identification of ILCs (CD45.2+lineage–Thy1.2+ lymphocytes), ILC2s (CD45.2+lineage– Thy1.2+ST2+CD127+), and ILC1s (CD45.2+lineage–Thy1.2+ST2–CD127–) lung cells were incubated with FITC–conjugated lineage cocktail (anti-CD3e, anti-GR-1, anti-CD11b, anti-B220, anti-Ter119), anti-CD11c, anti-NK1.1, anti-CD5, anti-FcεR1, anti-TCRβ, and anti-TCRγδ; PerCP-conjugated anti-CD45.2; eFluor 450-conjugated anti-Thy1.2 (ThermoFisher); APC-conjugated anti-ST2; and PE-Cy7-conjugated anti-CD127. In select experiments, ILCs were also stained with PE-conjugated anti-CD69, anti-ICOS, or anti-KLRG1. For the identification of NK cells (CD3-CD49b+NK1.1+ lymphocytes), lung cells were incubated with PerCP-conjugated anti-CD45.2, APC-conjugated anti-CD3, PE-conjugated anti-CD49b, and FITC-conjugated anti-NK1.1.

For transcription factor and STING staining, surface-stained cells were fixed and permeabilized with an intracellular staining kit (ThermoFisher) according to the manufacturer's protocol and stained for 30 min at 4°C with PE-conjugated anti-GATA-3 (ThermoFisher), anti-Ki67 (ThermoFisher), anti-T-Bet, or anti-STING (Sigma).

For ILC and NK cytokine staining, lung cells from WT and knockout mice were incubated for 3 h at 37°C with a phorbol 12-myristate 13-acetate, ionomycin, brefeldin A, and monensin cell stimulation cocktail (ThermoFisher) in RPMI 1640 media supplemented with penicillin/streptomycin, 10% FBS, glutamine, and 2-mercaptoethanol (ThermoFisher). Stimulated cells were surfaced stained for ILC2s and ILC1s as described above, fixed and permeabilized with an intracellular cytokine staining kit (BD) according to the manufacturer's protocol, and stained for 30 min at 4°C with PE-conjugated anti-IL-5 or anti-IL-13 and APC-Cy7-conjugated anti-IFNγ.

Reporter mice were used to assess cytokine staining directly *ex vivo* without stimulation. To visualize *in vivo* IL-13 in Smart13 mice, human CD4 was stained with PE-conjugated anti-human CD4 (28). To visualize *in vivo* IL-5 in Red5 mice, the PE channel was used without any requisite staining (29). ILCs were stained with DAPI (ThermoFisher) to discriminate live and dead cells. Finally, samples were analyzed with a Novocyte (Acea Biosciences) flow cytometer or sorted for *in vitro* stimulation experiments with a FACSAria II (BD) at the UCSD Human Embryonic Stem Cell Core Facility.

In vitro Stimulation

Sort purified ILCs were first allowed to rest *in vitro* for 48 h with 10 ng/ml IL-2 and IL-7. Following media change, ILCs were cultured with either IL-2 (10 ng/ml) and IL-7 (10 ng/ml); IL-2 (10 ng/ml), IL-7 (10 ng/ml), CDG (10 μM); IL-2 (10 ng/ml), IL-7 (10 ng/ml), and IL-33 (30 ng/ml); or IL-2 (10 ng/ml), IL-7 (10 ng/ml), IL-33 (30 ng/ml), and CDG (10μM). After 24 h of stimulation, supernatants were collected for ELISA.

ELISA

ELISAs for IL-5 (R&D), IL-13 (R&D), IFNγ (ThermoFisher), IFNβ (R&D), and IFNλ (R&D) were performed on BAL supernatants according to the manufacturers' protocols. ELISA plates were read on a model 680 microplate reader (Bio-Rad) at 450 nm.

RNAseq

Publicly available RNA sequencing of mouse lung ILC2s and ILC1s (GEO: GSE136156) was downloaded. Reads were then aligned to reference genome mm10 using TopHat. DUST scores were calculated with PRINSEQ Lite and low complexity reads (DUST > 4) were removed from the BAM files. Read counts to each genomic feature are obtained with the HTSeq count program. Differentially expressed genes were identified with DESeq2 and pathway analysis was performed with Metascape.

Data Analysis and Statistics

Flow cytometry data were analyzed using FlowJo version 10.4.1 (Flowjo). For all experiments (except RNAseq), statistical analysis was performed using GraphPad Prism software (GraphPad) using unpaired *t*-tests (2-tailed). *P*-values of < 0.05 were considered statistically significant.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by UC San Diego IACUC.

AUTHOR CONTRIBUTIONS

KC, JB, MA, LN, LL, AL, AS, SR, and SG performed the experiments. KC and TD designed and interpreted the experiments and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.618807/full#supplementary-material

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REVIEW
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Role of ILC2 in Viral-Induced Lung Pathogenesis

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Innate lymphoid type-2 cells (ILC2) are a population of innate cells of lymphoid origin that are known to drive strong Type 2 immunity. ILC2 play a key role in lung homeostasis, repair/remodeling of lung structures following injury, and initiation of inflammation as well as more complex roles during the immune response, including the transition from innate to adaptive immunity. Remarkably, dysregulation of this single population has been linked with chronic lung pathologies, including asthma, chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrotic diseases (IPF). Furthermore, ILC2 have been shown to increase following early-life respiratory viral infections, such as respiratory syncytial virus (RSV) and rhinovirus (RV), that may lead to long-term alterations of the lung environment. The detrimental roles of increased ILC2 following these infections may include pathogenic chronic inflammation and/or alterations of the structural, repair, and even developmental processes of the lung. Respiratory viral infections in older adults and patients with established chronic pulmonary diseases often lead to exacerbated responses, likely due to previous exposures that leave the lung in a dysregulated functional and structural state. This review will focus on the role of ILC2 during respiratory viral exposures and their effects on the induction and regulation of lung pathogenesis. We aim to provide insight into ILC2-driven mechanisms that may enhance lung-associated diseases throughout life. Understanding these mechanisms will help identify better treatment options to limit not only viral infection severity but also protect against the development and/or exacerbation of other lung pathologies linked to severe respiratory viral infections.

Keywords: ILC2, RSV, RV, influenza, SARS-CoV-2, asthma, COPD, IPF

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INTRODUCTION

Innate lymphoid type-2 cells (ILC2) are a rare population of lymphoid cells that unlike T cells or B cells do not contain an antigen receptor and therefore respond to the immune environment within the tissue compartment. Other ILC populations include ILC1 and ILC3 that differ by their transcriptional regulation and the cytokines that they produce. ILC2 can be differentially characterized as lineage negative, Sca-1+, GATA3+, ST2+, CD25+, ICOS+, and c-kit+ (1, 2). ILC2 differentiation can be driven by innate cytokines, such as TSLP, IL-25, and IL-33, and are characterized by expression of GATA3 and production of cytokines, including IL-4, IL-5, IL-9, IL-13, as well as amphiregulin (AREG). They develop from a common lymphoid progenitor (CLP) in the bone marrow that gives rise to the ILC2 lineage-specific progenitor (ILC2p). The development of this progenitor relies on GATA3 (3–7) and

the transcription factor retinoic acid receptor-related orphan nuclear receptor-α (RORα) (7, 8) which is also expressed in common ILC progenitors (7) (**Figure 1**). Mjosberg et al. identified that human ILC2 are also dependent on the transcription factor GATA3 and the innate cytokine, TSLP (5). In addition to inherent transcriptional properties, tissue-specificity has also been shown to play a strong role in ILC2 effector functions (9). Recent mouse studies have indicated that SCF/c-kit activation of ILCp/ILC2 populations within lungs of allergic mice induces important transcription factor expression, ID2 and GATA3, that lead to differentiation and production of IL-5 and IL-13 by ILC2 (10). Thus, the differentiation of ILC2, while not completely defined, appears to require multiple stimuli that likely help to dictate its tissue function under diverse disease responses.

ILC2 are considered the innate counterpart of Th2 cells based on their expression of the transcription factor GATA3 and the production of Th2-type cytokines. It has been suggested that ILC2 are far more potent than CD4+ T cells in their induction of type 2 cytokines; in fact, it is estimated that ILC2 produce 10 times more cytokine than T cells on a per cell basis (11). Innate lymphoid cells play key roles in lymphoid tissue development as well as the initiation of inflammation and more complex roles during the immune response, including the transition from innate to adaptive immunity and chronic inflammation (12, 13). Interestingly, ILC2 may be necessary during early-life for development of the lung and are known to assist in repair and/or remodeling of the lung following injury. However, the numbers increase in the lung during pathogen infections or damaging responses that may require repair of the epithelium. The increased numbers and activation of ILC2 can also lead to chronic disease phenotypes and lead to lung destruction (14–17). TSLP, a cytokine responsible for ILC2 differentiation, appears to be required for initiation/persistence of airway remodeling during chronic allergic asthma (18). The early over-expression of specific cytokines by ILC2 can lead to eosinophilia (IL-5), mucus production (IL-13) and lung remodeling (amphiregulin, AREG). Additionally, studies have suggested that there are subsets of ILC2 that perform different roles in repair and disease with differential contributions to disease phenotypes and ILC2 may have plasticity to allow them to respond appropriately into ILC2 subtypes based upon the immune environment. The effects of early-life pathogenic ILC2 induction may be long-lasting and have an impact on the lung environment well into adulthood as well as contributing to chronic diseases associated with aging. For example, early-life respiratory insults have been linked with an enhanced likelihood of developing asthma and chronic obstructive pulmonary disease (COPD) through long-term lung remodeling (19–21). In addition, ILC2 are known to lead to acute exacerbation of COPD through increased numbers of cells during viral infection leading to enhanced inflammatory damage and in some cases conversion from ILC2 to pro-inflammatory ILC1 (16, 22). Furthermore, as the lung ages, a loss of lung function occurs along with decreased elasticity and changes in the

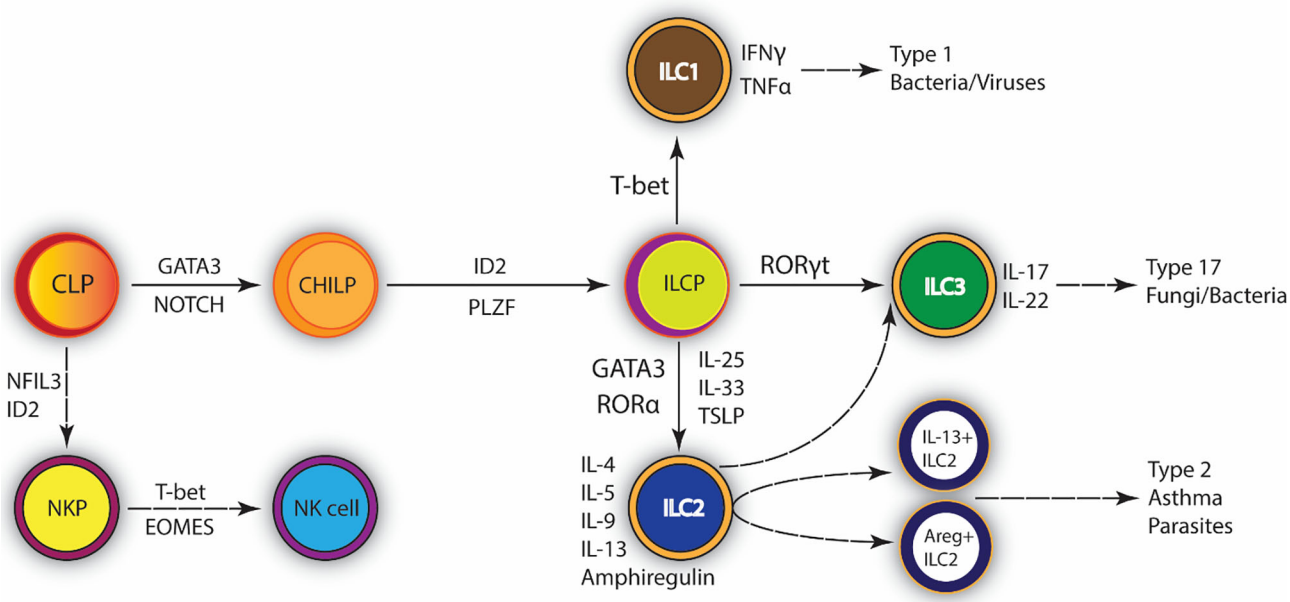

FIGURE 1 | Development of Type 2 Innate lymphoid cells (ILC2). ILC2 promote type 2 inflammation and tissue repair. They are mainly involved in the immune response against extracellular parasites. Common lymphocyte progenitor differentiates into ILC progenitor. GATA-3 is the central transcription factor required for their maintenance and survival. ILC2 are activated by IL-25, IL-33, and TSLP; upon infection they secrete IL-4, IL-5, IL-9, IL-13 and Amphiregulin (Areg). Exaggerated ILC2 immune responses can lead to the development of asthma. CLP, Common lymphoid progenitor; CHILP, Common helper and Innate lymphoid progenitor; ILCP, Innate lymphoid cell progenitor; GATA3, GATA-binding protein 3; ID2, inhibitor of DNA binding 2; NKP, NK precursor; NK, Natural killer cell; PLZF, promyelocytic leukemia zinc finger protein.

immune response (23) with ILC2 activation leading to enhanced viral-induced damage especially in patients with underlying disease. Interestingly, ILC progenitors in aged mice are increased in bone marrow, but reduced maintenance and function of the cells were observed in the lung (24). Several pulmonary diseases occur during aging and whether the prevalence of ILC2 activity alters the severity of these diseases is presently unclear.

In this review, we will focus on the role of ILC2 during respiratory viral exposures and their effects on the induction and regulation of lung pathogenesis to provide insight into ILC2-driven mechanisms that may enhance lung-associated diseases throughout life. Understanding these mechanisms will be crucial for developing therapeutic as well as prophylactic treatments to protect against initial viral disease as well as the development and/or exacerbation of other lung pathologies linked to severe respiratory viral infections.

ILC2 IN THE DEVELOPING LUNG

Lung ILC2 are tissue-resident lymphocytes that develop early, arising in the fetal mouse lung by embryonic Day 17.5 (25) persisting throughout life (25, 26). Studies have shown that newborn mouse lungs contain very few lymphocytes (27). During the first 3 weeks of life, T, B, and natural killer (NK) cells steadily increase to adult levels, while ILC2 increase more rapidly and reach the adult level by Postnatal (PN) Day 8 and further increase between 10 and 14 days of age when they return to adult equivalent levels (27). The most active phase of lung development (alveolar septation) occurs through the second postnatal week in mice and until 2 to 3 years in humans (28–30) which coincides with the early-life lung predisposition to a type 2 immune environment. ILC2 expansion begins soon after birth when neonatal mouse lungs are activated by IL-33 which persistently alters ILC2 activation and responsiveness throughout life (25, 27, 28, 31–34). Furthermore, Rock and colleagues have identified ILC2 production of IL-13 as being critical for lung regeneration following pneumonectomy lung injury (35). While ILC2 first appear during fetal hematopoiesis, their presence in tissues is established during early postnatal development through rapid expansion, priming, and acquisition of tissue-defining genes (25). This led to the discovery that there are three distinct waves of ILC2 development during early-life that include 1) dispersal into tissues, 2) expansion and activation of tissue-specific transcriptional programs, and 3) homeostatic maintenance with differences in local regulation of survival and turnover throughout life (25). Multiple other studies have shown that many of the ILC2 present in the adult lung were cells that developed during the perinatal period (27, 31, 33). Upon the first breath after birth, IL-33 is spontaneously released from the epithelium which leads to rapid expansion and activation of lung ILC2 (7, 28, 32, 33). In one study, IL-33 production led to a marked increase in ILC2 numbers by PN Day 7 that continued to increase until stabilized by 6 weeks of age (32). Additionally, it was determined that IL-13+ILC2 began to expand at PN Day 3, peaked around PN Day 10, and started to decline at PN Day 14 dependent on IL-33. A similar study by de Kleer et al. supports these findings by showing that type 2 immune cells, including ILC2, accumulate in the lungs at PN Day 3 which leads to enhancement of Th2 responses compared to weanling and adult mice dependent upon IL-33 signaling (28). Importantly, it has been determined that IL-33 activation of ILC2 during the mouse neonatal period has long-lasting effects on ILC2 activation into adulthood which leads to heightened IL-13 production (33). However, it was also observed that a lack of IL-33, using IL-33-deficient mice, had no obvious negative effects on alveolarization (32), indicating that the IL-33 signaling pathway during early-life may be more important for induction of the ILC2-driven type 2 immune response while lung development may be occurring through an alternative or compensatory pathway.

Utilizing an ROR-α lineage tracing model, it has been identified that the neonatal mouse lung consists of distinct ILC2 subsets that have either proinflammatory (Th2 cytokine-expressing) or tissue-repairing (AREG-expressing) properties (7). In addition to these two subsets, it was also shown that the neonatal lung contains an ILC progenitor population (IL-18Rα+/ST2-) that is similar to adult lung ILC progenitors capable of differentiating into multiple different ILC populations. The two distinct neonatal ILC2 effector subsets can be further identified by the genes expressed (7). Both subsets express equal amounts of *Id2, Gata3, Thy1*, and *Il1rl1* (*St2*) but can be further divided into *Il5+/Il13+/Arg1+/Klrg1+* proinflammatory ILC2 or *Areg+/Icos+* tissue-repairing ILC2. Interestingly, it has been determined that only the *Il5+/Il13+/Arg1+/Klrg1+* proinflammatory ILC2 subset is dependent on IL-33 regulation (7) and the activation pathway for the *Areg+/Icos+* tissue-repairing ILC2 subset has yet to be elucidated. These findings, along with those by Saluzzo et al. that determined that IL-33 is not required for alveolarization (32), suggest a possible role for *Areg+/Icos+* tissue-repairing ILC2 in the lung developmental process. Furthermore, a recent mouse study indicated that bronchopulmonary dysplasia (BPD), a severe complication of the respiratory system seen in preterm infants, was induced by IL-33/ILC2 signaling caused by an arrest in the process of alveolarization, determining a major destructive role of tissue-resident IL-13+ILC2 in the lung (36). This differentiation could help to identify mechanisms for divergent responses caused by alterations in ILC2 during early-life. It is realistic to assume that in some instances, a loss of AREG+ ILC2 may be detrimental whereby in other cases, the enhancement of IL-13+ ILC2 may be differentially regulated and detrimental. Thus, preferential differentiation of ILC2 that produce IL-13 may be inappropriate and pathologic leading to inflammation and altered lung development. Furthermore, these early developmental changes likely have long-term effects on childhood and adult lung function.

ROLE OF ILC2 IN TH2-INDUCTION DURING RESPIRATORY VIRAL INFECTION

Respiratory infections are responsible for significant healthcare burden throughout the world largely due to the development of

lower respiratory tract infections (LRTIs), including bronchiolitis and pneumonia. LRTIs are the leading cause of infectious death in children under the age of five (37). Approximately 80% of LRTI cases are caused by viruses. Among the most prevalent are infections with respiratory syncytial virus (RSV), rhinovirus (RV), and influenza virus. A study evaluating infants hospitalized due to bronchiolitis determined that disease correlated with increased numbers of ILC2 in the nasal passages (38). A mouse study evaluating the immune response to influenza was the first to identify the previously unknown non-T/B cell innate lymphoid "natural helper cell" (ILC2) involvement in respiratory viral infection (39). This latter study determined that IL-13-producing ILC2 were activated through IL-33 production from alveolar macrophages following NLRP3-inflammasome activation by the influenza virus leading to airway hyperreactivity (AHR) (39). Further studies using animal modeling, have discovered that ILC2 are increased in the lungs following other respiratory virus infections, such as RSV and rhinovirus infection (11, 40–48). Also, of note, early in life males are more susceptible to severe disease caused by respiratory viruses compared to females. In the case of early-life RSV, males are hospitalized at 2:1 ratio compared to females due to lower respiratory tract diseases, including bronchiolitis and pneumonia (49). Females tend to have stronger Th1 responses than males, with higher levels of inflammatory markers and viral infection clearance (50, 51) which may induce better protection against infection. Furthermore, clinical studies in children have identified increased atopic diseases in boys compared to girls (50, 52). In support of these findings, blood ILC2 numbers have been shown to be increased in neonates compared to adults with neonatal males having significantly higher levels of ILC2 than neonatal females although no differences were observed in adult men vs woman (53). Interestingly however, testosterone down-regulates ILC2 function, type 2 cytokine production, and expansion during asthma, possibly explaining a reduction in asthma in males post-puberty compared to females (54, 55). Supporting these observations are animal studies that supplement with testosterone to down-regulate ILC2 function and attenuate Th2 cytokine driven allergic disease (54). Thus, age-associated ILC2 function related to early-life viral responses may be mitigated later in life by sex-associated mechanisms in males.

Respiratory Syncytial Virus

The secretion of innate cytokines, such as IL-25, IL-33, and TSLP, following infection of airway epithelial cells by RSV, leads to the initial activation of the immune response, including the activation of ILC2 (56). While epithelial cells have been directly shown as the main source of these innate cytokines, the brush cells within the lung may also contribute to ILC2 induction in a similar manner as the IL-25 released by tuft cells in the intestine (57) and require further evaluation to expand upon lung ILC2 biology. Importantly, elevated levels of ILC2 were identified in nasal aspirates of infants hospitalized with severe RSV compared to infants with moderate disease and correlated with increased TSLP and IL-33 (58). Stier et al. (11), link the early induction of effector ILC2 to the development of AHR and mucus production associated with RSV through TSLP-driven induction of IL-13-producing ILC2 (11). Administering an anti-TSLP antibody after RSV infection significantly reduced the levels of IL-13-producing ILC2 suggesting a potential therapeutic target. Studies have also shown that RSV driven IL-33-activated ILC2 were crucial for the development of airway inflammation, including eosinophilia and AHR, through ILC2-specific IL-13 production (59). RSV-induced IL-33 appears to be regulated by the type 1 interferon (IFN)/STAT1 pathway with deletion of STAT1 promoting ILC2 activation and Th2 cytokine production (60). Interestingly, age-related IL-33 production was shown to be necessary to induce ILC2 following neonatal but not adult RSV infection that led to Th2-driven immunopathology (41). It has also been identified that ILC2 numbers remain increased in mouse lungs as far out as 4 weeks post-infection following neonatal RSV infection of 7 day old mice along with increased expression of *Il33* and *Tslp*, and the effector cytokines, *Il5* and *Il13* (43, 44). Studies by Fonseca et al. have identified that ILC2 upregulation following RSV in both neonates and adult mice can be driven by uric acid pathways and innate cytokines (e.g. IL-1β, CCL-2, IL-33) that promote ILC2/IL-13-driven Th2 response and immunopathology (43, 45). Finally, ILC2 may regulate RSV-induced CD4+T cell expansion and cytokine expression (especially IL-4/IL-5/IL-13) *via* OX40/OX40L interaction (61). These findings suggest a strong correlation between ILC2 and the development of severe respiratory disease following early-life RSV infection.

Rhinovirus

In addition to RSV-driven immunopathology, ILC2 have also been implicated in pathologies related to early-life rhinovirus (RV) infection (40, 42, 46–48). Similar to RSV studies, it was determined that rhinovirus infection in neonatal mice led to increased IL-13-producing ILC2 that was not seen in mature mice (47). However in this instance, IL-25 was shown to be the key cytokine responsible (47). Further evaluation of this pathway revealed that following neonatal RV infection both IL-33 and TSLP are required for IL-25-induced ILC2 production of IL-13 leading to immunopathology but TSLP was necessary for maximal ILC2 gene expression even in the presence of IL-25 and IL-33 (40). These results suggest that this group of alarmin cytokines cooperate and regulate each other during respiratory viral infections to expand and fully activate ILC2 populations. One suggested mechanism for the severe disease observed in neonates compared to adults is a lack of a strong Th1/IFN-γ immune response. Immature mice do not induce this pathway whereas adult mice induce strong IFN-γ following RV infection (48). This study showed that administration of IFN-γ immediately following RV infection of neonatal mice attenuated signs of immunopathology suggesting that the lack of IFN-γ is correlated with severe disease. Furthermore, a direct effect of IFN-γ on ILC2 was suggested since there was a reduction in IL-13-producing ILC2 without altering the expression of IL-25, IL-33 or TSLP (48). Importantly, studies that limit ILC2 induction, utilizing an ROR-α inhibitor (SR3335) or genetic deletion of ROR-α, either using complete knockout mice (ROR-$\alpha^{sg/sg}$) or conditional knockout mice to target only ILC2 (*Rora/*

Il7r^{Cre+}), led to protection from severe disease induced by RV infection (42, 46). Unlike RSV infection (43, 45), RV infection did not drive IL-1β, and neonatal infection in mice lacking IL-1β signaling (NLRP3-/- mice or chemical inhibition of IL-1β) led to enhanced RV-induced Th2 cytokine expression and mucus metaplasia (62). In contrast, another study indicated that NLRP3-inflammasome activity and IL-1β were required for RV-induced airway inflammation and AHR in adult mice (63). The discrepancy between these latter studies may be due to neonatal vs adult mice used in the studies as they also show that adult mice induce IL-1β to a greater extent than neonatal mice following RV infection.

Influenza Virus

Interestingly, while influenza infection is typically associated with Th1-type immune responses, one of the first studies to identify the role of lung ILC2 during viral-induction of Th2 cytokines was found using influenza (39). The authors identified a lymphoid cell of non-T/B cell origin within the lungs, termed "natural helper cells" which induced IL-13 in response to IL-33 following influenza A infection in mice that led to AHR (39). These results were confirmed by another mouse study that showed ILC2 produced significant Th2 cytokines following pandemic influenza infection (pH1N1) along with increased IL-33 in the lungs and were responsible for the development of AHR, independent of adaptive immunity (64). Another study revealed that c-kit^{+} IL-5-producing ILC2 were activated by IL-33 released from NK T cells and alveolar macrophages and led to eosinophil accumulation (65). A recent mouse study determined that type 1 IFN deficiency leads to ILC2 activation and type 2 immunopathology during influenza A virus infection (66). Furthermore, this study found that type 1 IFN directly negatively regulates both mouse and human ILC2 through regulation of IL-33. Additionally, the authors show that IFN-γ and IL-27 also regulate ILC2 in a STAT1 dependent manner (66) similar to that observed during RSV infection. However, a contradictory study showed that lack of IFN-γ led to protection from lethal infection with pandemic H1N1 and that this was dependent on ILC2 production of IL-5 and AREG which led to increased tissue integrity and reduced immunopathology (67). It is important to note that the latter study did not show differences in IL-13+ ILC2 populations whereas the previous study indicated reduction of IL-13 by the presence of type 1 IFN but not IFN-γ. These studies suggest that type 1 IFN and IFN-γ have different regulatory actions during influenza virus infection and that they appear to modify ILC2 with vastly different results supporting the possibility that there are subsets of ILC2 that are proinflammatory (IL-13+) and another that is responsible for lung repair (AREG+).

SARS-COV-2 Induced ILC2

Severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) is the newly identified β-coronavirus responsible for the pandemic viral pneumonia known as COVID-19. The risk for severe illness with SARS-CoV-2 increases with age, with older adults (>65 years) having five times more probability of developing severe COVID-19 disease (68). It has been reported that there is an increased level of systemic IL-33 in the serum and plasma of severe COVID-19 patients, together with an increased number of circulating ILC2 (69). Furthermore, it has been reported that increased levels of IL-18, IL-13, and IL-6 were increased along with accumulation of ILC2 during COVID-19 that could be linked with the severity of the diseases. A separate study reported increased circulation of ILC2 in moderate but not severe COVID-19 patients suggesting that ILC2 could be differentially regulated based on the severity of the diseases. Low ILC2 could be a marker of severe infection (70). This latter concept may be consistent with reports that ILC2 numbers are decreased by IFN-γ during influenza-induced disease and correlated to more severe disease phenotypes (67). In fact, an original report suggested that ILC2 were responsible for promoting epithelial repair post-influenza infection allowing tissue homeostasis to be reestablished (71). Thus, while ILC2 may provide signals that promote inflammation and pathology, such as IL-13, they likely also have important roles in development and tissue repair early in life and during severe viral-induced epithelial cell damage, perhaps through the differential production of AREG.

TRAINED ILC2 IMMUNITY AND EXACERBATED LUNG DISEASE

Lasting Impact of Activation of ILC2 in Lung Development and Viral Infections

A strong Th2-immune environment in early-life appears to promote preferential development of Th2 responses that may have long-term consequences on pulmonary diseases (6, 27, 32, 72, 73). Numerous studies suggest that IL-13+ILC2 are responsible for a persistent inflammatory lung environment following early-life viral infection that exacerbates secondary responses later in life (41, 43, 44). Studies have also shown that induction of IL-33 within the lung during early-life leads to enhanced activation of ILC2 which persists well into adulthood (33, 34) and therefore may promote lung structure and functional changes. Furthermore, recent studies have identified that frequent intranasal papain administration, or IL-33 administered in the presence of retinoic acid, may train ILC2 into an "exhausted" phenotype which induces IL-10 that could be harnessed as a potential treatment option for allergic responses to overcome the inflammatory effects of these cells (74).

The phenomenon of "trained immunity" or immunological recall of innate cells, such as myeloid cells (macrophages, dendritic cells, etc.), NK cells and innate lymphoid cells, has been explored in recent years. In the case of ILC2, neonatal "training" has been suggested to impact the activity of these cells during immune responses in adulthood (33, 34, 75). As noted previously, acute expansion and activation of ILC2 occurs shortly after birth and lineage tracing studies determined that 40-70% of these postnatally-derived ILC2 were present in the adult lung (25). This and many other studies have determined that the activity of these cells is dependent on their exposure during the

early postnatal period. Intranasal administration of allergen or IL-33 to mice led to ILC2 expansion and activation in the lung which persisted for up to 1 year after initial administration (34). Importantly, using 5-bromo-2′-deoxyuridine (BrdU)-labeling, the studies show that these were long-lived ILC2 and upon an unrelated allergen challenge or IL-33 administration later in life showed exacerbated responses due to ILC2 production of IL-5/IL-13. Likewise, Steer et al. showed that neonatal mouse lung ILC2 activation by IL-33 has significant effects on ILC2 activity during adulthood (33). Most neonatal lung ILC2 incorporated BrdU and persisted into adulthood. Adult lung BrdU+ ILC2 responded more intensely to IL-33 treatment compared with BrdU– adult lung ILC2. In IL-33-/- mice, lung ILC2 developed normally but because they are not activated during the neonatal period, they have a dampened response in adulthood compared with WT ILC2 (33). Together, these results suggest that activation of lung ILC2 by IL-33 during early-life may "train" ILC2 within the lung that become long-lived resident cells that have stronger type 2 responses to challenges later in life. As indicated in the previous sections, early-life respiratory viral infections likely enhance disease associated "training" that can influence later disease phenotypes (41, 43, 44, 46).

Viral-Induced Asthma Exacerbations

Asthma often starts during early childhood and according to the Centers for Disease Control, 1 in 12 children (~8%) had asthma in 2017. Asthma exacerbation can be life threatening and often requires hospitalization (76). Viral-induced exacerbation in established pediatric asthmatics is of great concern causing over 80% of exacerbation in these patients with RSV and RV as the most common causes (77). In a cross-sectional, analytical study of children 5-15 years of age that were admitted to the hospital for exacerbated asthma, 20% of those were due to viruses, especially RV and RSV (78). Furthermore, males were hospitalized to a greater extent than females, with boys accounting for ~57% of patients (78) supporting previous findings that males are more susceptible to both viral infection and childhood asthma. The role of ILC2 in asthma and airway disease has been studied recently and reviews describing their role have previously been published (79–82). In severe steroid resistant asthma, ILC2 numbers correspond to the severity of disease exacerbation demonstrating a systemic effect of the disease process (83, 84). The Th2 immune response has been correlated with these exacerbations and since respiratory viral infections enhance Th2 responsiveness of ILC2, it is likely that these cells play a crucial role in exacerbation of asthmatic disease (85). One study first identified that patients with asthma that were subsequently exposed to RV experienced greater RV-induced morbidity and had higher viral loads than their healthy counterparts with increased nasal and bronchial Th2 cytokine levels (86). In addition, asthmatic patients also had increased nasal IL-33 following RV infection. To further explore these findings, human bronchial airway epithelial cells were exposed to RV that led to the induction of IL-33. When the RV-infected epithelial cell supernatants were co-cultured with human ILC2, a strong induction of IL-5 and IL-13 was observed indicating an IL-33/ILC2 pathway activation following RV infection in humans (86).

Mouse models have been developed to begin to unravel the mechanisms of viral-induced asthma exacerbation (87–91). For example, studies have identified that RV leads to the exacerbation of previously established ovalbumin allergy through macrophage/epithelial CCL2-signaling (89) and eotaxin release from macrophages (88). Another study determined that influenza infection potentiates the exacerbation of house dust mite allergic responses (HDM) (91). However, so far, animal studies supporting direct ILC2 involvement in respiratory viral exacerbation of asthma have been limited and require further investigation as this could lead to discovery of new therapeutic targets. In a mouse study evaluating influenza-induced exacerbation of HDM allergic responses, it was determined that CD4+ T cells were the major source of IL-5 and IL-13 early during the exacerbation (Day 4) but at later timepoints (Day 7-11), ILC2 contributed more to the total number of IL-5/IL-13-producing cells (92). ILC2 appear to be a major source of IL-5 and IL-13 as assessed by the intensity of intracellular cytokine staining (92), indicating higher cytokine production per cell as previously suggested (11). These studies support a role for ILC2 at the time of severe exacerbation as complications and hospitalization during asthma exacerbation often occurs 7-10 days post-viral infection (76). Thus, targeting ILC2 promoting mechanisms may provide a therapeutic option for Th2-associated asthmatic disease. Recent clinical trials using either anti-TSLP or anti-IL-33, which should target ILC2 development and activation, are only now reaching Phase 2/3 human studies but show some promise possibly through regulation of ILC2 function.

Viral-Exacerbated COPD and ILC2

Chronic obstructive pulmonary disease (COPD) is characterized by severe chronic airway epithelial inflammation that leads to airway remodeling (93), characteristic thickening of the airway wall, increased layers of airway smooth muscle, and increased extracellular matrix (94). It has been reported that worldwide the prevalence of COPD in the population over 40 years is higher in smokers and ex-smokers, and the prevalence increases significantly among persons over 60 years of age (95). While continuous exposure to inhaled irritants that damage the airway function and structure are associated with all causes, the most significant risk factor for COPD is smoking, with ~50% of smokers developing COPD. Respiratory viral infections have been linked with worse outcomes than bacteria exacerbation episodes (96). A recent review of respiratory virus in COPD patients showed that the most common lower respiratory tract virus infections identified in acute exacerbation (AE-COPD) patients were RV (16.39%), RSV (9.9%), and influenza virus (7.83%). Overall, coronaviruses were more frequently detected in the upper respiratory tract than any other virus (97). A recent clinical study indicated increased serum IL-33 levels and numbers of peripheral blood ILC2 in AE-COPD when compared with COPD stable patients and healthy controls (22). When the signature transcription factors were analyzed, they observed increased expression of *Gata3* and *Rora* in the ILC2 sorted from AE-COPD compared with healthy controls and stable COPD patients. Moreover, the expression of these transcription factors was upregulated in the ILC2-sorted cells from stable

COPD patients compared with healthy controls (22), suggesting that the proliferation and activation of ILC2 are associated with AE-COPD and that active ILC2 could be involved in the pathogenesis of COPD. While ILC2 have been most notably explored in COPD patients and in animal modeling, ILC3 and production of IL-17 has also been suggested. Importantly, ILC2 exposure to IL-12 during viral exacerbation can mediate conversion to IFN-γ-producing ILC1 correlated with the severity of COPD exacerbations, demonstrating that ILC2 plasticity exists (16).

Lung ILC2-related enzyme arginase 1 (Arg1) is upregulated in asthmatic, idiopathic pulmonary fibrosis (IPF), and COPD patients and is a marker for lung ILC2 (98). Arg1 is part of the L-arginine metabolic pathway and drives collagen synthesis as well as bioenergetic pathways critical for cell proliferation (99). It has been shown that Arg1 ILC-intrinsic deletion abrogated type 2 lung inflammation by decreasing ILC2 proliferation and activation (100). Furthermore, the latter demonstrated that tissue samples from COPD and IPF patients presented ILC2-arginase positive staining, suggesting that ILC2 in COPD and IPF could be targeted with inhibitors of Arg1 to control ILC2-induced disease responses. To understand the role of ILC2 in the development of COPD, an experimental mouse model of COPD (exposure to cigarette smoke for 12 weeks) was performed using ILC2-deficient mice ($Rora^{fl/fl}/Il7r^{Cre}$). Cigarette smoke-exposed $Rora^{fl/fl}/Il7r^{Cre}$ mice were protected from emphysema development but interestingly presented increased IL-33, IL-13, and ILC2 numbers (101). However, it should be noted that there was no viral infection of these animals and the subsets of ILC2, such as Areg+ILC2, were not examined. Overall, the role of ILC populations in the development or exacerbation of COPD has not been clearly defined and will require future investigation but correlations suggest ILC subsets are involved in COPD pathogenesis, especially AE-COPD groups.

ILC2 and Idiopathic Pulmonary Fibrosis (IPF)

Interstitial lung diseases can cause significant morbidity primarily in older individuals when accompanied by compromised lung function. Idiopathic pulmonary fibrosis (IPF) is likely the most severe form of interstitial disease. Several risk factors have been linked with the development of IPF, including smoking, environmental inhaled exposures, chronic viral infections, genetics and comorbidities (102). Hallmarks of Aging: abnormal telomere shortening, mitochondrial dysfunction, cellular senescence, impaired autophagy, and epigenetic reprogramming, among others, are suggested to be essential during IPF pathogenesis (103). Furthermore, IPF has been linked with IL-13 and TGF-β production and ILC2 have been recognized as an essential source of these cytokines within the lung (10, 104). Increased levels of epithelial-derived cytokines IL-33, TSLP, and IL-25, critical activators, and recruiters of ILC2, have been detected in lung tissue and/or bronchoalveolar lavage (BAL) fluid of IPF patients (17, 105, 106). Together, these data suggest that ILC2 could play a critical role in the pathophysiology of IPF. A mouse model of bleomycin-induced pulmonary fibrosis showed a similar trend, with increased expression of IL-33 in the lung (107). In this latter study, bleomycin-induced pathology was not affected by deficiency of the IL-33 receptor (ST2) that is associated with ILC2 activity. Interestingly, the delivery of IL-33, using adenovirus-targeted delivery, during bleomycin treatment, showed a synergistic effect on airway inflammation, collagen accumulation, upregulation of heat shock protein 70 (HSP70), TGF-β, IL-6, CCL2/MCP-1, MIP-1α and TNF-α, but did not alter type 2 cytokines (107). Another bleomycin study in mice determined that fibrosis development was dependent on IL-33/ST2 signaling and that the adoptive transfer of ILC2 into the lung during treatment led to enhanced disease (108). In addition, systemic sclerosis patients were found to have increased tissue ILC2 that correlated with both fibrotic skin lesions as well as the presence of interstitial lung disease (109). Acute exacerbation of IPF (AE-IPF) is associated with increased mortality. In a clinical study where AE-IPF patients were tested to identify the pathogen (e.g. virus vs bacteria) involved in exacerbation, viral-positive nasopharyngeal swabs were reported in 60% of these patients (110), suggesting that respiratory virus incidence in the development of AE-IPF is higher than bacterial infection. As described above, RSV and RV immunopathology is highly linked to ILC2 activation and recruitment and therefore, these viruses could promote further acceleration of the fibrotic responses. Other viral infections including herpes viruses and cytomegalovirus have been implicated in the progression of IPF. However, no data have established a link of these latter viral infections with ILC2 biology. Additional investigations into the role of ILC2 in promoting lung remodeling may identify them as having an important role in the progression of interstitial lung diseases. Thus, while a causal effect of ILC2 for the development of pulmonary remodeling has not been established, the ability of ILC2 to produce IL-13 and/or AREG that can promote myofibroblast activation provide a rationale for further investigating their role in these chronic remodeling diseases.

STRATEGIES TO MITIGATE ILC2-INDUCED PATHOLOGY

Over the past several years a number of therapeutic targets have been advanced to clinical trials that can impact ILC2 activation, expansion, and mediator release that effect clinical disease. Two targets, TSLP and IL-33, appear to have significant impact on the development of chronic severe asthma responses, especially related to exacerbations. As indicated in the previous pre-clinical research, ILC2 likely play a critical role in not only maintaining a Th2 phenotype in the lung, but also for directing an inappropriate anti-viral response that leads to a worsening of the tissue responses. While the clinical studies have not reported ILC2-associated changes specifically, the biology would predict that there would be a significant effect on ILC2 over time, allowing a more appropriate immune environment. TSLP and IL-33 inhibition may alter ILC2 biology and therefore target their development, however, complementary targeting of ILC2

products may be more successful. The ability to target IL-13 biology has already been shown to be effective in chronic Th2-mediated disease, including asthma, with the IL-4Rα antibody, Dupilumab. While all of these ILC2-related targets hit many aspects of chronic disease biology, their role in long-term disease mitigation likely impacts ILC2 as one of the central components of disease severity. Future targets may depend upon the disease and the pathologic phenotype of the response, such as targeting AREG during chronic remodeling diseases, such as IPF. To properly identify viable ILC2 therapeutic targets, future studies examining the mechanisms of how ILC2 subsets impact the lung environment and how ILC (e.g. ILC1/2/3) differentiate will be defining for individual disease phenotypes.

CONCLUSION

Severe lung disease induction by respiratory viruses continues to be a significant healthcare burden and cause of morbidity and mortality worldwide. These diseases have been associated with the induction of strong Th2-type immune responses. ILC2 are now recognized as a significant contributor of dysregulated immunopathology within the lung following respiratory viral infection as well as during the pathogenesis of lung diseases, such as asthma. This review has highlighted the role of ILC2 during the development of the early-life lung and how subsets of ILC2 may become persistently altered following early-life infection to alter immune responses to future pathogens as well as how the effect of ILC2 responses on the aged lung later in life leads to enhanced complications (**Figure 2**). On the contrary, ILC2 may also play a protective role during lung disease pathogenesis by maintaining and/or repairing the lung epithelium. Therefore, critical targeting of specific ILC2 subsets [e.g. proinflammatory (IL-13) or tissue-repairing (AREG+)] will be crucial when evaluating potential therapeutic candidates. Elucidation of the mechanisms in which these cells may be damaging and/or protecting the lung immune and structural environment will help identify better treatment options to not only protect against initial disease but also reduce the development and/or exacerbation of other lung pathologies linked to severe respiratory viral infections.

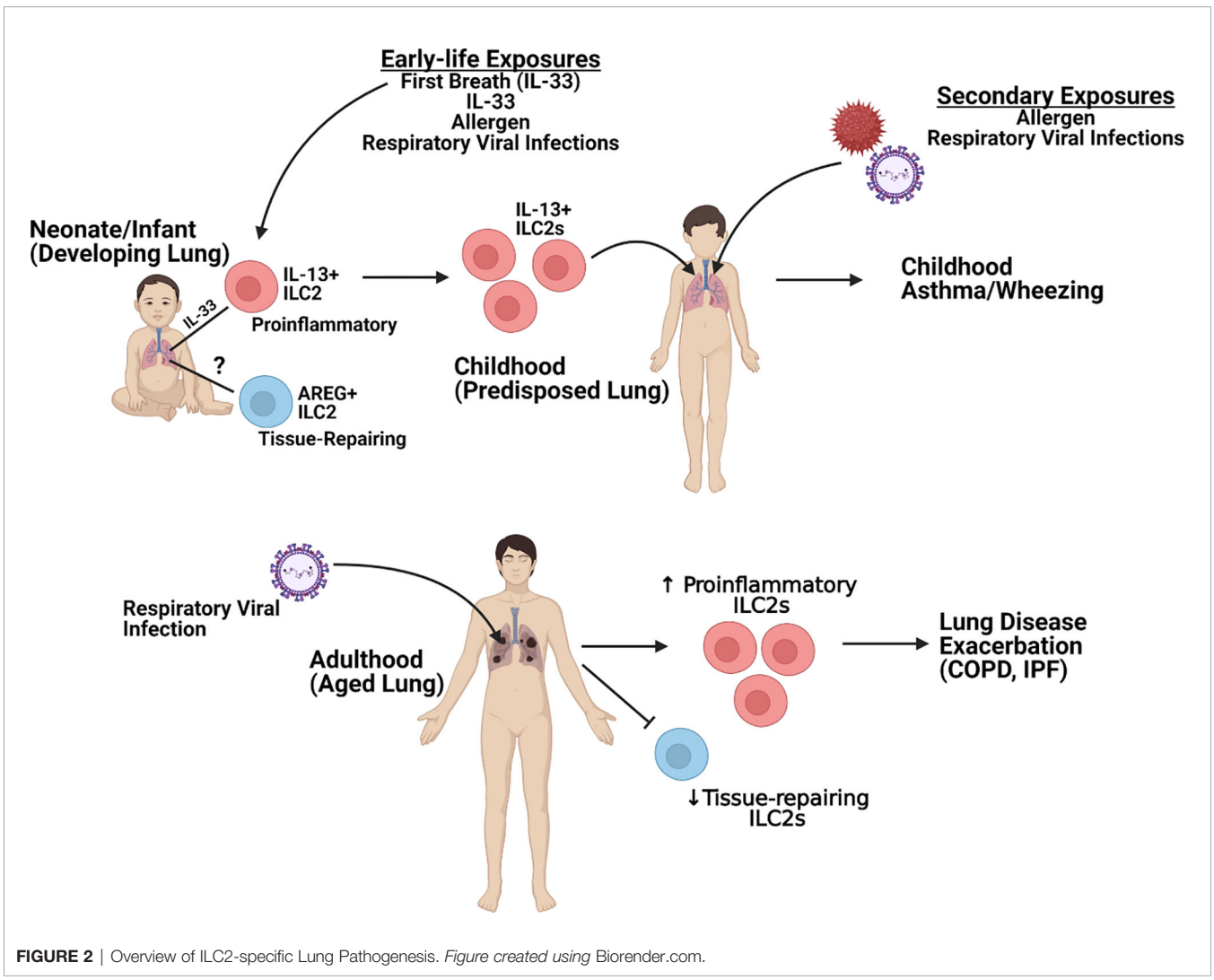

FIGURE 2 | Overview of ILC2-specific Lung Pathogenesis. *Figure created using* Biorender.com.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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The Fate of Activated Group 2 Innate Lymphoid Cells

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Group 2 innate lymphoid cells (ILC2s) reside in both mucosal and non-mucosal tissues and play critical roles in the first line of defense against parasites and irritants such as allergens. Upon activation by cytokines released from epithelial and stromal cells during tissue damage or stimulation, ILC2s produce copious amounts of IL-5 and IL-13, leading to type 2 inflammation. Over the past 10 years, ILC2 involvement in a variety of human diseases has been unveiled. However, questions remain as to the fate of ILC2s after activation and how that might impact their role in chronic inflammatory diseases such as asthma and fibrosis. Here, we review studies that have revealed novel properties of post-activation ILC2s including the generation of immunological memory, exhausted-like phenotype, transdifferentiation and activation-induced migration.

Keywords: innate lymphoid cells, type 2 inflammation, mucosal immunity, immunological memory, neonatal immunity, exhaustion, transdifferentiation, migration

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INTRODUCTION

Group 2 innate lymphoid cells (ILC2s) belong to the family of innate lymphoid cells that include natural killer (NK) cells, helper innate lymphoid cells (ILCs) and lymphoid tissue inducer (LTi) cells, which all share a common lineage. Helper ILCs are classified into three groups, based on their similarities to helper T cell subtypes with respect to the expression of transcription factors and effector cytokines: ILC1s are T-bet$^+$ and produce interferon (IFN) γ, ILC2s highly express GATA3 and release IL-5 and IL-13, while ILC3s are dependent on RAR-related orphan receptor (ROR) γt and secrete IL-22 and IL-17 (1). ILC2s were first identified in 2010 as a population of innate immune cells with a lymphoid morphology which lacks the expression of lineage markers commonly expressed by T, B and myeloid cells (2–4). They were initially termed natural helper cells (2), nuocytes (3) or innate helper type 2 (Ih2) cells (4), but unification of their nomenclature was proposed in 2013, and are now called ILC2s (5). Since their initial discovery in the gut, they have been identified in various other organs in mice, including the lung (6–8), skin (9–11), adipose tissues (12–14), liver (15), pancreas (16) and heart (17, 18), as well as in humans (6, 9, 10, 13, 19) and their pathologic and protective roles in multiple human diseases such as asthma, atopic dermatitis and fibrosis, and infections have been described. ILC2s also play a critical role in adipose tissue homeostasis by sustaining type 2 immunity and promoting beiging of white adipose tissue (12–14), while an ILC2-tuft cell circuit orchestrates intestinal homeostasis and remodeling (20, 21).

ILC2s are known to seed tissues early during development and adapt to environmental cues (22–27); consequently, their phenotype slightly differs depending on their residing organs (**Table 1**).

MouseILC2s express CD45, IL-7Rα (CD127), CD90 (Thy1) and IL-2Rα (CD25) (2–4), while CD103 (11) and IL-18Rα (22) are uniquely expressed by skin ILC2s. In contrast, the expression of IL-33 receptor (ST2) and IL-25 receptor varies depending on the organ. ST2 is expressed by the lung (6, 8), liver (15) and adipose tissue ILC2s (12–14), while ILC2s in the small intestine (SI) express low levels of this receptor (28) and the expression levels vary in the skin (9, 22, 28). In contrast, IL-25R is highly expressed by SI ILC2s (20), while adipose tissue ILC2s do not express IL-25R (22) and both IL-25R positive and low/negative ILC2s have been reported in the skin (11, 20, 22). Interestingly, we have previously shown that the majority of naïve lung ILC2s are negative for this marker, whereas its expression is induced by activation (29).

ILC2s in human peripheral blood (PB) are similar to mouse tissue ILC2s in that they express CD127 and CD25, but are uniquely identified by the expression of chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) and CD161 (19). Unlike mouse ILC2s, human PB ILC2s lack the expression of CD90, ST2, IL-25R and CD103 (19, 30, 31), whereas they are positive for IL-18Rα expression (31).

Due to the species and tissue differences in surface molecule expression and the lack of universal markers for ILC2s, identification of ILC2s can be challenging. Additionally, some surface molecules are downregulated or upregulated upon ILC2 activation or in disease conditions, making it more difficult to definitively identify them. However, the transcription factor GATA3 is highly expressed by mouse and human ILC2s, and therefore, it is a useful marker for ILC2 identification (32, 33).

ACTIVATION OF ILC2s

ILC2s lack antigen specific receptors and hence, their activation is finely regulated by a repertoire of molecules including cytokines, neurotransmitters and lipid mediators. Here, we review the activation of ILC2s by cytokines (direct) and allergens (indirect). The regulation of ILC2 activation and inhibition by other molecules is reviewed in great detail elsewhere (34, 35). Upon activation, ILC2s produce IL-5 and IL-13 among others, resulting in type 2 inflammation characterized by eosinophilia, alternative activation of macrophages, type 2 helper T (Th2) cell differentiation and IgE class switching.

Direct Activation by Cytokines

Alarmins are molecules that are normally present within cells and released upon tissue injury, environmental insults, physiological stress or necrosis (36). They induce activation of various immune cells, resulting in sterile inflammation. ILC2s express receptors for an alarmin cytokine, IL-33, and other secreted cytokines such as IL-25, thymic stromal lymphopoietin (TSLP) and IL-18, and are potently activated by these cytokines.

IL-33 is constitutively expressed in the nuclei of endothelial cells, epithelial cells, fibroblastic reticular cells and adventitial stromal cells (37–42), while IL-25 can be either constitutively expressed as seen in tuft cells (21), or its expression can be induced in immune cells, such as alveolar macrophages (43), mast cells (44), basophils, eosinophils (45) and Th2 cells (46). TSLP expression is induced in epithelial cells in the lung, such as alveolar type II cells (42), whereas it is constitutively expressed in the large intestine (47, 48). Stimulation of IL-33 and IL-25 signaling pathways through their cognate receptors consisting of ST2 and IL-1 receptor accessory protein (IL-1RAcP) (49), and IL-17RA and IL-17RB (50), respectively, induces activation of nuclear factor (NF) κB and mitogen-activated protein kinase (MAPK) (50, 51). This leads to phosphorylation of GATA3, promoting its binding to *Il5* and *Il13* promoters and ILC2 proliferation (52). TSLP binds to the receptor comprised of IL-7Rα and TSLPR (53) and activates a separate downstream signaling pathway involving Janus kinase (JAK) 1/2 and STAT5 (54). STAT5 binds to the *Gata3* gene and together, they regulate ST2 expression, enhancing IL-33 induced activation (55).

In vivo responsiveness of ILC2s towards cytokine stimulation varies depending on their residing tissues. Intranasal (i.n.) administration of recombinant IL-33 potently activates lung ILC2s (29, 56, 57). In contrast, naïve adult lung ILC2s do not respond to i.n. IL-25 or TSLP administration (29, 42, 56, 58, 59). Interestingly, lung ILC2s upregulate IL-25R as they acquire memory-like properties after allergen or IL-33 treatment and become responsive to IL-25 stimulation (29). We have also shown that naïve neonatal lung ILC2s are potently stimulated by IL-25 (27). Unlike lung ILC2s, ILC2s in mesenteric lymph nodes (mLN), spleen and liver expands upon intraperitoneal (i.p.) administration of IL-25 (3, 4). Moreover, IL-25 deficient mice have significantly reduced ILC2s in the SI at steady state and after worm infection, suggesting a critical role of IL-25 in maintenance and activation of intestinal ILC2s (21). IL-25 also serves as the predominant cytokine for skin ILC2 activation in BALB/c mice (10).

Despite their potency in activating ILC2s *in vivo*, IL-33 or IL-25 alone is not sufficient to induce ILC2 activation *in vitro*, suggesting that activation of ILC2s requires a secondary signal provided by co-stimulatory cytokines such as IL-2 and IL-7 (2, 7, 10).

It was recently shown that the majority of mouse skin ILC2s express IL-18Rα (22). Consequently, ILC2s isolated from skin are weakly activated upon *in vitro* stimulation by IL-18 + TSLP compared to TSLP only, which does not activate them (22). ILC2 stimulation through this pathway is physiologically significant, as IL-18 deficient mice have impaired skin ILC2 activation and type 2 inflammation in a mouse model of atopic dermatitis-like disease (22). Of note, IL-18 mediated activation of ILC2 is independent of IL-33, IL-25 and TSLP (22). Human PB ILC2s, unlike mouse ILC2s, respond very potently to *in vitro* IL-18 + IL-7 stimulation by producing type 2 cytokines (31), suggesting that IL-18 may play a more essential role in human ILC2 biology.

Indirect Activation by Allergens

ILC2s play a crucial role in airway allergic diseases induced by various allergens in experimental mouse models. Due to the lack of antigen specific receptors, ILC2s do not directly recognize and respond to allergens. Instead, they are stimulated by various

cytokines released upon irritation or tissue injury caused by allergen inhalation.

In murine models, various allergens including house dust mite (HDM) (56, 57, 60, 61), chitin (62), papain (29, 63), fungal allergens from *Alternaria* (64–66) and *Aspergillus* (29) species and ovalbumin (OVA) (56, 58) have been shown to activate lung ILC2s. Treatment with these allergens causes disruption of the barrier integrity and induces the release of ILC2-activating cytokines from the airway epithelial cells (7, 42, 59, 64, 66–69). As *Crlf2* (TSLPR), *Il25* and *Il1rl1* (ST2) triple-deficient mice have similar numbers of ILC2s as wild-type mice upon chitin treatment (62), and *Il33*$^{-/-}$ mice have normal ILC2 accumulation in bronchoalveolar lavage fluid (BALF) after HDM administration (70), these cytokines may not be necessary for ILC2 proliferation upon allergen stimulation. However, they appear to play a critical role in ILC2 activation as *Il33*$^{-/-}$ or *Il1rl1*$^{-/-}$ mice have reduced ILC2 cytokine production compared to wild-type mice in papain, *Alternaria* and HDM models (63, 64, 70), and mice deficient in one or more ILC2-activating cytokine signaling pathways show significantly reduced expansion of eosinophils and alternatively activated macrophages (AAM) after chitin treatment (62).

Interestingly, HDM driven ILC2 expansion requires T cell activation, as mice with impaired T cell activation do not mount ILC2-mediated type 2 inflammation upon HDM challenge (70), while adaptive lymphocytes are not necessary in the papain model, as i.n. papain treatment into *Rag1*$^{-/-}$ mice leads to normal ILC2 activation and eosinophilia (7). In contrast, ILC2s are not required for allergic inflammation in an OVA model, in which Th2 cells are stimulated by OVA plus adjuvant alum, as ILC2 deficient mice show no impairment of type 2 immune responses (61). These data suggest that immunological environment induced by these allergens seems to vary and the role of ILC2s in type 2 inflammation may be slightly different in each model.

FUNCTION OF ILC2s

Upon activation, lung ILC2s secrete copious amounts of type 2 cytokines, IL-5 and IL-13 but also IL-9 and amphiregulin (6, 7, 42, 71, 72). IL-5 induces eosinophil development and recruitment, resulting in eosinophilia in the lung (73). IL-13 causes goblet cell hyperplasia, followed by mucus hyperproduction, smooth muscle contraction and subepithelial fibrosis, resulting in tissue remodeling and airway hyperresponsiveness (74). Moreover, together with IL-4, IL-13 provides cues for macrophage differentiation into AAM (74). ILC2-derived IL-13 has been shown to disrupt tight junctions in the epithelial lining of the lung causing leakiness of the airways (75). It also facilitates differentiation of helper T cells into Th2 type by inducing migration of activated dendritic cells to the draining LN (63), further enhancing type 2 inflammation. In addition to type 2 cytokines, ILC2s transiently produce IL-9 early during inflammation (42, 71, 72), which provides signals for mast cell accumulation and airway remodeling by inducing goblet cell hyperplasia (76, 77). IL-9 also promotes survival (72) and/or activation (42) of ILC2s in an autocrine/paracrine manner (42, 71), resulting in amplification of IL-5 and IL-13 production.

More recent studies have shown that IL-10 producing ILC2s, termed "$ILC2_{10}$" and "exhausted ILC2s" (discussed in more detail later) are generated in the mouse lung during chronic inflammation or in response to IL-2 (78, 79). The release of IL-10 can also be induced by mouse lung and SI ILC2s *in vitro* by various stimulants, including IL-2, IL-10, IL-27, IL-4, retinoic acid (RA) or neuromedin U (78, 80). Interestingly, blocking IL-10 results in a reduction in IL-10 production from ILC2s, suggesting an autocrine/paracrine regulation by IL-10 (80). In humans, a KLRG1$^+$ subset of ILC2s secrete IL-10 when stimulated in the presence of RA (81, 82). While GATA3 expression confirms their ILC2 lineage identity, IL-10$^+$ ILC2s express CTLA4 and CD25, resembling regulatory T cells (81, 82). Consequently, they inhibit various CD4$^+$ helper T cell subsets and ILC2s in an IL-10 dependent manner (81, 82), suggesting previously unappreciated immune regulatory role of ILC2s.

In addition to soluble mediators like cytokines, ILC2s directly interact with other ILC2s and lymphocytes through a repertoire of surface molecules. ILC2s have been shown to directly facilitate Th2 polarization through PD-1/PD-L1 interaction during *Nippostrongylus brasiliensis* infection (83), while IL-33

TABLE 1 | Summary of ILC2 phenotypes.

	Mouse					Human
	Lung	Intestine	Skin	Liver	Fat	Blood
CD127	+	+	+	+	+	+
CD90	+	+	+	+	+	–
CD25	+	+	+	+	+	+
CD103	ND	ND	+	ND	ND	–
IL-18Rα	–	–	+	–	–	+
ST2	+	low	+/low	+	+	–
IL-25R	low	+	+/low	ND	–	–
CRTH2*						+
CD161*						+
GATA3	+	+	+	+	+	+

*"+", expressed; "–", not expressed; "low", expressed at low levels; "+/low", various reports exist; "ND", not determined. *human ILC2 markers. Human ILC2 phenotype is based on peripheral blood ILC2s.*

activated ILC2s stimulate Th2 and regulatory T cells *via* OX40/OX40L interaction (84). Activated ILC2s, expressing ICOS ligand (ICOSL), also promote accumulation of $ICOS^{high}$ regulatory T cells through ICOS/ICOSL interactions (85). Interestingly, ILC2s express both ICOS and ICOSL and provide survival signals to each other (86).

Due to ILC2's potent capacity to produce cytokines, they initiate robust type 2 immune responses upon epithelial injury or environmental insults, and consequently, they are implicated in pathogenesis of various airway diseases, including asthma (87, 88), chronic rhinosinusitis with nasal polyps (CRSwNP) (19, 89–93), allergic rhinitis (94) and pulmonary fibrosis (95–97), and respiratory infections caused by influenza (8) and respiratory syncytial (98, 99) viruses. In contrast, ILC2s play a crucial protective role during influenza virus infections by producing amphiregulin, which facilitates the repair of damaged airway epithelium and restores the impaired lung function caused by influenza (6). Moreover, human $IL\text{-}10^{+}$ ILC2s prevent the loss of epithelial barrier integrity upon allergen exposure (81), suggesting a protective role in airway allergic diseases. Overall, the role of ILC2s in type 2 inflammation seem to vary in different models and diseases.

THE FATE OF ACTIVATED ILC2s

Once activated and having proliferated, lung ILC2s propagate a series of inflammatory events by production of type 2 cytokines as described above. The majority of activated lung ILC2s remain in the lung (23, 100, 101) and presumably die once their task is completed, although activation-induced death of ILC2s has not yet been studied. A small proportion of activated lung ILC2s persists in the lung as memory-like ILC2s (29), or becomes exhausted (79). Some ILC2s may transdifferentiate into other types of ILCs under specific conditions (102–106), whereas it was recently found that a subset of ILC2s leaves the residing organs and circulates (101, 107) (**Figure 1**, **Table 2**).

ILC2 Memory

Immunological memory is the ability of immune cells to recall a previous encounter with a specific antigen and mount robust responses upon subsequent exposures to the same antigen. The acquisition of immunological memory is a hallmark of adaptive immunity and innate leukocytes have long been thought to not possess the ability to remember previous activation. However, with the discovery of memory NK cells (110–113), the potential

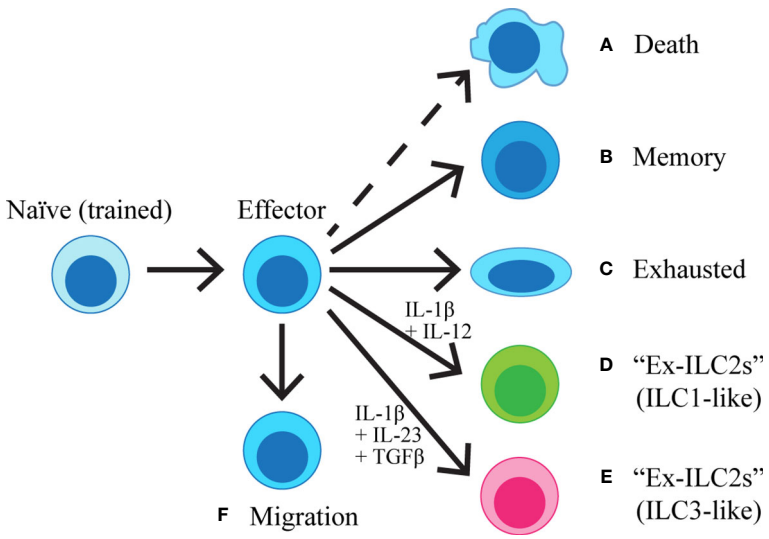

FIGURE 1 | Naïve ILC2s are exposed to IL-33 during the neonatal period and become "trained". Once activated, ILC2s become effector cells, producing cytokines and initiating the inflammatory cascade. The majority of the effector cells are predicted to die **(A)**, while a proportion of ILC2s acquires immunological memory **(B)**. Some ILC2s may become exhausted **(C)**, whereas others can also transdifferentiate into IFNγ-producing ILC1-like cells **(D)** or IL-17-producing ILC3-like cells **(E)** upon stimulation with IL-1β + IL-12 or IL-1β + IL-23 + TGFβ, respectively. A subset of ILC2s migrates out of the lung and enter circulation **(F)**.

TABLE 2 | The fate of activated ILC2s.

	Models (mouse/human)	References
Immunological memory/trained immunity	Mouse	(27, 29, 108)
Exhaustion	Mouse	(79)
Transdifferentiation to ILC1s	Human	(102–104)
	Mouse	(103)
Transdifferentiation to ILC3s	Human	(105, 106)
Activation-induced migration	Mouse	(101, 107, 109)

of innate lymphocytes to acquire memory-like properties have been unraveled. Detailed reviews of immunological memory of ILC2s and innate leukocytes can be found elsewhere (114, 115).

Memory ILC2s in Mouse

We have recently shown that lung ILC2s can acquire immunological memory (29). Upon allergen inhalation, mouse lung epithelium releases alarmins such as IL-33, which activates lung resident ILC2s. This results in a vigorous expansion of ILC2s, followed by a contraction phase and resolution of lung inflammation as indicated by the decrease in lung eosinophils and BALF type 2 cytokines. While the number of ILC2s continues to decrease after the peak of inflammation, some of the activated ILC2s survive for a long time as shown by persistence of ILC2s labelled with bromodeoxyuridine (BrdU), which was administered at the time of initial activation (29). These ILC2s are more responsive to a secondary stimulus compared to naïve ILC2s, which is a feature of immunological memory, and hence, were termed "memory-like ILC2s" (29). However, unlike antigen specific T and B cells, ILC2s do not recognize specific antigens and consequently, memory-like ILC2s are able to mount robust immune responses to subsequent stimulation by unrelated allergens or cytokines. Memory-like ILC2s are defined by changes in the expression of a small subset of genes compared to naïve or effector ILC2s (29). These changes include the upregulation of *Il17rb*, a subunit of the IL-25 receptor complex, which enables them to uniquely react to *in vivo* IL-25 stimulation, unlike naïve lung ILC2s. Although adoptive transfer of memory-like ILC2s and gene expression data indicate that the acquisition of immunological memory is cell-intrinsic (29), we cannot rule out the possibility that environmental components may be required to mount an efficient recall response.

A similar memory-like ILC2 population has been demonstrated in mouse models of nematode infections. Yasuda et al. found that ILC2s persist in BALF for at least 1 month after infection of mice with the migratory helminth *Strongyloides venezuelensis*, while other immune cell numbers go back to similar levels as in naïve mice (108). Upon challenge with the unrelated nematode *N. brasiliensis* a month later, mice pre-infected with *S. venezuelensis* demonstrate protective effects against *N. brasiliensis* infection, accompanied by increased number of ILC2s and cytokines in the BALF of pre-infected mice compared to those without primary infection. The protective effect is not specific to *S. venezuelensis*, as similar results are obtained when the order of infection is switched (108). In contrast, IL-33 pre-treatment fails to induce a similar protection (108). The resistance against *N. brasiliensis* infection is independent of $CD4^+$ T cells, suggesting a primary role of ILC2s, whereas IL-33 and eosinophils are critical. Unlike the memory-like ILC2s we have previously described (29), the enhanced responsiveness of ILC2s upon challenge infection by nematodes is not mediated by IL-25R upregulation and hence, the authors named these cells "trained ILC2s (108)."

Seehus and colleagues have defined a new subset of IL-10 producing ILC2s in mouse lungs, termed "$ILC2_{10}$" (78). These cells are generated upon i.p. administration of high dose IL-33 or chronic stimulation with i.n. papain treatment and act as regulatory cells with an immunosuppressive function. Interestingly, although $ILC2_{10}$ contracts quickly upon withdrawal of stimulation, administration of a single dose of IL-33 a month after the initial stimulation induces an increased number of $ILC2_{10}$ in pre-treated mice compared to untreated mice (78). Therefore, it is likely that immunological memory can be acquired by different subsets of ILC2s and this may have implications not only in the exacerbation but also in regulation of lung inflammation.

It is important to note that *in vitro* or *in vivo* primed Th2 cells can also demonstrate innate-like properties by responding to cytokines or unrelated antigens (116). Such innate-like behavior of Th2 cells is TCR independent and requires IL-33, suggesting that it is an antigen non-specific response (116). Th2 cells in mice inoculated with *N. brasiliensis* can mount an efficient airway eosinophilic inflammation upon HDM challenge 3-4 weeks later, suggesting their resemblance to the innate immunological memory of ILC2s (116). Therefore, immunological memory should be defined as the capacity of immune cells to remember previous activation and respond more efficiently upon reactivation despite antigen specificity (114).

Memory ILC2s in Human

Although immunological memory-like properties have been described in human NK cells (117, 118), there is little evidence of memory ILC2s in human. A recent paper by van der Ploeg identified the human counterpart of the inflammatory ILC2s (iILC2s) (119), which is a subtype of ILC2s induced during inflammation (discussed in more detail later) (109). These human iILC2s, characterised by the expression of CD45R isoform CD45RO, are enriched in the blood and nasal polyps of patients with CRSwNP and in the blood of asthma patients, and are highly activated. $CD45RO^+$ ILC2s differentiate from resting $CD45RA^+$ ILC2s isolated from PB upon *in vitro* cytokine stimulation. While CD45RO is a marker for human memory T cells (120), whether or not some of these highly activated iILC2s are able to retain the expression of CD45RO and become memory ILC2s remains to be explored.

Trained ILC2s (Neonatal ILC2s)

In vivo lineage-tracing experiments have revealed that a portion of steady-state ILC2s in various mouse tissues originates in the neonatal period and persists into adulthood (23, 27). ILC2s are undetectable in the lung of newborn mice, but ILC2 numbers rapidly increase in the following 10 days and peak on postnatal days 10-14 when the numbers reach to ~2-3 times of those in adult mouse lungs (24–26). In parallel to the ILC2 expansion, the level of IL-33 expression is significantly higher in neonatal lungs than adult lungs (24, 26, 27). It is thought that endogenous IL-33 is released from stromal cells in the neonatal period (41) due to postnatal mechanical stress from the first newborn breaths (26), development (25), or hyperoxia (24, 121). At the peak of their expansion, neonatal ILC2s upregulate activation-related genes such as *Mki67, Il13, Il5*, and *Il1r2*, increase intracellular expression of IL-13 and IL-5, and expression of Ki67 and IL-25R (24, 27). Neonatal lungs also have ILC2-dependent

eosinophilia (24, 26, 27). In IL-33 deficient pups, ILC2s develop normally but they do not expand as in wild-type pups and their activation is not observed (24, 26). Administration of recombinant sST2, a decoy receptor that blocks IL-33 signaling, also inhibits the activation of neonatal lung ILC2s (25). These studies together showed that neonatal lung ILC2s are activated by endogenous IL-33.

While the neonatal lung ILC2s contract to adult levels around 3 weeks of age, ILC2s labeled by i.n. BrdU administration during the expansion phase persist into adulthood (27). Schneider et al. also irreversibly marked neonatal ILC2s by tamoxifen-induced Cre expression and red fluorescent protein (RFP) and showed that RFP-marked neonatal ILC2s persist for many months and are only very slowly replaced by RFP-negative ILC2s in adulthood (23). Therefore, ILC2s that develop in the neonatal period become tissue resident cells in adulthood. Interestingly, i.n. injections of IL-33 into adult mice previously given BrdU in the neonatal period more intensely activate BrdU-positive than negative lung ILC2s (27). Lung ILC2s in adult IL-33 knock out (KO) mice do not respond as intensely to i.n. IL-33 stimulation as wild-type ILC2s, and i.n. administration of IL-33 in the neonatal period reverses the impaired response of ILC2s in IL-33KO mice in adulthood (27). Ricardo-Gonzalez et al. also showed that absence of IL-33 signaling leads to downregulation of adult ILC2 type 2 signatures (22). These studies suggested that activation of lung ILC2s by endogenous IL-33 in the neonatal period has long lasting effects on ILC2 functions in adulthood and the effect was termed "ILC2 training (27)." The idea of trained ILC2s implies that lung resident ILC2s in adult mice are more responsive to stimuli than ILC2s that develop in adulthood as the former are trained in the neonatal period whereas the latter are untrained, similar to ILC2s in IL-33KO mice.

It should be noted that there are significant differences between memory-like ILC2s and neonatally trained ILC2s. Neonatal ILC2s transiently upregulate IL-25R but the levels diminish into adulthood and adult lung ILC2s do not respond to i.n. IL-25 stimulation (27). In contrast, memory-like ILC2s maintain IL-25R expression and are activated by i.n. IL-25 treatment (29).

Exhausted ILC2s

Exhaustion of lymphocytes occurs upon persistent antigen stimulation, where immunological memory generation fails and cells are also unable to execute effector functions (122). Miyamoto et al. reported that ILC2s lacking core binding factor (Cbf) β showed an activated phenotype at steady state but a hyporesponsive phenotype upon *in vitro* stimulation (79). The gene expression profiles of Cbfβ deficient ILC2s stimulated by IL-33 *in vitro* resemble those of exhausted $CD8^+$ T cells, and hence, these cells were termed "exhausted-like ILC2s". A similar population of exhausted-like ILC2s, defined by their expression of IL-10 and TIGIT, are generated in a severe subacute asthma model, where wild-type mice are treated with high doses of papain short-term (79). These ILC2s, which express killer cell lectin-like receptor G1 (KLRG1), programmed cell death protein 1 (PD-1) and glucocorticoid-induced TNFR-related (GITR), appear in the BALF, where the inflammation is the most severe, but not in the lung. They do not proliferate well nor do they produce much type 2 cytokines. The exhausted-like ILC2s are also generated in the lung as well as in the BALF when wild-type mice receive a high-dose papain treatment every 3 days over a month period (79). The exhausted phenotype of ILC2s is further enhanced by Cbfβ deficiency, suggesting that the ILC2 exhaustion process is inhibited by Runx/Cbfβ complex. It is important to note that exhausted-like ILC2s are different from previously-described $ILC2_{10}$ (78), as the former lacks the ability to efficiently produce type 2 cytokines, whereas the latter is a great producer of type 2 cytokines.

Ex-ILC2s

Plasticity of ILC2s towards IFNγ producing ILC1s (102–104) and IL-17 producing ILC3s (105, 106) have been documented. An indication of ILC2's capacity to transdifferentiate into ILC1s came from the observation that PB and lung samples collected from chronic obstructive pulmonary disease (COPD) patients are enriched in ILC1s whereas the frequency of ILC2s is reduced compared to healthy subjects or less severe COPD patients (102, 103). In vitro stimulation of ILC2s isolated from PB of healthy donors in the presence of IL-2, IL-1β (or IL-33 + TSLP) and IL-12 causes the loss of ILC2 signatures, such as GATA3, IL-5 and IL-13, and upregulation of ILC1 features, T-bet and IFNγ expression (102, 104). Priming of ILC2s with IL-1β is necessary, during which IL-1β epigenetically modifies the transcriptome of ILC2s, shifting it to a more ILC1-like profile (104). Interestingly, the ILC2 phenotype is restored when ex-ILC2s are stimulated with IL-4, whereas true ILC1s do not covert to ILC2s upon IL-4 stimulation (102). A similar transdifferentiation of ILC2s to ILC1s have been shown in mice using a model of influenza infection, where immunodeficient mice adoptively transferred with GFP^+ ILC2s were infected with influenza virus (103). GFP^+ ILC2s underwent downregulation of GATA3 and upregulation of IL-18Rα and IL-12Rβ2, and produced IFNγ upon ex vivo stimulation with IL-12 and IL-18 (103).

Human ILC2s have also been shown to have the capacity to convert into IL-17 producing $Ckit^+$ $NKp44^-$ ILC3-like cells when cultured in the presence of IL-2 + IL-1β + IL-23 + TGFβ (105, 106). This transdifferentiation is associated with downregulation of GATA3, followed by upregulation of RORγt expression and IL-17 production (105). IL-1β is required for IL-17 production, while TGFβ further promotes transdifferentiation by enhancing IL-17 production and RORγt expression, and inhibiting IL-5 production (105, 106). ILC2 phenotype and function are partially restored in $IL\text{-}17^+$ ILC2s upon IL-4 stimulation. A marked increase in $NKp44^-$ ILC3s is observed in psoriatic lesions and nasal polyps collected from cystic fibrosis patients (105, 106), suggesting that ILC2 transdifferentiation into ILC3s is physiologically relevant in the presence of the appropriate cytokine milieu.

Notably, the majority of studies demonstrating ILC2 plasticity have been performed using human samples. It is most likely due to the fact that human ILCs are constantly exposed to various stimuli and hence, their identities as different subsets of ILCs are

more ambiguous than in mice, which are maintained relatively sterile in animal facilities. Therefore, it is more feasible to induce transdifferentiation of human ILC2s into ILC1s or ILC3s upon stimulation with type 1 or type 3 skewing cytokines, respectively, compared to mouse ILC2s.

Migration of ILC2s

The majority of mouse lung ILC2s are generated during the neonatal period and there is a limited amount of *de novo* generation of ILC2s during adulthood (23). The idea of ILC tissue residency was first proposed by Gasteiger and colleagues, where they examined the composition of ILC pools at homeostasis and during inflammation using a parabiosis model (100). At steady state, more than 95% of ILCs are host-derived in all tissues analyzed, including the SI, salivary gland, lung and the liver (100). Upon infection with the migratory helminth, *N. brasiliensis*, the composition of donor and host-derived ILC2s in the lung, SI and mLN remains unchanged during the early stage of helminth infection, indicating that ILC2s are tissue resident lymphocytes unlike circulatory T and B cells.

While tissue residency of ILC2s is a well-established concept in the mouse system, in humans we can extrapolate from the presence of elevated ILC2s in asthmatic patients' PB (88) that ILC2s may have the capacity to circulate or migrate. In mice, despite limited influx of hematogenous sources of ILC2s at homeostasis or during inflammation, i.p. IL-25 treatment or *N. brasiliensis* infection causes circulation of iILC2s, an inflammation-induced population of ILC2s identified as Lin^- $KLRG1^+ST2^{lo}$ cells (107, 109). iILC2s originate in the SI, from which they enter the lymphatic and blood circulation before reaching the peripheral organs. We have also previously shown that activation of ILC2s in the lung by i.n. IL-33 or papain treatment causes accumulation of ILC2s in the draining mediastinal LN (29). Although we did not specifically investigate their migration from the lung to the LN, these data together with iILC2 migration indicate that migratory behavior of ILC2s may be induced upon activation (**Figure 2**).

A recent report by Ricardo-Gonzalez et al. examined the source of circulating ILC2s upon *N. brasiliensis* infection using fate-mapping models (101). Upon subcutaneous administration, *N. brasiliensis* first migrates to the lung, where it is coughed up and swallowed, entering the digestive tract, reaching the intestine (123). Early during the parasite infection (day 5), the majority of circulating ILC2s are dependent on IL-25 signaling and are of intestinal origin. In contrast, lung ILC2s, dependent on IL-33 signaling appear in circulation later during infection (day 12). These results demonstrate that in spite of their tissue residency at homeostasis and during inflammation, local activation of ILC2s can cause systemic dissemination of type 2 inflammation by inducing migratory behavior of ILC2s. It remains to be determined whether the circulating ILC2s return to their residing organs or migrate to other tissues.

CONCLUDING REMARKS

The past few years of research on ILC2s at mucosal and non-mucosal sites has provided a wealth of knowledge regarding the

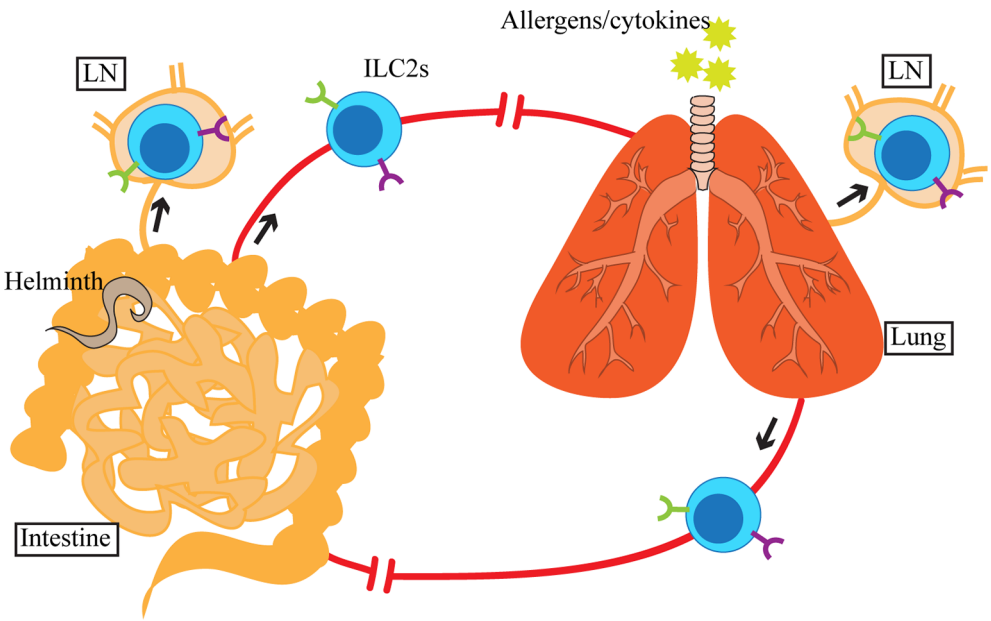

FIGURE 2 | Upon activation by allergens, cytokines or helminths in the lung or small intestine (SI), ILC2s and inflammatory ILC2s (iILC2s) appear in circulation as well as in the tissue-draining lymph nodes (LN). SI-derived iILC2s migrate to the lung, whereas the destination of activated SI and lung ILC2s is currently unclear. Although ILC2s and iILC2s are detected in blood and LN, which suggests that their migration involves lymphatics and blood, the exact route of migration has yet to be determined.

regulation and functions of tissue resident ILC2s in various inflammatory conditions. In contrast, our understanding of what happens to ILC2s after activation and resolution of inflammation is still limited. While the majority of activated ILC2s are expected to die as the ILC2 population contracts after the peak of inflammation, some may become memory-like ILC2s, exhausted ILC2s, ex-ILC2s, or circulatory ILC2s, as summarized in this review. Considering that humans are continuously exposed to extrinsic insults, our ILC2s are presumably activated at some point. Therefore, we will likely benefit from future research on the dynamics of ILC2s after activation in various organs and the functional significance of previously activated ILC2s in inflammation at local and distant sites. It remains to be elucidated how and why activated tissue resident ILC2s take different pathways, namely death, memory, exhaustion, transdifferentiation or migration and whether it is predetermined by neonatal training or decided during activation. Finding specific markers for individual ILC2 populations will help us understand the processes.

AUTHOR CONTRIBUTIONS

LM wrote and edited the manuscript and generated the figures and tables. IM-G and CS wrote and edited the manuscript. FT reviewed the drafts, provided critical input and edited the manuscript and figures. All authors contributed to the article and approved the submitted version.

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Rhinovirus C Infection Induces Type 2 Innate Lymphoid Cell Expansion and Eosinophilic Airway Inflammation

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Rhinovirus C (RV-C) infection is associated with severe asthma exacerbations. Since type 2 inflammation is an important disease mechanism in asthma, we hypothesized that RV-C infection, in contrast to RV-A, preferentially stimulates type 2 inflammation, leading to exacerbated eosinophilic inflammation. To test this, we developed a mouse model of RV-C15 airways disease. RV-C15 was generated from the full-length cDNA clone and grown in HeLa-E8 cells expressing human CDHR3. BALB/c mice were inoculated intranasally with 5 x 10^6 ePFU RV-C15, RV-A1B or sham. Mice inoculated with RV-C15 showed lung viral titers of 1 x 10^5 $TCID_{50}$ units 24 h after infection, with levels declining thereafter. IFN-α, β, γ and λ2 mRNAs peaked 24-72 hrs post-infection. Immunofluorescence verified colocalization of RV-C15, CDHR3 and acetyl-α-tubulin in mouse ciliated airway epithelial cells. Compared to RV-A1B, mice infected with RV-C15 demonstrated higher bronchoalveolar eosinophils, mRNA expression of IL-5, IL-13, IL-25, Muc5ac and Gob5/Clca, protein production of IL-5, IL-13, IL-25, IL-33 and TSLP, and expansion of type 2 innate lymphoid cells. Analogous results were found in mice treated with house dust mite before infection, including increased airway responsiveness. In contrast to *Rora*$^{fl/fl}$ littermates, RV-C-infected *Rora*$^{fl/fl}$ *Il7r*^{cre} mice deficient in ILC2s failed to show eosinophilic inflammation or mRNA expression of IL-13, Muc5ac and Muc5b. We conclude that, compared to RV-A1B, RV-C15 infection induces ILC2-dependent type 2 airway inflammation, providing insight into the mechanism of RV-C-induced asthma exacerbations.

Keywords: asthma, rhinovirus, innate cytokine, viral infection, exacerbation, ILC2

INTRODUCTION

First reported in 2006 (1, 2), rhinovirus C (RV-C)[4] has been associated with severe respiratory illnesses in children and adults, often requiring hospitalization (3–15). Infections with RV-C are more likely to occur in children with a history of asthma or who develop asthma (6, 10–14). In addition, compared to RV-A, children with RV-C have been reported to have severe lower respiratory tract infections including wheezing, oxygen supplementation and intensive care unit admission (7, 8, 10, 11, 15).

Despite increasing recognition of RV-C as a cause of severe exacerbation, little is known about the pathogenesis of RV-C infections. Thus far, 55 RV-C genotypes have been reported which are believed to be synonymous with serotypes (16, 17). In contrast to major and minor group RV-A and RV-B viruses, RV-C does not utilize intercellular adhesion molecule (ICAM)-1 or low density lipoprotein family receptors (LDL-R). This is likely due to the fact that the hydrophobic pocket in VP1 is filled with multiple bulky residues (18). Instead, RV-C utilizes cadherin related family member 3 (CDHR3) as a receptor (19). Individuals with CDHR3 C529Y variants (AG and AA genotype) appear to be more susceptible to RV-C infection, as this variant is localized on the airway epithelial cell surface, where it is accessible to viral infection, in contrast to the more common GG genotype which is mostly localized to the cytoplasm (19–21).

RV-C has been refractory to study in part because it is difficult to grow *in vitro*. RV-C has been grown in primary mucociliary-differentiated human airway epithelial cells grown at air-liquid interface (22, 23) and HeLa cells transduced with the CDHR3 AA allele (HeLa-E8 cells) (24).

The mechanisms by which RV-C promotes severe respiratory illness are unknown. Since type 2 inflammation is an important disease mechanism in a large subgroup of individuals with asthma [reviewed in (25)], we hypothesized that RV-C infection, in contrast to RV-A infection, preferentially stimulates type 2 inflammation, leading to exacerbated eosinophilic inflammation. Mouse models have been utilized to study the host response against respiratory enteroviruses such as major group RV-A16 (26), minor group RV-A1B (26, 27) and enterovirus D68 (28). To study underlying mechanisms, we obtained cDNA encoding RV-C15 and HeLa-E8 cells from James Gern and Yury Bochkov (University of Wisconsin). We inoculated mature BALB/c mice with RV-C15, comparing inflammatory responses to those induced by RV-A1B. In addition, we compared the response of allergen-sensitized and challenged mice to the two viruses.

MATERIAL AND METHODS

Generation of RV-C15 and RV-A1B

Full length cDNA encoding RV-C15 and HeLa-E8 stable cells expressing human CDHR3 C529Y (19) were provided by James Gern and Yury Bochkov, University of Wisconsin. The cDNA was reverse transcribed and resulting full-length vRNA transfected into HeLa-H1 cells (ATCC, Manassas, VA) using lipofectamine (ThermoFisher Scientific, Waltham, MA). Virus was harvested from the HeLa-H1 cell supernatants and used to infect HeLa-E8 cells. Initial RV-C15 from transfected HeLa-H1 cells does not cause obvious cytopathic effects or form plaques in HeLa-E8 cells. However, upon close inspection, areas of cellular damage matched staining with Alexa Fluor 555-conjugated anti-mouse EV-D68 VP3 (**Figure 1A**). Anti EV-D68 VP3 (GeneTex, Irvine, CA) recognizes VP3 from EV-D68, RV-A1B and RV-C15 (**Figure 1B**). RV-C15 replicated in HeLa-E8 cells but not HeLa-H1 cells (**Figure 1C**). RV-A1B (ATCC), a minor group virus that

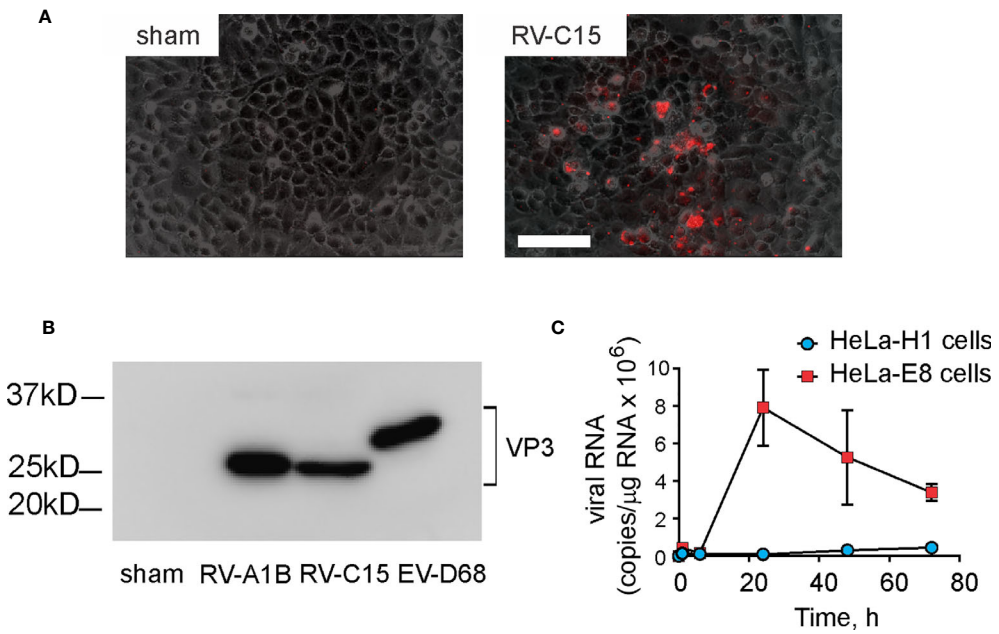

FIGURE 1 | Generation of RV-C15. Full-length cDNA encoding RV-C15 was reverse transcribed, and the resulting vRNA transfected into HeLa-H1 cells. Virus was harvested from the cell supernatants and used to infect HeLa-H1 cells or HeLa-E8 cells expressing human CDHR3 C529Y. **(A)** RV-C15 induces cytopathic effects on HeLa-E8 cells. Areas of cell damage matched staining with AlexaFluor-labeled anti-VP3. The white bar is 100 μ **(B)** Anti-VP3 immunoblots of HeLa cell lysates infected with RV-A1B, RV-C15 and enterovirus D-68. Concentrated viral preparations were resolved by SDS-PAGE and probed with anti-VP3. **(C)** Infection of Hela-E8 and HeLa-H1 cells with RV-C15. RV copy number was determined by qPCR. Data shown are mean ± SD, *n* =3.

infects mouse cells (29) was grown in Hela-H1 cells. The two viruses were propagated, concentrated and partially purified from infected HeLa cell lysates by means of ultrafiltration with a 100-kDa cutoff filter assay, as described previously (27). Similarly concentrated and purified HeLa cell lysates were used for sham infection. With propagation and concentration needed to produce sufficient quantities for animal experiments, we observed amplified cytopathic effects in RV-C15-infected HeLa-E8 cells. We took advantage of this result to test for live virus in the lungs of infected mice (see Results below).

RV-C15 Infection and Treatment

All animal usage was approved by the Institutional Animal Care and Use Committee of University of Michigan and performed under National Institutes of Health guidelines. Eight-to-twelve-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were inoculated with 5 x 10^6 PFU equivalents (ePFU) of RV-C15 in 50 µL PBS by intranasal instillation. RV-C ePFU was calculated based on a calibrated standard curve for RV-A1B (18). Additional mice were inoculated with 5 x 10^6 ePFU RV-A1B (in 50 µL PBS) or an equal volume of sham HeLa cell lysate. In additional experiments, we examined RV-C15-induced mRNA expression in *Rora*$^{fl/fl}$*Il7r*^{Cre} mice and *Rora*$^{fl/fl}$ littermates (from Dr. Andrew McKenzie, MRC Laboratory of Molecular Biology, Cambridge, UK). Based on the requirement of RORα for ILC2 development (30), *Rora*$^{fl/fl}$*Il7r*^{Cre} mice are ILC2 deficient (31). *Il7r*^{Cre} mice were originally generated by Dr. Hans-Reimer Rodewald (Division of Cellular Immunology, German Cancer Research Center, Heidelberg) (32).

Using specific forward and reverse primers, RNA from passaged virus was used to produce random primed first strand cDNA and overlapping products using Phusion high fidelity DNA polymerase (New England Biolabs, Ipswich, MA) of about 2 kB each. These products were agarose gel purified and processed for Sanger sequencing (University of Michigan DNA Sequencing Core). Primers used for PCR production and sequencing are shown in the **Supplemental Table**. Sequencing through nucleotide 7042 of the W-10 RV-C15 reference sequence GU219984, we did not detect the previously described mutations in VP1 or 3A (24).

Model of Allergic Airways Disease

BALB/c mice were sensitized through the intranasal route with 100 µg *D. pteronyssinus* house dust mite (HDM) extract in 50 µl PBS (Greer Labs, Lenoir, NC) by intranasal installation on day 1 and challenged with 10 µg HDM on days 11 and 12 (33). On day 13, mice were inoculated through the intranasal route under Forane anesthesia with RV-C15, RV-A1B or sham, as noted above.

Real-Time Quantitative PCR

Lungs were harvested at different points and RNA was extracted with Trizol (Invitrogen, Carlsbad, CA). Lung RNA was isolated using an RNAeasy kit (Qiagen). cDNA was synthesized from 2 µg of RNA using high capacity cDNA synthases kit (Applied Biosystems, Foster City, CA) and subjected to quantitative real-time PCR using specific primers for mRNA (**Table 1**). The level of gene expression for each sample was normalized to GAPDH unless otherwise specified. RV copy number (vRNA) was determined by qPCR using previously published primers (34).

TABLE 1 | Primer sequences for real-time PCR.

Gene	Primer sequences
CCL2	Forward:5'-GCTCTCTCTTCCTCCACCAC-3'
	Reverse:5'-GCGTTAACTGCATCTGGCT-3'
CCL24	Forward:5'-ACCTCCAGAACATGGCGGGC-3'
	Reverse:5'-AGATGCAACACGCGCAGGCT-3'
CXCL1	Forward:5'-TGCACCCAAACCGAAGTCAT-3'
	Reverse:5'-CAAGGGAGCTTCAGGGTCAAG-3'
CXCL2	Forward:5'-GCGCTGTCAATGCCTGAAG-3'
	Reverse:5'-CGTCACACTCAAGCTCTGGAT-3'
CXCL10	Forward:5'-GCTGCAACTGCATCCATATC-3'
	Reverse:5'-TTTCATCGTGGCAATGATCT-3'
GAPDH	Forward:5'-GTCGGTGTGAACGGATTTG-3'
	Reverse:5'GTCGTTGATGGCAACAATCTC-3'
GOB5	Forward:5'-CTGTCTTCCTCTTGATCCTCCA-3'
	Reverse:5'-CGTGGTCTATGGCGATGACG-3'
IFN-α1	Forward:5'-CCATCCCTGTCCTGAGTG-3'
	Reverse: 5'-CCATGCAGCAGATGAGTCCTT-3'
IFN-β	Forward:5'-CAGCCCTCTCCATCAACTATAAG-3'
	Reverse:5'-CCTGTAGGTGAGGTTGATCTTTC-3'
IFN-γ	Forward:5'-ACGCTACACACTGCATCTTGG-3'
	Reverse:5'-GTCACCATCCTTTTGCCAGTTC-3'
IL12b	Forward:5'-CTCCTGGTTTGCCATCGTTT-3'
	Reverse:5'-GGGAGTCCAGTCCACCTCTA-3'
IL13	Forward:5'-CCTGGCTCTTGCTTGCCTT-3'
	Reverse:5'-GGTCTTGTGTGATGTTGCTCA-3'
IL-17a	Forward:5'-GCCTGAGAGCTGCCCCTTCAC-3'
	Reverse:5'-GGCTGCCTGGCGGACAATCG-3'
IL-5	Forward:5'-CTCTGTTGACAAGCAATGAGACG-3'
	Reverse:5'-TCTTCAGTATGTCTAGCCCCTG-3'
IL-25	Forward:5'-ACAGGGACTTGAATCGGGTC-3'
	Reverse:5'-TGGTAAAGTGGGACGGAGTTG-3'
IL-33	Forward: 5'-GGCTGCATGCCAACGACAAGG-3'
	Reverse: 5'-AAGGCCTGTTCCGGAGGCGA-3'
Muc5ac	Forward:5'-AAAGACACCAGTAGTCACTCAGCAA-3
	Reverse:5'-CTGGGAAGTCAGTGTCAAACC-3'
Muc5b	Forward:5'-GAGCAGTGGCTATGTGAAAATCAG-3'
	Reverse:5'-CAGGGCGCTGTCTTCTTCAT-3'
TNF-α	Forward:5'-GCAGGTTCTGTCCCTTTCAC-3'
	Reverse:5'-GTCGCGGATCATGCTTTCTG-3'

Generation of a Peptide Directed Anti-CDHR3 Antibody

The CDHR3 protein has extracellular calcium binding domains and a sialic-acid modified Asn186 important for RV-C15 binding (19). Hopp-Wood hydrophilicity analysis (DNASTAR, Madison WI) of the NIH BLAST sequence alignments of NP_689963.2 (human) and NP_001019649.1 (mouse) revealed a short conserved peptide (human amino acids 154-167, YQVEAFDPEDTSRN) in the second calcium binding domain representing a possible selective antigen for both human and mouse CDHR3. A rabbit polyclonal antibody was produced and purified using affinity chromatography (Genscript, Piscataway, NJ).

HeLa-H1 cells, HeLa-E8 cells, and mouse lungs were lysed, cellular proteins resolved by 10% SDS-PAGE, and proteins transferred to a nitrocellulose membrane. Membranes were probed with anti-CDHR3. Signals were amplified and visualized with horseradish peroxidase-conjugated secondary antibody

(BioRad, Hercules, CA) and chemiluminescence solution (Pierce, Rockford, IL). To determine the specificity of the observed bands, primary antibody was incubated with cysteine-conjugated YQVEAFDPEDTSRN peptide. Anti-CDHR3 (1 μg/mL) recognized 100 kD bands in CDHR3-expressing HeLa-E8 cells and mouse lung lysate but not HeLa-H1 cells (**Supplemental Figure 1A**). Recognition of these bands was abolished by addition of YQVEAFDPEDTSRN peptide (10 μg/mL). Other bands, perhaps representing proteins with calcium binding domains, remained. HeLa-E8 cells, but not HeLa-H1 cells on glass coverslips stained positively with AF488-conjugated anti-CDHR3. Staining was blocked with addition of either unlabeled rabbit IgG or YQVEAFDPEDTSRN (**Supplemental Figure 1B**).

Lung Histology and Immunofluorescence

Lungs were harvested at different points, fixed with 10% formaldehyde overnight and paraffin embedded. Blocks were sectioned at 500 μm intervals at a thickness of 5 μm, and each section was deparaffinized, hydrated and stained. To visualize inflammation, sections were stained with H&E. Other lung sections were incubated with anti-CDHR3, anti-EV-D68 VP3, mouse anti-acetyl α-tubulin (MilliporeSigma, Burlington, MA), rat anti-IL-25 (Biolegend), goat anti-IL-33 (eBioscience), rat anti-TSLP (Biolegend), rat anti-CD123 (Biolegend) or isotypic IgG. Antibodies and IgGs were labeled with AlexaFluor NHS esters (ThermoFisher) according to manufacturer's instructions. ILC2s were identified as IL-13- and GATA3-positive, and T cells were identified as IL-13-, GATA3- and CD3-positive. Images were visualized using an ApoTome microscope (Carl Zeiss, Thornwood, NY) or a Leica SP5 inverted laser confocal microscope (Buffalo Grove, IL).

Flow Cytometric Analysis

Lungs were harvested 2 days after sham, RV-C15 and RV-A1B treatment. Lungs were perfused with PBS containing EDTA and minced and digested in collagenase IV. Cells were filtered and washed with red blood cell lysis buffer, and dead cells were stained with Pacific Blue Live/Dead fixable dead staining dye (Invitrogen). Nonspecific binding was blocked by 10% FBS with 1% LPS-free BSA and 5-μg rat anti-mouse CD16/32 (BioLegend, San Diego, CA) added. To identify ILC2s, cells were then stained with FITC-conjugated antibodies for lineage markers [CD3ε, T-cell receptor- β (TCR β), B220/CD45R, Ter-119, Gr-1/Ly-6G/Ly-6C, CD11b, CD11c, F4/80, and FcεRIα; all from BioLegend], anti-CD25-peridinin- chlorophyll-protein complex (PerCP)-Cy5.5 (eBioscience), anti-CD127-allophycocyanin (APC; eBioscience) and anti-ST2-phycoerythrin (PE)-Cy7 (BioLegend), as described (35). Cells were fixed, subjected to flow cytometry, and analyzed on a LSR Fortessa (BD Biosciences, San Jose, CA). Positive/negative staining was determined using fluorescence minus one (FMO) controls. Data were collected using FACSDiva software (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed using 1 ml PBS aliquots. Cytospins were stained with Diff-Quick (Dade Behring, Newark, DE) and differential counts determined from 200 cells (36).

ELISA

Mouse lungs were harvested, homogenized in PBS plus Roche Complete Protease Inhibitors (MilliporeSigma), and snap frozen in liquid nitrogen. After thawing and resuspension at 4°C, particles were centrifuged at 10,000 x *g* for 30 min, and the supernatant was diluted serially in the homogenization buffer for ELISA of IFN-β, IFN-λ, IL-5, IL-13, IL-25, IL-33 and TSLP according to the manufacturer's instructions (R&D Systems, Minneapolis, MN and eBioscience).

Measurement of Airway Responsiveness

Mice were anesthetized, intubated, and ventilated with a Buxco FinePointe System (Wilmington, NC). Mice were administered nebulized saline and increasing doses of nebulized methacholine to assess airways responsiveness (27).

Airway Epithelial Cell Culture

Mouse airway epithelial cells were purchased from Cell Biologics (Chicago, IL). Primary airway epithelial cells were cultured in Transwells at air-liquid interface as described previously, with some modifications (37). Briefly, airway epithelial cells were cultured under submerged conditions in complete PneumaCult-Ex Plus medium (Stemcell Technologies, Vancouver, CA) for 1 week. Cells were transferred to Transwells and cultured with complete medium in both basal and apical wells until confluence was reached. Cells were then maintained at air-liquid interface for three weeks in PneumaCult-ALI maintenance medium. Cells were infected with sham, RV-C15 or RV-A39 at a multiplicity of infection (MOI) of 2 for 12 hrs. RV-A39 was purchased from ATCC and purified from infected HeLa-H1 cell lysates by ultrafiltration with a 100-kDa cutoff filter. Selected cell cultures were fixed and stained with AlexaFluor 488-conjugated anti-acetyl α-tubulin (MilliporeSigma, Burlington, MA) and AlexaFluor 555-conjugated anti-VP3. Immunoreactivity was visualized with a NikonA1 laser confocal microscope.

Data Analysis

Normality was tested using the Shapiro-Wilk test. Group mean data are represented as mean ± SEM or median ± interquartile range as appropriate. Statistical significance was assessed by unpaired t-test, Mann-Whitney test, one-way analysis of variance (ANOVA) or Kruskal-Wallis test as appropriate. Group differences were pinpointed by the Tukey or Dunn's multiple comparison test.

RESULTS

RV-C15 Persists in Lungs at Similar Levels as RV-A1B

Female adult BALB/c mice were inoculated sham HeLa cell lysate, 5 x 10^6 PFU RV-C15 or 5 x 10^6 PFU RV-A1B (50 μl at 10^8 PFU/ml) by intranasal instillation. Mice were sacrificed,

and the lungs were analyzed by qPCR at 0-96 h after infection for the presence of viral RNA. Viral RNA levels peaked 12 h after inoculation (**Figure 2A**). RV-A1B and RV-C15 infectivity was assessed by homogenizing lungs from virus- or sham-inoculated mice, overlying this material onto confluent monolayers of HeLa-H1 or HeLa-E8 cells, and assessing viral infectivity. Clarified supernatants from lung homogenates of RV-C15-infected mice caused cytopathic effects in HeLa-E8 cells. Fifty percent tissue culture infectivity doses ($TCID_{50}$) of RV-A1B and RV-C15 were determined by the Spearman-Karber method (38). Lung RV-A1B and RV-C15 titers peaked 24 h after inoculation (**Figure 2B**, left panel). There was no difference in lung viral titers 48 h after inoculation (**Figure 2B**, right panel). In addition, lungs from RV-A1B- and RV-C15-infected mice showed significant increases in IFN-α, β and l2 mRNA expression (**Figure 2C**) and IFN-β and IFN-l protein expression (**Figure 2D**), consistent with the presence of viral RNA. Lung homogenates of RV-C15-infected mice also formed small plaques on HeLa-E8 cells up to 48 h after infection, but homogenates from sham-infected mice did not (**Figure 2E**).

Lungs were also formalin-fixed and paraffin-embedded 24 h after exposure, and sections stained with fluorescent tagged anti-VP3, anti-CDHR3 and acetyl-α-tubulin. RV-C15 was localized to CDHR3+ ciliated airway epithelial cells (**Figure 3A**). Confocal microscopy using anti-YQVEAFDPEDTSRN showed colocalization of CDHR3 with RV-C15 but not RV-A1B (**Figure 3B**). However, it is important to note that this antibody did not block RV-C15 replication *in vitro* or *in vivo* (not shown).

Infection of Cultured Mouse Airway Epithelial Cells With RV-C15

To confirm that RV-C15 infects mouse airway epithelial cells, we cultured differentiated mouse airway epithelial cells at air-liquid interface with sham, RV-C15 or RV-A39, a major group virus which does not infect mouse cells. Selected cell cultures were fixed and stained for AlexaFluor-conjugated anti-acetyl α-tubulin and anti-VP3. Cultures stained for acetylated tubulin indicating the presence of cilia (**Figure 4**, upper panel). Cultured inoculated with RV-A39 showed no VP3 present (middle panel). Cultures inoculated with RV-C15 showed colocalization of VP3 and acetyl α-tubulin, indicating infection of ciliated cells (lower panel).

RV-C15 Induces Neutrophilic and Eosinophilic Inflammation and Expression of Type 2 Cytokines

Forty-eight h post-inoculation, lungs were stained with hematoxylin and eosin. Sham-inoculated mice showed no inflammation (**Figure 5A**). However, RV-C15 exposed mice showed leukocyte infiltration around large airways, which was similar to RV-A1B. Next, we determined bronchoalveolar lavage inflammatory cell counts in RV-C15- and sham-infected mice 24 and 48 h after treatment. We also examined the effects of replication-deficient UV-irradiated virus. Selected RV-C15 preparations were irradiated with ultraviolet (UV) light at 1200 mJ/cm^2 for 30 mi using a UVB CL-1000 cross-linker (39). Forty-eight h after inoculation, RV-C15-infected mice had significantly greater monocyte, neutrophil, lymphocyte and eosinophil recruitment into the bronchoalveolar fluid than sham-treated mice (**Figure 5B**). Mice infected with UV-irradiated virus showed significantly reduced viral copy number and fewer neutrophils, lymphocytes and eosinophils in the airways (**Figures 5B, C**).

Lungs were also harvested for mRNA expression 24 and 48 h after treatment, as measured by qPCR. Compared to sham treatment, RV-C15 infection significantly increased mRNA expression of IFN-γ, CXCL10, CXCL1, CXCL2, TNF-α, IL-12, IL-25, IL-13 and IL-5 (**Figure 5D**). UV irradiation significantly reduced RV-C15-induced IFN-γ, CXCL10, CXCL1, CXCL2, IL-13 and IL-5 transcript levels, and UV-irradiated virus failed to induce TNF-α or IL-25 mRNA expression.

Comparison of RV-C15 and RV-A1B Responses

We compared lung inflammatory responses 48 h after infection with RV-C15 and RV-A1B, a minor group virus which infects mouse cells (29, 40) and constitutes a well-established model of RV infection (26, 27). As noted above, RV-C induced significant eosinophilic inflammation, whereas RV-1B did not (**Figure 6A**). Compared to RV-A1B, RV-C15 infection induced significantly higher mRNA expression of CXCL1, CXCL2, IL-5 and IL-13 (**Figure 6B**). Only RV-C15 induced significant mRNA expression of the IL-13-responsive genes Muc5ac, Muc5b and Gob5/Clca1. Finally, only RV-C15 significantly increased protein expression of the type 2 cytokines IL-5 and IL-13 (**Figure 7A**).

We also examined lungs for elaboration of IL-25, IL-33 and TSLP, innate cytokines that promote ILC2 expansion (41–47). Compared to RV-A1B, RV-C15 infection induced significantly higher mRNA expression of IL-25 (**Figure 6B**). Only RV-C15 significantly increased mRNA expression of IL-33 and TSLP. In addition, compared to RV-A1B, RV-C15 infection induced significantly higher protein expression of IL-25, IL-33 and TSLP (**Figure 7A**). Lungs were formalin-fixed and paraffin-embedded 2 days post-exposure, and sections stained for IL-25, IL-33 and TSLP immunofluorescence. Abundant staining for IL-25, IL-33 and TSLP was seen in the airways of mice inoculated with RV-C15 but not RV-A1B (**Figure 7B**). IL-25 and TSLP were localized to the airway epithelium whereas IL-33 was found in both airway epithelial and peribronchial cells.

RV-C15 Inoculation Enhances mRNA Expression of Type 2 Cytokines, Mucus Genes and Airway Responsiveness in Allergen-Challenged Mice

RV-C15-induced respiratory illnesses have been associated with a previous history of asthma (3–6, 11, 13). We therefore

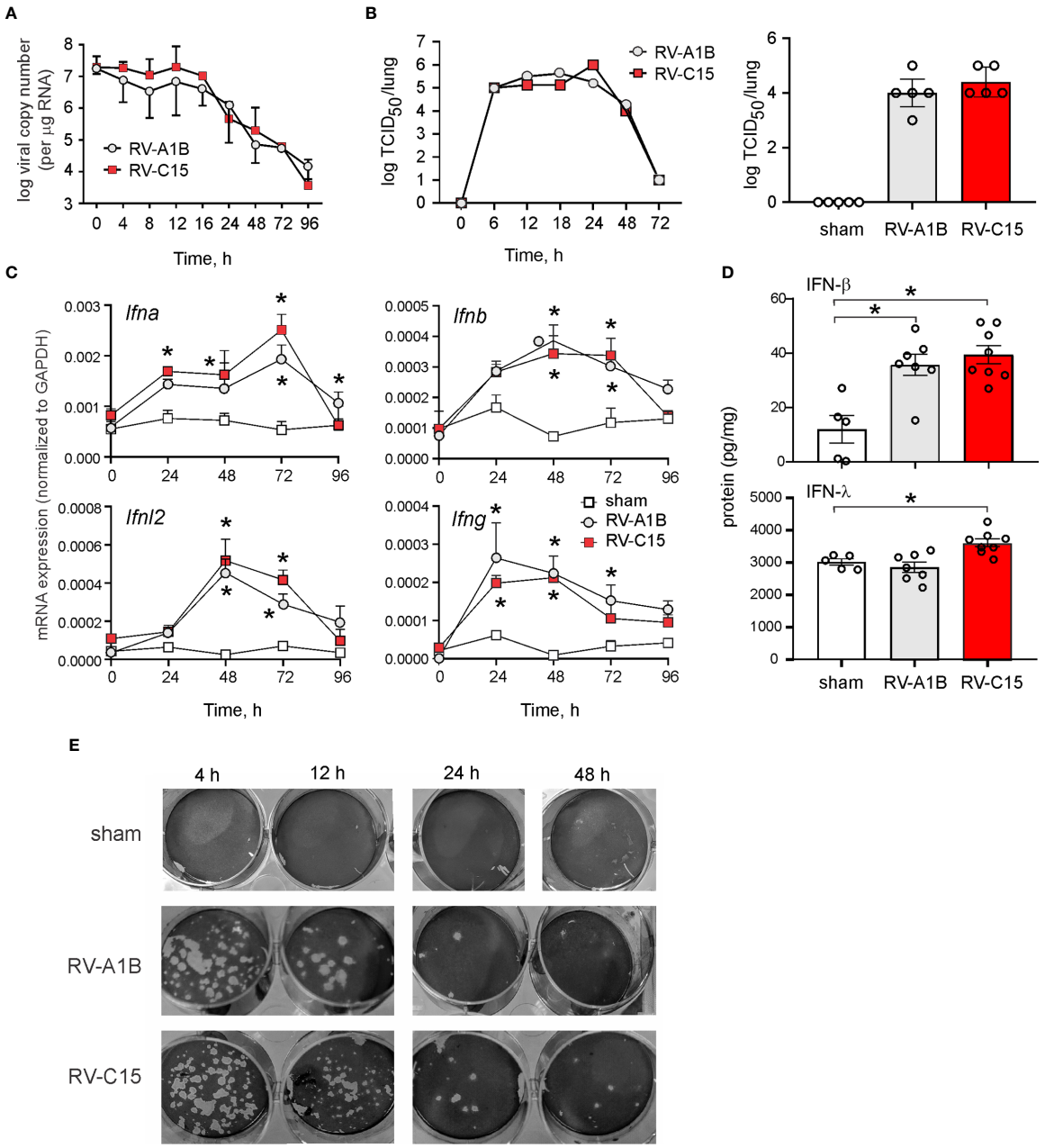

FIGURE 2 | Viral RNA is detectable in the lungs of RV-C15 treated mice. **(A)** Female 8-10-week-old BALB/c mice were inoculated with 5 x 10^6 ePFU of RV-C15 or RV-A1B by intranasal instillation and lungs were examined by RT-PCR for viral RNA at the indicated time points. Graph showing RV-C15 and RV-A1B copy number at the indicated time points up to day 4. Data are mean ± SEM, n = 2-16 mice/group from five different experiments. **(B)** Left panel. Time course of lung viral titers in mice infected with RV-C15 and RV-A1B (7 mice per virus). Viral titer was assessed by $TCID_{50}$. Right panel. Group mean data from an additional 5 mice at the 48 hr time point are also shown. Median ± interquartile range, n = 5 mice per group from one experiment are shown. **(C)** Graphs showing IFN mRNA expression analysis at indicated time points. The level of gene expression for each sample was normalized to GAPDH. Data represent mean ± SEM, n = 6 mice in each group from two different experiments, *P<0.05 by two-way ANOVA. **(D)** Graphs showing IFN expression analysis 48 hr after infection. Data represent mean ± SEM, n = 7 mice in each group from one experiment, *P<0.05 by one-way ANOVA. **(E)** Plaque assays show live virus in the lungs of RV-A1B and RV-C15-infected mice, as evidence by plaque formation in HeLa-H1 and HeLa-E8 cells, respectively.

determined the response to RV-C15 infection in mice with allergic airways disease and compared the responses with RV-A1B. As described previously (33), wild-type BALB/c mice were sensitized with house dust mite (HDM) and challenged with HDM 10 and 11 days after sensitization. Two days later, mice were inoculated with sham, RV-C15 or RV-A1B. Forty-eight h

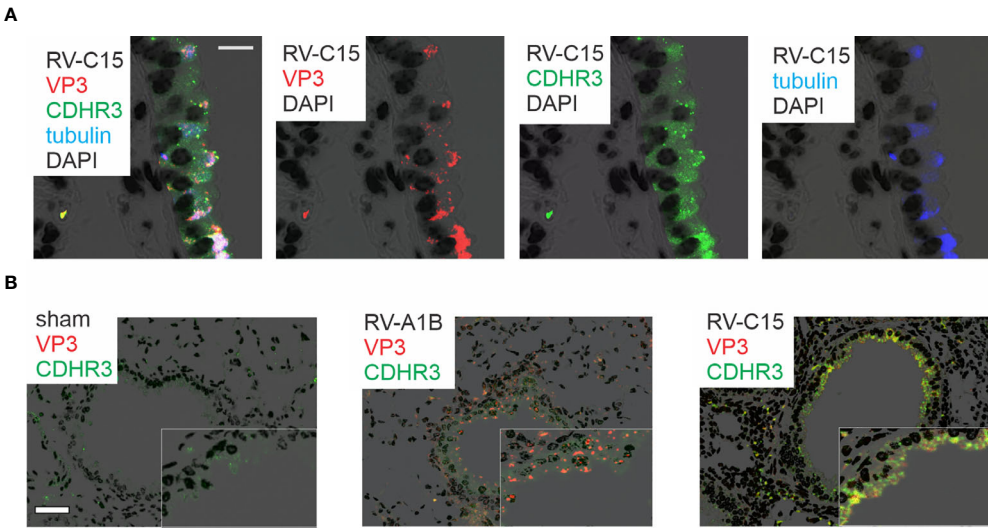

FIGURE 3 | Colocalization of RV-C15 and CDHR3 in airway tissues of RV-C15-infected mice. **(A)** Airways from RV-C15-infected mice were stained with anti-VP3 (red), anti-acetyl α-tubulin (blue) and anti-CDHR3 (green). To stain mouse CDHR3, we identified a peptide (YQVEAFDPEDTSRN, human AAs 154-167) in the second extracellular calcium-binding domain identical in mouse and human. Polyclonal antiserum was generated and purified by affinity chromatography. Acetyl α-tubulin was localized to the epithelial cell apical surface. Colocalization is white. RV-C15 was localized to CDHR3+ ciliated airway epithelial cells. The white bar is 10 μ. **(B)** Airway sections from sham-, RV-A1B and RV-C15-infected mice stained for viral protein 3 (VP3, shown in orange/red) and CDHR3 (green). Colocalization is yellow. The white bar is 50 μ.

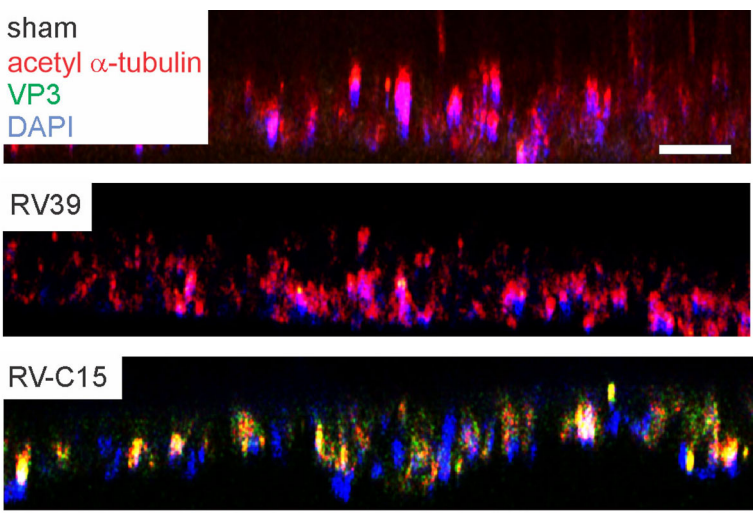

FIGURE 4 | RV-C15 co-localizes with mouse epithelial cell acetyl-α-tubulin and induces expression of pro-inflammatory cytokines. Mouse airway epithelial cells were differentiated at air-liquid interface for 21 days. Cells were infected with sham HeLa cell lysate, RV-C15 or RV-A39 at MOI 2 and harvested 5 min after infection for immunofluorescence staining with anti-mouse acetyl α-tubulin (red) and anti-VP3 (green). Nuclei are stained with DAPI (blue). Confocal microscopy shows colocalization of cilia and RV-C15 (yellow). The white bar is 50 μ.

after infection, mice were sacrificed for bronchoalveolar lavage and lung mRNA determination or anesthetized and endotracheally intubated for measurement of methacholine responsiveness. As noted above, RV-C15 infection of naïve mice increased BAL monocytes, neutrophils, eosinophils and lymphocytes (**Figure 8A**), while inducing mRNA expression of IFN-γ, CXCL10, IL-17A, IL-13 and IL-5 (**Figure 8B**). HDM sensitization and challenge induced lung infiltration with monocytes, lymphocytes and eosinophils (**Figure 6A**), and lung mRNA expression of the eosinophil chemoattractant CCL24, the type 2 cytokines IL-5 and IL-13 and the mucus-related genes Muc5AC and Gob5 (**Figure 8B**).

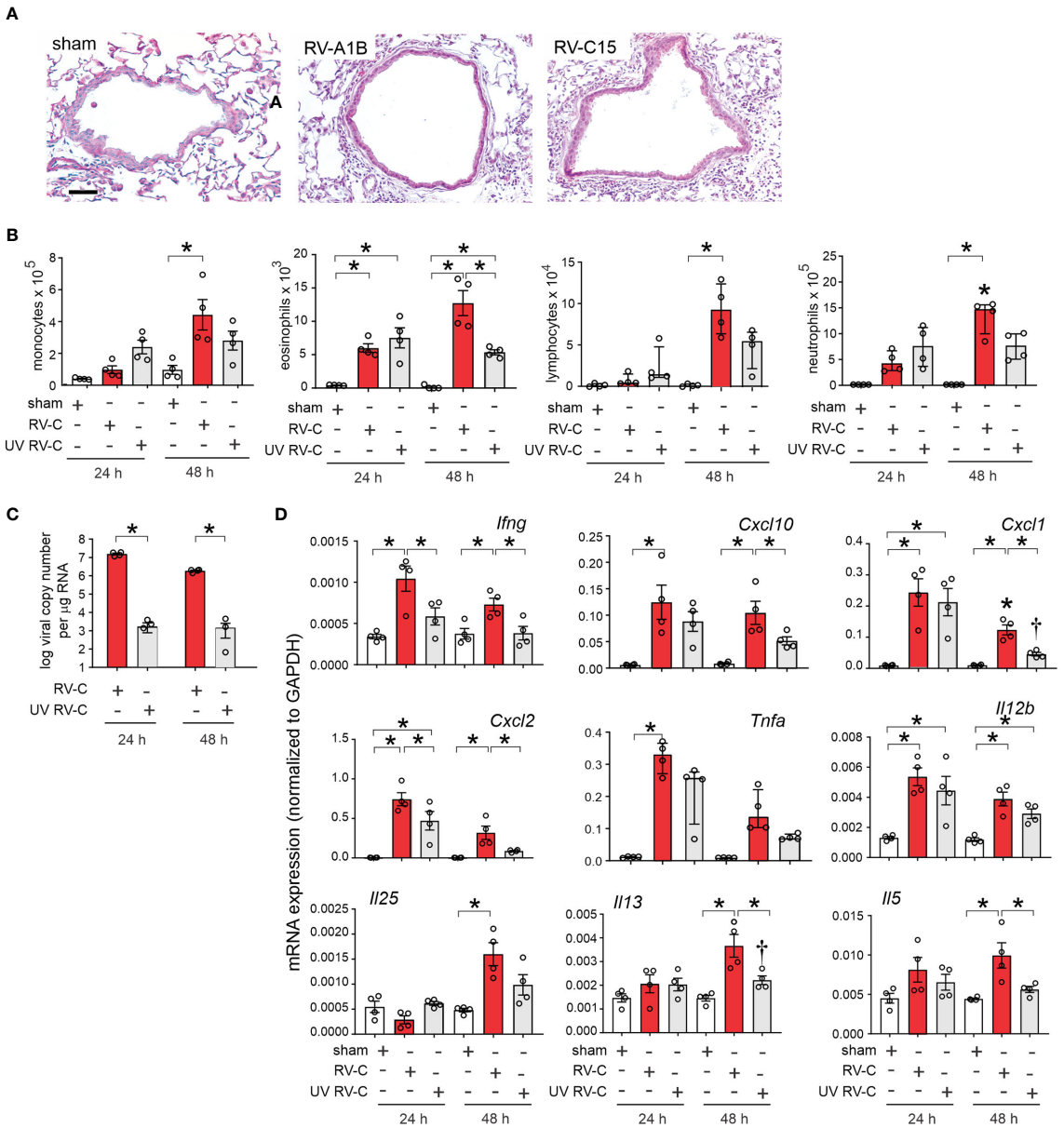

FIGURE 5 | RV-C15 induces airway inflammation in naïve mice that is partially dependent on viral replication. Female 8-10-week-old BALB/c mice were treated with sham HeLa cell lysate, 5 x 10^6 ePFU RV-C15 or UV-irradiated RV-C15. **(A)** Hematoxylin- and eosin-stained lung tissue. Bar = 50 µ. **(B)** Lungs were harvested 24 or 48 h after inoculation and processed for BAL inflammatory cell counts. **(C, D)** Twenty-four or 48 hr after inoculation, lungs were harvested for vRNA and mRNA expression. For mRNA, the level of gene expression for each sample was normalized to GAPDH. (For panels B-D, data are mean ± SEM except for lymphocytes, neutrophilis and *Tnfa, n* = 3-4 mice in each group from one experiment, **P* < 0.05 by one-way ANOVA; for lymphocytes, neutrophils and *Tnfa*, data are median ± IQR, **P* < 0.05 by Kruskal-Wallis test).

Infection of HDM-treated mice with either RV-A1B or RV-C15 had additive effects on airway inflammation (**Figure 8A**). RV-A1B and RV-C15 each increased neutrophilic and eosinophilic inflammation; however, the increase in eosinophils was significantly greater in mice infected with RV-C15. In addition, RV-C15 infection of mice with allergic airways disease had additive effects on BAL lung IL-13, IL-5 and CCL24 expression, which were greater than induced by RV-A1B (**Figure 8B**). Thus, RV-C15 infection enhanced allergen-induced type 2 inflammation to a greater extent than RV-A1B. On the other hand, RV-C15-induced neutrophilic inflammation and mRNA expression of CXCL10 and IL-17 tended to be lower in allergen-challenged mice.

Next, we examined the effects of RV infection on the airway responsiveness in naïve and HDM-sensitized and -challenged mice. Increasing doses of nebulized methacholine were given by

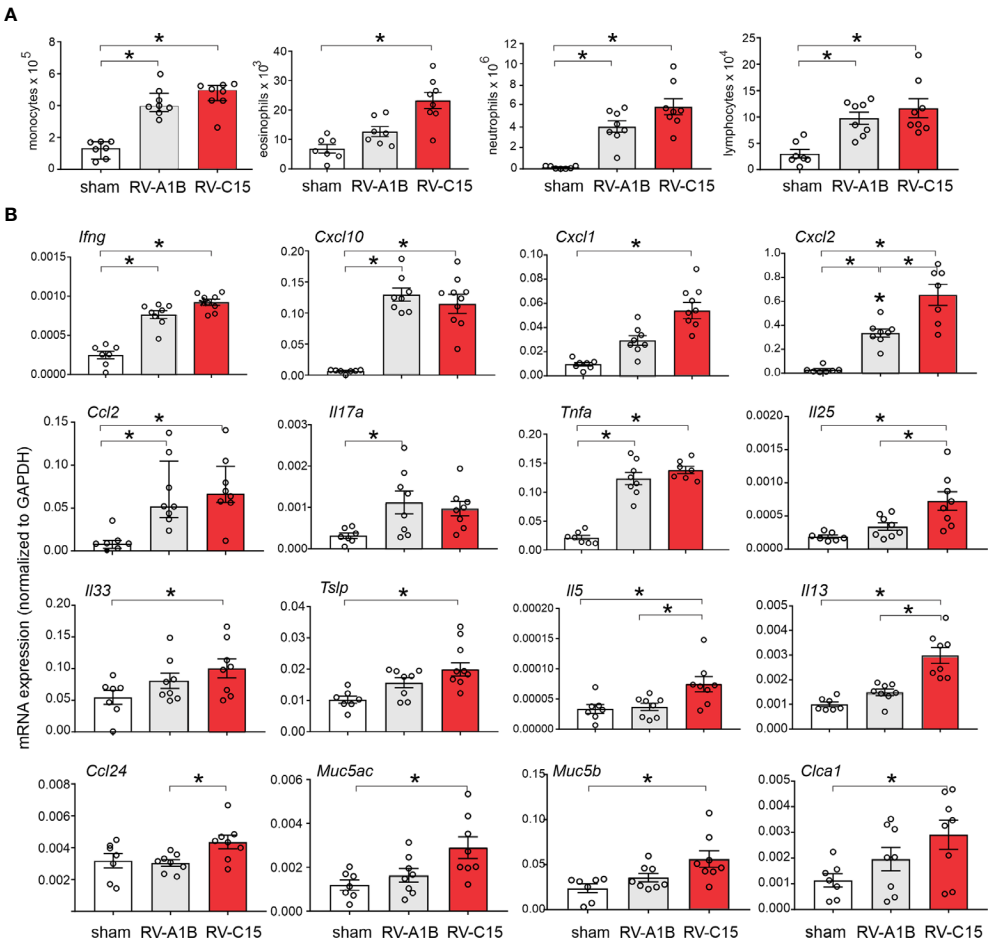

FIGURE 6 | Comparison of RV-C15- and RV-A1B-induced airway inflammation in naïve mice. Female 8-10-week-old BALB/c mice were treated with sham HeLa cell lysate, 5 x 10^6 ePFU RV-C15 or 5 x 10^6 ePFU RV-A1B. **(A)** Lungs were harvested 48 hr after inoculation and processed for BAL inflammatory cell counts. **(B)** Forty-eight h after inoculation, lungs were harvested for mRNA expression. The level of gene expression for each sample was normalized to GAPDH. For panels **(A, B)** data are mean ± SEM except for monocytes and *Ccl2*, n = 7-8 mice in each group from two experiments, $^*P < 0.05$ by one-way ANOVA; for monocytes and *Ccl2*, data are median ± IQR, $^*P < 0.05$ by Kruskal-Wallis test).

inhalation and respiratory system resistance measured. In naïve mice, RV-C15 increased airways responsiveness compared to RV-A1B (**Figure 8C**). In HDM-treated mice, only RV-C15 increased airway responsiveness compared to HDM alone.

Potential Contribution of ILC2s to RV-C-Induced Airway Inflammation

Next, we examined lung ILC2s by flow cytometry. Cells were sorted for size, live/dead and surface markers for the various hematopoietic lineages (**Figure 9A**). Lungs from sham-treated mice showed a large number of lineage- CD25- CD127- cells, likely representing lung structural (epithelial and mesenchymal) cells. Two days after infection, RV-C15 infection was associated with a small but significant increase the number of lung lineage-negative, CD25-, CD127-double positive ILC2s (**Figures 9B, C**). There was no increase in the lungs of RV-A1B-infected mice. Identification of ILC2s was confirmed by co-staining with ST2, the IL-33 receptor (**Figures 9D, E**).

We examined RV-C15-induced cytokine responses in ILC2-deficient *Rora*$^{fl/fl}$*Il7r*^{Cre} mice and *Rora*$^{fl/fl}$ littermates. We previously found that six day-old *Rora*$^{fl/fl}$*Il7r*^{Cre} mice fail to show ILC2 expansion after RV-A1B infection despite a small increase in viral load, demonstrating the effectiveness of this knockout (48). RV-C15 infection increased airway eosinophilic inflammation (**Figure 10A**) and mRNA expression of IL-13, Muc5ac and Muc5b in *Rora*$^{fl/fl}$ mice but not *Rora*$^{fl/fl}$*Il7r*^{Cre} littermates (**Figure 10B**). In contrast, RV-C15-induced neutrophils, lymphocytes and mRNA expression of IFN-γ and CXCL10 were preserved.

DISCUSSION

Despite increasing recognition of RV-C as a cause of severe asthma exacerbation, little is known about the pathogenesis of RV-C infections. To accomplish this, we infected BALB/c mice with RV-C15, and compared responses to our previously established

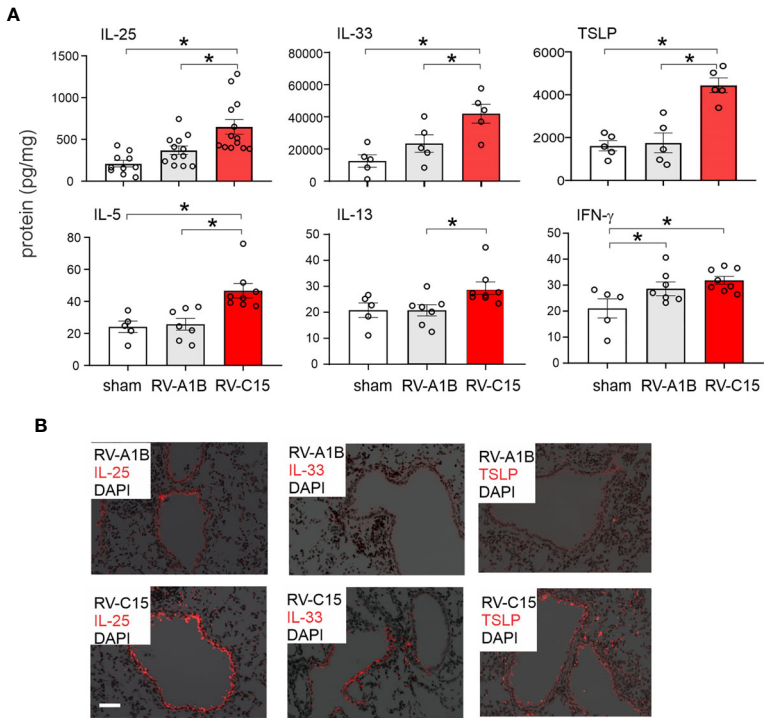

FIGURE 7 | Comparison of RV-C15 and RV-A1B-induced cytokine expression in naïve mice. **(A)** Effects of RV-C15 and RV-A1B on cytokine expression measured by ELISA. Data are mean ± SEM, n = 5-12 mice in each group from one or two experiments, *$P < 0.05$ by one-way ANOVA. **(B)** Immunofluorescence images of mouse lungs infected with 5 x 10^6 ePFU RV-C15 or 5 x 10^6 ePFU RV-A1B. Staining for IL-25, IL-33 and TSLP is shown. Scale bar is 50 μm. These images are representative of four mice.

RV-A1B model (27). Following intranasal inoculation with RV-C15, we isolated positive-strand viral RNA from the lungs of mice up to four days after exposure. We detected RV-C15 protein in airway epithelial cells. We also showed that lung homogenate from RV-C15-exposed mice 48 hr after infection causes cytopathic effects and plaque formation in HeLa-E8 cells. RV-C exposure induced a robust type I and type III interferon response which peaked 48-72 hrs after infection, evidence of viral replication (49). UV-irradiated virus had significantly reduced effects on airway inflammation and cytokine expression. RV-C15 exposure induced airway inflammation, as demonstrated by lung histology, increased BAL cells, and increased cytokine expression. Airways inflammation was accompanied by a functional state of hyperresponsiveness. Finally, RV-C15 but not RV-A39 infected ciliary-differentiated mouse airway epithelial cells cultured at air-liquid interface. Together, these data suggest that mouse lower airways may be infected with RV-C15. However, the steep reduction in viral RNA we observed in our model, similar to that previously observed for RV-A16 (26), minor group RV-A1B (26, 27) and enterovirus D68 (28), speaks against a substantial replicative infection.

In addition, we found that RV-C15 infection induced quantitatively and qualitatively different airway responses than RV-A1B. RV-C15-infected mice showed significantly higher CXCL1 and CXCL2 mRNA expression than RV-A1B-infected mice. In addition, only RV-C-infected mice showed increases in lung mRNA expression of IL-5, IL-13 and CCL24, indicating a type 2 inflammatory response. While at times the differences between RV-C15 and RV-A1B responses were small, RV-C-induced type 2 cytokine responses were sufficient to generate robust airway eosinophilia and mucous gene expression. We also found increased mRNA expression and peribronchial deposition of the innate cytokines IL-25, IL-33 and TSLP. This response is distinctly different from the response to RV-A1B or RV-A16, which have been shown by five different laboratories to be a classic type 1 antiviral response (26, 27, 50–55). Only Tbet-deficient mice show IL-13 expression after RV-A1B-infection (56). Similarly, after human experimental RV-A16 infection, only subjects with asthma, but not controls, show elevation of IL-5, IL-13, IL-25 or IL-33 (51, 57, 58). Similar differential IL-25 and TSLP expression has been noted in asthmatic epithelial cells (51, 59). This pattern of increased type 2 and innate cytokine expression could explain why infections with RV-C are more likely to occur in children with a history of asthma or who develop asthma (6, 10, 11, 13, 14).

The precise mechanism by which RV-C15 induces greater type 2 and innate cytokine expression than RV-A1B is unclear. A previous study examining the effects of various respiratory viruses on airway epithelial cell replication kinetics, cell tropism, tissue integrity, and cytokine secretion showed no differences between RV-C15 and other RVs (23). Similarly, we did not notice more cell loss in cultures infected with RV-C15 (data not shown). We speculate that differential innate cytokine

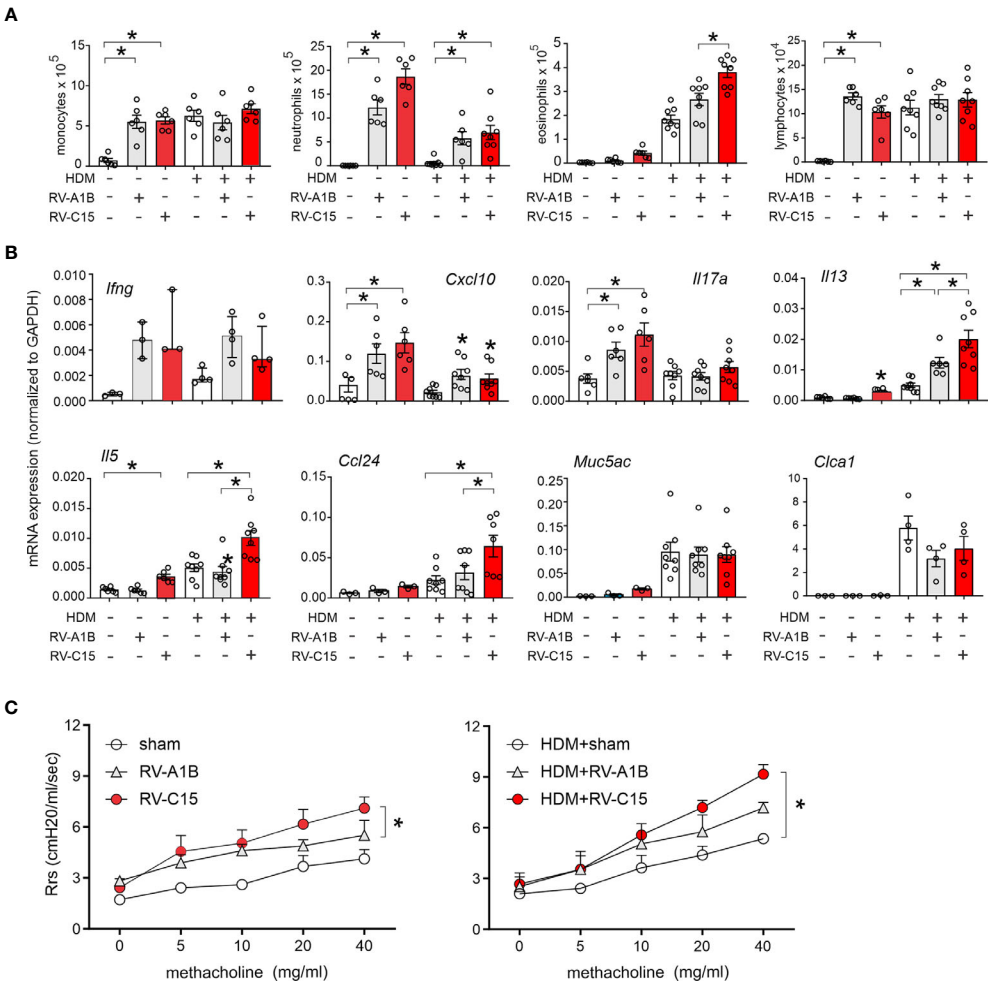

FIGURE 8 | Comparison of RV-C15- and RV-A1B-induced airway inflammation in house dust mite-sensitized and -challenged mice. Female 8-12 week-old BALB/c mice were challenged with house dust mite (HDM) and treated one day after the last HDM treatment with sham HeLa cell lysate, 5 x 10^6 ePFU RV-C15 or 5 x 10^6 ePFU RV-A1B. Forty-eight hrs later lungs were harvested for BAL analysis and qPCR. A separate set of mice were similarly treated and were anesthetized and endotracheally intubated for measurement of airways responsiveness. **(A)** Graphs showing BAL cell counts. **(B)** Graphs showing qPCR analysis of lung mRNA expression. The level of gene expression for each sample was normalized to GAPDH. For panels **(A, B)**, data are mean ± SEM except for *Ifng*, *n* = 3-8 mice/group for 1-3 experiments, *p<0.05, one-way ANOVA; for *Ifng*, data are median ± IQR. **(C)** Airways methacholine responsiveness of the indicated treatment groups. Data are mean ± SEM of 3-4 mice/group from two experiments, *$P < 0.05$, two-way ANOVA.

expression following RV-C15 reflects the receptor-linked signaling pathways that establish the inflammatory response. In support of this hypothesis, differences in macrophage gene expression between two rhinovirus serotypes, RV-A16 and RV-A1A, have been traced to differential kinase and transcription factor phosphorylation following initial RV binding (60). Also in support of this hypothesis are the robust increases in cytokine expression we observed in mice treated with UV-irradiated RV-C15, more than we observed previously with RV-A1B (27). Responses to replication-deficient virus are likely to reflect receptor binding and endocytosis of virus, rather than later replication-dependent responses. Previous *in vitro* studies have also shown responses to replication-deficient RV (61–63), suggesting that binding and endocytosis of RV is sufficient, and viral replication unnecessary, for a subset of inflammatory responses, to be followed by a second set of replication-dependent responses.

We found colocalization of RV-C and acetyl α-tubulin, a microtubule protein that is enriched in the axonemes of most cilia. Thus, these data confirm that RV-C binds to ciliated airway epithelial cells (64). We found that CDHR3 preferentially colocalizes with RV-C15 compared to RV-A1B. Mouse CDHR3 is highly homologous to human CDHR3 and includes the N186 glycosylation site (19) and EC-1 domain (65) required and sufficient for RVC15 binding to the human protein. However, we were unable to block RV-C binding or replication with anti-CDHR3 (not shown). We are therefore unable to state with certainty that CDHR3 is the RV-C receptor in mice. Since structural analysis suggests that, similar to enterovirus-D68, RV-C15 binds to a sialic acid moiety of a CDHR3-bound glycan (18),

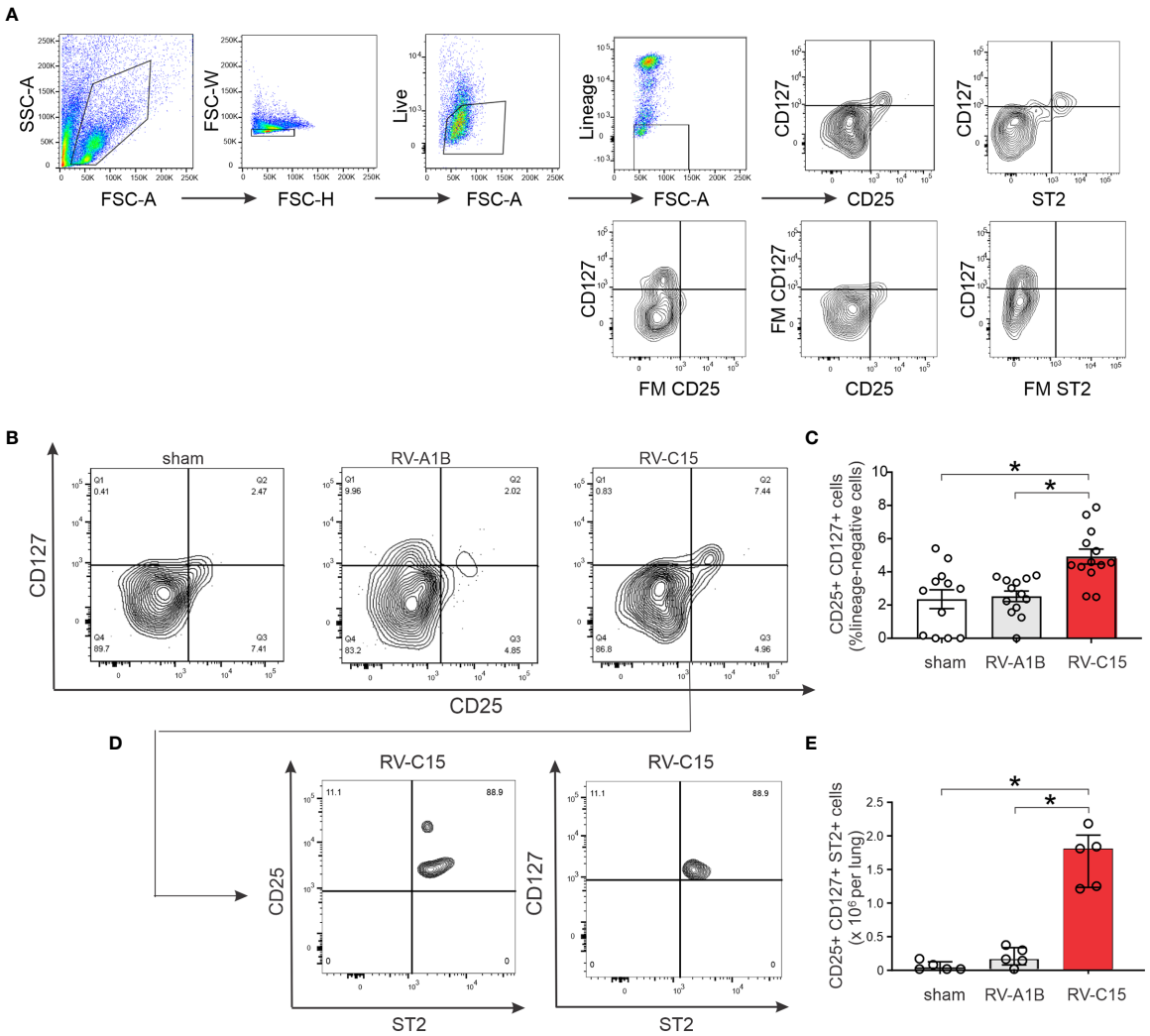

FIGURE 9 | Flow cytometric analysis **(A–E)** for ILC2s was carried out in RV-A1B- and RV-C15-infected mice. For flow cytometry, ILC2s were identified as lineage-negative, CD25- and CD127-double positive cells **(B, C)** and lineage-negative, CD25+, CD127+, ST2+ cells **(D, E)**. For **(C)**, data are mean ± SEM, $n = 12$ mice in each group from three different experiments, $^*P < 0.05$ by one-way ANOVA. For **(E)**, data are median ± IQR, $n = 5$ mice in each group from one experiment, $^*P < 0.05$ by Kruskal-Wallis test.

it is conceivable that RV-C binds to other glycan-binding proteins. A recent study in human airway epithelial cells showed that, while CDHR3 knockdown blocks RV-C replication, it does not affect binding of RV-C (66), suggesting that a co-receptor is required for binding of the virus.

As noted above, RV-C15 infection induced airway expression of IL-25, IL-33 and TSLP, innate cytokines responsible for activation of IL-5- and IL-13-producing ILC2s expansion (41–47). IL-25 production was mostly limited to airway epithelial cells, whereas IL-33 was noted in epithelial and subepithelial cells. A similar pattern of IL-33 deposition was observed in children with steroid-resistant asthma (67). Accordingly, we found increased lineage-negative, ST2+, CD25- and CD127 ILC2s in the lungs of RV-C15-, but not RV-A1B-infected mice. ILC2 depletion blocked RV-C15-induced mRNA expression of IL-13 and the mucus-related genes Muc5ac and Muc5b. Previously we noted expansion of ILC2s in RV-A1B-infected 6 day-old mice but not mature mice (35). Lung ILC2 expansion has also been shown after influenza infection (68, 69) and allergen-induced airway inflammation (44–46, 70–72).

A limitation of our study is the transient nature of viral infection in our model. Species differences restrict viral replication, requiring a high inoculum. However, we have previously shown that infection with RV-A1B increases lung type 1 IFN and negative-strand viral RNA expression (27), markers of viral replication. In addition, MDA5 is required for RV-A1B-induced inflammatory responses (73), inferring a role for double-stranded RNA, a form of viral RNA which is only made during viral replication. Inhibition of inflammasome activation (55) and corticosteroids (74) each increase viral load in RV-infected mice, consistent with the notion

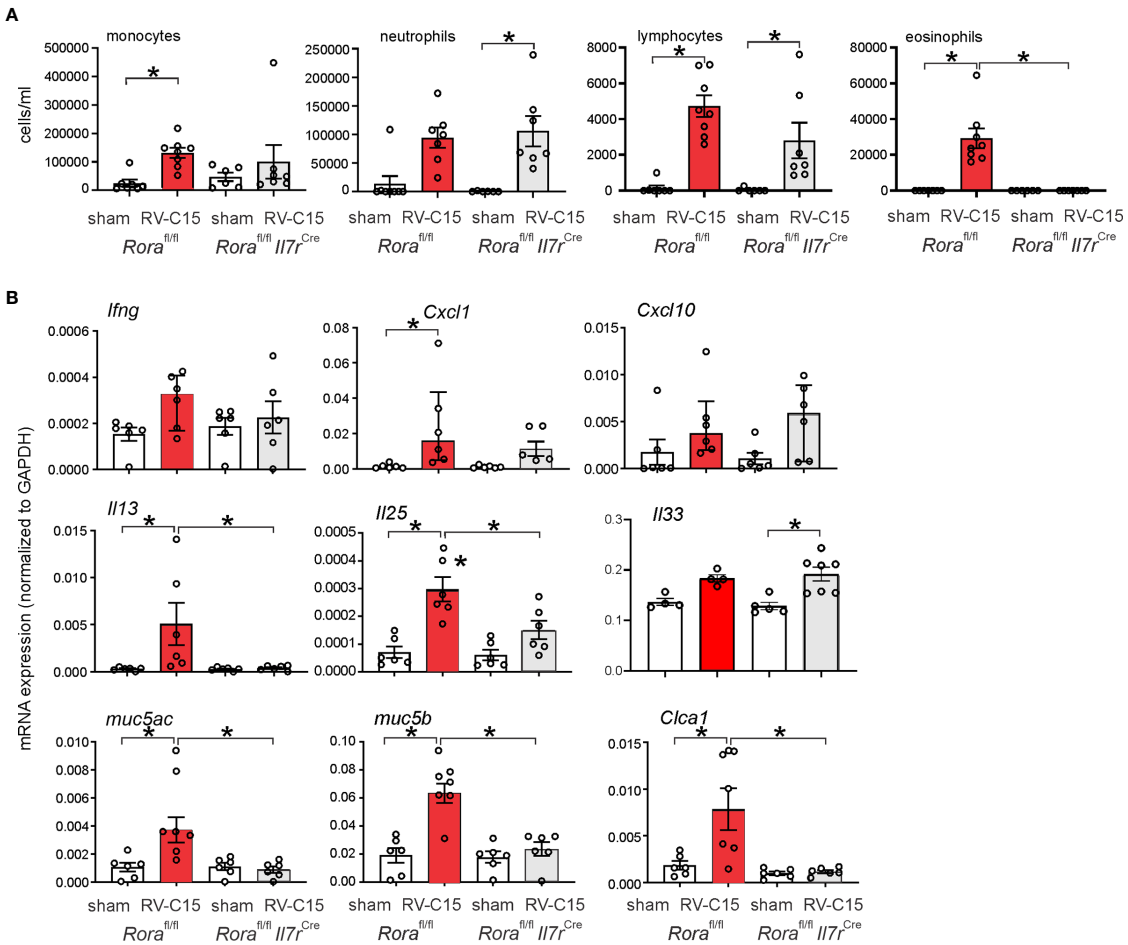

FIGURE 10 | Requirement of ILC2s for RV-C15-induced airway inflammation and mRNA expression. **(A)** Rora$^{fl/fl}$ Il7r^{Cre} mice infected with RV-C show lower eosinophils compared to Rora-$^{fl/fl}$ littermates. Data are mean ± SEM, n = 6-8 mice/group from one experiment, *P < 0.05 by one-way ANOVA. **(B)** Rora$^{fl/fl}$ Il7r^{Cre} mice infected with RV-C show lower *Il13, Muc5ac, Muc5b* and *Clca1* mRNA expression compared to Rora-$^{fl/fl}$ littermates. Data are mean ± SEM except for IFN-γ and CXCL10, *n* = 6 mice/group from three different experiments, *$P < 0.05$ by one-way ANOVA; for *Ifng* and *Cxcl10*, data are median ± IQR.

that antiviral immunity plays a significant role in our model. In addition, we have observed differences in the inflammatory response to RV-A1B, RV-C15 and EV-D68 (28) which are qualitative in nature and resemble responses in human subjects. Taken together, these results suggest that while replication of human RV is minimal in mice, the resulting host-induced innate immune response and immunopathology is worthy of study. Indeed, replication-deficient viral vectors are a useful tool for studying the innate immune response to acute viral infection without ongoing cytopathic effects (75).

We conclude that RV-C15 exposure induces airways inflammation in mice, binding to ciliated airway epithelial cells. Compared to RV-A1B infection, the inflammatory response to RV-C15 is characterized by greater eosinophils, epithelial-derived innate cytokines and IL-13-producing ILC2s. Further characterization of this model, combined with studies of human subjects, will provide insight into the pathogenesis of rhinovirus C infections.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of University of Michigan and performed under National Institutes of Health guidelines.

AUTHOR CONTRIBUTIONS

CR performed experiments, analyzed the data, and drafted the manuscript. MYH performed experiments, analyzed data, and

edited the manuscript. TI performed experiments and analyzed data, JL performed experiments. AG performed experiments and analyzed data. SJ performed experiments. CS performed experiments. JB performed experiments, analyzed data, and edited the manuscript. MBH supervised all aspects of the project, interpreted the data, and write the final draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.649520/full#supplementary-material

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MINI REVIEW
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Regulation and Function of ILC3s in Pulmonary Infections

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Lower respiratory infections are among the leading causes of morbidity and mortality worldwide. These potentially deadly infections are further exacerbated due to the growing incidence of antimicrobial resistance. To combat these infections there is a need to better understand immune mechanisms that promote microbial clearance. This need in the context of lung infections has been further heightened with the emergence of SARS-CoV-2. Group 3 innate lymphoid cells (ILC3s) are a recently discovered tissue resident innate immune cell found at mucosal sites that respond rapidly in the event of an infection. ILC3s have clear roles in regulating mucosal immunity and tissue homeostasis in the intestine, though the immunological functions in lungs remain unclear. It has been demonstrated in both viral and bacterial pneumonia that stimulated ILC3s secrete the cytokines IL-17 and IL-22 to promote both microbial clearance as well as tissue repair. In this review, we will evaluate regulation of ILC3s during inflammation and discuss recent studies that examine ILC3 function in the context of both bacterial and viral pulmonary infections.

Keywords: ILC3, innate lymphoid cell (ILC), pneumonia, SARS-CoV-2, COVID-19, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*

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INTRODUCTION

Th1 and Th2 helper cell subsets were initially defined by cytokine secretion (1, 2) and this was expanded to other T cell subsets including Th17 cells in the early 2000s, which demonstrated that IL-17 secreting CD4+ T cells arise independent of transcription factors of Th1 (STAT4) and Th2 (STAT6) cells (3–5). However, shortly after the expansion of these Th subsets, it was recognized that many of these cytokines could also be produced by non-T-cell receptor bearing innate cells such as IFNγ-producing NK cells. Th2 cytokines, such as IL-5 and IL-13, were found to be expressed in innate lymphoid cells (ILCs) by several groups (6, 7) and these cells have been subsequently termed group 2 or ILC2 cells. Similarly, cytokines associated with Th17 lineage, IL-17 and IL-22, were originally found in ILCs within tonsils and the gastrointestinal tract (8, 9) and termed group 3 or ILC3 cells. A key early finding showing the functionality of ILC3 cells was demonstrating that they could mediate colitis in mice lacking T cells (9). As opposed to the gastrointestinal tract, where ILC3 cells are abundant, ILC2 cells predominate in the lung, seeding tissues during fetal development (10, 11). However, it has been increasingly recognized that ILC3s play a role in lung immunity. Like other ILC populations, ILC3s require IL-7R signaling and derive from Id2 expressing progenitor cells (12). ILC3s also express the transcription factor RORγt, which differentiates them from ILC1 or ILC2 cells. This review will highlight recent advances in ILC3 biology in the lung.

REGULATION OF ILC3 IN THE LUNG

ILC3s localized to the lung are ideally positioned to regulate mucosal immunity within the context of constant exposure to environmental insults. Indeed, in newborn mice, insulin-like growth factor 1 (IGF1)-dependent maturation and expansion of ILC3 precursors in lungs was essential for protection against respiratory pathogens (13). Importantly, disruption of ILC3 development in neonates resulted in increased susceptibility to infection into adulthood, emphasizing the importance of early establishment of these surveyors of lung health. Several chemokines may facilitate ILC3 positioning in the lung. The CXCL13-CXCR5 axis has been implicated in localization of ILC3 to inducible bronchial associated lymphoid tissue (iBALT) structures that develop in the lungs in *Mycobacterium tuberculosis* infection in mice (14), while both CCR6 and CXCR5 were expressed by ILC3s recruited to sites of lung tumors in patients (15). Trafficking of ILC3s to the lung during pneumonia was attributed to CCR4 expression, as deficiency of CCR4 in adoptively transferred ILC3s abrogated homing to the lungs in newborn mice (16). This study also demonstrated the possibility that some lung ILC3 populations may derive from circulating ILC3s. Finally, the CXCR6-CXCL16 axis, which is critical for ILC3 precursor localization to the mouse lamina propria (17), enabled homing of ILC1 and ILC2 cells to the lung under inflammatory conditions (18).

Further studies that define mechanisms for how ILC3s or their progenitors traffic to and function in the lung are much-needed, as research on ILC3s largely centers on the gastrointestinal tract where large populations of these cells reside. This gap in knowledge likely stems from the difficulty of studying these cells, as ILC3s comprise < 5% of total ILCs in the mouse lung (19). Similarly, in human lungs, frequencies of IFNγ+ ILCs were higher than IL-17+ or IL-22+ ILCs, though ILC3 and ILC3-like cells encompassed the highest percentage of total ILCs. However, in chronic obstructive pulmonary disease that is associated with iBALT and chronic infection, the percentage of ILC3 cells were increased compared to healthy lung tissue (20), suggesting that environmental exposures may be key drivers of ILC3 accumulation and that studies in mice will need to include modeling such exposures to study ILC3 biology.

Once in the lung, ILC3s are believed to primarily reside in their resident tissue. Parabiosis studies in uninfected mice revealed that > 95% of all ILCs from various tissues were of host origin (21), though ILC3s were not analyzed in the lungs, perhaps due to the low numbers of these cells at rest (19). However, evidence also supports an increase in circulating ILCs can occur during inflammation. Indeed, helminth infection of mice induced an increase in circulating ILC2s derived from the small intestine and lung (22, 23), though only lung-derived ILC2 were able to migrate back into the lung (23). Commensal bacteria in the intestines of newborn mice were also found to induce expression of CCR4 on ILC3s, enabling subsequent migration to the lungs during pneumonia (16). Nevertheless, analysis of human blood found low numbers of ILC3s in circulation at rest (24), suggesting that while mature ILC3s may migrate during inflammation, the majority of ILC3s may reside and proliferate within their resident tissue.

As ILC3s do not bear T-cell receptors, other factors including cytokines, alarmins, and co-stimulatory molecules can mediate ILC3 stimulation to induce effector function. ILC3s provide immune surveillance of the lung at the steady state, delivering immediate innate protection after host exposure to pathogens. During inflammation, IL-1β and IL-23 stimulate ILC3s to produce IL-17 and IL-22 (8, 25), which in turn regulate epithelial barrier function and mediate host response to infections (8, 26, 27). In the lungs, these cytokines function to enhance production of antimicrobial proteins, facilitate barrier repair through promotion of epithelial cell proliferation, and augment neutrophil recruitment, resulting in increased clearance of pulmonary pathogens (28–30). Thus, activated ILC3s are well-poised to provide immediate and direct action toward foreign antigens. Importantly, ILC2s may also provide IL-17-mediated benefits upon infection. Indeed, ILC2s from nasal polyps of cystic fibrosis patients or skin lesions of psoriasis patients transdifferentiated to an ILC3-like cell—expressing RORγt and producing IL-17—upon ex vivo stimulation with IL-1β, IL-23, and TGFβ (31, 32). Therefore, given the abundance of ILC2 in the lung, their potential for plasticity could contribute to ILC3-attributed functions during inflammation.

The inducible T cell costimulatory molecule (ICOS) may also stimulate ILC3s in the lung. Differential expression of ICOS, which is also expressed on T cells, has been observed in both mouse and human ILC3s (33, 34). However, the role of this molecule in regulating ILC3 function remains to be fully determined. Mice deficient in ICOS had no change in the total amount of ILC3 in the lung at rest compared to their wild-type counterparts (19), though < 2% of ILCs studied were ILC3s. As not all ILC3s express ICOS, it is possible in the deficient background the balance between ICOS+ and ICOS- ILC3s is shifted, especially since the total population of ILC3s in the lung at rest is very low (19, 20). Indeed, we have shown administration of a neutralizing anti-ICOS antibody to mice prior to bacterial infection resulted in a decrease in *Icos* expression as well as expression of *Il17* and *Il22* after infection with *Klebsiella pneumoniae* (33). In addition, ex vivo stimulation of ILC3s isolated from *K. pneumoniae* infected mice with ICOS ligand (ICOSL) resulted in proliferation of cells (33), indicating the ICOS : ICOSL pathway may be important in mediating ILC3 function and proliferation (**Figure 1**). Interestingly, mouse and human ILC2s express both ICOS and functional ICOSL (35) which can stimulate ICOS+ Treg cells (36), raising the possibility that a coordinated interplay could also exist between ILC3 and ICOSL-expressing ILC2. Such interactions could play a key role in regulating ILC3 activation and providing a swift response upon pathogen presentation in the lungs.

Recent data also suggests a role for the aryl hydrocarbon receptor (AhR) in mediating ILC3 function in the lungs. AhR is an environmental sensor expressed in barrier tissue cells that is critical for ILC3 maintenance and function in the gut (37). Within the environment of the lungs, AhR tunes immune responses to a variety of insults through regulation of ILC3s.

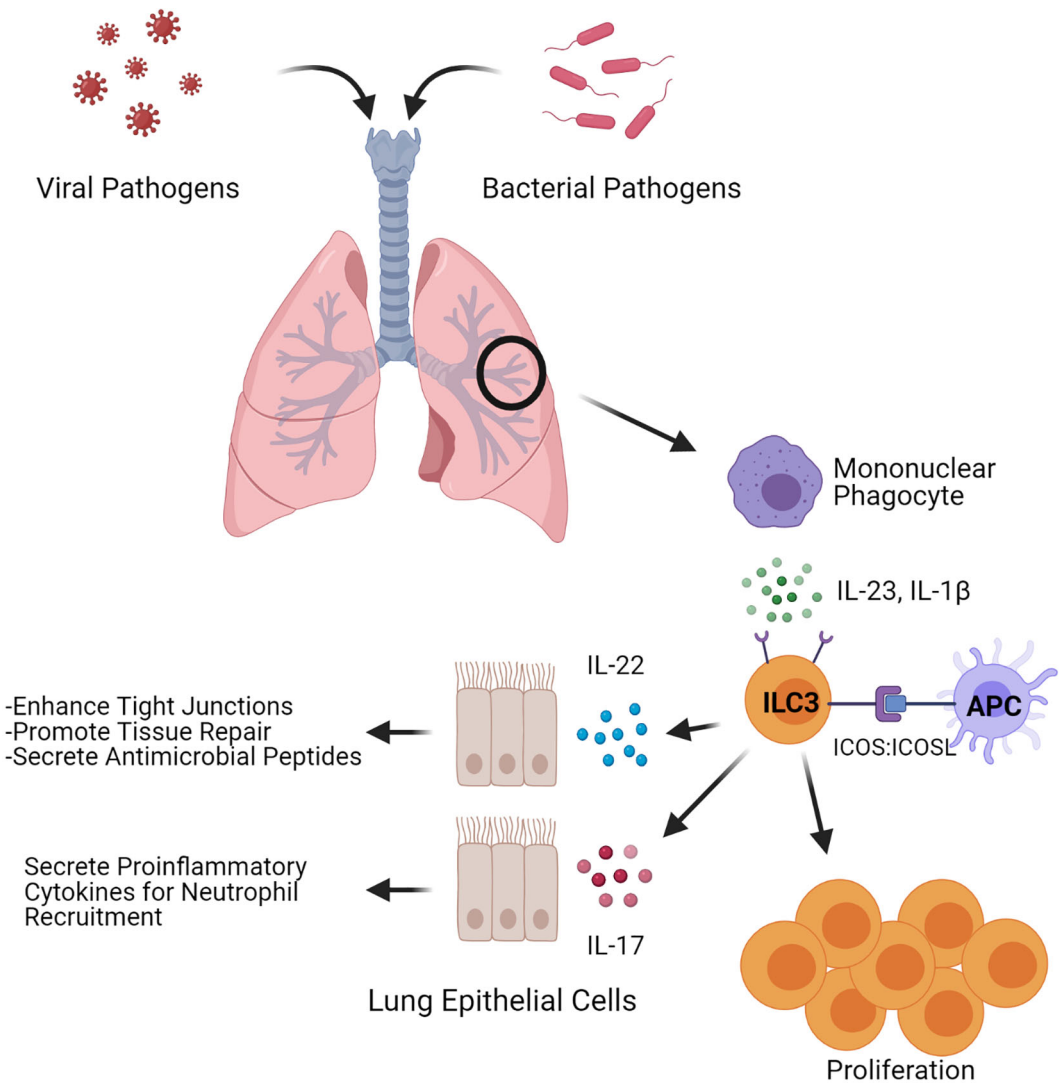

FIGURE 1 | ILC3-induced antimicrobial and tissue regenerative responses. Depiction of factors that influence both inflammatory and regenerative responses in ILC3s upon microbial pulmonary infections. During an infection, ILC3s can be stimulated through ICOS: ICOSL interactions or by the cytokines IL-1β and IL-23. Stimulated ILC3s expand and secrete the cytokines IL-22 and IL-17. Figure created with BioRender.com.

Indeed, *Ahr*$^{-/-}$ mice displayed a decrease in ILC3s during pulmonary paracoccidioidomycosis (38), indicating this receptor could be critical for expansion or recruitment of ILC3 to sites of infection within the lung. In support of this, activation of AhR resulted in recruitment of ILC3 during chronic exposure of mice to ozone (39).

ILC3s IN BACTERIAL PNEUMONIA

Even in an era of antimicrobial treatments, pneumonia remains the leading cause of morbidity and mortality in children aged 28 days to 5 years (40, 41). Among the most common etiological bacterial agents in these cases of pneumonia are pathogens such as *Streptococcus pneumoniae, Streptococcus pyogenes, Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (41–43). Since the discovery of the cytokine IL-17 in 1993 and its receptor in 1995, IL-17 has been demonstrated to be critical in protection against extracellular bacteria and fungi. Further, the coregulated cytokine IL-22 has been shown to promote epithelial integrity and tissue repair at barrier surfaces such as in the gut and lung following inflammation (44). As ILC3s are maintained in lung tissue and can rapidly produce IL-17 and IL-22 upon stimulation, it is clear that they play an important role in the innate immune response against bacterial pneumonia (45).

Though the discovery of ILC3s is recent, there have been numerous studies that have linked ILC3s to bacterial clearance in lung infections. A recent study illustrated that antibody depletion of IL-17 decreases mouse survival against *K. pneumoniae* by 50%. Further, IL-17 induction occurs within the first 3 hours of infection

suggesting it is mediated by innate cells such as ILC3s or γδ T cell rather than Th17 cells. In T- and B-cell-deficient *Rag2*$^{-/-}$ mice, the dominant source of IL-17 is ILC3 cells. Depletion of these cells using anti-CD90 or *Rag2*$^{-/-}$ mice that also are deficient in *Il2rg* (a common cytokine receptor for IL-7, among other cytokines) ablated IL-17 expression and exacerbated pulmonary infection with *K. pneumoniae* (46). It is important to recognize that due to limited ILC3 depletion models, these studies only illustrate that ILC3s are sufficient to clear infection in T-cell-deficient mice. Further studies are required to determine whether ILC3s are required for clearance. Using single cell RNA sequencing, we determined IL-17+, IL-22+, and ICOS+ ILC3s are imperative to protection against carbapenem resistant *K. pneumoniae* in a murine challenge model (33). This study also demonstrated that lung burdens in *Rag2/Il2rg*$^{-/-}$ mice can be significantly reduced through the addition of exogenous IL-22.

ILC3s also play a role in the clearance of *S. pneumoniae* in murine models of lung infection. One group found that lethal challenge with intranasal *S. pneumoniae* resulted in increased IL-22, IL-17A, and IL-17F expression in lung tissue within 24 hours suggesting a rapid innate response (45). ILC3s appeared to be sufficient for this response as infected *Rag2*$^{-/-}$ mice had no change in IL-22 levels compared to wild-type controls, while *Rag2/Il2rg*$^{-/-}$ mice were unable to produce IL-22 upon *S. pneumoniae* infection. While these models demonstrate the utility of ILC3s in response to *S. pneumoniae*, future studies are required to demonstrate their necessity. Boosting of ILC3 function could prove therapeutic, as treatment with flagellin at the time of *S. pneumoniae* infection enhanced IL-22 expression in ILC3s and decreased bacterial burdens in mice (45). This was supported by previous findings that treatment with flagellin enhanced ILC3 production of IL-17 and IL-22 in the intestinal lamina propria and spleen (47, 48). The importance of ILC3s in *S. pneumoniae* infections in the neonatal period was demonstrated by Gray et al. This group found that in newborn mouse lungs, 90% of the cells producing IL-22 carried the phenotypic markers of ILC3. Depleting ILC3s by administering diphtheria toxin to $ROR\gamma t^{iDTR}$ newborn mice dramatically increased their susceptibility to *S. pneumoniae* and all mice succumbed to infection within 20 hours. Adoptive transfer of ILC3s into the ILC3-depleted mice restored their resistance to *S. pneumoniae* (16). More recently, it was found that intranasal administration of interleukin 7, an important factor for RORγt+ cell survival and homeostasis, increased the number of RORγt+ innate T cells in the lung, enhanced expression of IL-17A, and reduced bacterial burdens upon *S. pneumoniae* challenge (49).

Many additional studies since the early 2000s characterize the importance of early IL-17 and IL-22 expression in the clearance of bacterial lung infections. Though these studies do not specifically investigate ILC3s, the ability of ILC3s to readily produce IL-17 and IL-22 upon stimulation suggests they are integral in an early IL-17/IL-22 response (44, 50). Additionally, IL-17 evolutionarily predates RAG, the protein needed for adaptive immune responses, as demonstrated by its function in invertebrates (51). This suggests that animals developed IL-17-dependent innate immune responses against bacterial pathogens before the advent of Th17 cells. In one of our previous studies using a murine model of *K. pneumoniae* infection, we found that IL-17R knockout mice had significantly higher bacterial burdens 24 hours post-infection than their wild-type counterparts (52). A similar study infected IL-17R knockout and wild-type mice with *K. pneumoniae* intratracheally and found that 100% of the IL-17R knockout mice succumbed to infection within 48 hours. By comparison, only 20% of the wild-type mice succumbed to infection within the same time frame (53). Interestingly, intratracheal administration of recombinant CXCL5 was able to decrease bacterial burdens in IL-17R knockout mice within 2 hours of treatment (52). Though CXCL5 is predominantly expressed in epithelial cells, ILC3s have been shown to secrete CXCL5 upon stimulation, further implicating them in bacterial immunity. It has also been demonstrated that IL-23-dependent IL-17 production was the most important for survival against *K. pneumoniae* challenge in adult mice (54). This is noteworthy as IL-23 is a potent activator of ILC3s (55). Highlighting the importance of IL-17 and IL-22 in response to *K. pneumoniae* challenge, we demonstrated that bacterial burdens in the lungs dramatically increase upon depletion of both IL-17 and IL-22 (30).

IL-17 has also been demonstrated to play a key role in immunity to *S. pneumoniae*. In a murine model it was demonstrated that systemic depletion of IL-17 at the time of infection resulted in persistent bacterial burdens in the nasopharynx detectable at day 21 post infection (56), suggesting that IL-17 expression at the time of infection may prevent pneumococcal colonization. Another study found that following pneumococcal vaccination, 95% of mice that produced > 0.3 ng/mL IL-17A upon antigen stimulation were protected from bacterial colonization (57). A murine intranasal vaccination study further illustrates the importance of IL-17A in pneumococcal immunity, as IL-17A neutralization abolished all vaccination protection while IFNγ neutralization had no impact on vaccine efficacy (58). Though this is likely indicative of a Th17 response, ILC3s do not produce IFNγ suggesting they are playing a role in the IL-17 dependent protection (12).

Both IL-17 and IL-22 have proven critical in clearing *P. aeruginosa* lung infections. A recent study using human sputum samples found that patients with the highest levels of IL-17 prior to being placed on a mechanical ventilator did not get ventilator-associated pneumonia (59). The importance of IL-17 was supported in a murine lung infection model using *P. aeruginosa*-infused agar beads. In this study, IL-17R knockout mice had inhibited clearance of *P. aeruginosa* infections. Of note, the authors used a clinical isolate from a cystic fibrosis patient and found that IL-17R knockout mice had exacerbated bacterial burdens and increased weight loss 14 days post-infection. Further, in innate immune cells, IL-17 production appeared to be dependent on ILC3s as 90% of the IL-17-producing CD3- cells in the lung carried the phenotypic markers for ILC3s (60). Regarding IL-22, it was demonstrated in mice that absence of IL-22 in *P. aeruginosa* pneumonia enhanced neutrophil recruitment thus exacerbating lung pathology (61). Supporting this, a recent study found that IL-22 upregulated IFNλ expression in a murine *P. aeruginosa* pneumonia model. Increased IFNλ correlated with decreased neutrophil

recruitment and knocking out IFNλ led to exacerbated lung inflammation and pathology (62).

ILC3s IN VIRAL LUNG INFECTIONS WITH IMPLICATIONS FOR COVID-19

Studies investigating ILC3s in viral lung infections are very limited, with much of the focus centered on ILC2s (63). However, there have been numerous studies on the effects of IL-22, a key cytokine of ILC3s, in influenza infections. Though IL-22 does not appear to reduce viral titers, it has been demonstrated to reduce disease severity though its functions in tissue repair and regeneration (64, 65). A recent study by Hebert et al. found that inflammation from influenza infection could be significantly reduced by knocking out IL-22 binding protein (IL-22BP, *Il22ra2*), a soluble inhibitor to IL-22 (66). Additionally, this group showed that *Il22ra2*$^{-/-}$ knockout mice had enhanced tight junctions during influenza infection promoting tissue integrity (67). Supporting this was the finding that addition of exogenous IL-22 in a murine model of influenza infection limited tissue damage (68).

Expression of IL-22 and IL-17 during viral lung infections also promotes prevention of secondary bacterial infections. Secondary bacterial infections commonly occur after moderate to severe respiratory viral infections and are a significant contributor to morbidity and mortality (69). During the 2009 H1N1 influenza pandemic, up to 26% of case-patients had a bacterial co-infection, which was associated with longer stays in the ICU and a need for mechanical ventilation (70, 71). Currently it is believed that primary infection with a virus impairs the function of mucus and cilia in clearing otherwise normally nonpathogenic bacteria resulting in opportunistic infection (69). As such, it stands to reason that the regenerative properties of IL-22 secreted by ILC3s may function in prevention of secondary bacterial infections. This was recently demonstrated using a murine model of influenza A (IAV) viral infection followed by a secondary *S. pneumoniae* bacterial infection. The group found that within 2 days of IAV infection there was upregulation of IL-1β, IL-23, and most importantly IL-22. Further, the group found an increase of RORγt cells and IL-22+ ILC3s in the lung. While IL-22-deficient mice had no change in viral clearance, these same mice had dramatically impaired survival after *S. pneumoniae* secondary infection (68). A similar study used transgenic IL-22BP knockout mice infected with influenza followed by *Staphylococcus aureus* or *S. pneumoniae* challenge. The study found that IL-22BP knockout mice had increased bacterial clearance and decreased mortality from secondary bacterial infection, and improved airway epithelial integrity (72).

IL-17 production during influenza infections was also demonstrated to promote the clearance of secondary bacterial infections. One study using a murine model of IAV infection followed by *S. aureus* challenge found that overexpression of IL-23 during infection resulted in enhanced production of IL-17 and IL-22 and promoted bacterial clearance (73). It was subsequently demonstrated that IAV infection prior to secondary *S. aureus* pneumonia inhibited IL-1β production, decreasing IL-22 and IL-17 expression and worsening the *S. aureus* infection. Overexpression of IL-1β during IAV infection rescued the generation of IL-17 and IL-22 and promoted bacterial clearance (74).

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes the acute respiratory disease COVID-19, has been one of the most severe pandemics and public health crises of the last century. As a newly emerged virus, much remains to be elucidated on effective immune responses distinguishing severe and mild disease. As COVID-19 is a respiratory disease that shares some symptoms with influenza, it is possible that ILC3 production of IL-17 and IL-22 also may serve to limit disease severity. In addition to benefits in influenza infection (65, 72), it was recently demonstrated that IL-22 promotes immunity against respiratory syncytial virus; these benefits of IL-22 may also extend to SARS-CoV-2 infection (75). Similar to influenza, it has also been demonstrated that secondary bacterial infections are common in COVID-19 patients. One study found that of 3,338 total COVID-19 patients, 14.3% developed a secondary bacterial infection (76). It is likely IL-17 may also play a role in preventing secondary bacterial infections in COVID-19 patients, though its role has yet to be demonstrated.

Recently, a function for ILCs has been demonstrated in COVID-19. Evaluation of the blood from COVID-19 patients found that severely infected individuals had fewer ILC1, ILC2, and ILC precursor cells than those with mild disease. Additionally, ILCs in severely infected individuals had higher expression of CD69, a marker for activation and tissue homing. The decrease in blood ILCs coupled with the increase in CD69+ ILCs in severely infected individuals suggests that there is more tissue homing to the lungs in severe infections (77). Silverstein et al. corroborate these findings by establishing that a higher ILC abundance in the blood was associated with less time spent in the hospital. Further, hospitalized individuals with COVID-19 had 1.78-fold fewer ILCs in the blood (78). Taken together, these studies illustrate a correlation between decreased ILCs in the blood periphery and severe SARS-CoV-2 infection. As current data regarding ILCs in COVID-19 infections are obtained though analysis of blood, further studies are required to elucidate their exact role in COVID-19 infection since ILC3s are predominantly tissue resident.

DISCUSSION

Overall, regulation of ILC3s in both normal and disease states remains an understudied area of research. It is clear these cells contribute significantly to mediating disease as an imbalance of ILC3 has been linked to both COPD and asthma (20, 25). Given their role in barrier protection from invading pathogens, ILC3s or their cytokines could be an ideal target for development of immunotherapies. For example, several groups have FDA approval to study IL-22 in COVID-19 (79, 80) (completed, findings pending). Therefore, it is imperative to develop a complete understanding of how these cells are regulated within

the microenvironment of the lungs, which should enable discovery of novel targets for immunotherapeutic development.

AUTHOR CONTRIBUTIONS

JH, JK, and JM all contributed to writing and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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NK Cells in the Pathogenesis of Chronic Obstructive Pulmonary Disease

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Chronic obstructive pulmonary disease (COPD) is a prevalent chronic airway disease with varied frequencies of acute exacerbations, which are the main cause of morbidity and mortality of the disease. It is, therefore, urgent to develop novel therapies for COPD and its exacerbations, which rely heavily on understanding of the pathogenesis and investigation for potential targets. Current evidence indicates that natural killer (NK) cells play important roles in the pathological processes of COPD. Although novel data are revealing the significance of NK cells in maintaining immune system homeostasis and their involvement in pathogenesis of COPD, the specific mechanisms are largely unknown. Specific and in-depth studies elucidating the underlying mechanisms are therefore needed. In this review, we provided a brief overview of the biology of NK cells, from its development to receptors and functions, and outlined their subsets in peripheral blood and lungs. Then we reviewed published findings highlighting the important roles played by NK cells in COPD and its exacerbations, with a view of providing the current state of knowledge in this area to facilitate related in-depth research.

Keywords: natural killer cells, chronic obstructive pulmonary disease, COPD, acute exacerbations, cigarette smoke, pathogenesis

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a prevalent chronic airway disease with increasing morbidity and mortality globally (1–3). The global incidence of COPD was estimated to achieve 3.9%, and caused a mortality of 41.9 per 100 000 individuals in 2017 (2). A recent cross-sectional study in mainland China revealed an overall prevalence of 8.6% of spirometry-defined COPD in adult populations (≥20 years old) (3). COPD is a preventable and treatable disease featured by persistent airway inflammation and parenchymal destruction, mostly caused by cigarette smoking. Clinically, COPD is usually manifested as exertional dyspnea, cough and/or sputum production (4, 5), and acute exacerbation of these symptoms, i.e., AECOPD, represents the main cause of COPD-related disabilities and mortality and accelerates disease progression (6, 7). Currently, main therapeutic interventions for COPD include non-pharmacological management (smoking cessation, influenza and pneumococcal vaccinations, rehabilitation), and pharmacological therapies with bronchodilators and/or inhaled corticosteroids. However, none of the available medications has been shown to modify disease progression and reduce mortality (5). It is, therefore, urgent to develop novel therapies for COPD and its exacerbations, which rely heavily on understanding of the pathogenesis and identification of potential targets.

Studies have shown that immune-infiltrating cells play a pivotal role in the airway inflammation and lung destruction of COPD, including neutrophils, macrophages, lymphocyte subsets, and dendritic cells (8, 9). NK cells, as innate immune cells, are considered to be the first line of defense mechanism for the human body against infections and tumors (10). More recent evidence has implicated NK cells in maintaining immune homeostasis and in pathogenesis of COPD (11, 12). However, the specific mechanisms are still elusive (13, 14). Specific and in-depth studies elucidating the underlying mechanisms are therefore needed. In this article, we provided an overview of the biology of NK cells, their subsets in peripheral blood and the lungs, and current knowledge on the potentially important roles played by NK cells in COPD and its exacerbations.

HUMAN NK CELL DEVELOPMENT

Over the past 40 years, great achievements have been made in the field of research on NK cells. Studies in humans revealed that the bone marrow (BM) and secondary lymphoid tissues (SLTs) such as tonsils, spleen, and lymph nodes (LNs) are essential organs for the differentiation and development of NK cells (15, 16). Different stages of this process were determined by unique surface biomarkers (15). During the initial phase, hematopoietic stem cells (HSCs) give rise to $CD34^+CD133^+CD38^-CD90^-CD45RA^+$ lymphoid-primed multipotential progenitor (LMPP) (17), and then, LMPP progress into common lymphoid progenitors (CLPs) with the expression of CD38, CD7, CD10, and CD127 (18–21). Hereafter, CD122 marks the irreversible transition of CLPs into NK lineage, and then develop into its mature form *via* CD56 expression (22). Some works have also categorized the development of NK cells into six stages according to the studies pertaining to BM and LNs development (15, 16). $CD3\epsilon^-CD7^+CD127^+$cell subgroup defines the transformation of NK cells from stage 1 through stage 2a. Expression of IL-1R marks the initiation of stage 2b. Then NK cells come to stage 3 with the expression of activating receptors involving NK cell group 2D(NKG2D), CD335 and CD337. During stage 4, NK cell development is comprised of two sections: stage 4a and stage 4b. NK cells in Stage 4a are $NKP80^-$ that have high expression of NKG2D, CD335, CD337, inhibitory NKG2A and CD161. Besides, this stage also exhibits high expression of CD56 ($CD56^{bright}$). On the other hand, NK cells in stage 4b are $NKP80^+$ while sustain $CD56^{bright}$ status (23, 24). Stage 5 of development is denoted by a proportion of NK cells with downregulated CD56 ($CD56^{dim}$) and upregulated CD16 (25). Finally, stage 6 of the development, also known as terminal maturation, occurs following expression of CD57 (26).

SUBGROUPS OF HUMAN NK CELLS IN PERIPHERAL BLOOD AND LUNGS

NK cells are defined as $CD3^-CD56^+$ cells, which make up approximately 5–15% of the circulating lymphocytes. NK cells are subdivided into two main subpopulations, $CD56^{bright}$ $CD16^-$ and $CD56^{dim}$ $CD16^+$, based on expression of the surface marker CD56 and CD16. $CD56^{bright}$ $CD16^-$ NK cells, accounting for about 10% of peripheral blood (PB) NK population, mainly produce cytokines, including interferon-gamma (IFN-γ), interleukin (IL)-10, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor. $CD56^{dim}$ $CD16^+$ NK cells are the predominant (90%) peripheral blood (PB) NK population, which are highly cytotoxic by producing a large amount of perforin and granzymes, expressing Ig-like receptors (KIRs), and inducing antibody-dependent cytotoxicity (ADCC) effects, but with limited ability to produce cytokines (27–29).

In recent years, emerging evidence indicates that NK cells also reside in peripheral tissues including liver, lung, spleen, BM, intestine and uterus under steady-state conditions (**Figure 1**). Studies indicated that the majority of NK cells in the lung, BM, LNs and spleen are $CD56^{dim}$ $CD16^+$, while $CD56^{bright}$ $CD16^-$ NK cells predominate in the liver, gut and uterus (30–34). NK cells in the spleen, BM and LNs comprise 5-20%,4% and 2-5% of the total lymphocyte population, respectively. $CD56^{bright}CD69^+$ $CXCR6^+$ NK cells define the BM and spleen resident NK cells, which make up a proportion of 9-51% in BM NK cells, and 28-69% in spleen NK cells, respectively. While $CD56^{dim}$ $CXCR6^+CD69^+NKP46^+$ NK cells define LN resident NK cells, accounting for 43-67% of the LN NK cells (34, 35). Dogra et al. found that the frequencies of NK cells in BM, spleen and lung were higher than those in LNs and gut, and interestingly, they also demonstrated that tissue sites shaped the functions of NK cells, especially the potential for cytokine production (31). In humans, NK cells constitute approximately 5-20% of the CD45-positive lung lymphocytes, and are located in lung parenchyma (28). Most lung NK cells show a mature $CD56^{dim}CD16^+$ phenotype which takes a proportion of 80% (29), whereas $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^-$ cells make up the remaining 20%, in equal ratios (12, 36). However, it should be noted that the majority of lung NK cells are circulating cells, though hypofunctional, otherwise are similar to PB NK cells. A minority of the lung NK cells are tissue-resident, and could be specifically defined by $CD49a^+CD69^+CD103^+$ cells, which constitute 20% of lung NK cells (34). Due to the difficulty of obtaining human lung NK cell samples, the research in human lung NK cells is very limited. Thus, further investigations are needed to fill the gap in understanding of human lung NK cells in pulmonary diseases.

HUMAN NK CELL RECEPTORS AND THEIR FUNCTIONS

Human NK cells express a variety of receptors, including inhibitory, activating, cytokine, chemokine receptors and death receptors (**Figure 2**). Inhibitory receptors are expressed when immune surveillance is normal, among them are killer KIRs such as KIR2DL1, KIR2DL2/3, KIR3DL1, KIR3DL2, KIR2DL5 (expressed by subsets of $CD56^{dim}$ NK cells) and KIR2DL4 (expressed by all mature $CD56^{dim}$ NK cells), CD94: NKG2A heterodimers and immunoglobulin-like transcript 2(ILT-2).

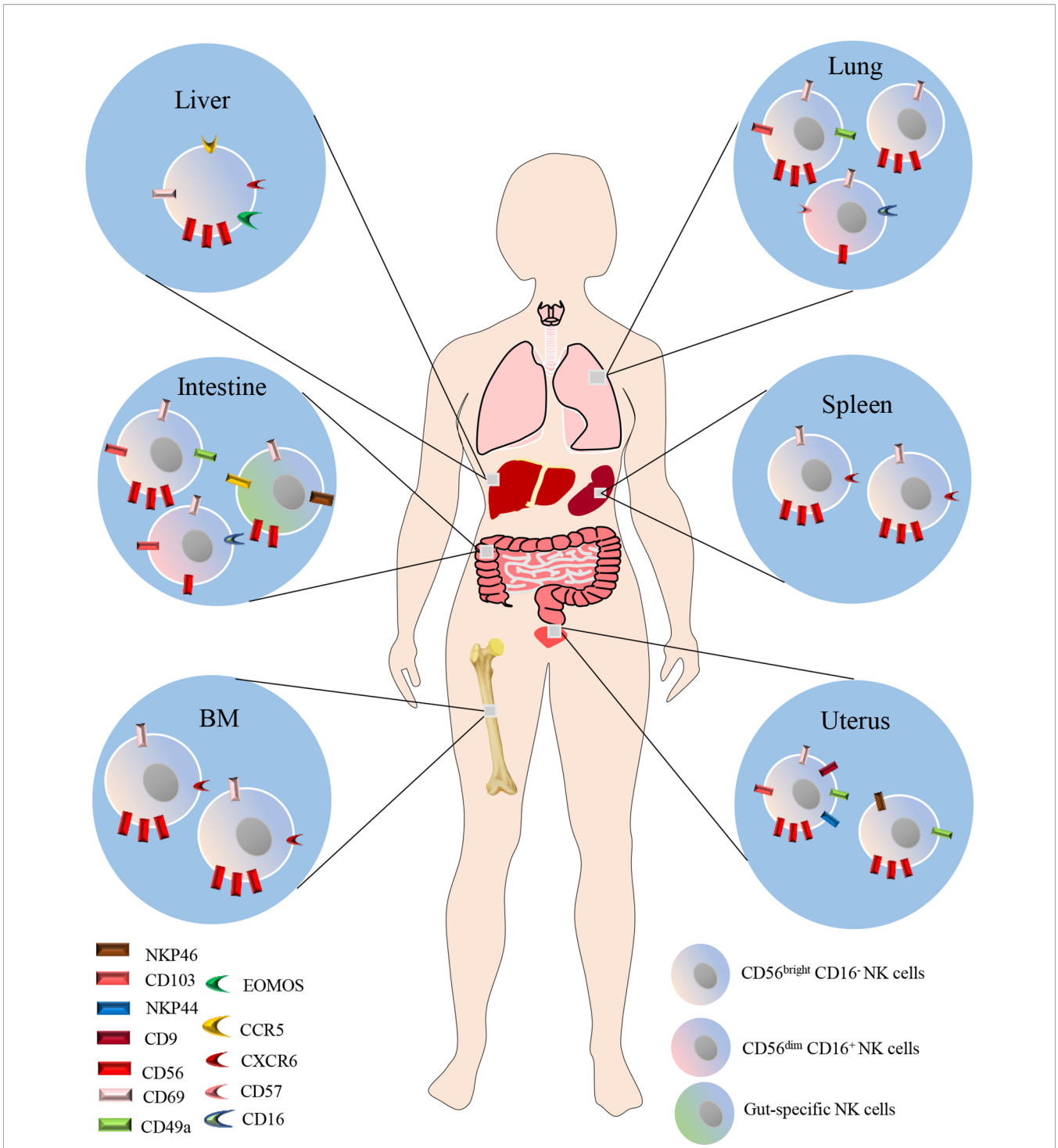

FIGURE 1 | NK cells also reside in peripheral tissues including liver, lung, spleen, BM, intestine and uterus under steady-state conditions. The majority of NK cells in the lung, BM, LNs and spleen are $CD56^{dim}$ $CD16^{+}$, while $CD56^{bright}$ $CD16^{-}$ NK cells predominate in the liver, gut and uterus. $CD56^{bright}CD69^{+}$ $CXCR6^{+}$ NK cells define the BM and spleen resident NK cells. While $CD56^{dim}CXCR6^{+}CD69^{+}NKP46^{+}$ NK cells define LN resident NK cells. In humans, NK cells constitute approximately 5-20% of the CD45-positive lung lymphocytes, and are located in lung parenchyma. Most lung NK cells show a mature $CD56^{dim}CD16^{+}$ phenotype which takes a proportion of 80%, whereas $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{-}$ cells make up the remaining 20%, in equal ratios. A minority of the lung NK cells are tissue-resident, and could be specifically defined by $CD49a^{+}CD69^{+}CD103^{+}$ cells, which constitute 20% of lung NK cells.

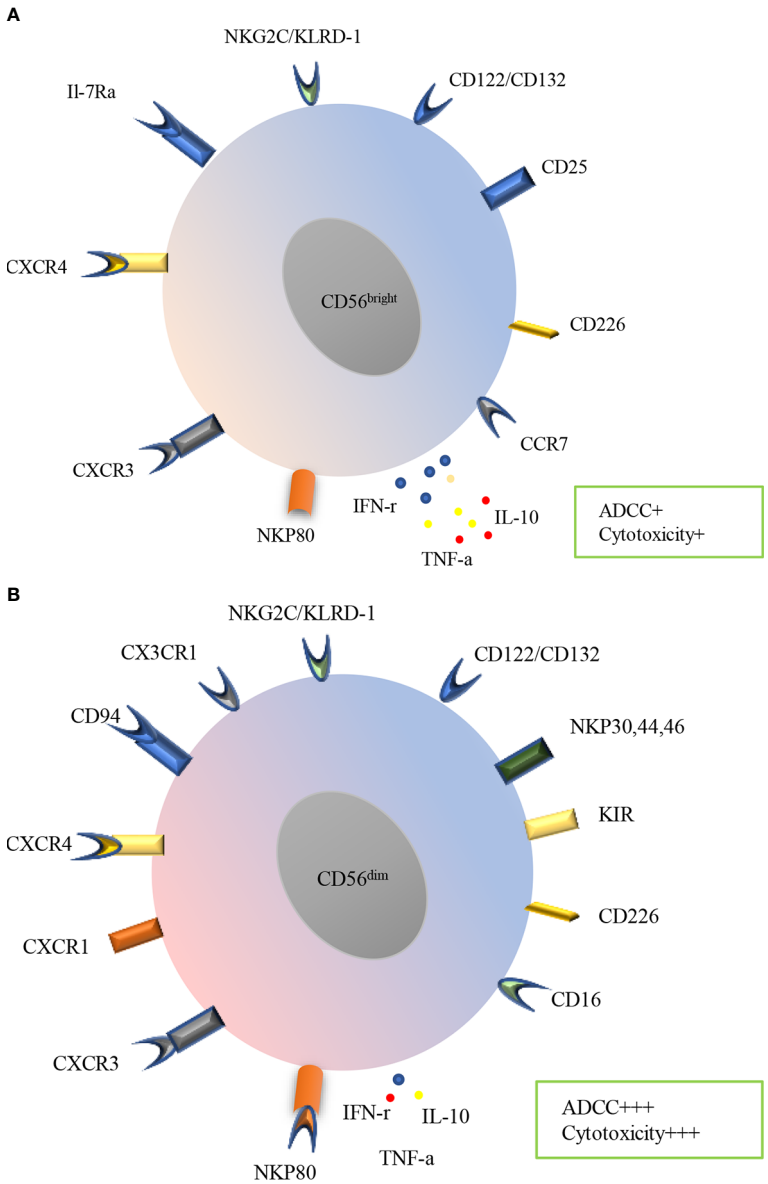

FIGURE 2 | Surface receptors and functions of PB human NK cells subtypes. Human NK cells are divided into $CD56^{bright}$ **(A)** and $CD56^{dim}$ **(B)** subsets in PB, which express a variety of receptors, including inhibitory, activating, cytokine, chemokine receptors and death receptors. $CD56^{bright}$ subset mainly expresses CCR7, CD226, CD25, CD122/132, NKG2C/KLRD-1, IL-7Ra CXCR4, CXCR3and NKP80, meanwhile, $CD56^{dim}$ subset mainly expresses CD16, CD226, KIR, NKP30, NKP44, NKP46, CD122/CD132, NKG2C/KLRD-1, CX3CR1 CD94, CXCR4, CXCR1, CXCR3. $CD56^{bright}$ subset produces more IFN-γ, TNF-α and IL-10 while less ADCC and cytotoxicity. In contrast, $CD56^{dim}$ subset produces less cytokines, however prominent ADCC and cytotoxicity.

Recently, several other inhibitory receptors have also been reported, including programmed cell death protein 1 (PD-1), T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT), cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), killer cell lectin-like receptor subfamily G member 1 (KLRG1), CD161, T cell immunoglobulin domain and mucin domain-3 (TIM-3) (37). All mature NK cells may also express the inhibitory receptor IRp60 and p75/AIRMI. Inhibitory receptors mainly recognize classical and non-classical major histocompatibility complex (MHC) class I molecules (38, 39). Regulation of the balance between inhibitory and activating receptors determines NK cell effects. NK cells are activated by tumor or infected cells lacking inhibitory receptor ligands or having abundant activating receptor ligands. NK cells will undergo activation if the activating signals surpasses the MHC class I inhibitory receptors (40–42). Activating receptors include natural cytotoxicity receptors (NKp30, NKp44, and NKp46), natural killer group 2C (NKG2C), NKG2D, CD16, KIR2DS1, KIR2DS2/3, KIR2DL4, KIR3DS1, KIR2DS5, KIR2DS4, NKRP-1,

CD226 as well as coreceptors (2B4, NTB-A, and NKp80) (10, 38, 39). NK cells may also express many other cytokine and chemokine receptors, including IL-2Ra, IL-2Rb/IL-2Rc, c-Kit, IL-7Ra, CXCR1, CXCR3, CXCR4, CCR4, CCR7, IL-18R, ChemR23 and CX3CR1. In addition, death receptors such as TNF-related apoptosis-inducing ligand (TRAIL), Fas and Apo1 as well as ligands including Fas ligand and CD40L have also been reported in NK cells (39). Knowledge of these receptors and their functions is essential for understanding the role of NK cells in diseases including COPD.

NK CELLS IN VIRAL AND BACTERIAL LUNG INFECTIONS

NK cells play a vital role in lung immune response to respiratory viral and bacterial infections. During the early phase of influenza infection in mice, NK cells accumulate in the lung to clear the virus *via* IFN-γ production, adaptive immune cell activation, ADCC and direct lysis (43, 44). Studies have demonstrated that patients with genetic deficiency with loss of NK cell function suffered from recurrent viral infection (45–47). However, NK cells may not always protective. Deletion of NK cells promote the survival of mice infected with high-dose influenza viruses *via* mitigating lung immunopathology, suggesting the destructive role of NK cells in influenza infection. It seems likely that NK cells play a dual role in influenza infection.

Recent evidence indicates that NK cells may play an increasingly vital role in bacterial infections. During infection with *Mycobacterium tuberculosis*, NK cells upregulated CD69, IFN-γ and perforin expression for promotion of host defense against the bacteria. However, it is still unclear whether NK cells employ cytotoxic lysis to restrict *Mycobacterium tuberculosis*, and therefore further investigations are needed (48–51). In another study, lung NK cells protect the host against *K. pneumoniae* infection *via* IL-22 and IFN-γ production. Similarly, lung NK cells defend against *Pseudomonas aeruginosa* infection *via* NKG2D expression and IFN-γ production (52, 53). Yoshihara et al. found that NK cells facilitate host defense against *Staphylococcus aureus* infection through IFN-γ and TNF production (54, 55). Similarly, lung NK cells could also protect against *Haemophilus influenzae* through IFN-γ production. Overall, current knowledge shows that NK cells play a beneficial role in the restriction of bacterial infection (56).

NK CELLS IN ANIMAL MODELS OF COPD

Frequency of NK Cells in COPD

An earlier study by Motz et al. found no significant change of lung NK cells after 6-month cigarette smoke (CS) exposure in a mouse model of COPD (57). Similar results were confirmed by Wortham et al. who observed no difference in the number of lung NK cells between 6-month CS-exposed and air-exposed mice (58). Additionally, another study reported that short-term CS exposure (4 days) did not increase the frequency of NK cells in the lung (59). On the contrary, Stolberg et al. found that lung NK cells showed elevated frequency after 4-day CS exposure (60). The reasons for conflicting results from these studies are not clear, but may be explained by differences in experimental protocols, CS exposure times and doses.

Activating Receptors of NK Cells in COPD

The study by Wortham et al. found that the activating receptors of NK1.1, NKG2D, and CD244 in lung and spleen NK cells did not differ between 6-month CS-exposed and air-exposed mice (61). In contrast, Stolberg et al. found that after a short-term of 4-day CS exposure, lung NK cells showed elevated CD69 expression, which could be mitigated by CCR4 deletion. Additionally, they found that short-term CS exposure could significantly induce the expression of retinoic acid early transcript 1 protein (RAET1) (NKG2D ligand) in lung airway epithelium (60). Other studies also confirmed that long-term CS exposure could significantly induce the expression of NKG2D ligand expression including RAET1 and Mult1, which were shown to contribute to pulmonary emphysema (58, 62). Similarly, in a spontaneous COPD mouse model, Finch et al. observed increased percentage of $CD69^+$ lung NK cells (14). These discrepancies in activating receptors of NK cells in COPD models maybe explained by differences in CS exposure times and doses, the receptors studies, and the compartments of NK cells. Collectively, these data indicate that activating receptors of NK cells are altered and may play an important role in lung inflammation and emphysema of COPD.

Inhibitory Receptors of NK Cells in COPD

Thus far, there has been limited data on the inhibitory receptors of NK cells in COPD models. In the study by Wortham et al., the inhibitory receptor CD94 as heterodimerized with NKG2A/C subunits in the lung and spleen showed no difference between 6-month CS-exposed and air-exposed mice (58). Indeed, further experimental evidence is needed to elucidate the potential role of NK cell inhibitory receptors in the pathological mechanisms of COPD.

Altered Effector Functions of NK Cells in COPD

The study by Wortham and his colleagues found that NK cells from 6-month CS-exposed mice showed elevated cytotoxicity toward NKG2D ligand RAET1ε (58). Similarly, Motz et al. found that lung and spleen NK cells from 8-week CS-exposed mice demonstrated elevated cytotoxicity following Ly49D or NK1.1 challenge. In this study, they also found that lung and spleen NK cells in CS-exposed mice showed higher IFN-γproduction following IFN-γ-inducing cytokine challenge including IL-12 or IL-18, or both. Particularly, they found that IFN-γ^+ NK cells decreased significantly after IL-12 treatment following smoking cessation (57). In another study, Finch et al. observed that lung NK cells from 8-week CS exposed mice showed elevated cytotoxicity toward lung epithelial cells in relative to control group. In this article, they also found that DCs were necessary for the priming of NK cells to acquire cytotoxicity potential following CS exposure, which could be abrogated by blocking IL-15 (14). Additionally, another study demonstrated that CS

could prime NK cells for the production of IL-17A (59). Stolberg et al. found that lung NK cells from mice exposed to CS for 4 days showed elevated IFN-γand CXCL10 production, which could be abrogated by CCR4 deletion. Also, they found that CCR4 was required for NK cells' contacting with lung $CD11c^+$and $CD11^+MHCII^+$ cells (60). In summary, these studies imply that the CS exposure may exaggerate lung epithelial cell injury through increased NK cell cytotoxicity and cytokine production.

NK Cells in AECOPD of Mice

Other studies demonstrated that NK cells produced much more IFN-γ in CS-induced COPD models after viral infection or after viral pathogen-associated molecular patterns (PAMPs) challenge (63). Additionally, IFN-γ^+lung NK cells frequency from in CS exposed mice for 8 weeks responded differently to different bacterial PAMPs: increase for LPS, no difference for HKLM and ST-FLA, decrease for pam3CSK4 (57). Another study found that infection of mice with virus in six-month CS exposure showed exaggerated inflammation and airway epithelial damage. However, this augmented damage could be mitigated in NKG2D deletion mice, indicating the pivotal role of NKG2D in viral infection induced COPD exacerbations (58). In contrast, one study by Mian et al. showed that CS inhibited IL-15 mediated NK cells activation, manifested as decreased CD69, NKG2D and Granzyme B expression as well as NK cell cytotoxicity following poly I:C stimulation (64). In line with this, Pichavant et al. found that the NK cells in 12-week CS-exposed mice showed defective immune response upon *S. pneumoniae* challenge (65). In all, the study of NK cells in AECOPD of mice is still in its infancy, there is an urgent need to assess the role of NK cells in AECOPD with more innovative approaches.

NK CELLS IN HUMAN COPD

Evidence indicates that immune-infiltrating cells play a pivotal role in the pathogenesis of COPD (8, 9). NK cells act as a proinflammatory population during the immune response (12). Theoretically, NK cells could cause the injury to the lung, and thus COPD pathogenesis. However, their precise role in the pathogenesis of COPD remained elusive with discrepant studies in humans (**Table 1**). Alterations of NK cells, as manifested by surface marker including inhibitory receptors, activating receptors (**Table 2**), as well as effector functions occur in stable COPD patients and smokers.

Frequency of NK Cells

As the disease progresses, the frequency of NK cells may also change. One study by Richard et al. indicated that the percentage of induced sputum NK cells increased significantly in COPD patients compared with healthy non-smokers (HNS) and smokers. $CD56^{bright}CD16^-$ induced sputum NK cells subset also increased significantly in COPD patients in relative to the two other groups (74). Another study observed an elevated percentage of PB NK cells in COPD patients compared with HNS (69). Similarly, Chen et al. also observed an elevated percentage of PB NK cells in cigarette smoking-related COPD patients (67). In addition, another study by Olloquequi et al. showed increased NK cells in lung lymphoid follicles (75). However, NK cells may not always increase. Several studies gave opposite results showing decreased frequencies of PB $CD16^+$ NK cells in smokers as compared to nonsmokers, which persisted years after ceasing smoking (76, 77). Similarly, one study demonstrated that the percentage of NK cells decreased significantly in COPD patients compared with HNS. The $CD56^{dim}CD16^+$ PB NK cells subset also reduced in COPD patients in relative to smokers and HNS with a corresponding elevation of the $CD56^{bright}CD16^-$ cells (78). Interestingly, Pascual et al. pointed out that PB NK cell frequency showed no difference between HNS and COPD patients disregarding disease severity (68). Hodge et al. confirmed that there were no changes in the percentage of PB NK cells between HNS, smokers, current smoker COPD and ex-smoker COPD. However, this study observed an elevated frequency of bronchoalveolar lavage fluid (BALF) NK cells in COPD patients compared with HNS (13). Taken together, currently available data indicate that the NK cell population increases in the pulmonary compartment of COPD, but varies in the blood circulation.

Activating Receptors of NK Cells

There are a variety of activating receptors on the surface of NK cells, which are crucial for the function of NK cells (39). Wang et al. observed elevated frequency of activated ($CD69^+CD25^-$; $CD69^+CD25^+$; $CD69^-CD25^+$) NK cells in PB of healthy smokers, current smokers and ex-smokers with COPD in relative to healthy non-smokers, and this was positively correlated with the number of cigarettes smoked. Additionally, they found increased proportion of activated induced sputum NK cells in current smokers with COPD and ex-smokers with COPD compared with healthy non-smokers. Interestingly, the proportion of activated NK cells was elevated in current smokers with COPD compared healthy smokers. These data implied that activating receptors in NK cells were close related with COPD state and cigarettes smoked. However, no differences were observed in NKG2D expression between groups (66). More recently, it was demonstrated that there were no differences in the percentage of $NKG2C^+$ PB NK cells between COPD patients and HNS, and no correlation was observed with disease severity, smoking status. However, they found decreased $NKG2A^+$ NK cells in COPD patients in relative to HNS. Yet, $NKG2A^+$ PB NK cells level were in no correlation with disease severity, smoking status and exacerbations frequency (68). Additionally, Hodge et al. and his colleagues reported that there were no changes in the percentage of $CD69^+$ PB NK cells between COPD patients and HNS groups (13). Overall, these studies gave absolute different observation, thus a profiling of NK cells receptors are urgently needed.

Inhibitory Receptors of NK Cells

Studies have found a variety of crucial inhibitory receptors in the surface of NK cells which are crucial for NK cells function (39) One study by Wang et al. demonstrated that the proportion of PB NK cells expressing CD158e1 was significantly decreased in healthy smokers and current smokers with COPD in relative to HNS (66). In line with this, Hodge et al. found that both PB and

TABLE 1 | Studies of human NK cells in COPD.

References	Patients	Compartment	Functional assay	Observations in NK cells	Key findings
Wang et al. (66)	HNS 21 Smokers 21 CuS-COPD 14 ExS-COPD 10	PB	Yes	Decreased CD158e1 expression in smokers and CuS-COPD compared with HNS	Hyperfunction of NK cells in COPD
Chen et al. (67)	HNS 16 COPD 40	PB	Yes	Increased NK cells in COPD, decreased FPR3 expression	Immune imbalance in COPD
Pascual et al. (68)	HNS 13 COPD 66	PB	No	Increased NK cells in severe-to-very severe COPD VS mild-moderate COPD	Immune imbalance in COPD
Hodge et al. (13)	HNS 25 Smokers 16 CuS-COPD 30 ExS -COPD 41	PB	Yes	Increased CD94 and Granzyme B expression in COPD compared with HNS; no difference of CD69 between COPD and HNS group	Hyperfunction of NK cells in COPD
Fang et al. (69)	HNS 12 COPD 19	PB	Yes	Elevated NK cells in COPD patients, decreased IFN-γ production	Hypofunction of NK cells in COPD
Finch et al. (14)	ExS 4 CuS 15 CuS-COPD 14 ExS -COPD 16	Lung	Yes	Increased NK cells cytotoxicity in COPD compared with HNS	Hyperfunction of NK cells in COPD
Wang et al. (66)	HNS 5 Smokers 10 CuS-COPD 5 ExS -COPD 6	Induced sputum	Yes	Increased $CD69^+$ and/or $CD25^+$ NK cells in CuS-COPD and ExS -COPD patients compared with HNS	Hyperfunction of NK cells in COPD
Hodge et al. (13)	HNS 19 Smokers 12 COPD 33	BALF	Yes	Increased NK cells frequency, cytotoxicity, Granzyme B, CD94 in COPD patients compared with HNS and smokers	Hyperfunction of NK cells in COPD
Prieto et al. (70)	HNS 50 COPD 60	PB	Yes	Decreased cytotoxicity in COPD patients	Hypofunction of NK cells in COPD
Hughes et al. (71)	HNS 32 Smokers 14	PB	Yes	Decreased cytotoxicity in heavy smokers, but normal ADCC	Hypofunction of NK cells in COPD
Phillips et al. (72)	HNS 22 Light/moderate smokers 12 Heavy smokers 12	PB	Yes	Declined cytotoxicity in heavy smokers, normal in mild smokers	Hypofunction of NK cells in COPD
Freeman et al. (73)	Smokers 6 Mild COPD 14 Severe COPD 15	lung	Yes	Elevated cytotoxicity in COPD	Hyperfunction of NK cells in COPD
Urbanowicz et al. (16)	HNS 5 Smokers 10 COPD 11	Induced sputum	Yes	Elevated frequency and higher perforin and granzyme B production in $CD56^{bright}CD16^-$ subset of COPD patients compared with HNS and smokers.	Hyperfunction of NK cells in COPD

TABLE 2 | Activating and inhibitory receptors in COPD of human.

Receptor type	Name	Compartment	Findings	Reference
Activating receptors	CD69 and or CD25	PB/Induced sputum	Increased $CD69^+$ and/or $CD25^+$ in smokers CuS-COPD and ExS-COPD compared with HNS	(66)
	NKG2C	PB	Elevated frequency $NKG2C^+$ NK cells in frequently exacerbation COPD compared with occasional exacerbated COPD	(70)
	NKG2C/NKG2A	PB	No difference of $NKG2C^+$ NK cells between COPD and HNS, decreased $NKG2A^+$ in COPD compared with HNS	(68)
	CD69	PB	No changes of $CD69^+$NK cells between COPD and HNS	(13)
Inhibitory receptors	CD158e1	PB	Decreased $CD158e1^+$NK cells in smokers and CuS-COPD compared with HNS	(66)
	CD94	PB	Decreased $CD94^+$ NK cells in CuS-COPD compared with HNS	(13)
	CD94	BALF	No difference between smokers and HNS, decrease in CuS-COPD compared with HNS	(13)
	CD158a/ CD158b	PB	Decreased $CD158a^+$ and $CD158b^+$ in COPD compared with HNS	(69)

BALF NK cells' inhibitory receptor CD94 was also significantly decreased in current smoker COPD patients compared with HNS. However, there was no alteration of PB NK cells CD94 in healthy smokers compared with HNS. Meanwhile, no difference was observed in BALF NK cells regarding CD94 expression between ex-smoker COPD and HNS (13). In contrast, Tang et al. found increased inhibitory receptor $CD158a^+$and $CD158b^+$ PB NK cells in COPD patients, which

were negatively correlated with pulmonary function, indicating that NK cell inhibitory receptors may contribute to COPD progression (69). There is still no consensus on reliable observation about inhibitory receptors of NK cells concerning COPD. which may due to different locations of NK cells, receptor types, smoking status, disease severity. Thus, in-depth research is urgently need.

Altered Effector Functions of NK Cells

NK cells may perform effector functions through producing perforin, granzyme indirectly and killing target cells through cytotoxicity directly (12). Urbanowicz et al. found that the $CD56^{bright}CD16^{-}$ NK cells subpopulation in induced sputum produced significantly more perforin and granzyme B in COPD patients compared with HNS and smokers. However, they observed no difference in the percentages of $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells producing only perforin and no granzyme B (74). One study by Hodge et al., who reported that there were a higher percentage of PB and BALF NK cell expressing granzyme B in COPD patients compared with HNS (13). Similarly, there were significantly higher percentage of PB $CD56^{dim}CD16^{+}$subset expressing only perforin and no granzyme B in COPD patients compared with the two other group (78). In contrast, Tang et al. found decreased IFN-γ production in PB NK cells of COPD patients compared with HNS (69) Additionally, the percentage of PB NK cells expressing both perforin and granzyme B decreased obviously in COPD patients in relative to smokers and HNS, while not the $CD56^{bright}CD16^{-}$ subset. However, no difference between the percentage of PB $CD56^{bright}CD16^{-}$ or $CD56^{dim}CD16^{+}$subsets expressing only granzyme B and no perforin was observed. Similarly, the proportion of $CD56^{bright}CD16^{-}$ PB NK expressing only perforin and no granzyme B showed no differences between the three groups (78). Additionally, no changes were observed regarding Granzyme A and perforin expression in PB NK cells between COPD patients and HNS (13). Even though these results involving cytokines production are of great promising, they are preliminary and definitely need further investigation.

In addition to cytokine production, it is worth mentioning that cytotoxicity, another mechanism through which NK cells perform direct killing effect, play a vital role in COPD pathogenesis. In one article, Urbanowicz et al. found that the cytotoxicity of PB NK cells from COPD patients was lower than those from smokers and HNS, and similarly decreased cytotoxicity was observed in smokers compared with HNS. Particularly, they observed a positive correlation between the cytotoxicity of PB NK cells and the lung function (78). The decreased cytotoxicity of PB NK cells in COPD patients was also confirmed by Prieto and his colleagues, and this decline could be rescued by Glycophosphopeptical treatment (70). In line with this, Hughes et al. found that heavy smokers showed decreased PB NK cell cytotoxicity, but normal ADCC (71). Similarly, Phillips et al. also reported that PB NK cells from heavy smokers displayed declined cytotoxicity, which was normal in mild smokers (72). However, several studies observed contrary findings. One study by Hodge et al., found that BALF NK cells showed elevated cytotoxicity in COPD patients compared with HNS (13). Similarly, a higher cytotoxicity of lung NK cells was also observed in COPD patients compared with smokers, and these NK cells induced severer injury of the lung epithelium. This article indicated that increased cytotoxicity in COPD was driven by lung NKs, not lung epithelial cells. The study also demonstrated that dendritic cells (DCs) from COPD patients could significantly enhance NK cell cytotoxicity through IL-15Rαtrans-presentation, suggesting that blocking IL-15 may protect lung epithelial cells from injury (14). Another study reported a positive correlation between enhanced stress-induced NK cell cytotoxicity and COPD severity, suggesting the contribution of heightened lung NK cell cytotoxicity in emphysema progression (73). All these observations implies that the intervention of cytotoxicity, cytokine release may be potential targets of COPD treatment.

Adhesion Molecules of NK Cells

Richard et al. found that CXCR3 and very late antigen-4(VLA-4) expression in $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells elevated significantly in COPD patients compared with smokers (74). Another study by Chen et al., observed that intracellular expression of formyl peptide receptor (FPR3) in PB NK cells was markedly decreased in COPD patients compared with HNS. Particularly, this FPR3 expression showed a positive correlation with pre-bronchodilator FEV1/FVC ratio and predicted FEV1 percentage. FPR3 expression in COPD patients showed obvious elevation after 1-year treatment, suggesting the promising role of NK cell FPR3 in COPD therapy (67). Thus, searching for potential responsible molecule may be promising in the therapy of COPD.

NK Cells in AECOPD of Human

Exacerbations are vital events in the management of COPD for their negative effect on health status, hospitalization and readmission rates as well as disease progression, which are primarily triggered by viral and/or bacterial infections (5). In one study, higher levels of PB $NKG2C^{+}$ NK cells were closely associated with the number of exacerbations, implying a potential role in predicting COPD exacerbations (68). Another study reported that NK cells from COPD patients were not responsive to S. *pneumoniae*, suggesting function defect of NK cells in COPD patients (65). Up till now, the role of NK cells in AECOPD remains under-appreciated, and in-depth investigation holds promise for better understanding the immune mechanisms underlying infection-related acute exacerbation and deterioration of the disease.

Concluding Remarks

As innate immune cells, NK cells are considered to be the first line of defense mechanism for the human body against infections and tumor. Available data demonstrate that NK cells probably play a pivotal role in COPD and its exacerbations. Though advances have been made in revealing the potential involvement of NK cells in COPD, there still remain discrepancy involving NK cells frequency, activating and inhibitory receptors, effector functions, which may be attributable to different compartment of NK cells,

different stages of disease in human and different dose of CS exposure in mouse model of COPD. Thus, further studies are urgently needed to elucidate the mechanisms of NK cells in the pathogenesis, endotypes and acute exacerbations of COPD. Particularly, the investigators should pay more attention to cross talks between adaptive immune cell and NK cells, NK cells and epithelial cells, which may shed light on the mechanisms of lung damage (emphysema), a hallmark of COPD. Also, investigators should put emphasis on researches involving inhibitory, activating receptors and chemokine receptor in NK cells and its ligand in epithelial cells, thus develop more potent agonists/antagonists, which may pave the way to facilitate the translation of such a promising strategy into clinical use for therapy of COPD and AECOPD.

AUTHOR CONTRIBUTIONS

YR analyzed the data and prepared the manuscript. YS contributed to the scientific expertise and manuscript preparation. YL, JX, and YP participated in the preparation of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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ILC-You in the Thymus: A Fresh Look at Innate Lymphoid Cell Development

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The discovery of innate lymphoid cells (ILCs) has revolutionized our understanding of innate immunity and immune cell interactions at epithelial barrier sites. Their presence and maintenance are critical for modulating immune homeostasis, responding to injury or infection, and repairing damaged tissues. To date, ILCs have been defined by a set of transcription factors, surface antigens and cytokines, and their functions resemble those of three major classes of helper T cell subsets, Th1, Th2 and Th17. Despite this, the lack of antigen-specific surface receptors and the notion that ILCs can develop in the absence of the thymic niche have clearly set them apart from the T-cell lineage and promulgated a dogma that ILCs develop directly from progenitors in the bone marrow. Interestingly however, emerging studies have challenged the BM-centric view of adult ILC development and suggest that ILCs could arise neonatally from developing T cell progenitors. In this review, we discuss ILC development in parallel to T-cell development and summarize key findings that support a T-cell-centric view of ILC ontogeny.

Keywords: T-cell development, innate lymphoid cell development, layered hematopoiesis, neonatal, fetal, origins of lymphocytes

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INTRODUCTION

While hints of innate type immune cell subsets, including NK cells and lymphoid tissue inducer (LTi) cells, were discovered as early as 1970s and early 2000s respectively (1, 2), a more detailed and full characterization of the innate lymphoid cell (ILC) family emerged in the late 2000s (3). Undoubtedly, their classification marks a formative breakthrough that changed our perception of the immune system and immune homeostasis (4–6). In just over a decade, ILCs were shown to be important in allergic disease, autoinflammation and immune tolerance (7, 8). Previous and on-going studies have highlighted them as key drivers of inflammation and fibrosis in inflammatory bowel disease, inducers of chronic airway inflammation, and active players in other disorders such as obesity and cancer (9–14). In general, ILCs are tissue-resident and are triggered through relatively broad spectrum receptors for pathogens or inflammatory cues rather than specific-antigen receptors (BCRs or TCRs). Upon appropriate alarmin signaling, they orchestrate downstream responses by communicating with neighboring stromal and immune cells to adjust the cytokine microenvironment in a fashion that promotes protection, health and homeostasis at mucosal barrier sites (5, 15). In addition, they have also emerged as regulators of homeostasis and tissue repair in non-barrier organs (16).

Molecularly, ILCs are extremely heterogenous but, for convenience, have been grouped into subsets that resemble the classification of T cells based on their surface marker, cytokine and transcription factor profiles during development and activation (ILC1/NK, ILC2 and ILC3/LTi) (17, 18). Thus, like type 1, 2 and 17 helper T cells, ILC1, 2 and 3 are categorized according to the class of immune response they invoke upon perturbation. ILC1s include conventional NK cells and "helper ILC1s" and are defined by the production of interferon γ (IFN-γ) in response to IL-12, IL-15 and IL-18 (19). The transcription factor, T-bet, functions as their master regulator with Eomes being present in NK cells and a small subset of ILC1s (20, 21). ILC2s, on the other hand, depend on the expression of GATA3, and are responsible for generating type 2 cytokines such as IL-5, IL-9, IL-13 and amphiregulin upon stimulation by the alarmins, IL-25, IL-33 and TSLP (12, 22, 23). Lastly, ILC3s are defined by production of IL-17 and IL-22 in response to IL-23 and IL-1β signaling and are maintained by the transcription factors RORγt and RORα (24, 25). Within the ILC3 group, there also exists an LTi family that arises during embryogenesis and facilitate the formation of secondary lymphoid tissues (26). Broadly speaking, ILC1s are involved in the clearance of intracellular pathogens, ILC2s are associated with helminth infection and chronic airway inflammation in response to allergens (27), and ILC3s are predominantly implicated in gut immunity and responsible for establishing tolerance and mucus secretion (17).

Because ILCs closely resemble helper T cells, they are often regarded as the innate counterparts of Th1, Th2 and Th17 cells. Despite this, current dogma suggests that the ontogeny of ILCs and T cells are separate and distinct, and that the thymic microenvironment is dispensable for ILC maturation (19). This concept arose from three distinct observations made from seminal studies that identified ILCs and their function. First, unlike T cells, all subset of ILCs do not depend on the expression of surface antigen-specific T-cell receptors (TCR) for their development and activation (18). Therefore the thymus, the site which provides the appropriate niche for TCR gene rearrangements and signaling through TCR and other co-receptors, was not considered to be important in ILC biology. Second, they are present, expanded and functional in mice with profound lesions in T-cell development including *Foxn*$^{nu/nu}$ (nude) and in *Rag*$^{-/-}$ mice (28). Lastly, the characterization of early ILC progenitors in the adult BM and their restricted T-cell potential as they differentiate downstream of common lymphoid progenitors (CLP) have left an impression that the unique microenvironment of the thymus is dispensable for ILC development (29–32). As a result, investigations of ILC development in adult animals have focused predominantly on the key progenitor populations originally described in the adult BM, and the concept of ILCs arising during early T-cell development, until recently, has remained largely unexplored.

With the identification of BM ILC progenitors, the mapping of signaling pathways and factors that contribute to ILC development became the topic of intense investigation. Interestingly, these studies found that although ILCs do not undergo TCR-dependent development or activation, the factors required for their commitment and maturation are virtually indistinguishable from those found in T-cell development with the noteworthy exception of NFIL3 expression in early BM ILC progenitors (18, 32–34). Furthermore, recent reports have highlighted the ontogeny and presence of ILCs in embryonic/neonatal thymi (35, 36), suggesting that the bifurcation of T- and ILC-driven lineages, at least during neonatal life, is at the committed T-cell progenitor stage in the thymus. Here, we revisit ILC development in the context of neonatal T-cell development with a focus on recent literature highlighting layered ontogeny of ILC2s and TCR gene rearrangements and propose a model that more closely aligns the development of ILC and T-cell lineages during neonatal life.

AN OVERVIEW OF THE BM MODEL OF ILC DEVELOPMENT

The current framework of ILC development was built on the identification of CXCR6$^+$ α-lymphoid progenitors (α-LP), early innate lymphoid progenitors (EILP), common helper ILC progenitors (CHILP) and ILC precursors (ILCP) in the adult BM (**Figure 1**). These were discovered and classified through a series of differentiation assays and transcription factor analyses that determined whether these precursors could competently become either ILC1, ILC2 or ILC3 (37). Briefly, it is thought that ILC lineage commitment occurs at the level of CXCR6$^+$ α-LPs and EILPs. These progenitors are direct descendants of CLPs in that they retain both ILC and NK cell potential, but lack the ability to become functional T or B cells. Transcriptomic and protein analyses revealed that transcription factors NFIL3, TOX and TCF-1 (*Tcf7*) are critical in ILC lineage specification as they precede the expression of downstream ILC progenitor genes (29, 32, 38). In contrast, CHILPs and ILCPs represent further restricted downstream progenitors that have lost the ability to form T, B and NK cells. CHILPs and ILCPs are defined by the expression of transcription factors Id2 and PLZF, which reaffirms their commitment to helper ILC subsets (30, 39). Subsequently, based on the upregulated expression of T-bet (ILC1), GATA3, RORα, Bcl11b (ILC2) and RORγt (ILC3), the precursors become one of three mature ILC subsets (18) (**Figure 1**). Using this model as a foundation, later studies examined other possible sources of tissue-resident ILCs in adult and during embryonic development. Similar to the adult BM, it was shown that ILC development occurs in the fetal liver from Id2, PLZF and Arginase-1 (Arg-1) expressing ILC progenitors (40, 41). Fetal ILC development contributes predominantly to the generation of CD4$^+$ LTi cells and NKp46$^+$ ILC3s which then migrate to the gut and guide Peyer's patch development (42). NK1.1$^+$ ILC1 precursors are also found in the fetal liver. However, their gene expression signature resembles that of adult hepatic ILC1s suggesting that, perhaps, they represent liver-specific precursors that are long-lived, maintained, and self-renewed after their genesis in early liver development (43, 44). To date, the exact timepoint in which ILC progenitors colonize the periphery is unclear. Surprisingly,

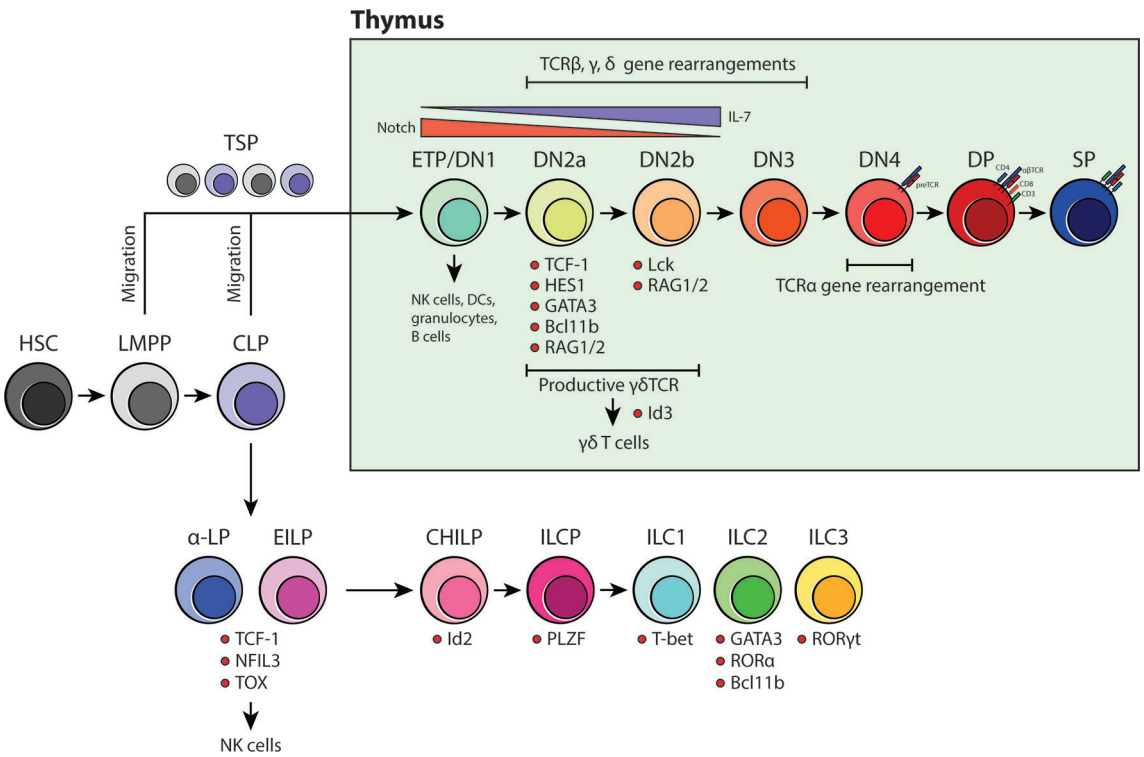

FIGURE 1 | An overview of BM-dependent ILC and thymus-dependent T-cell development. Current models for both ILC and T-cell development suggest that they are of distinct lineages and that the site for maturation do not overlap beyond the CLP stage. ILCs have been proposed to develop in the BM whereas committed T-cell progenitors undergo intense development processes that are heavily influenced by the thymic niche.

elegant recent parabiosis and pulse-label lineage tracing studies showed that the majority of tissue-resident ILC1, ILC2, ILC3 and LTi cells residing in the mucosal barrier tissues are not replenished through steady-state BM lymphopoiesis but instead self-renewed locally in their tissue of residence (44–47). Furthermore, tissue-resident lung Il18r1^{+}ST2^{-} ILC2 progenitors have been recently shown to produce ILC2s locally upon immune challenge and independent of *de novo* production of ILCs in the BM (48). These simple observations then call into question the concept and significance of BM ILC generation and its contribution to the peripheral ILC pool.

NORMAL THYMIC DEVELOPMENT OF T CELLS

In contrast to most other hematopoietic lineages, T-cell development is critically dependent on the ability of developing thymocytes to undergo a strict maturation process in the thymic microenvironment before colonizing the peripheral tissues (**Figure 1**). Originally, CLPs were thought to be the only branchpoint in which T-cell fate restriction occurs. However, with the discovery of lymphoid-primed multipotent progenitors (LMPP), the classical view of T-cell development was revised to include a secondary pathway that was independent of CLPs (49). LMPPs are defined by their expression of the fms-like tyrosine kinase 3 (Flt3) and are positioned upstream of CLPs in the differentiation hierarchy (50, 51). Like CLPs, they are restricted to the T-, B-cell and NK cell lineage, but are different in that they retain the potential for granulocyte/monocyte development. It has been shown that LMPPs are much more akin to ETPs in the thymus, suggesting that the thymus-seeding progenitor (TSP) pool includes both LMPPs and downstream CLPs lacking Ly6D^{-} expression (49, 52). Once in the thymus, CD4^{-}CD8^{-} TSPs enter the double negative (DN) stage in T-cell specification. This stage is divided into four major compartments: DN1/ETP to DN4 (**Figure 1**). Depending on the differential expression of CD24, CD25, CD44 and CD117 (KIT) and the state of the TCR loci, developing DN thymocytes are classified as either DN1/ETP, DN2a, DN2b, DN3 or DN4 (49, 53). DN1 cells represent 0.01% of the total T-cell progenitors in the thymus and are surprisingly multipotent as they retain the ability to differentiate into cells of the myeloid and lymphoid lineages. However, upon arrival at the corticomedullary junction, Notch signaling induces a genetic program that secures their commitment to the T-cell fate and prepares them for TCR gene rearrangement (49). As these cells progress to the DN2 stage, they become localized within the subcapsular zone of the thymic cortex. Here, they begin rearranging their TCRβ, γ and δ loci *via* the activation of *Rag1* and *Rag2* genes. It is expected that DN2

cells become more dependent on IL-7 produced by thymic epithelial cells (TEC) as it is vital for their proliferation, survival and differentiation (54). There are two subtypes of DN2 cells, DN2a and DN2b, and they are characterized based on the expression of lymphocyte-specific protein tyrosine kinase (Lck) and the ability to suppress NK, myeloid and dendritic cell (DC) potential. Although they are more restricted than DN1/ETPs, DN2a cells are still relatively fluid in terms of their differentiation potential. This however, is lost as they continue through the process of TCR gene rearrangement and transition towards DN2b (55). At DN3, developing thymocytes extensively rearrange their DNA at the TCRβ, γ and δ loci and are selected for survival based on the expression of functional γδ or preTα/β (preTCR) chains. γδTCR expression, along with transcription factor Id3, promotes γδ T-cell development whereas preTCR expression guides the remaining DN3 cells to enter the DN4 stage. Once at the DN4 stage, thymocytes begin migrating back towards the medulla and initiate TCRα gene rearrangements upon preTCR signaling. After the formation of a functional αβTCR, thymocytes then upregulate CD4 and CD8 co-receptors to become double positive (DP) thymocytes. From here, DP cells are positively selected for reactivity with MHC, becoming either CD4 or CD8 single positive (SP) cells. Shortly thereafter, the surviving SP cells undergo negative selection against autoreactivity and become mature naïve T cells (49, 53, 54) (**Figure 1**).

ILCs ARE LONG LIVED TISSUE-RESIDENT CELLS IN ADULT AND COLONIZE TISSUES DURING FETAL/NEONATAL DEVELOPMENT

The working model of adult hematopoiesis argues that the BM is responsible for generating and replenishing all blood and immune cells required for the lifetime an individual. Although this is true for some short-lived circulating leukocytes (56), it has become increasingly clear that tissue-resident cells such ILCs, macrophages, mast cell subsets and γδ T cells persist throughout life and, for the most part, expand and contract locally in their tissue of residence, largely independent of hematopoietic activity in the BM (45, 57–60). As outlined in Elsaid et al. (61), the developmental pathways for most tissue-resident cells do not fit the rudimentary model of adult hematopoiesis, but instead, follow a highly conserved and layered approach during ontogeny. These cells arise in distinct waves in coordination with tissue development, thus accounting for both the spatial and temporal aspects of embryogenesis. It is thought that highly coordinated programs and interactions between the stromal and immune cells facilitate tissue development, in addition to providing early immune protection specific to a given tissue type (61).

Considering the fact that BM ILC progenitors provide minimal contributions to tissue-resident ILC pools, it stands to reason that tissue-resident ILCs perhaps colonize during early stages in ontogeny rather than constantly being restored through BM lymphopoiesis. Intriguingly, the idea of layered ontogeny in ILC development has recently been explored in a lineage tracing study that closely monitored ILC2 development and turnover (47). Through elegant pulse-labeling of putative BM and fetal ILC precursors ($Id2^+$ and Arg-1^+ respectively) (30, 62), this group showed that development of ILC2s is temporally controlled and that they follow the model of layered lymphopoiesis similar to the one described in early macrophage development. These findings argue that ILC2s rapidly colonize peripheral tissues during the first week or two of postnatal life in mice and at a time when the bone marrow (BM) is still establishing itself as the sole source of hematopoietic progenitor activity. Strikingly, this study also concludes that once they establish residence in peripheral tissues, ILC2s turn over very slowly within the specific peripheral tissue microenvironments. This includes BM ILC2s, which appears to turnover with a kinetics of weeks to months rather than days. Thus, the evidence to suggest that BM ILC2s seed peripheral tissues to any large degree is increasingly scant and it is likely that the ILC2s present within the BM represent tissue-resident cells with tissue-specific function (Schneider et al., 2019). Indeed, a subsequent fate-mapping study using polychromic reporter mice expressing Id2, Bcl11b, GATA3, RORα and RORγt revealed co-differentiation of ILCs and developing thymocytes in embryonic thymi, strengthening the concept that ILCs develop early in life parallel to early T-cell development (36).

TCR GENE REARRANGEMENTS IN ILCs

V(D)J recombination at the TCR loci is an extraordinary process reserved for committed thymocytes at their DN stage. It is thought to occur in sequence as DN cells progress towards more committed DP state. TCR gene expression, as a unique hallmark of T-cell development, has hinted at a strikingly close relationship between ILCs and T cells in the past with both EILPs and ILCPs expressing high levels of sterile TCR transcripts even though they were originally identified in the adult BM (63). In order to concretely address whether ILCs stem from embryonic/neonatal T-cell development, we recently performed detailed genetic analyses of all TCR loci in mature tissue-resident ILCs. Single-cell analyses of cecal ILC1, 2 and 3s and lung ILC2s showed abundant expression of TCR constant region transcripts. Specifically, Cβ transcripts are expressed in all ILC subsets while Cα, γ and δ transcript levels are more closely associated with specific ILC subsets. Subsequent in-depth genomic analyses revealed that although lung ILC2s do not show evidence of DNA rearrangements at the TCRβ loci, they exhibit clear evidence of rearrangements at their TCRγ loci in a pattern that is strikingly similar to mature $Vγ2^+$ γδ T cells. In addition, qPCR analysis shows that at least one of the TCRδ alleles is frequently deleted. Lastly, when sequenced, Vγ2-Jγ1 rearrangements were found to be largely out-of-frame, thus precluding their ability to express a functional TCRγ subunit even if this locus was actively transcribed and translated (64).

The detailed characterization of TCR gene rearrangements performed in our study offers a fascinating window into the life history of ILC2s. The preferential expression of TCRγ constant regions and preponderance of non-productive TCRγ and δ gene rearrangements without the VDJ recombination of the TCRβ locus suggest the possibility that ILC2s may abortively arise from DN2-DN3 transition stage during γδ T-cell development. As reviewed in Spidale et al. (59), the development of tissue-resident γδ T cells occurs early in life in a time-sensitive manner. Unlike adult αβ T cells, γδ T cells emerge in progressive waves that are defined by the specific gene usage of Vγ and Vδ segments during fetal/neonatal development (58). Vγ3$^+$ γδ T cells (also known as DETCs) are the first T cell subset to arise from the fetal liver during embryogenesis. They preferentially migrate to the epidermis and provide early immunity in developing skin. Subsequent γδ T cells that follow this vanguard wave of DETCs are Vγ4$^+$ and Vγ2$^+$ γδ T cells. Vγ4$^+$ γδ T cells seed non-lymphoid tissue sites such as the uterus, lung, adipose tissue and skin dermis during late fetal development while Vγ2$^+$ γδ T cells appear during the late fetal/neonatal stage, colonizing various mucosal and non-mucosal sites (59). In line with the layered model of ILC-genesis, it is possible that abortive Vγ2-Jγ1 locus rearrangements in tissue-resident ILC2s represent an ontogeneologic relic of "failed" Vγ2$^+$ γδ T-cell development (**Figure 2**). When viewed in the context the aforementioned neonatal lineage tracing experiments (47) and the presence of ILCs in the embryonic thymus and their dependence on early T-cell transcription factors (36), a compelling case emerges for the development of ILCs from abortive T-cell development and as an offshoot from neonatal T-cell progenitors.

In addition to ILC2s, it is noteworthy that earlier studies showed the TCR loci are also frequently rearranged in NK cells. Comprehensive genomic analyses of adult and neonatal splenic NK cells revealed that the TCRγ locus is rearranged while TCRβ locus maintains its germline configuration. Intriguingly, unlike ILC2s, NK cells were reported to express rearranged TCRγ transcripts; however, the sequencing data exhibited a variable degree of productive rearrangements despite being isolated from mRNA products. Minimal TCRδ locus rearrangements (Vδ4-Jδ1) were detected and only in neonatal NK cells, suggesting that similar to ILC2s, at least one allele could have been deleted due to an abortive gene rearrangement event at the TCRα/δ loci (65).

Further evidence for TCR gene rearrangements in other ILC subsets has recently been demonstrated in human studies through sophisticated single-cell transcriptome analyses that revealed gene expression patterns associated with tissue-residency and migration in human ILCs (66). In this report, blood ILC1s (EOMES$^{-/+}$) are shown to uphold the expression of T-cell related-genes such as CD3, CD4, CD5, CD6, CD27, LEF1. As such, the group investigated whether these cells and other ILC subsets express rearranged TCR transcripts, despite lacking surface TCRs. They showed that blood EOMES$^+$ ILC1s express rearranged αβ chains and putative blood EOMES$^-$ ILC1s exhibit rearrangements in all four TCR chains. Within this pool, ILC1s with rearranged δ/γ transcripts associated with *Ikzf2* expression while ILC1s with rearranged α/β transcripts clustered closely

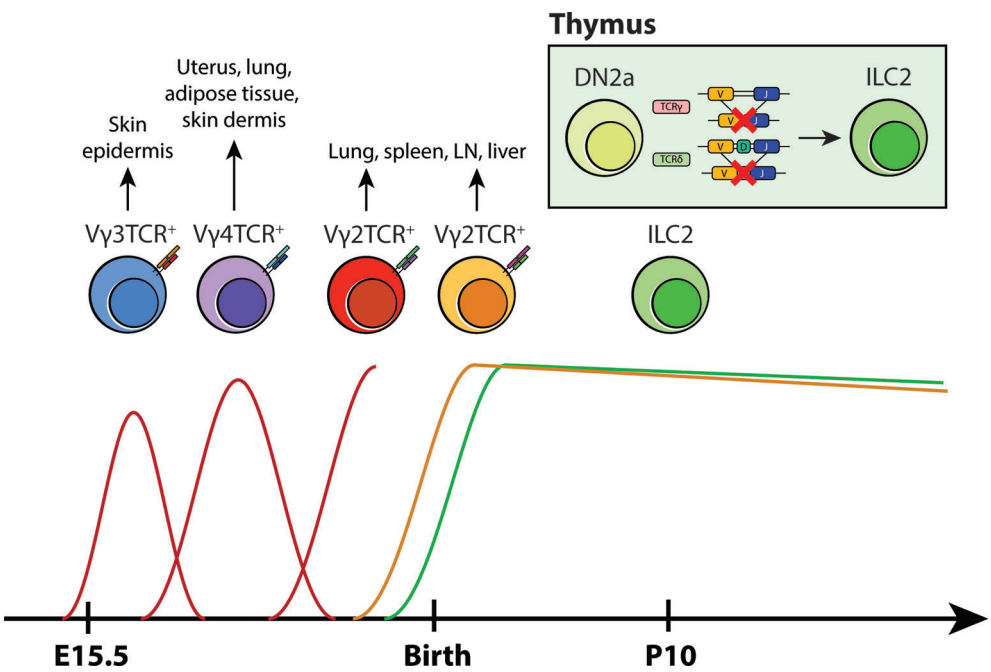

FIGURE 2 | A new model for layered ontogeny of ILC2s along with fetal and postnatal waves of γδ T cells. Like most tissue-resident cells, the developmental timepoint in which ILC2s arise and colonize tissues coincides with γδ T cells early in life (based on layered ontogeny in B6 mice). Taken together with TCR locus sequence tracing data and pulse-labeling lineage tracing studies, it is highly likely that tissue-resident ILC2s arise perinatally from developing DN2s that have ineffectively rearranged their γ/δ loci, rather than from progenitors in the BM. These cells then take up residence at their designated tissue sites and self-renew locally.

with CXCR3$^+$ Th1 cells (66). These observations suggest that ILC1s are more akin to CD4$^+$ and CD8$^+$ T cells and that in their lifetime, they have undergone a maturation process in the thymus. Although it is noteworthy that, these authors failed to detect rearranged TCRγ chain expression in human ILC2s, it is also important to bear in mind that these studies only evaluated TCR *transcripts* rather than the genomic loci. Indeed, in our previous studies of murine ILC2 genomic loci and transcripts, we found that the rearranged loci are transcriptionally silent in murine ILC2s as well, potentially reflecting an attempt to silence alleles that have failed productive in-frame rearrangement; a process that naturally occurs in developing thymocytes. With this in mind, it would be of interest to now evaluate genomic TCR loci in human ILC subsets. Although human and murine ILC/T-cell biology exhibit significant differences, the use of TCR gene rearrangements as an indelible mark of the lineage of origin in both of these studies points to a thymic origin of the tissue-resident ILCs.

NOTCH SIGNALING, THE INITIAL FATE DETERMINATOR IN THE THYMUS

In addition to abortive V(D)J recombination, limited Notch signaling in the thymic microenvironment may also determine whether thymocytes continue developing as T cells or shunt away from the T-cell lineage and into ILC lineages. Notch activity was discovered in the early 20th century from strains of *Drosophila* that exhibited serrated "notched" wings (67). Its signaling pathway is evolutionarily conserved and is imperative for regulating cell fate decisions, survival, proliferation and niche formation (68). In the context of T-cell development, Notch1 and its ligand, Delta-like 4 (DLL4), serve as an essential checkpoint signal that imprints T-cell identity in TSPs entering the thymus. Their signaling is mediated by direct cell-cell interaction between TSPs and TECs and promotes the acquisition of T-cell fate by upregulating T-cell specific genes while gradually repressing myeloid and B-cell potential (49, 69). Upon interaction, a series of proteolytic cleavage events release the cytoplasmic domain of Notch, which then binds to recombination binding protein-J (RBP-J) in the nucleus and activates transcription of genes associated with T-cell development including *Tcf7*, *Hes1*, *Gata3* and *Bcl11b* (54, 69, 70). Loss-of-function studies of Notch1 and Cre-mediated deletion of DLL4 revealed aberrant proliferation of B cells in the thymus, and argues that their presence is indeed necessary for T-cell commitment (49). In contrast, the importance of Notch signaling in ILC development has been quite controversial. Deletion of RBP-Jκ in hematopoietic cells leads to a noticeable reduction in the frequency of lamina propria NKp46$^+$ILC3, but not CD4$^+$LTi-like cells (71, 72). Moreover, culturing EILPs on OP9-DLL1 stroma results in enhanced generation of ILC2s, yet the development of all ILC subsets is unaffected in the absence of Notch signaling (29). Lastly, multiple groups have concluded that committed ILC progenitors and precursors have diminished dependency on Notch (29, 31, 73), thus creating the notion that its signaling is dispensable in ILC development. More recently however, an *in vitro* assay performed using a Tet-inducible cell system, which allows for fine-tuning of DLL1 and DLL4 expression under doxycyclin, revealed that the strength and duration of Notch signaling influences the development of different ILC subsets. In this study, CLPs that were exposed to robust Notch-DLL signaling differentiated predominantly into T cells, LTi cells and ILC3s, whereas those receiving intermediate signaling preferentially became ILC1s/NK cells and ILC2s. Expectedly, CLPs with minimal to no Notch signaling led to B-cell differentiation (**Figure 3**). The same study also showed that specific deletion of DLL4 on TECs results in an abnormal expansion of ILC2 in the thymus (38), suggesting that the gradient of Notch signaling may influence ILC fate in the thymic microenvironment.

Notch signaling is not binary, but rather it is dose- and time-dependent during ontogeny and in cell fate decisions (68). It is widely known that TSPs/ETPs are multipotent, and that T-cell potential is acquired progressively through DN to DP stage. Considering the fact that Notch signaling is facilitated by cell-cell interaction, it is highly likely that the size of initial thymic niche for ETPs is limited. Therefore, the degree and duration of Notch signaling would vary from cell to cell, leaving the option for other lineages to appear in the embryonic/postnatal thymus similar to the ones observed in the dose-dependent *in vitro* system (**Figure 3**). Addressing the redundancy of Notch in later ILC progenitors requires careful consideration of its unique purpose. Notch and its ligand interaction are often described in the context of development and regulation of cell fates. In both T-cell and ILC development, Notch establishes T-cell and ILC identity through upregulation of TCF-1 and its related transcription factors. As DN2 cells mature into DN3 cells, their dependency on Notch signaling decreases, eventually becoming redundant (74). Since a similar behavior is observed in downstream ILC progenitors, it is possible that TCF-1$^+$ EILPs and CHILPs are already committed and downstream of developing thymocytes that share the same hierarchical level as DN3 cells in the differentiation hierarchy.

E-Id PROTEINS AND Bcl11b DETERMINE THYMOCYTE FATE

The transcription factors involved in positive and negative regulation of ILC differentiation have been discussed in great detail in other reviews (19, 69, 75). Emerging studies have highlighted the fact that differential expression of factors associated with T-cell development, HES1, TCF1 and GATA3, RORα, E and Id proteins, and Bcl11b, are all critical for ILC maturation (19, 36, 76–78). Among these, E-Id proteins and Bcl11b are especially interesting because they have been shown to play a role in inducing ILC development in the thymus. E proteins are under the class I basic helix-loop-helix (bHLH) family of transcription factors that are historically described in early T-cell lineage commitment and specification in coordination with Notch signaling. They form

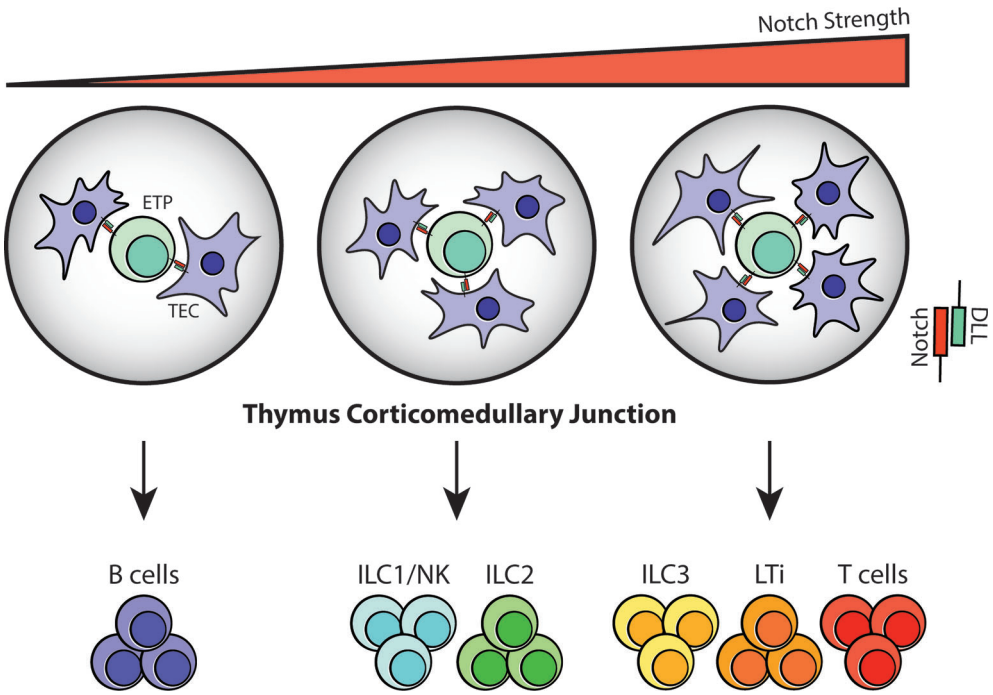

FIGURE 3 | Notch signaling at the thymus corticomedullary junction may determine T-cell/ILC fate. Notch signaling is mediated through direct cell-cell contact in the thymus; therefore, the number of TECs interacting with TSPs/ETPs at the corticomedullary junction will vary depending on cell position and niche availability. Based on the observations from *in vitro* assays, it is highly likely that ETPs that receive zero to low Notch-DLL interaction will differentiate into B cells. In contrast, strong Notch signaling will guide ETPs towards the T-cell and ILC3/LTi lineages while intermediate Notch signaling will result in ILC1/NK and ILC2 commitment during fetal and postnatal periods.

homo- or heterodimers (e.g., E2A-HEB) with other bHLH or HLH proteins, which then turns on the T-cell-specific program in developing thymocytes. E protein function is regulated by class IV HLH family inhibitor of DNA binding (Id) factors. Id proteins lack the DNA binding domain; thus, they sequester E protein function by forming a heterodimer complex that cannot bind to DNA (69). Intriguingly, hindering the activity of E proteins by deleting E2A and HEB or ectopically expressing Id1 or Id2 blocks the generation of functional T cells, and instead promotes ILC2 proliferation in adult thymi (76, 79, 80). Furthermore, fetal ETPs lacking E2A and HEB *in vitro* can also generate ILC1- and ILC3-like cells (76); therefore, it is highly likely that the strength of Notch signaling, and E-Id protein levels may influence ETP fate in the thymus (**Figure 4**). Bcl11b, on the other hand, belongs to a family of Kruppel-like C_2H_2 type zinc finger transcription factors and is expressed in late DN2a thymocytes and essential for DN2-DN3 transition in T-cell development (69, 81). *Bcl11b*-deficiency causes arrest of T cells at the DN2a stage and, fascinatingly, allows aberrant differentiation of NKp46$^+$ ILC1/NK cells and myeloid cells in the thymus (82, 83). Bcl11b has also been implicated ILC2 biology and development. It is widely accepted that Bcl11b is important for overall ILC2 maintenance and function; however, in the original study that used heterozygous germline knockout and conditional knockout of *Bcl11b*, only the ST2$^+$ BM ILC2 compartment was reduced upon *Bcl11b* deletion. Surprisingly, the frequency of lung-resident ST2$^+$ ILC2s was not affected by this genetic manipulation, but rather there was an incredible increase in the KLRG1$^+$ ILC2 population (84). It would be extremely interesting to revisit these transgenic mice models and examine their thymus-residing cells to determine if there is an abnormal expansion of KLRG1$^+$ ILC2s or perhaps other ILC subsets in the thymus through high fidelity single-cell sequencing. Considering this and the evidence for layered ontogeny of ILCs, it is quite possible that the origin of BM ILC2s and lung ILC2s is completely independent of each other and that E-Id proteins and Bcl11b may be the transcriptional checkpoints that initiate reprogramming of T cells that have neither received adequate Notch signaling nor undergone productive TCR gene rearrangements (**Figure 4**).

ADDRESSING NORMAL DEVELOPMENT OF ILCs IN T-CELL KNOCKOUT MODELS

The presence of functional ILC subsets in mice with genetic lesions in T-cell development raises an interesting conundrum because if ILCs are indeed associated with T-cell development, one might predict there would be a reduction in their frequency upon interfering with thymocyte maturation. In contrast, earlier characterization of ILCs has indicated that ILCs can develop and

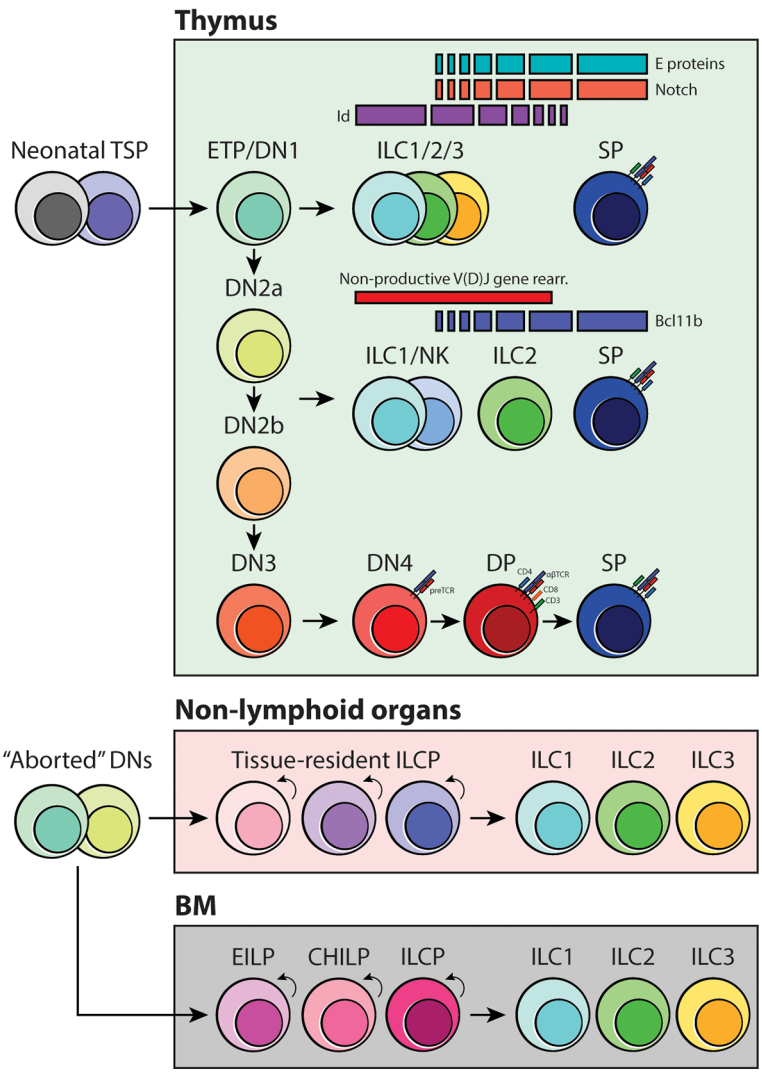

FIGURE 4 | Schematic of ILC development in coordination with thymocyte specification and maturation. As early T-cell progenitors are known to be multipotent, we propose that the branchpoint in which ILC differentiation occurs is at the DN1/ETP and DN2-DN3 transition stage. Depending on the status of the TCR loci, strength of Notch signaling and activities of E-Id proteins and Bcl11b, developing thymocytes may acquire innate-like properties and give rise to one of three ILC subsets. Furthermore, we suggest that tissue-resident ILC progenitors, including the BM, originate from failed T-cell development and locally maintain the mature ILC pool.

function normally in *Rag*$^{-/-}$ and nude mice, thus highlighting them as distinct subsets, independent of the T-cell lineage (85). With the evidence of non-productive TCR rearrangement, however, it is now critical to revisit this hypothesis and re-evaluate it from the perspective of abortive T-cell development. In maturing thymocytes, RAG genes are typically expressed during the DN2-DN3 transition (49) (**Figure 1**). Therefore, without active RAG1 or RAG2 proteins, thymocytes cannot rearrange their TCR loci and progress further into latter stages of T-cell development (86–88). Taken together with the fact that pre-DN3 cells are multipotent, there is room for *Rag*-deficient ETPs and DN2a cells to shunt away from the T-cell lineage and immediately choose the innate cell fate as part of a salvage mechanism. Indeed, our characterization of ILC2s in *Rag1*$^{-/-}$ mice revealed that there is a heightened frequency of ILC2s in the thymus, indicating active generation of ILC2s upon blocking TCR gene rearrangements. Likewise, other studies have also characterized the heterogeneous populations of DN cells in RAG mutants containing putative NK cells (89), and suggesting that this pathway may apply to other ILC subsets. Similar to RAG mutants, the mature T-cell population is also substantially decreased in nude mice carrying null mutations of the forkhead transcription factor (FOXN1), which is essential for TEC differentiation during development (90). The nude mouse vestigial thymic microenvironment is detrimental for early T-cell progenitors as it cannot provide sufficient Notch signaling for their specification and survival. Considering the evidence of dose-dependent Notch signaling in determining cell fate, it is

highly likely that all T-cell progenitors are co-opted into the ILC fate program immediately after entering this non-functional thymic niche. This would increase the total frequency of ILCs in the peripheral tissues, and certainly, previous studies have observed heighten ILC counts at various barrier sites (91, 92). In future studies, it would be extremely interesting to purposefully disrupt TCR gene rearrangement at specific stages in development and investigate whether this influences the total number of ILCs in the periphery.

FUNCTIONAL SIGNIFICANCE OF BM ILCs

The vast majority of ILCs within the BM are affiliated with the ILC2 subset. Given the evidence that ILCs can develop neonatally from thymic precursors and become tissue-resident thereafter, the functional significance of BM ILC2s then comes into question. Likewise, the fact that these cells show a remarkably slow turnover (47) would argue against them serving as a precursor pool for peripheral ILCs and might instead suggest that, like peripheral ILCs, these cells fulfill an important tissue-resident function. Consistent with this observation, it has been shown that self-renewal and maintenance of ILCs is facilitated by a pre-existing pool of tissue-resident ILCPs under inflammatory conditions (45, 48). Specifically, during the acute phase of *Nippostrongylus brasiliensis* helminth infection, ILC2s in the lung, gut and mesenteric lymph node proliferate locally without significant contribution from the BM (45, 47, 93). The signs of recruitment and redistribution of ILC2s from other tissues including the BM only begin to appear after day 15 post-infection where the acute inflammatory conditions turn chronic and overt (45, 93, 94). Recent studies have shown that their contribution is rather minor (<10%) and that immature *Il18r1* expressing BM ILCPs are responsible for seeding BM-derived ILC2s in the lung to generate the full phenotypic spectrum of ILC2s (47, 93). Indeed, there is a clear precedent for a selective BM-resident function for several mature hematopoietic lineages. Macrophage-like osteoclasts, for example, play a key role in bone remodeling and, together with osteoblasts regulate bone homeostasis (95). Similarly, the BM serves as a long-term reservoir and archive for antigen-specific, antibody-secreting plasma cells and possibly a unique population of isotype-switched, affinity-mature memory B cells that could be called into service upon reinfection with specific pathogens (96, 97). With these examples in mind, it is worth considering a BM specific role for resident ILC2s. Intriguingly, several studies have suggested that BM ILC2s can, in fact, play key roles in stimulating eosinophilopoiesis in response to system Th2 inflammatory insults (98, 99). Likewise, recent studies suggest that through secretion of GM-CSF ILC2s can stimulate the recovery of BM hematopoiesis in response severe chemically induced stress (100). In aggregate, these studies suggest that, like there peripheral tissue counterparts, BM ILC2s may have colonized this tissue early in development and serve a tissue-resident purpose thereafter. Taken together with previous TCR gene rearrangement data, it will now be important to test whether these cells, too, show genetic marks of deviation from early thymic progenitors.

CONCLUDING REMARKS

The recent discovery of ILCs has been transformative in our understanding of the development of finally orchestrated and appropriate immune responses to the appropriate pathogens and in bridging the division of labor between innate and adaptive immune responses. More recently, their functional significance has been expanded to include roles in non-barrier organs and key roles in tissue and organ homeostasis and repair. Despite this attention and these insights, a deep understanding of their ontogeny and development has lagged behind. Accumulating evidence now suggests remarkable conservation of molecular, transcriptional, and developmental parallels between these cells and neonatal T cells and, indeed, that in some instances these cells can develop from T cells that have failed to appropriately rearrange their antigen specific receptors. Studies of layered ontogeny of T cells and ILCs point towards the fact that long-lived, tissue-resident ILCs are likely to be thymus-derived and that during steady-state or mild immune challenge, they expand and respond appropriately to remaining inflammation. However, in hematopoietic crisis, for example chronic inflammation, complete hematopoietic ablation, sepsis, or severe viral infections, BM stem cell derived ILCs may also be called into service and enter the peripheral niches to support the existing pool of tissue-resident ILC subsets. Certainly, future studies are needed to further clarify the relative contributions of these pools, their lineage relationships and whether they can be harnessed for improved treatment of clinical disease.

AUTHOR CONTRIBUTIONS

SBS and KMM wrote the manuscript and SBS designed the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Functional Contribution and Targeted Migration of Group-2 Innate Lymphoid Cells in Inflammatory Lung Diseases: Being at the Right Place at the Right Time

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During the last decade, group-2 innate lymphoid cells (ILC2s) have been discovered and successfully established as crucial mediators of lung allergy, airway inflammation and fibrosis, thus affecting the pathogenesis and clinical course of many respiratory diseases, like for instance asthma, cystic fibrosis and chronic rhinosinusitis. As an important regulatory component in this context, the local pulmonary milieu at inflammatory tissue sites does not only determine the activation status of lung-infiltrating ILC2s, but also influences their motility and migratory behavior. In general, many data collected in recent murine and human studies argued against the former concept of a very strict tissue residency of innate lymphoid cells (ILCs) and instead pointed to a context-dependent homing capacity of peripheral blood ILC precursors and the inflammation-dependent capacity of specific ILC subsets for interorgan trafficking. In this review article, we provide a comprehensive overview of the so far described molecular mechanisms underlying the pulmonary migration of ILC2s and thereby the numeric regulation of local ILC2 pools at inflamed or fibrotic pulmonary tissue sites and discuss their potential to serve as innovative therapeutic targets in the treatment of inflammatory lung diseases.

Keywords: Innate lymphoid cells, ILC2, airway inflammation, immune cell trafficking, tissue migration

INTRODUCTION

The lungs are the major organs of the respiratory system in mammals and birds and constantly provide the body with essential amounts of oxygen. Gas exchange in the human lung requires the consumption of enormous amounts of air each day and thus exposes the thin pulmonary mucosal surface with quantities of rather harmless organic and inorganic particulate matter such as aerosols, smoke and pollen (1). At the same time however, there is inevitable exposure to viruses, bacteria and

other respiratory pathogens that could compromise proper lung function and overall body wellbeing. As a result, complex and tightly regulated immunological networks evolved to provide both effective tolerance to environmental antigens and protection against potentially invasive airborne pathogenic threats. Many different cell types, such as epithelial cells, myeloid cells and innate and adaptive lymphocytes contribute to cellular and humoral immunity in the lung (2). While the lung already harbors numerous immune cells in the steady-state, their frequencies and relative proportions strongly change during infections, tissue damage or chronic inflammatory diseases. At homeostasis, different subsets of lung resident myeloid cells including bronchial, interstitial and alveolar macrophages as well as dendritic cells comprise the vast majority of pulmonary leucocytes and have been shown to largely support pathogen clearance, tolerance mechanisms and tissue repair (3). However, more recently increasing evidence suggests that innate lymphoid cells (ILCs) substantially contribute to innate immune surveillance of the lung in the steady-state and also play vital roles during physiological or dysregulated chronic inflammatory reactions (4).

ILCs comprise heterogeneous groups of developmentally related lymphocytes that characteristically lack recombination-activating gene (Rag)-dependent rearranged antigen receptors. Mainly based on functional measures, developmental trajectories and transcription factor/cytokine expression patterns, they are broadly categorized into five major subgroups [natural killer (NK) cells, ILC1s, ILC2s, ILC3s, lymphoid tissue-inducer cells (LTi)] that functionally resemble analogous T cell counterparts (5). In both mice and humans, most subsets of ILCs are particularly enriched in tissues harboring barrier functions such as gut, lung and skin and possibly their main biological function is related to their capacity to rapidly respond to danger and stress signals derived from e.g. epithelial or stromal cells. In lungs of naïve mice, ILC2s represent the most frequent ILC population. Upon exposure to alarmin-like proteins released during tissue damage or infections, lung ILC2 numbers rapidly increase and substantial amounts of the type-2 signature cytokines IL-5, IL-9 and IL-13 and also IL-4 are released (6). ILC2 development from common hematopoietic progenitor populations depends on the transcription factors GATA3 and retinoic acid receptor-related orphan nuclear receptor-alpha (RORα). It was shown in parabiosis experiments with genetically distinguishable mice that in the steady-state and during parasitic infections lung ILC2s are largely tissue resident (7) and mostly renew from local pools established in the neonatal period (8). However, as in detail outlined in the following chapters, the extent to which lung ILC2s are strictly tissue resident is currently discussed, as recent studies also suggest that ILC2s can migrate into the lungs from other tissue sites under certain circumstances.

Here, we summarize recent advances in our understanding of the involvement of ILC2s to lung homeostasis and diseases with a particular focus on the immunological aspects that regulate their migratory capacity and thereby their pulmonary accumulation during inflammatory processes.

ILC2s AS GATEKEEPERS OF LUNG HOMEOSTASIS

ILC2 Expansion in the Postnatal Period

How ILC progenitors (ILCP) disseminate into peripheral tissues such as throughout embryonic development remains incompletely understood. However, the profound tissue compartmentalization of different ILC subsets suggests that tissue-specific niches exist that drive the establishment of local ILC pools. Although at low frequencies, ILC2s have been described to populate the human lung already in the second trimester of the fetal period (9). In line, some ILC2s were found in murine embryonic lungs just prior birth. Remarkably, the postnatal window from birth to weaning is characterized by a rapid and pronounced accumulation of ILC2s and other type 2 innate cells such as eosinophils, mast cells and basophils. Because their numbers again decline after weaning and this was not observed in other postnatal organs (10), it was proposed that ILC2s might inhibit overwhelming immune reactions to airborne particles that naïve lungs are exposed to in the context of alveolarization. De Kleer et al. also found that these postnatal lung ILC2s all expressed the IL-33 receptor ST2, proliferated in response to stimulation with IL-33 and produced IL-5 and IL-13. In line with an important role of innate type 2 immunity for lung tissue repair and remodeling, increased production of IL-33 by the lung epithelium was observed (11). Although it is currently unclear which factors trigger neonatal IL-33 production, it is feasible that mechanical and oxidative stress initiated by exposure to air after birth is of primary importance (12). Recent studies using IL-33 reporter mice also confirmed that, in addition to epithelial type 2 pneumocytes, adventitial stromal cells produce IL-33 in lungs of neonatal and adult mice (13). Notably, scRNA-seq analysis of neonatal lung ILC2s revealed the existence of ILC2 subsets with distinct proinflammatory and tissue-repairing functions that potentially depend on the nature of activation signals (14). Taken together, these recent data clearly indicate that the neonatal period is critical for the establishment of pulmonary ILC2 populations and may already prime lung homeostasis and host defenses during adulthood.

Regulation of Pulmonary ILC2 Activity

It is believed that type 2 responses evolved to monitor damage to barrier surfaces and to contain or eliminate the pathologic agent in a way that minimizes tissue damage and provides rapid resolution of inflammation. As early sentinels of a lost barrier integrity, lung ILC2s are endowed with a large spectrum of cellular receptors enabling rapid and effective detection of micro-environmental changes and intercellular communication with epithelial-, stromal- and hematopoietic cells *via* soluble mediators or direct cellular interactions.

Regulation by Cytokines and Chemokines

Characteristically, ILC2s strongly respond to stimulation with the alarmin-like molecules IL-25, IL-33 and thymic stromal

lymphopoietin (TSLP), which are mainly released by type 2 pneumocytes, tuft cells and subsets of lung myeloid cells. As a result, ILC2s are able to secrete on a per cell basis large amounts of IL-5, IL-13, IL-9 and IL-4 thereby mediating eosinophil recruitment, alveolar macrophage polarization, mast cell activation, goblet cell mucus production and smooth muscle contraction (15, 16). By promoting ILC2 survival, IL-9 initiates a positive feedback loop that amplifies ILC2 cytokine production (17). While in the steady-state murine lung "natural" ILC2s (nILC2s) were shown to be largely tissue resident and more reactive to stimulation with IL-33 than IL-25, more recent studies identified a circulating "inflammatory" ILC2 (iILC2) subset. These ILC2s are dependent on the AP1 family transcription factor BATF (18) and can transiently accumulate in the lung after mobilization in the gut or bone marrow *via* systemic IL-25 administration or nematode infection (19). Compared to nILC2s, iILC2s were phenotypically characterized by higher expression of the IL-25 receptor chain IL17RB and KLRG1 and lower expression of the IL-33 receptor chain ST2 and the enzyme arginase 1 (Arg1) (18, 20). Recently, a human inflammatory subset with transcriptional and functional similarities to mouse iILC2s was described in inflamed mucosal tissues. These cells expressed CD45RO, were linked to severe atopic diseases and displayed resistance to corticosteroid therapy, thereby representing potential targets for therapeutic intervention (21).

Reflecting the presence of several alternative activation pathways, lung ILC2s are known to be activated by several further soluble mediators in addition to alarmin-like cytokines. While IL-7 is important for their lineage commitment and drives their proliferation, ILC2s express high levels of the IL-2 receptor chain CD25 and IL-2 derived mainly from T cells, but also other cellular sources is vital for lung ILC2 survival and supports their alarmin-dependent production of IL-5 and IL-13 (22). Furthermore, ILC2s express death domain receptor 3 (DR3), a member of the tumor necrosis factor (TNF)-receptor superfamily, which is activated by its cognate ligand TL1A (23). In mice, ILC2 proliferation and activation were increased upon *in vivo* stimulation with TL1A in a DR3-dependent manner. DR3 is also expressed on human ILC2s and TL1A binding was able to induce IL-5 and IL-13 production *in vitro* suggesting that the TL1A/DR3 axis is an important pathway for ILC2 co-stimulation. Notably, mouse and human ILC2s can not only produce copies amounts of IL-4, but also express a functional IL-4 receptor. Consistent with an important role of IL-4 for ILC2 regulation, Motomura et al. demonstrated that basophil-derived IL-4 stimulated their pulmonary cytokine and chemokine production (24). In humans, IL-4 stimulation of lung ILC2s in the presence of alarmins was important for induction of GATA3 and CRTH2 expression and the secretion of IL-5 and IL-13 (25), suggesting that IL-4 could serve as a critical costimulator of ILC2s.

Interestingly, ILC2s display characteristic chemokine receptor expression patterns and were recently shown to be subject of substantial chemokine dependent regulation. In this context, the C-C motif chemokine receptor 8 (CCR8) was shown to be a critical regulator of ILC2s during parasitic infections and allergic lung diseases. Notably, the two known cognate chemokines CCL1 and CCL8 are produced by different cells in the lung and can have divergent functions. ILC2s are an important *in vivo* source of CCL1 and support their proliferation and effector functions *via* an auto-regulatory positive feedback loop (26). By contrast, CCL8 was shown to be primarily produced by inflammatory $CD11c^+$ mononuclear phagocytes (27) and controls the local positioning of ILC2s within the lung during type 2-mediated inflammation (28).

ILC2s are subject of substantial negative regulation and several cytokines turned out to be important for the control of overshooting type 2 responses in the lung. For example, ILC2s express receptors for both type I and type II interferons on their surface (29) and their prototypic functions are strongly impaired by type I IFNs and IFN-γ *in vitro* and *in vivo* (30, 31). A further inhibitory mediator is the IL-12-related cytokine IL-27 that suppresses lung ILC2 activation *in vivo via* STAT-dependent signal transduction pathways (32). Interestingly, studies also identified Treg derived IL-10 and TGF-β as strong inhibitor of murine and human ILC2 effector cytokine production (33, 34). Conversely, TGF-β production of pulmonary epithelial cells rather seems to drive ILC2 proliferation and chemoactivity of murine ILC2s (35), indicating that cell type- or context-specific roles of TGF-β may control ILC2 responses.

Regulation by Lipid Mediators

A plethora of lipid mediators including prostaglandins and leukotrienes are present in lungs during type 2 inflammatory reactions and there is growing evidence that several of them regulate biological functions of murine and human ILC2s. Xue et al. demonstrated that the prostaglandin D2 (PGD2) binds to CRTH2 on human ILC2s and induced strong production of type 2 cytokines as well as upregulation of the receptor chains of IL-33 and IL-25 (36). These data were later confirmed in mice (37). Notably, more recent findings demonstrated that activated human blood and tonsillar ILC2s produce PGD2 *in vitro* indicating that the PGD2/CRTH2 axis represents an important autocrine/paracrine pathway of ILC2 stimulation (38). Human ILC2s also express receptors for the leukotrienes CysLT1 and CysLT2 and respond to stimulation with leukotriene C4 (LTC4) and leukotriene D4 (LTD4) and potentially other related ligands (39, 40). Other studies indicated that a similar mechanism is functional in lungs of mice and demonstrated that LTC4 signaling promotes nuclear translocation of the transcription factor NFAT (41). Importantly, LTD4 was shown to promote IL-4 secretion by ILC2s more efficiently than the alarmins IL-25 and IL-33 (39, 42). Notably, some lipid mediators such as lipoxin A4, PGI2 and PGE2 were shown to bind to their receptors on ILC2s to inhibit cytokine production (43–45). Overall, these findings indicate that lipid mediators can act in concert with or independent of alarmins to fine tune lung ILC2 responses in specific tissue contexts and represent interesting therapeutic targets to modulate ILC2 functions in atopic diseases.

Interactions of ILC2s with the Nervous System

The lung is densely innervated by different sympathetic, parasympathetic and sensory nerve fibers. Given the well-known implication of neuro-immune interactions in several lung inflammatory conditions and the anatomical proximity of ILC2s and neuronal cells (46), many recent studies investigated molecular mechanisms underlying the functional crosstalk of the nervous system with ILC2s. Thereby, a number of receptors for neuronal transmitters including the neuropeptides neuromedin U (NMU) and neuromedin B (NMB), vasoactive intestinal peptide (VIP), calcitonin gene–related peptide (CGRP) and β2 adrenergic receptor (β2AR) activators are expressed in human and mouse ILC2s, suggesting that ILC2s are a central target of the nervous system's capacity to rapidly control innate type 2 responses. Compared to other immune cells, ILC2s were identified as the immune cell subset possibly expressing the highest levels of the neuromedin U receptor 1 (NMUR1). Indeed, several parallel studies described a strong *in vitro* responsiveness of lung and gut ILC2s to NMU stimulation that was independent of IL-33 co-stimulation. *In vivo* studies with NMUR1 deficient and NMU-treated mice confirmed a role of NMUR signaling in ILC2s in models of parasitic infections and asthma (47–49).

CGRP released from lung neuroendocrine cells also promoted ILC2 activation in combination with IL-33 or IL-25 in lung inflammation induced by ovalbumin (OVA) challenge (50). However, three subsequent reports employed single cell sequencing as well as *in vitro* and *in vivo* approaches to identify CGRP signaling as a negative regulator of at least subsets of ILC2s, indicating that CGRP may control ILC2s in a context-dependent manner (51–53). While the role of CGRP thus seems to be ambiguous, signaling through the β2AR has been shown to negatively regulate ILC2 proliferation and cytokine production. Consistently, mice lacking β2AR were more prone to excessive type 2 mediated lung inflammation, whereas treatment of wildtype mice with an β2AR agonist was associated with impaired ILC2 responses and reduced lung pathology (54).

Moreover, the bombesin-related peptide NMB, a molecule previously implicated in smooth muscle contraction and metabolic processes, was shown to curb ILC2 responses. Interestingly, this effect was related to direct interactions with basophils as their presence was required for upregulation of the NMB receptor on ILC2s (55). Collectively, these studies clearly suggest that signals from peripheral neuronal cells can potentiate or inhibit type 2 immune responses in the lung *via* ILC2s.

Role of ILC2s in Lung Infectious Diseases

The respiratory tract continuously interacts with the environment and is therefore possibly the body's most common target for infectious diseases. Indeed, a large spectrum of different pathogens including large metazoan parasites, fungi, viruses and bacteria can infect the lung and cause worldwide millions of deaths per year. ILC2s are sentinels of damage and mediators of tissue repair and are thus critically involved in the pathogen-directed pulmonary immune response.

Viral Infections

Already early after initial description of ILC2s, an accumulation of ILC2s in mouse models of influenza virus infection was observed, which most likely is a direct consequence of alarmin release in the context of infection-dependent epithelial disturbance and necrosis. In 2011, Monticelli et al. observed increased lung numbers of ILC2s after influenza infection. Production of the epidermal growth factor ligand amphiregulin (AREG) by ILC2s supported epithelial restoration in this model (56). Conversely, a further study identified activated ILC2s as a driver of virus-induced airway hyperreactivity after infection with H3N1 influenza (57). During resolution of experimental influenza infection, ILC2s were identified as the major cellular source of the cytokine IL-5 thereby inducing a progressive pulmonary accumulation of eosinophils (58). Additional studies identified influenza infection-dependent plasticity of ILC2s towards ILC1-like cells characterized by low expression of Gata3 and upregulated expression of IL-18Rα, IL-12Rβ2 and IFN-γ (59). Importantly, IFN-γ production during viral infections suppressed ILC2s and their tissue protective capacity (59). IL-33-dependent lung ILC2s were also shown to play a role during infections with respiratory syncytial virus (RSV). Although RSV infections typically provoke dominant Th1 type responses that mediate viral clearance, type 2 cytokines expression early in life may support exacerbated disease activity and even susceptibility to asthma development later in life. In RSV-infected neonatal mice, lung IL-33 expression is upregulated and linked to ILC2 accumulation. In line with this, blockade of IL-33 signaling improved lung pathology without altering viral loads (60, 61). Interestingly, anti-IL-33 treatment was more effective in reducing ILC2 numbers in infected neonates, whereas in adults, secretion of TSLP was shown to more prominently activate lung ILC2s (62). A potential role of dysregulated ILC2 activation also seems to contribute to respiratory tract infections with rhinoviruses (RV). Experimental RV infection of neonatal but not adult mice was associated with the development of asthma-like symptoms, which were associated with alarmin-dependent lung accumulation and activation of ILC2s (63, 64). Studies with ILC2-deficient $Il7r^{Cre}Rora^{flox}$ mice indicated that ILC2s are largely responsible for eosinophilia, mucous metaplasia and airway hyperresponsiveness in six-day-old mice infected with RV. Furthermore, in a model based on two time-shifted infections with heterologous RV strains, the ILC-dependent type 2 polarized immune response in infected premature mice resulted in a more severe disease outcome (65). Thus, similar to the conclusions derived from RSV infection models, these data indicate that lung ILC2 activation very early in life may significantly shape pulmonary immune responses later in life and could be related to an increased susceptibility for asthma development. Recently, the pandemic situation caused by the worldwide spread of the SARS-CoV2 virus has prompted a strong interest in respiratory virus research and immunomodulatory strategies to prevent and treat severe COVID-19 disease. Consistent with the strong epithelial damage observed in SARS-CoV2 lungs, global transcriptomic analyses of cells within the bronchoalveolar fluid indicated that

the expression of IL-33 is upregulated in lungs of patients suffering from COVID-19 disease (66). In line with this, IL-33 blood concentrations were reported to be increased during active disease (67, 68). Moreover, the expression of IL-33 after stimulation of COVID-19 convalescent peripheral blood mononuclear cells with SARS-CoV2 peptides correlated with seropositivity for the viral spike glycoprotein (69) indicating that ILC2s or other $ST2^+$ cells such as T cells, B cells and macrophages are functionally linked to the disease. Repeated intranasal treatment of mice with the protease allergen papain drives IL-33 release from the pulmonary epithelium and is a commonly used model to study the functions of lung ILC2s. Because the SARS-CoV2 genome encodes for the essential papain-like protease PLpro (70), Gomez-Cadena et al. administered this protein intranasally on five consecutive days to mice (71). Indeed, this treatment led to increased lung numbers of $IL\text{-}5^+$ ILC2s and subsequent eosinophilia indicating that SARS-CoV2 could directly drive ILC2 responses in infected human lungs *via* PLpro. In the same study, flow cytometric analysis revealed a relative increase of ILC2 frequencies in the peripheral blood in mild to severe COVID-19 disease, while the pool of total ILCs was lower compared to healthy controls (71). Interestingly, within ILC2s, a subset expressing low levels of the receptor tyrosine kinase cKit was significantly expanded in patients with severe COVID-19 disease, possibly consistent with an accumulation of mature ILC2s. In addition to cKit, ILC2s of patients with severe COVID-19 disease displayed higher expression of the C-type lectin-like molecule NKG2D but lower expression of the classical ILC2 markers KLRG1 and CD25. An approach to stratify patients according to the median expression of NKG2D revealed that patients with low numbers of $NKG2D^+$ ILC2s required more mechanical ventilation and longer hospitalization, albeit the numbers of analyzed patients was very low (71). Noteworthy, a further study found decreased frequencies of circulating ILC2s in severe but not moderate COVID-19 disease along with an increased presence of damage markers, suggesting that low ILC2 numbers could be indicative of a more severe COVID-19 manifestation (72). Moreover, recent data suggest that increased serum concentrations of IL-13 are seemingly correlated to severe COVID-19 outcomes and IL-13 blockade reduced disease severity in a mouse model of SARS-CoV2 infection (73). However, it remains to be determined, whether ILC2s represent a significant source of this cytokine in COVID-19. Further experiments using *in vitro* studies with human lung cells, respiratory tissue of COVID-19 patients, rodent infection models and larger patient cohorts are required to prove the functional relevance of ILC2s in the pathogenesis of COVID-19 lung disease.

Bacterial Infections

Contrary to the rather intensively described functional roles of ILC2s during viral lung infections, studies linking ILC2s to bacterial infections of the lung are scarce. Saluzzo et al. were able to demonstrate that ILC2-deficient and IL-13-deficient mice showed a superior capacity to control *Streptococcus pneumoniae* infections (12). This observation was linked to activated ILC2s induced by postnatal upregulation of IL-33 expression in lung epithelial cells. By the production of IL-13, these ILC2s may shape the resident alveolar macrophages towards an anti-inflammatory M2-like phenotype promoting a quiescent steady-state immune environment, which, however, at the same time delays the immune response to *S. pneumoniae*. It was furthermore found that intratracheal IL-33 administration protected mice from systemic *Staphylococcus aureus* infection. This effect was dependent on the presence of lung ILC2s and the accumulation of eosinophils at the expense of pulmonary neutrophils (74).

Fungal Infections

Infections with fungal pathogens typically manifest at mucosal surfaces, particularly the lung, before they possibly spread systemically. It was demonstrated for many fungal pathogens that the formation of well-balanced immune responses is of critical importance for the host to be either resistant or susceptible (75). In recent years, changes in lung ILC2 numbers were described in the context of fungal infections or after exposure to fungal components. During *Aspergillus fumigatus* infection, increased pulmonary production of IL-25 was described, which resulted in the induction of an innate cell type secreting the cytokines IL-5 and IL-13 (76). In subsequent studies, fungal products such as the common cell wall constituent chitin or substances in extracts of the opportunistic pathogen *Alternaria alternata* were recognized as strong inducers of alarmin production and subsequent ILC2 activation after intranasal delivery (77, 78).

Cryptococcus neoformans is a fungal pathogen that can cause severe disease in immunocompromised individuals. Dominant lung Th1/Th17 immunity and the activation of classical macrophages are believed to ultimately facilitate fungal clearance, whereas highly polarized type 2 responses were observed to be rather detrimental. During the onset of pulmonary cryptococcosis, IL-33 expression is elevated and ILC2 numbers were strongly increased in lungs of these mice (79). Mice deficient in IL-33 signaling alone or in combination with IL-25 receptor deficiency developed less profound type 2 immunity in this model (80). In an intranasal infection model with a highly virulent *C. neoformans* strain, ILC2-deficiency in mice was associated with increased production of Th1 cytokines, elevated pulmonary frequencies of classically activated macrophages, improved fungal control and prolonged survival (79).

Overall, the here summarized data emphasize the significant role of ILC2s during viral lung infections, strongly suggest that ILC2s can orchestrate also pulmonary immune responses against fungal infections and even implicate a potential, yet not fully defined, involvement in bacterial lung infections.

ILC2s AS DRIVERS OF HUMAN CHRONIC LUNG DISEASES

Besides their before described crucial involvement in the immunological responses directed against lung tissue-

affecting infectious pathogens, ILC2s also represent important cellular modulators of non-infectious chronic inflammatory and fibrotic diseases of the upper and lower airways. Here we will in particular discuss the disease-modifying influence of local ILC2 pools on the pathogenesis of asthma, cystic fibrosis and idiopathic pulmonary fibrosis, but reference should also be made to the role of these cells in the course of inflammatory disorders of the upper airway such as chronic rhinosinusitis with nasal polyps, which has been extensively summarized by other review articles (81, 82).

ILC2s as Potential Drivers of Human Asthma

Asthma is a common inflammatory disorder of the airways typically characterized by airway hyperreactivity (AHR), mucus overproduction, IgE production and remodeling of the airways. It has been known for a long time that allergic asthma, the most common subtype, is characterized by the overproduction of the prototypical type 2 cytokines IL-4, IL-5 and IL-13 that promote the hallmark features of the disease (83). Consistent with a role of epithelial-derived alarmins as activators of type 2 cytokine production in the context of the disease, genome wide association studies identified the genes of IL-33 and its receptor IL1RL1 as highly replicated asthma susceptibility loci (84). In addition, several other studies found that IL-33, TSLP and IL-25 were upregulated on the RNA and protein level in lungs of patients with various forms of allergic asthma (85). Traditionally, allergic asthma was considered as a disease largely orchestrated by Th2 cells and their multifaceted interactions with other immune cells including IgE-producing B cells, eosinophils and mast cells. However, with the discovery of ILC2s as a novel lymphocyte subset with large functional similarities and profound pro-asthmatic roles in asthma models, e.g. induced by intranasal administration of plant proteases, house dust mites, fungal extracts or alarmins, this concept was recently revisited. Accordingly, the functional contribution of ILC2s to the development of human asthma is a subject of extensive investigation. Indeed, the frequencies and the activation status of ILC2s, which are in most studies distinguished from other ILCs by their expression of CRTH2, have been shown to be increased in blood, bronchoalveolar lavage and lung tissue of patients with asthma (86–88). Here ILC2 frequencies were particularly increased in eosinophilic asthma compared with non-eosinophilic asthma and ILC2 numbers positively correlated with surges in eosinophils and M2-like macrophages (89, 90). Seemingly, women with severe asthma have an even higher increase in the numbers of circulating ILC2s compared to men, likely due to sex hormone-mediated ILC2 suppression (91). Consistent with their role as drivers of asthma symptoms, ILC2s were increased during the allergy season (92) and their local numbers in the lung increased significantly after segmented allergen challenge and went along with an increased expression of genes related to type 2 immunity (93). Noteworthy, it was also found in the latter study that systemic ILC2 frequencies decreased after allergen challenge indicating that ILC2s can be actively recruited from the blood. Similarly, ILC2s increased in the lungs of patients with moderate allergic asthma after allergen inhalation challenge albeit the increase was transient (94). Importantly, further indirect evidence for potential pathogenic roles of ILC2s in asthma was generated in studies reporting decreased ILC2 frequencies after successful therapeutic interventions (95, 96). Because loss of epithelial barrier integrity is common in asthmatic lungs, Sugita et al. performed *in vitro* co-culture experiments with human bronchial epithelial cells and ILC2s and *in vivo* experiments in mice to study direct interactions of ILC2s with epithelial cells (97). Overall, these data indicated that ILC2s, mainly through production of IL-13, were potent inducers of bronchial epithelial barrier leakiness, although further studies are needed to determine whether a similar mechanism is functional in the human pulmonary environment. Finally, a recent study demonstrated a role of human KLRG1$^-$ ILC2s in reducing grass-pollen allergy. This novel subtype of ILC2s produces immunomodulatory IL-10, was reduced in allergic patients, but notably was restored in such patients receiving grass-pollen sublingual immunotherapy (98).

Collectively, an increasing number of studies in human asthma supports the notion that alarmin-dependent ILC2 activation is a major driver of the disease. However, further studies elucidating their relative disease-promoting importance compared to Th2 cells and identifying specific ways for their targeted modulation are urgently needed.

Role of ILC2s in Human Fibrotic Lung Diseases

Fibrosis is characterized by excessive tissue accumulation of extracellular matrix components, resulting from chronic inflammation and dysregulated tissue repair. The pathogenesis of fibrosis depends on complex mutual interactions between different immune cell subpopulations, mesenchymal and parenchymal cells. Given the key pathogenic roles of type 2 cytokines in experimental models of fibrotic diseases, studies recently addressed the specific role of ILC2s for fibrosis development in the human lung (15, 99). Pulmonary fibrosis is a feature of several lung diseases including cystic fibrosis (CF), idiopathic pulmonary fibrosis (IPF) and also chronic allergic diseases. Although, the contribution of ILC2s in pulmonary fibrosis is supported by several data from animal models, their precise role in humans has been addressed only in few studies and thus remains largely unclear.

In bronchoalveolar lavage fluid and sputum of patients with CF, the presence of IL-33 as well as IL-5, IL-9 and IL-13 was upregulated, suggesting that local type 2 responses may somehow contribute to CF pathogenesis (100–102). In line with this, the risk for manifestation of allergic asthma was reported to be higher in patients with CF (103). Morelli et al. reported an association of a single nucleotide polymorphism in the IL-9 gene with high *Aspergillus*-specific IgE levels in females with CF (101). Based on additional studies in mice, demonstrating that ILC2-derived IL-9 triggers CF-associated

inflammation *via* a complex vicious cycle that includes activation of mast cells, it was speculated that a similar mechanism could support the infection-dependent disease exacerbation in CF. Moreover, decreased numbers of $CCR6^+$ ILC2s in peripheral blood of CF significantly correlated with advanced pulmonary failure (104), suggesting a pathophysiologically relevant alteration of the ILC2 migration patterns in patients with severe CF. In patients with IPF, increased levels of IL-25 and also ILC2s were observed. Most recently, ILC2 frequencies in IPF were shown to correlate negatively with Regnase-1 expression levels, a factor important for posttranscriptional processes in immune cells. In addition, a high number of ILC2s was associated with poor IPF prognosis (105). Because a similar functional mechanism was described also in lungs of Regnase-1 deficient mice subjected to bleomycin-treatment, these data suggested that dysregulated ILC2s may accelerate the progression of IPF.

NUMERIC REGULATION OF LOCAL ILC2 POOLS AT INFLAMED OR FIBROTIC PULMONARY TISSUE SITES BY ILC2 RECRUITMENT AND TRAFFICKING

Context-Dependent Tissue Homing and Interorgan Trafficking of ILCs and ILC Precursors

As already discussed in the previous chapters, based on important pioneering experiments in parabiotic mice with a joint blood circulation, ILCs have long been assumed to represent strictly organ resident immune cells, whose maintenance in secondary lymphoid and non-lymphoid organs in the adult organism is guaranteed by local expansion and self-renewal (7). However, the life-long existence of ILCs and precursor ILCs in the peripheral blood in humans (9, 106–108), altered frequencies of circulating ILCs under inflammatory conditions (72, 104, 109–111), an at least moderate infiltration of donor-derived ILCs in mesenteric lymph nodes, lung and intestinal tissue even in the parabiotic mouse model under chronic inflammatory conditions (7) and the observation that IL-25-induced inflammatory ILC2s (iILC2s) behave like circulating cells, strongly argue for a maintained and context-dependent capacity of ILCs to traffic between different organs (112). In accordance with this concept, Ai Ing Lim et al. successfully established the model of tissue ILC differentiation, which is based on the identification of circulating ILC precursors in the peripheral blood. Once infiltrated into peripheral tissue, these ILC precursors are able to undergo differentiation into all helper ILC subsets triggered by local environmental signals (107). In this context, it is also interesting to note that results from several studies indicated that the analogy between innate helper ILCs and the adoptive T helper cell compartment is not exclusively limited to their cytokine, transcription factor and effector function profiles but also concerns the expression of tissue homing receptors (e.g. S1PR1, CCR9, CCR4, LFA-1, $\alpha4\beta7$ integrins) and specific aspects of their migratory behavior (19, 109, 110, 113–115). Indeed, *in vivo* and *ex vivo* experimental studies clearly demonstrated that both T cells and ILCs use CCR7 and S1PR1 for reaching and egressing secondary lymphoid organs, respectively (113, 114, 116, 117). Being especially well studied in ILC1s and ILC3s, it has been documented that these ILC subpopulations are obviously able to undergo an adaption of their tissue homing receptor expression profile once they have entered into secondary lymphoid organs, thus predisposing them for trafficking towards specific non-lymphoid organs, like the skin or gut (113, 118, 119). In contrast, the homing receptor profile of ILC2s and precursor ILC2s appeared to be predetermined already in the bone marrow and ILC2s are thus able to bypass secondary lymphoid organs and instead traffic directly from the bone marrow towards the peripheral target organ (113, 118). Besides the peripheral distribution of bone marrow-egressed ILCs and precursor ILCs, several studies also described the migration of mature ILCs between different peripheral non-lymphoid organs (19, 110). A landmark study published by Yuefeng Huang et al. convincingly demonstrated that for instance lung infiltrating IL-25-induced iILC2s originated from ILC2s that naturally reside in the intestinal mucosa (19). In a similar way, data acquired in a cohort of patients diagnosed for ankylosing spondylitis implicated the existence of an ILC3 homing axis between the gut and the inflamed joints, which obviously allows a targeted distribution of gut-derived and disease-modulating $\alpha4\beta7$-expressing ILC3s (110).

In addition to determining the interorgan trafficking of ILCs, the integrin and chemokine receptor expression profile turned out to predispose specific ILC subsets for the accumulation within a particular anatomical niche of the target organ. For instance, in analogy to CD8 Trm cells, intraepithelial $NKp44^+CD103^+$ ILC1s could be characterized by an increased surface expression of the $\beta7$ integrin, which together with CD103 forms the $\alpha E\beta7$ heterodimer as a potent binding partner for epithelial E-cadherin and, thus, a marker for epithelial retention (120).

Regulation of ILC2 Trafficking in the Context of Inflammatory Lung Diseases

As described before, there is continuously increasing experimental evidence for the concept that tissue migrating ILCs together with the enforced proliferation of organ resident ILCs contribute to the inflammation-triggered expansion and modulation of local ILC pools (16, 93, 107). However, the existing data strongly implicate that the functional and numeric relevance of newly recruited ILCs for local immune responses as well as the pattern of involved chemokines and adhesion molecules depend on the respective target organ, the pathological context and on the ILC subtype (7, 112, 121, 122). As ILC2s have been established as the predominant helper ILC population in the context of allergic and inflammatory lung diseases (9), we will here describe in detail mediators and signaling pathways regulating their lung homing capacity and

pulmonary recruitment upon inflammatory conditions (**Figure 1**), while the mechanisms underlying the inflammation-triggered expansion of lung resident ILC2s in the human and murine system have been reviewed elsewhere (16, 123). As alarmin-like cytokines (e.g. IL-25, IL-33, TSLP) are very potent inducers of ILC2 proliferation, survival and activation (16, 32) and are released by damaged lung epithelial cells in direct proximity to lung resident ILC2s as a very early response to exogenous or endogenous inflammatory triggers, it appears likely that expansion of local ILC2 pools is of predominant importance during initial phases of acute inflammatory disorders, while the relevance of pulmonary ILC2 recruitment might increase with the subsequent development of an inflammatory milieu (including increased expression of chemokines). Moreover, further arguing for a slightly delayed involvement of newly recruited ILC2s, most blood-derived ILCs enter their peripheral target tissue as precursor ILCs, which still have to undergo final differentiation induced by the local inflammatory micromilieu (107). However, it will be important to define the pathogenic relevance of lung ILC2 recruitment versus expansion of local ILC2 pools more precisely for specific pulmonary diseases in future studies.

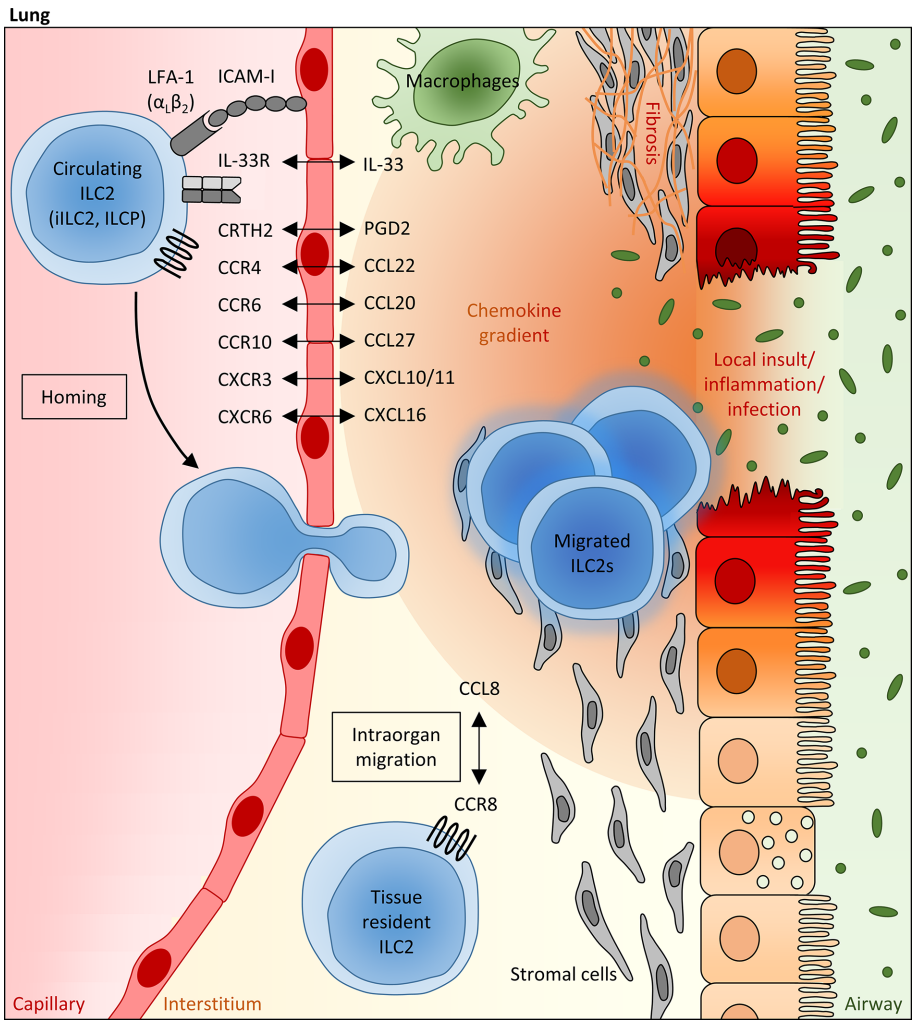

FIGURE 1 | Mechanisms regulating the pulmonary recruitment and spatial distribution of ILC2s during adulthood. Infectious or allergic lung inflammation and fibrosis are able to trigger the pulmonary recruitment of circulating blood ILC2s, including precursor ILCs (ILCP) egressed from the bone marrow, which is at least partly mediated *via* IL-33, as well as mature "inflammatory" ILC2s (iILC2s) primed in the gut. In this scenario, the local pulmonary milieu has been attributed to a crucial modulating function, which can influence the migratory behavior of systemic ILC2s and their intraorgan distribution within the lung tissue. In particular, lipid mediators (PGD2), cytokines (e.g. IL-33) and chemokines (mainly CCL20, CCL22, CCL27, CXCL1, CXCL10 and CXCL16), whose local expression levels are markedly altered under inflammatory conditions and which can be largely attributed to epithelial and stromal cells as well as innate immune cells (representatively shown as macrophages), are assumed to function as potent numeric regulators of pulmonary ILC2 pools, allowing the adaption to local requirements. Besides the chemokine receptor profile of ILC2s, the presence of specific integrins (e.g. LFA-1) on their surface influences the lung homing capacity of circulating ILC2s. Within the lung, the CCR8/CCL8 axis has been identified as a crucial regulator of the spatial distribution of pulmonary ILC2s, promoting their peribronchial enrichment in a niche defined by stromal cells.

IL-33-Mediated Pulmonary Recruitment of Bone Marrow-Derived ILC2s

Experimental induction of allergic lung inflammation was found to result in a decreased ILC2 frequency in the bone marrow, while the number of pulmonary ILC2s increased subsequently, strongly implicating a targeted ILC2 migration from the bone marrow towards the inflamed tissue site (124). In this context, it was interesting to learn that the IL-1 family member IL-33, whose serum levels are markedly elevated in asthma patients (125), does not only function as a key activator of ILC2s in peripheral organs, but also crucially impacts on the inflammation-triggered egress of ILC2s from the bone marrow and their subsequent lung-directed hematogenous migration (126). Indeed, it could be demonstrated *in vivo* that the intravenous delivery of recombinant IL-33 into wild type mice resulted in a decreased total number of ILC2 precursor cells in the bone marrow, while the *de novo* generation of ILC2 precursor cells in the bone marrow turned out to be independent from IL-33 signaling (126). Regarding the underlying mechanism, the IL-33-induced downregulation of the bone marrow retentive chemokine receptor CXCR4 has been identified as a relevant factor for the bone marrow egress of ILC2 precursor cells at least during the early postnatal phase (126). Moreover, performed *in vivo* and *in vitro* studies based on the use of neutralizing antibodies directed against specific integrins were able to demonstrate that the bone marrow-to-lung route depends on the binding of the ILC2-expressed integrin heterodimer LFA-1 to the endothelial adhesion molecule ICAM-I (124).

Role of Lipid Mediators and Chemokines for the Lung Migration of Circulating Blood ILC2s

Focusing on the subsequent pulmonary recruitment of bone marrow-egressed and systemically circulating blood ILC2s, an elegant clinical study in a small cohort of patients with mild to moderate asthma was able to describe that allergen challenge resulted in a simultaneous but opposite regulation of ILC2 counts in the peripheral blood and lung tissue: While the number of peripheral blood ILC2s significantly decreased 24 hours after antigen provocation, there was a parallel enrichment of ILC2s in the bronchoalveolar lavage (93). A similar decrease of ILC2 counts in the peripheral blood was also described in patients infected with tuberculosis, although here this phenomenon was not restricted to ILC2s, but also included circulating ILC1s and ILC3s (111). In accordance with these observations in the human system, intravenously transferred murine ILC2s were found to successfully migrate to the alveoli in a mouse model of cytokine-induced lung inflammation, while this was not the case in the absence of inflammatory triggers (28, 31). Thus, these *in vivo* acquired data strongly supported the concept that local ILC2 pools in the lung are replenished by hematogenous ILC2s in the presence of inflammatory stimuli. Taking into account the impact of the local inflammatory milieu on this phenomenon, the authors of the aforementioned clinical study interestingly observed an inverse correlation between the number of ILC2s remaining in the blood circulation and the levels of the mast cell-released lipid mediator PGD2 and the chemokine CXCL12 in the bronchoalveolar lavage of allergen challenged patients, whereby both molecules could be characterized as potent inducers of ILC2 chemotaxis (36, 93). It is also worth mentioning here that the leukotriene E4 (LTE4), which represents a biomarker in asthma (127) and whose receptor $CysLT_1$ is present on the surface of a relevant fraction of circulating ILC2s, was able to mediate additive effects on the PGD2-induced ILC2 migration in an *in vitro* chemotaxis assay (40). Besides LTE4, the family of CysLT leukotrienes also includes LTC4 and LTD4, whereby LTC4 represents the parent CysLT leukotriene and is converted to LTD4 and LTE4 after being released to the extracellular compartment. Indeed, also LTC4 and LTD4 were found to induce chemotaxis of ILC2s *in vitro*, although to a lower extend than LTE4, and LTC4 could be identified as a potentiator of IL-33-induced lung inflammation (40). Accordingly, mice challenged with a combination of LTC4 plus IL-33 showed a significantly increased pulmonary accumulation of ILC2s (128). However, it remains open, whether the observed phenomenon might at least partly be due to a potential influence of LTC4 on the *in vivo* lung recruitment of ILC2s or mainly depends on the described capacity of LTC4 to trigger IL-33-mediated proliferation of local ILC2 pools (128). In contrast to CysLT leukotrienes, the leukotriene B4 (LTB4) seems to impact on the number and activation status of lung ILC2s predominantly *via* indirect and IL-33-dependent mechanisms, although a minor direct influence of LTB4 on ILC2 chemotaxis could at least be demonstrated *in vitro* (129).

In view of the broadly established general role of chemokine signaling for lung homing of different lymphoid immune cell populations (130, 131), it appears worth to pay more detailed attention to the chemokine receptor repertoire of ILC2s in the context of pulmonary inflammation. Even under steady-state conditions, the vast majority of peripheral blood ILC2s show a relevant surface expression of the chemokine receptor CCR4, whose ligand CCL22 represents a well-established Th2 cell attractant and was found to potently trigger *in vitro* ILC2 migration (26, 132, 133). It is thus strongly assumed that locally enhanced levels of CCL22 in the respiratory tract, as for instance observed in patients diagnosed for idiopathic pulmonary fibrosis or allergic rhinitis and in experimental asthma models (134–136), are able to promote pulmonary ILC2 recruitment. Accordingly, intravenously transferred CCR4-deficient ILC2s failed to migrate into the lung of mice intranasally challenged with the protease allergen papain (26). As an interesting side note, it should be mentioned that CCR8, which also represents a Th2-associated chemokine receptor and is closely related to CCR4, seemed to be dispensable for the lung migration of ILC2s in the same experimental *in vivo* system despite its relevant expression in pulmonary ILC2s (26). Besides a marked surface expression of CCR4, circulating ILC2s in the peripheral blood often express the chemokine receptor CCR6 (9, 109), which turned out to be of particular relevance for the lung homing capacity of this helper ILC subpopulation in the context of lung inflammation (104). Accordingly, adult patients suffering from CF could be characterized by a decreased frequency of circulating $CCR6^+$

ILC2s in the peripheral blood and this phenomenon interestingly correlated with the severity of respiratory dysfunction, strongly implicating a CCR6-mediated migration of systemic ILC2s into the inflamed lung tissue of these patients (104). Interestingly, a similar decrease of peripheral blood $CCR6^+$ ILC2s and a significant inverse correlation between the number of circulating ILC2s and serum levels of the CCR6 ligand CCL20 have recently been described in patients suffering from COVID-19 disease (72), further pointing to the involvement of CCR6/CCL20-initiated signaling cascades in the inflammation-triggered lung recruitment of ILC2s. The altered frequency of $CCR6^+$ ILC2s in the peripheral blood of COVID-19 patients turned out to be accompanied by a significant reduction of the overall small population of $CXCR3^+$ ILC2s. Together with the observation that high serum concentrations of CXCL10 and CXCL11 (CXCR3 ligands) associated with decreased $CXCR3^+$ ILC frequencies in the blood of these patients, it appears likely that also CXCR3-ligand-dependent effects are able to impact on the pulmonary enrichment of blood-derived ILC2s in the context of viral lung infections (72). Analyses in murine asthma models further point to CXCR6 as an additional chemokine receptor with direct chemoattractant effects on systemic ILC2s (137, 138). Indeed, the CXCR6 ligand CXCL16 is constitutively expressed by human lung epithelial cells and alveolar macrophages (139, 140) and even increased CXCL16 levels have been detected in OVA- or IL-33-challenged mice (137). In accordance with the *in vitro* demonstrated capacity of recombinant CXCL16 to induce chemotaxis of lung tissue-derived murine ILC2s, antibody-mediated *in vivo* neutralization of CXCL16 was able to successfully dampen the pulmonary accumulation of ILC2s in the context of experimentally induced asthma (137). Although classically described as a skin homing receptor, the chemokine receptor CCR10 was found to be expressed on the surface of more than one third of pulmonary ILC2s in non-inflamed human lungs and, moreover, asthma patients could be characterized by a markedly increased frequency of $CCR10^+$ ILC2s and elevated levels of CCL27 in the peripheral blood, implicating a potential impact of CCR10 and its ligands CCL27 on the inflammation-dependent enrichment of pulmonary ILC2 pools. In accordance with this hypothesis, *in vivo* blockade of CCR10 ligands indeed abolished the pulmonary presence of $CCR10^+$ ILC2s in allergen-exposed mice (141). Taking into account the described capacity of $CCR10^+$ ILC2s to ameliorate allergic lung inflammation, the CCR10-mediated recruitment of pulmonary ILC2s might be of particular relevance for maintenance or restoration of the balance between the pro-inflammatory effects of lung ILC2s and their capacity to enhance resolution of inflammation.

Intrapulmonary Distribution of ILC2s

Notably, local inflammatory conditions in the lung turned out to not exclusively impact on the recruitment and tissue migration of systemic ILC2s or precursor ILCs, but apparently also influenced their spatial distribution within the inflamed pulmonary tissue (**Figure 1**). Indeed, accumulation of ILC2s in the peribronchial and perivascular space could be demonstrated in murine lungs after intranasal administration of recombinant IL-33, which was obviously dependent on the CCL8-CCR8 ligand-receptor interaction (28). Upregulation of the chemokine receptor CCR8 has been described upon IL-33 exposure in lung-infiltrating ILC2s and turned out to be accompanied by increasing pulmonary levels of CCL8, as the respective ligand. Indicating functional evidence, antibody-mediated *in vivo* blockade of CCR8 was able to significantly dampen the inflammation-induced accumulation of ILC2s in peribronchial tissue areas (28). At first glance, these data seem to contradict the before described dispensability of CCR8 for the pulmonary recruitment of ILC2s from the peripheral blood circulation and the inefficacy of the CCR8 ligand CCL8 to promote *in vitro* migration of ILC2s (26). However, this seeming controversy might most likely be explained by the fact that ILC2s in blood and lung tissue are surrounded by different microenvironments and the functional outcome of the CCR8/CCL8 axis for ILC2s might thus be critically influenced by additional, yet to be defined, co-factors. In addition to chemokine signaling, the perivascular presence of adventitial stromal cells has been identified as another factor, which relevantly promotes the spatial accumulation of pulmonary ILC2s around lung arteries upon inflammatory conditions (13, 138). Involving adventitial stromal cell-derived IL-33 and TSLP as well as ILC2-derived IL-13, the bi-directional adventitial stromal cell-ILC2 crosstalk was found to be triggered by inflammatory stimuli and to generate an optimized niche for enhanced ILC2 enrichment and activation (13).

DISCUSSION OF CLINICAL IMPLICATIONS

In view of the increasingly perceived relevance of interorgan trafficking of non- or not yet organ resident ILCs and precursor ILCs, the cytokine- and chemokine-driven recruitment of this innate cell population towards local inflammatory tissue sites has drawn our attention also with regard to resulting therapeutic implications for allergic or inflammatory diseases. In the following section, we will thus discuss the therapeutic potential and targetability of selected signaling cascades with a described relevance for pulmonary ILC2 recruitment in patients suffering from allergic, inflammatory or fibrotic lung diseases. In this context, it is obvious to mention the successful development of the fully human antibody REGN3500, which specifically recognizes human IL-33, is able to efficiently block the IL-33 downstream signaling and is currently under clinical development for the treatment of asthma and COPD (142). In addition to REGN3500, etokimab represents a second clinically applicable anti-IL-33 antibody, whose suppressive *in vivo* effect on the function of IL-33 has successfully been validated in phase 1 and phase 2a clinical trials (143). As the alarmin IL-33 represents a well-established promotor of the bone marrow-to-lung recruitment of ILC2s under inflammatory conditions and also serves as a key activating cytokine for pulmonary ILC2

pools (125, 126), its antibody-mediated blockade can be expected to relevantly dampen the local involvement of ILC2s in the induction or maintenance of lung inflammation. Due to the rather broad expression profile of the IL-33 receptor ST2 on different immune cell subtypes, targeting IL-33 signaling does not represent an ILC2-specific strategy and can more be seen as a global approach to efficiently block type 2 immunity, including also marked inhibitory effects on lung infiltrating Th2 cells, eosinophils, mast cells and macrophages (144–148). Besides the advanced clinical validation of blocking IL-33 antibodies in inflammatory lung diseases, it will thus be exciting to define in how far the assumed therapeutic efficacy of anti-IL-33 antibodies depends on their capacity to interfere with the activation and recruitment of pulmonary ILC2s.

Due to the local upregulation of CCL22 in inflamed and/or fibrotic lung tissue, for instance in patients diagnosed for asthma or idiopathic pulmonary fibrosis (149, 150), its proven capacity to chemoattract ILC2s (26, 132) and the presence of the corresponding chemokine receptor CCR4 on the vast majority of circulating ILC2s (109), the CCL22/CCR4 axis emerged as an attractive target for controlling the additional pulmonary recruitment of peripheral ILC2s in the course of inflammatory lung diseases. Interestingly, molecular docking studies indicated that the anti-malarial hydroxychloroquine, whose potential benefit for patients suffering from COVID-19 disease had been under intensive debate (151), was able to interact with the active site of CCR4, implicating that an inhibitory effect on CCR4 signaling might, at least partly, underly the reported therapeutic and immunomodulatory capacity of this drug in the clinical context of asthma (152, 153). However, despite the availability of the humanized anti-CCR4 antibody mogamulizumab, which has been clinically approved for the treatment of adult T cell lymphoma (154, 155), till today, there exist no clinical or preclinical *in vivo* data confirming a clinical benefit of antibody-mediated CCR4 blockade in the therapeutic context of allergic or inflammatory lung disease. Indeed, intravenous injection of a blocking anti-CCR4 antibody into guinea pigs previously sensitized with OVA, was not able to relevantly decrease the inflammatory cell infiltrates and chemokine levels in the lung after local antigen challenge (156). It might be assumed that therapeutic strategies based on the use of CCL22-neuralizing antibodies or small molecules are superior to a direct inhibition of CCR4, because they would imply the feasibility and potential efficacy of a topic inhalative drug application and also prevent partial agonistic or inverse agonistic activities, which might otherwise be associated with the use of receptor antagonists (157, 158). Indeed, the CCL22-neutralizing decoy molecule GPN136 was able to successfully decrease the level of lung inflammation in an experimental *in vivo* model of allergic asthma. However, considering that CCR4 is expressed on various immune cell populations besides ILC2s, such as Th2 cells, dendritic cells and eosinophils (133), it is difficult to estimate in how far this GPN136-mediated therapeutic effect depends on a particular blockade of the pulmonary ILC2 recruitment.

In the field of classic inflammatory lung pathologies, therapeutic inhibition of CCR4 signaling has mainly been discussed as a potential treatment strategy for allergic asthma so far, while the interplay between CCR6 and its ligand CCL20 recently arose as another promising target for a numeric regulation of local ILC2 pools in the inflamed lung tissue, which might be of particular relevance for the clinical management of CF. Indeed, significantly increased levels of CCL20 could be detected in the bronchoalveolar lavage of CF patients (159) and advanced pulmonary failure in this disease turned out to be accompanied by decreased frequencies of systemically circulating CCR6-expressing ILC2s (104). Moreover, based on preclinical analyses in a humanized mouse model, blood-derived human ILC2s could be identified as potent inducers of eosinophil and neutrophil accumulation in the lung and relevantly impacted on the pulmonary tissue structure (104). Taken together, these data strongly implicated that increased CCL20/CCR6-driven ILC2 recruitment from the blood towards the inflamed lung tissue promotes the clinical exacerbation of CF (104), making it an attractive future research objective to experimentally investigate the therapeutic capacity of specific and already available pharmacological inhibitors of the CCL20-CCR6 axis, such as anti-CCR6 or anti-CCL20 antibodies or small molecular inhibitors (160), in this lung-affecting disease. However, following this concept, it is inevitable to take into account the relevant expression of CCR6 also on adaptive immune cells (mainly Th17 and Treg cells, but also γδ T cells and B cells) and the resulting and intensively described influence of the CCL20-dependent immune cell recruitment on the immunological tissue homeostasis of various organ systems besides the lung, such as the gastrointestinal tract and the nervous system (160–163). Although the development of topic lung-selective administration routes (e.g. *via* inhalation) might potentially allow to minimize systemic effects, experimental data acquired in several *in vivo* model systems strongly suggest that therapeutic blockade of the CCL20/CCR6-mediated pulmonary immune cell recruitment might not exclusively decrease the local enrichment of ILC2s (72, 104), but will also relevantly impact on the Th17/Treg cell ratio in the lung (160, 163, 164) and thereby potentially target another important aspect in the immunopathogenesis of CF, which is a pathological predominance of Th17 cells (165, 166). At the current state, all this remains speculative and further studies are needed to preclinically validate the therapeutic efficacy of CCL20/CCR6 inhibitors in CF and to define the particular relevance of CCR6-expressing blood-derived ILC2s in this context.

Regardless of whether one considers IL-33/ST2, CCL22/CCR4 or CCL20/CCR6 as target structure for a potential therapeutic modulation of the lung recruitment and local activation of ILC2s, it becomes obvious that none of these strategies will allow an exclusive interference with this innate cell population. In general, the main unsolved obstacle in realizing the idea of ILC-based therapeutic concepts is that fully ILC-specific target structures are still lacking. This phenomenon can mainly be explained by the broad analogy between ILCs and Th cells, which encompasses their responsiveness to key activators of effector function and chemotaxis (16). As a highly innovative future attempt, strategies implementing cellular therapy and thus the adoptive

transfer of *ex vivo* expanded and potentially pre-selected ILC populations might be able to overcome this limitation and allow to exclusive modulate the ILC compartment. Further following this hypothetical idea, CCR10$^+$ ILC2s can be considered as interesting candidates for cellular therapy of patients suffering from severe asthma. Based on the combined interpretation of descriptive human data acquired in blood- and lung tissue-derived ILC2s and functional murine analyses in an experimental model of intranasal allergen-challenge, a suppressive function of lung infiltrating CCR10$^+$ ILC2s on allergic airway inflammation has been strongly suggested, which was obviously due to ILC1-like properties of this specific ILC2 subfraction and their increased capacity for IFNγ secretion (141). Provided that it will be feasible to *ex vivo* expand CCR10$^+$ ILC2s under the stable maintenance of their functional properties, it can thus be assumed that asthma patients might benefit from the adoptive transfer and the subsequent intended pulmonary enrichment of these suppressive ILC2s. In this context, it is worth mentioning that human CCR10$^+$ ILC2s also differed from their CCR10-negative counterparts with regard to several other surface markers and could for instance be characterized by a decreased expression of the inhibitory molecules CTLA-4 and PD-L1. This makes it interesting to compare different subfractions within the CCR10$^+$ ILC2 population on a functional level and, thereby, potentially further narrow down the surface marker profile indicative for high suppressive and anti-allergic activity. Very recently, IL-10-producing KLRG1$^+$ ILC2s emerged as other hypothetically interesting candidates for therapeutic cell transfer in the context of allergic asthma therapy (98). In particular the observation that patients with allergy showed a decreased frequency of this Th responses-attenuating ILC2 subpopulation and that the induction of IL-10-secreting ILC2s in these patients by allergen immunotherapy was associated with clinical response (98) strongly implicated that targeted enrichment of IL10$^+$KLRG1$^+$ ILC2s could be an attractive, albeit technically challenging therapeutic target to strive for in the future clinical management of asthma patients.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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c-Rel Is Required for IL-33-Dependent Activation of ILC2s

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Group 2 innate lymphoid cells (ILC2s) are emerging as important cellular regulators of homeostatic and disease-associated immune processes. The cytokine interleukin-33 (IL-33) promotes ILC2-dependent inflammation and immunity, with IL-33 having been shown to activate NF-κB in a wide variety of cell types. However, it is currently unclear which NF-κB members play an important role in IL-33-dependent ILC2 biology. Here, we identify the NF-κB family member c-Rel as a critical component of the IL-33-dependent activation of ILC2s. Although c-Rel is dispensable for ILC2 development, it is critical for ILC2 function in the lung, with c-Rel-deficient (*c-Rel*$^{-/-}$) mice present a significantly reduced response to papain- and IL-33-induced lung inflammation. We also show that the absence of c-Rel reduces the IL-33-dependent expansion of ILC2 precursors and lower levels of IL-5 and IL-13 cytokine production by mature ILC2s in the lung. Together, these results identify the IL-33-c-Rel axis as a central control point of ILC2 activation and function.

Keywords: ILC2, c-Rel, allergic lung inflammation, IL-33, papain

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INTRODUCTION

Innate lymphoid cells (ILCs) are important regulators of innate and adaptive immune responses, including inflammatory and allergic responses, as well as in homeostatic processes at barrier tissues (1). ILC subsets are distinguished by distinct developmental pathways, transcription factor expression and production of effector cytokines. Group 1 ILCs (ILC1s) produce IFN-γ and express T-BET, group 2 ILCs (ILC2s) express IL-5, IL-13 and GATA-3, and group 3 ILCs (ILC3s) produce IL-17 and IL-22, and express RORγt (1). Of all ILC subsets, ILC2s are the most important subtype for regulating type 2 immune responses and thus serve key roles in mucosal homeostasis, allergy and anti-helminth immunity. ILC2s primarily reside at mucosal barrier surfaces, highlighting their importance as a first line of defense against invading pathogens. Upon epithelial cell damage, the alarmin IL-33 is released by epithelial cells and binds to a heterodimeric receptor expressed on immune cells, including ILC2s that results in cellular activation (2). Furthermore, IL-33 has been shown to regulate ILC2 mobilization from the bone marrow to the lungs (3). In addition to IL-33, the epithelial cell-derived cytokines IL-25 and TSLP also have important roles in ILC2 activation and function in response to helminth infections (4), as well as type 2 inflammatory responses such as allergy and asthma (5–7). However, the precise molecular mechanisms of IL-33-induced ILC2 activation remain unclear.

NF-κB comprises a group of transcription factors that play diverse and often critical roles in innate and adaptive immune responses. In mammals, there are five NF-κB family members, namely RelA, RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p100), all of which share a Rel homology domain (8). NF-κB proteins exist as either homodimers or heterodimers that normally reside within the cytoplasm in an inactive state. These proteins are rapidly activated in response to diverse upstream signals that typically engage one of two activation pathways: the canonical and non-canonical pathways (9). The canonical pathway activates NF-κB *via* the Iκκβ-dependent phosphorylation and subsequent degradation of the cytoplasmic inhibitory protein IκBα, thereby to allow RelA, c-Rel and p50 homo- and heterodimers to translocate to the nucleus and regulate gene expression. In contrast, the non-canonical pathway relies upon IKKα phosphorylation-dependent activation of the NF-κB family members p100 and RelB, allowing translocation of p52/RelB heterodimers to the nucleus (10). Although IL-33 has been shown to activate NF-κB in a wide variety of immune cells (2), the precise molecular mechanisms involved are unknown, as are the composition of activated dimers in different cell types, including ILCs.

ST2 is one of the subunits of the IL-33 receptor that signals *via* MYD88-dependent NF-κB activation to modulate distinct gene expression programs (2, 11, 12). Canonical NF-κB signaling has been shown to regulate GATA3 expression in T helper 2 (Th2) cells (13), and T regulatory (Treg) cells (14, 15). Furthermore, IL-33-ST2 signaling is associated with the development and function of Th9 cells (16), dendritic cells (17, 18), and macrophages (19). IL-33-ST2 signaling is also critical for ILC2 function, although whether canonical NF-κB is required remains unclear (20, 21). Recently, non-canonical NF-κB signaling has been shown to be required for IL-33-dependent ILC2s in adipose tissue following death receptor 3 engagement (22), as well as in pulmonary ILC2s upon tumor necrosis factor receptor 2 binding (23). However, adiponectin treatment used to activate the energy sensor AMP-activated protein kinase inhibits the phosphorylation of IKKα/β and IκBα in IL-33-activated adipose-resident ILC2s and impairs IL-13 production (24), suggesting that canonical NF-κB signaling may also play an important role in ILC2 activation and function.

In this present study, we identify a role for the canonical NF-κB family member c-Rel in ILC2 biology under both homeostatic and inflammatory conditions. Our results suggest that while c-Rel is dispensable for ILC2 development in the bone marrow (BM), it is required for peripheral IL-33-dependent ILC2 activation and the development of allergic lung inflammation. These findings point to c-Rel as a potential therapeutic target for treating ILC2-dependent lung inflammation.

MATERIALS AND METHODS

Mice

C57BL/6J mice (wild-type), c-Rel-deficient mice on C57BL/6J background (c-Rel$^{-/-}$), NF-κB1 (p105/p50)-deficient mice on C57BL/6J background (NF-κB1$^{-/-}$) (25) were bred and kept at Monash University. Animals used in this study were 7 to 10 weeks old, with mice maintained under specific-pathogen-free conditions (SPF) conditions. All studies were performed at Monash Biomedicine Discovery Institute (BDI), Monash University in accordance with Monash Animal Ethics Committee (AEC) and Australian National Health and Medical Research Council (NHMRC) guidelines for animal experimentation.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extract preparation was performed as previously described (26). 1-2 μg of nuclear extracts prepared from IL-33-restimulated (6 h) BM-derived ILC2 precursors (ILC2Ps) were incubated with a κB3-specific ^{32}P-dATP end-labelled probe, as previously described (27). For supershift analysis, antibodies against p50, c-Rel and RelA were incubated with nuclear extracts on ice for 30 min before adding the radiolabeled probe (27). The samples were incubated for 20 min at room temperature, then 2 μl of gel loading dye Ficoll was added, and the samples fractionated on 5% non-denaturing polyacrylamide gels. Gels were dried and exposed to autoradiography.

Preparation of Single Cell Suspension

BM cells were isolated from femur and tibia by flushing the BM using a 25G needle and passing the cells through a 70-μm strainer to form a single cell suspension. For some experiments, lungs were cut in small pieces and digested in 400 U/ml collagenase IV (Sigma Aldrich), followed by incubation at 37°C for 45 min in complete RPMI media (Life Technologies). The digested lungs were then passed through a 70-μm strainer. Red blood cells (RBCs) in organ cell suspensions were lysed in 1 ml RBC lysis buffer (eBioscience) for 1 min. After two washes in FACS buffer, the samples were resuspended in a 30% Percoll separation solution and centrifuged at 400 x *g* to enrich the leukocytes, followed by staining for flow cytometry analysis.

Flow Cytometry

Cells were first blocked with purified rat anti-mouse CD16/CD32 (2 μg/ml) (eBioscience) and rat serum (20 μg/ml) (Stem Cell). Cells were then stained with specific antibodies of interest in the FACS buffer (**Supplementary Table 1**) (2% FCS, 1 mM EDTA, and 0.05% azide in PBS). For BM-derived ILC2Ps FACS sorted for culture, the cells were stained in ILC media. Viable cells were identified using the viability dye 7-AAD (eBioscience). The samples were either resuspended in the FACS buffer for acquisition, or fixed overnight at 4°C for intracellular staining the next day using a FOXP3 kit (Tonbo Biosciences). The staining was performed according to the manufacturer's instructions.

Cell Culture

Bone marrow-derived ILC2Ps or lung ILC2s were sorted by flow cytometry following standard protocols. In short, 5,000 ILC2s were cultured in round bottom plates containing ILC media in the presence of IL-2 (50 ng/ml), IL-7 (10 ng/ml) and IL-25

(100 ng/ml) at d0. Cells were split 1/2 with fresh ILC media plus IL-2 (50 ng/ml), IL-7 (10 ng/ml) and IL-25 (100 ng/ml) at d3 and every second day thereafter over a period of 14 days. On d15, 200,000 cells were plated per well in ILC media in the presence of IL-2 (50 ng/ml) and IL-7 (10 ng/ml) for 24 h. On the following day, the cells were restimulated with IL-33 (10 ng/ml) for 6 h. For some experiments, 5,000 lung-derived ILC2s were cultured overnight in ILC media plus IL-2 (50 ng/ml), IL-7 (10 ng/ml), IL-25 (100 ng/ml) and IL-33 (10 ng/ml) with pentoxifylline (500 ng/ml).

Lung Inflammation Model of Asthma

Mice were intranasally instilled with 10 μg of papain (Sigma-Aldrich) in 40 μl of PBS under isoflurane anesthesia daily for 3 days. For rIL-33-induced lung inflammation, mice were intranasally injected with 500 ng IL-33 (eBiosciences) daily for 3 days, as previously described (21). 24 h after the last treatment, mice were sacrificed, and their bronchoalveolar lavage (BAL) fluid and lung tissues were collected for flow cytometry analysis and RNA extraction. The left lobe of the lung was obtained and fixed in 10% Formalin solution. The lung tissue was embedded into paraffin blocks and were stained with periodic acid-Schiff (PAS) stain, as previously described (28).

qPCR

RNA from homogenized tissue was isolated using phenol-chloroform extraction as per standard protocol. RNA from cultured ILC2s was extracted using a NucleoSpin RNA Kit according to manufacturer's instructions (MACHEREY-NAGEL). The concentration and purity of extracted RNA was measured using a Spectrophotometer NanoDrop 1000 (Thermo Fisher Scientific). 1 μg of RNA was used for cDNA generation using a cDNA conversion kit (Thermo Fisher Scientific). qPCR was performed using a SYBR green chemistry (Qiagen) on a qPCR system (Rotor-Gene Q Qiagen) using specific primers (**Supplementary Table 2**). Samples were standardized using *Actb*.

ELISA

ELISA plates were coated with primary antibodies at 1 μg/ml in PBS at 4°C overnight. The plates were washed 4 times using a washing buffer (PBS containing 0.05% Tween 20) (Sigma Aldrich) with 1 min rests between washes. Subsequently, the plates were blocked for 1 h at room temperature with 200 μl PBS containing 10% newborn calf serum (Bovogen). The samples were loaded, followed by a two-hour incubation at room temperature. The plates were washed 5 times, and the secondary antibodies (biotinylated) were loaded (0.5 μg/ml), and incubated for 1 h at room temperature, followed by 8 final washes with washing buffer. TMB substrate (Invitrogen) was added, and the reaction was stopped with HCL. The samples were read at 450 nm using a microplate Epoch spectrophotometer (BioTek) and analyzed using Gen5 2.0 (data analysis software).

Statistics

All statistical calculations were performed with GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA). All graphs represent mean ± SEM. Statistical significance was determined by 2-tailed Student's t test or one-way-ANOVA. Results were considered statistically significant with $p \leq 0.05$.

RESULTS

Canonical NF-κB Activity in ILC2s

To begin investigating NF-κB involvement in the molecular requirements for IL-33-dependent activation of ILC2s, we examined IL-33-dependent NF-κB activation in ILC2s by electrophoretic mobility shift assay (EMSA). We used flow cytometry to isolate Lin^- $CD45^+$ $c\text{-}KIT^-$ $Sca\text{-}1^+$ $CD127^+$ $CD25^+$ $\alpha^4\beta^{7+}$ ILC2Ps from WT mice and expanded them *in vitro* with IL-2, IL-7, IL-25 and IL-33 for 2 weeks. Expanded ILC2s were rested for 48 h in IL-2 and IL-7, and subsequently restimulated with IL-33 for 6 h. IL-33 restimulation led to increased nuclear translocation and DNA binding of three distinct NF-κB complexes in ILC2s that are denoted as C1 (lower band), C2 (middle band) and C3 (upper band) (**Figure 1A**). Antibody super-shifts revealed that the three complexes were comprised of the canonical NF-κB family members p50 (i.e. NF-κB1 component) (C1), RelA (C2) and c-Rel (C3) (**Figure 1B**). Thus, ILC2s respond to IL-33 stimulation by activating the canonical NF-κB pathway.

We next examined the frequencies of ILC2s in the lungs of c-Rel-deficient ($c\text{-}Rel^{-/-}$) and p105/p50-deficient ($NF\text{-}\kappa B1^{-/-}$) mice. We did not examine the role of RelA as RelA-deficient mice are perinatal lethal (29). We found the frequencies of lung ILC2s were subtly, but significantly reduced by the absence of c-Rel, while $NF\text{-}\kappa B1^{-/-}$ mice had a non-significant reduction in ILC2 frequency and numbers (**Figures 1C–E**). We also found that the absence of c-Rel or NF-κB1 had no effect on the expression level of the IL-33 receptor component ST2 in ILC2s (**Figures 1F, G**). Thus, deficiency of the NF-κB family member c-Rel has a minimal yet significant impact on the homeostatic levels of ILC2s in the lungs.

c-Rel Is Required for IL-33-Dependent ILC2 Expansion but Not ILC2 Development in the Bone Marrow

We focused our investigation on c-Rel and started by examining the development of immature ILC2s (iILC2s) in the bone marrow of c-Rel-deficient mice during steady state hematopoiesis. Flow cytometric analysis revealed equivalent frequencies of Lin^- $CD127^+$ $\alpha_4\beta_7^+$ $CD25^+$ $c\text{-}KIT^{low}$ ILC2Ps (30–32) in the BM of control and $c\text{-}Rel^{-/-}$ mice (**Figures 2A, D**). Further, cells upstream of ILC progenitors, including common lymphoid progenitors (CLPs), α-lymphoid progenitors (αLPs) and common helper innate lymphoid progenitors (ChILPs), were also equally represented in the BM of control and $c\text{-}Rel^{-/-}$ mice (**Figures 2A–C** and **Supplementary Figure 1A**). Because GATA3 is crucial for ILC2 development (33), we also assessed GATA3 expression in *ex vivo* BM ILC2s. Similar levels of GATA3 expression in BM ILC2s in both control and $c\text{-}Rel^{-/-}$ mice coincided with normal development of BM ILC2 observed in $c\text{-}Rel^{-/-}$ mice (**Supplementary Figures 1B, C**). This is also consistent with the phenotype observed in our ILC2 cultures whereby a majority of both control and $c\text{-}Rel^{-/-}$ cells remained

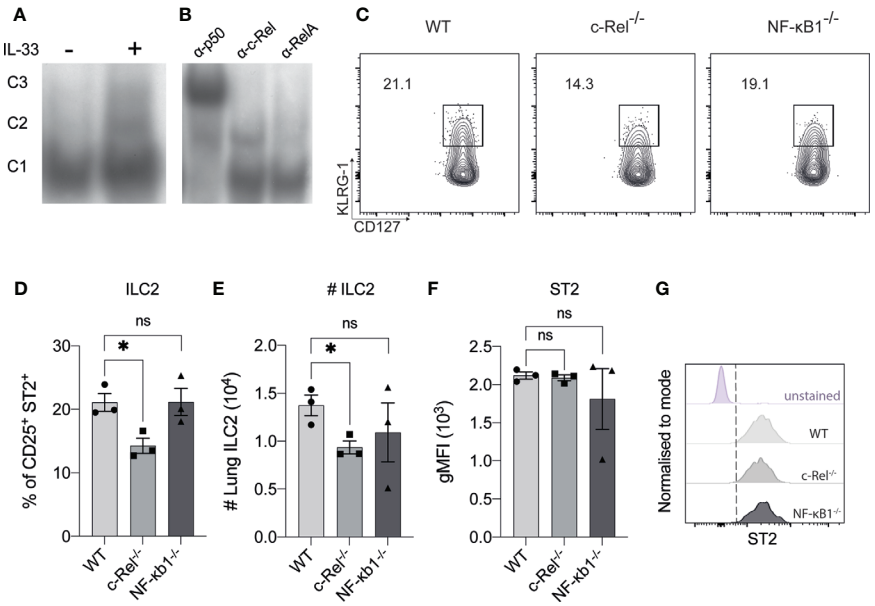

FIGURE 1 | The presence of canonical NF-κB activity in ILC2s. **(A)** Electrophoretic mobility shift assay (EMSA) analysis in IL-33-stimulated BM-derived ILC2Ps. **(B)** The specific members of NF-κβ were detected *via* antibody supershift analysis by using specific antibodies against p50, cRel and RelA. C1 is composed of p50, C2 is composed of RelA and C3 is composed of c-Rel protein. **(C)** Representative flow cytometry plot of CD127$^+$ KLRG-1$^+$ lung ILC2s (gated on viable CD45$^+$ Lin$^-$ CD90$^+$ CD25$^+$ ST2$^+$ cells) from WT, c-Rel$^{-/-}$ and NF-κB$^{-/-}$ naive mice. **(D)** Quantification of lung ILC2 among parent CD25$^+$ ST2$^+$ cells. **(E)** Absolute numbers of lung ILC2s per animal. **(F)** Quantification of ST2 in ILC2s, expressed as geometric mean fluorescence intensity (gMFI). **(G)** Representative histogram plots of ST2 expression in ILC2s. All plots and graphs are representative of at least two independent experiments with at least three mice per group. Error bars represent ± SEM. *p ≤ 0.05. ns, non-significant.

Lin$^-$ and CD45$^+$ (>70%) with equal representation of CD25 and Sca-1 expression (**Supplementary Figure 2A**). In steady state, *cRel* is expressed in both *ex vivo* WT BM and lung ILC2s in comparison to c-Rel-deficient control cells and the levels of *cRel* expression are comparable in these two ILC2 populations (**Figure 3A**). To assess the correlation between changes in *cRel* expression in response to IL-33 stimulation, we cultured WT BM-derived ILC2s for 6 h in the presence of IL-33. *cRel* expression was dramatically increased in the presence of extracellular IL-33 (**Figure 3B**). In accordance with increased *cRel* expression in ILC2s to IL-33 stimulation, we did find that c-Rel was critical for the IL-33-dependent expansion of ILC2Ps. We isolated ILC2Ps from BM of control and c-Rel$^{-/-}$ mice and stimulated them *in vitro* with IL-2, IL-7 and IL-25. Under this condition, we observed equivalent expansion of control and c-Rel-deficient ILC2Ps (**Figure 3C**), although to a significantly lesser extent than when IL-33 was present. However, unlike WT ILC2Ps, c-Rel-deficient ILC2Ps failed to expand in response to IL-33. Thus, c-Rel is critically involved in the IL-33-responsiveness of ILC2Ps.

c-Rel Regulates Papain-Induced Lung Inflammation

IL-33 is maintained in the nucleus of epithelial cells under homeostatic conditions, but is released following tissue damage (2). To determine if c-Rel was required for ILC2 function under inflammatory conditions, we used a model of allergic lung inflammation. We treated control WT and c-Rel$^{-/-}$ mice intranasally with the protease allergen papain on day 0, 1 and 2, and examined the acute, ILC2-dependent inflammatory response in the lungs on day 3 (32, 34, 35). Strikingly, c-Rel$^{-/-}$ mice showed reduced papain-induced lung inflammation, with a significantly diminished influx of lymphocytes into BAL and significantly fewer eosinophils (**Figures 4A–C**). Like homeostatic conditions in the BM, we did not find any significant difference in the frequencies and numbers of lung ILC2s in c-Rel$^{-/-}$ and control mice in response to papain (**Figures 4D, E**). However, we observed significantly reduced *Il5* and *Il13* expression in the absence of c-Rel, suggesting that c-Rel plays an important part in the expression of these type 2 inflammatory proteins. (**Figures 4F, G**). As damaged epithelial cells also produce IL-25 and IL-33 in response to injury, which could in turn activate ILC2s in a paracrine manner (2) to produce IL-5 and IL-13, we examined their involvement in this model. Importantly, we found that expression levels of *Il25 and Il33* were equivalent between c-Rel$^{-/-}$ mice and control mice (**Figures 4H, I**), showing that c-Rel does not regulate *Il25* and *Il33* expression in epithelial cells in our experiments. Collectively, these data suggest that c-Rel is an important regulator of ILC2-dependent lung inflammation.

c-Rel Is Indispensable for IL-33-Dependent Lung Inflammation and Activation

As papain treatment induces expression of the ILC2-inducing cytokine IL-33, and given that IL-33 treatment of ILC2s leads to

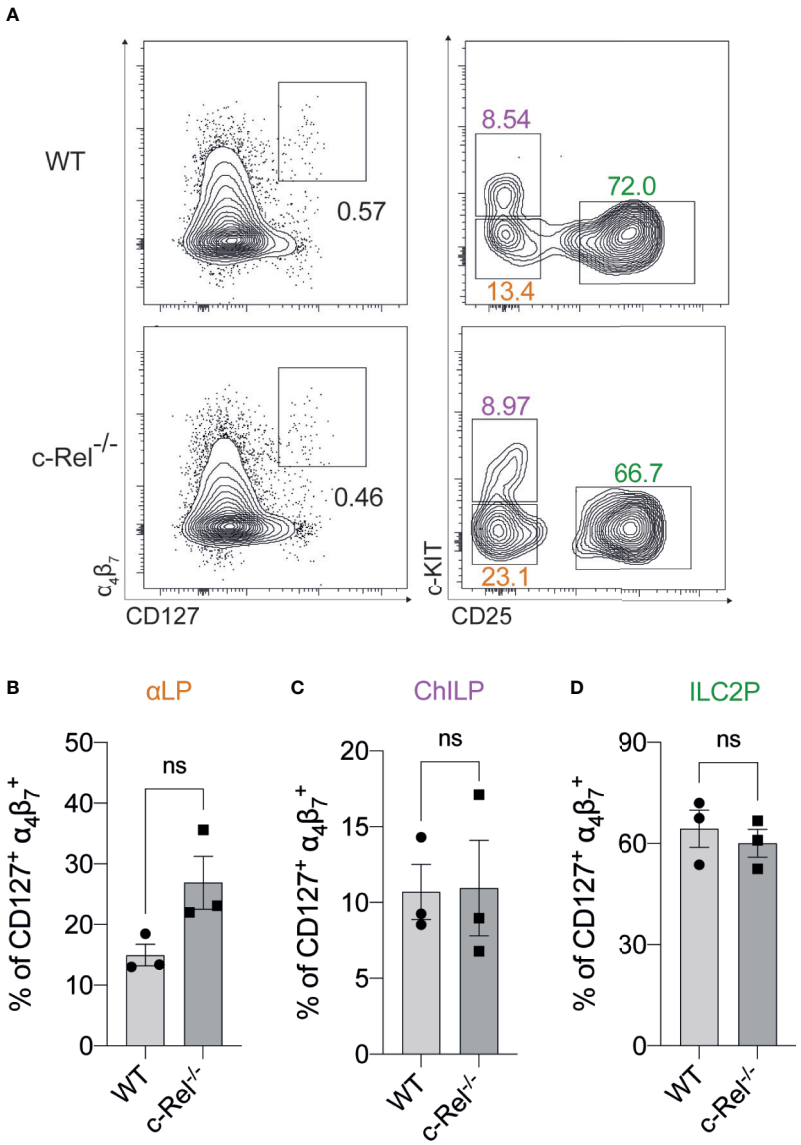

FIGURE 2 | The development of BM-derived immature ILC2s are independent of c-Rel **(A)** Representative flow cytometry plot of $\alpha_4\beta_7^+$ CD127$^+$ immature BM ILC2s (left) (gated on viable CD45$^+$ Lin$^-$ cells) and specific ILC2 progenitors (gated on parent $\alpha_4\beta_7^+$ CD127$^+$ cells). α-lymphoid progenitors (αLPs) were defined as c-KIT^{low} CD25$^-$, whereas common helper innate lymphoid progenitors (ChILPs) and ILC2 precursors (ILC2Ps) were c-KIT^{high} CD25$^-$ and c-KIT^{low} CD25$^+$, respectively. **(B–D)** Quantification of αLP, ChILP and ILC2P among $\alpha_4\beta_7^+$ CD127$^+$ cells, respectively. All plots and graphs are representative of at least two independent experiments with at least three mice per group. Error bars represent ± SEM. ns, non-significant.

c-Rel activation, we next tested whether c-Rel was required for the direct activation of ILC2s by IL-33 *in vivo*. We treated control WT and c-Rel$^{-/-}$ mice intranasally with rIL-33 on day 0, 1 and 2, and analyzed lung responses on day 3. Consistent with our lung inflammation model using papain, c-Rel$^{-/-}$ mice displayed reduced IL-33-dependent inflammation in the lungs, with reduced infiltration of lymphocytes and eosinophils into the BAL (**Figures 5A–C**), reduced frequencies of ILC2s in the lungs (**Figure 5D**), and lower expression levels of *Il5* and *Il13*, but not *Il25*, in lung tissue (**Figures 5E–G**). IL-33-induced goblet cell hyperplasia and mucus production in the lungs lacking c-Rel was also severely reduced (**Figure 5H**). Further, *ex vivo* activation of lung-derived ILC2s with IL-2, IL-7, IL-25 and IL-33, resulted in the c-Rel-dependent production of IL-5 and IL-13 (**Figures 5I, J**). Additionally, in the presence of pentoxifylline, an NF-κB inhibitor with selectivity for c-Rel over other family members (36, 37), c-Rel-sufficient ILC2s failed to produce IL-5 and IL-13, in a similar manner to c-Rel-deficient ILC2s. Together, these observations strongly suggest that c-Rel is critical for IL-33-dependent activation of ILC2s during lung inflammation.

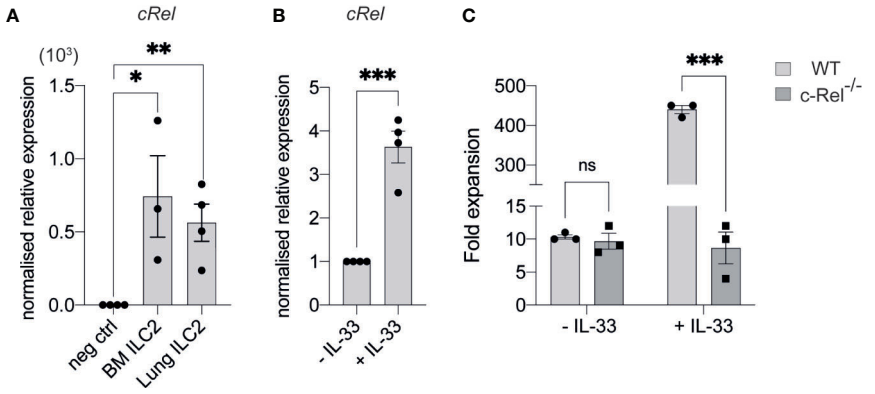

FIGURE 3 | c-Rel is required for IL-33-dependent expansion of ILC2s. **(A)** qPCR analysis of *cRel* in *ex vivo* FACS-sorted BM-derived ILC2 and lung-derived ILC2 from naive WT mice (fold change over negative control). **(B)** qPCR analysis of *cRel* in BM-derived ILC2 cultures restimulated with IL-33 for 6 h. **(C)** Quantification of fold expansion derived from cell count on *ex vivo* BM-derived ILCP cultures. FACS-sorted BM ILC2Ps were cultured in ILC media in the presence of IL-2, IL-7 and IL-25 for 14 days. On d15, expanded cells were cultured in only IL-2 and IL-7 overnight. On d16, expanded cells were re-stimulated with or without IL-33 (control) for 6 h, followed by cell count. All plots and graphs are representative of at least two independent experiments with at least three mice per group. Error bars represent ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. ns, non-significant.

DISCUSSION

Although IL-33 has been shown to be a potent activator of ILC2s during inflammatory responses, the molecular mechanisms responsible for its regulation are poorly understood. While the primary source of IL-33 is epithelial cells (2), other cell types such as natural killer T cells (NKT) cells and alveolar macrophages (38) have also been shown to produce IL-33. Previous studies have established a role for several factors for a number of transcription factors such as GFI1 (39), ETS1 (40), TCF1 (41, 42), G9a (32), and non-canonical NF-κB factors (22, 43) as important regulators of ILC2 biology; however, the roles of canonical NF-κB proteins have not been directly examined in ILC2s. The present study identified the presence of canonical NF-κB family members p50, RelA and c-Rel activity in ILC2s following IL-33 stimulation. We found that while c-Rel is dispensable for early ILC2 precursor development in BM, it is required for optimal ILC2P expansion and proliferation. Furthermore, our results suggest that c-Rel regulates ILC2 activation and function in response to papain- or rIL-33-induced lung inflammation. Overall, the present study identifies the transcription factor c-Rel as a regulator of IL-33-dependent ILC2 function, particularly during lung inflammatory responses.

We observed an increase in nuclear localization and DNA binding of each canonical NF-κB family member p50, RelA and c-Rel in ILC2s upon IL-33 stimulation. Typically, NF-κB can act as a homodimer (e.g., c-Rel/c-Rel), and/or a heterodimer (e.g. c-Rel/p50) (2, 9). Thus, further characterization of the functional roles in different combinations of NF-κB family members in IL-33-stimulated ILC2s is needed. In response to IL-33, the expression of canonical NF-κB in our murine activated ILC2s is consistent with previous findings for human IL-1β-primed ILC2s that require IKK-mediated activation of NF-κB (44). In addition to canonical NF-κB signals, non-canonical NIK-dependent NF-κB signaling involving p100 and RelB activation occurs in pulmonary ILC2s, specifically in response to alveolar macrophage-derived TNF-α *via* TNFR2 (43). Notably, the expression of the canonical NF-κB genes in TNF-α-stimulated ILC2s is either unchanged or reduced (43). Thus, we speculate that the activation of canonical NF-κB signals in pulmonary ILC2s is largely dependent on the pathway downstream of IL-33 mediated by c-Rel, whereas the non-canonical signals are activated in response to other activating cytokines such as TNF-α.

Unlike conventional cytokines, IL-33 is typically released by epithelial cells following damage to barrier tissue and binds to ST2 that forms a heterodimer with IL-1RAcP (45, 46) to serve as an alarmin in response to inflammation and infection (47–49). During the steady state, IL-33 is constitutively expressed in both human (50) and mouse airway epithelial cells (51). Our results show that in steady-state conditions, the absence of either c-Rel or p50 subunit of NF-κB1 does not impair the proportion of ILC2s expressing ST2. However, we observed that the homeostatic development of pulmonary naive ILC2s is somewhat compromised when c-Rel is absent. This indicates that c-Rel-dependent immature ILC2s in the lungs develop independently of ST2 expression. Alternatively, c-Rel may be a prerequisite for IL-33-mediated egress of ILC2s from BM to the lungs (3), which can potentially explain why the accumulation of naive lung ILC2s is slightly reduced in the absence of c-Rel. Notably, there is only a minimal reduction in ILC2s in the lungs of naive mice lacking c-Rel, suggesting that c-Rel is not absolutely required for normal naive pulmonary ILC2 development. This is in line with our earlier observation, in which minimal NF-κB activity is detected in non-activated ILC2s in the absence of IL-33, but its activation is increased upon IL-33 stimulation. Such an observation is consistent with prior studies that have demonstrated the expression and nuclear activity of NF-κB in resting, naive T cells is minimal (52–54).

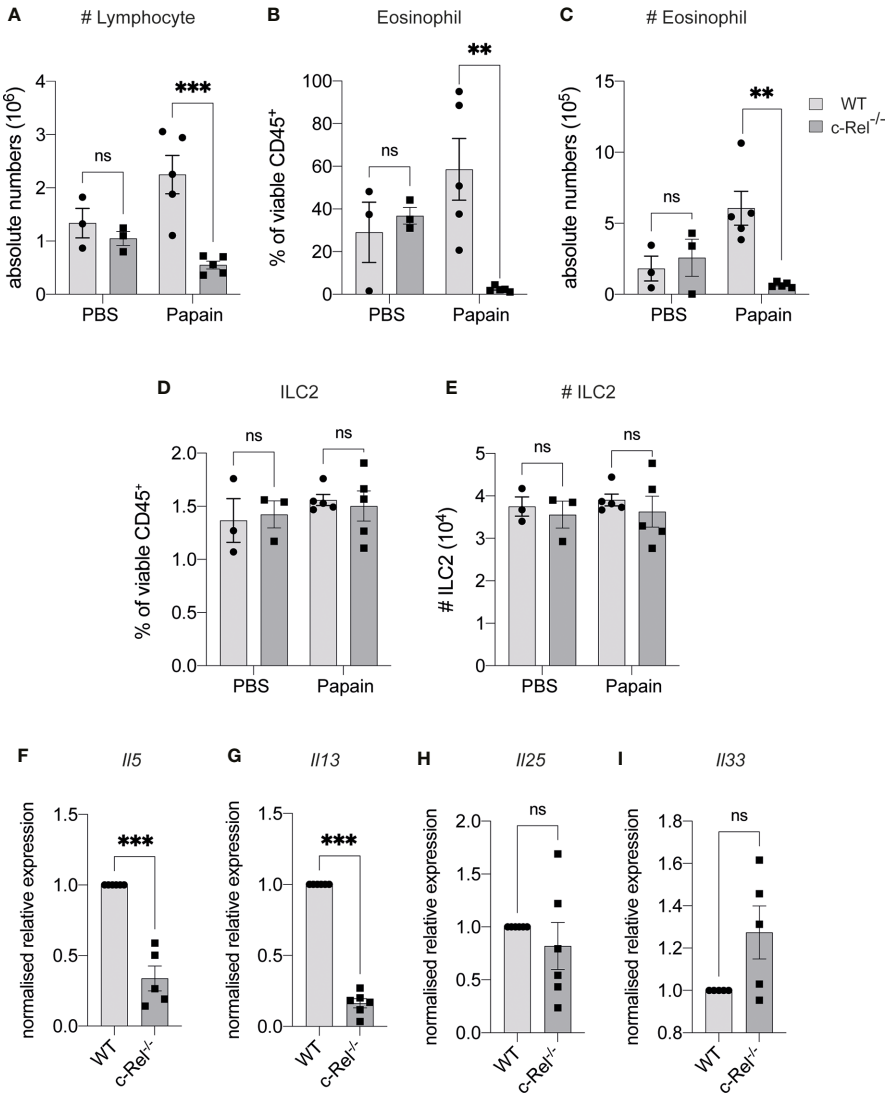

FIGURE 4 | c-Rel deficiency reduces papain-induced lung inflammation. **(A)** Absolute numbers of CD45$^+$ lymphocytes in the bronchoalveolar lavage (BAL) fluid following PBS (control) or papain challenge for three days. **(B)** Quantification of Siglec-F$^+$ CD11c$^-$ eosinophils in BAL among viable CD45$^+$ cells. **(C)** Absolute numbers of BAL eosinophils. **(D)** Quantification of CD127$^+$ KLRG-1$^+$ lung ILC2s (gated on Lin$^-$ CD90$^+$ CD25$^+$ ST2$^+$) among viable CD45$^+$ cells. **(E)** Absolute numbers of lung ILC2s per animal. **(F–I)** qPCR analysis of the indicated genes from lung tissue following papain challenge. All graphs are representative of at least two independent experiments with 3-6 mice per group. Error bars represent ± SEM. **p ≤ 0.01, ***p ≤ 0.001. ns, non-significant.

Numerous studies have demonstrated that both human (55–57) and mouse ILC2s are strongly associated with respiratory and allergic diseases, including asthma and that activation of ILC2s requires IL-33 signals (58, 59). Expression of *Il33* or *Il1rl1* genes also correlates with susceptibility to asthma (19, 60–62), as well as the type 2 response in murine experimental asthma models (2, 58, 59), further supporting a crucial role of IL-33-mediated ILC2s activation during allergy. In addition, blockade of IL-33 signaling has been shown to limit the development of ILC2-mediated chronic asthma (63), with the administration of IL-33 activating ILC2-dependent lung inflammatory responses and goblet cell hyperplasia (12). As the loss of c-Rel impairs *ex vivo* IL-33-dependent ILC2 proliferation and expansion, we questioned whether c-Rel could also be responsible for ILC2-mediated allergic lung responses. Both papain and IL-33 have been shown to induce ILC2-dependent IL-5 and IL-13 production and airway eosinophilia (32, 34, 35). We found that c-Rel is critical for the development of papain- and IL-33-induced allergic lung inflammation. We observed a significantly reduced airway eosinophilic response and lower levels of *Il5* and *Il13* expression in the lungs of c-Rel-deficient mice. Surprisingly, following papain but not IL-33 treatment, the frequencies of lung ILC2s lacking c-Rel remain at the equivalent levels seen in control mice, implying that c-Rel is critically required for ILC2 activation in producing IL-5 and IL-13, but not ILC2 development or survival in response to papain. Collectively,

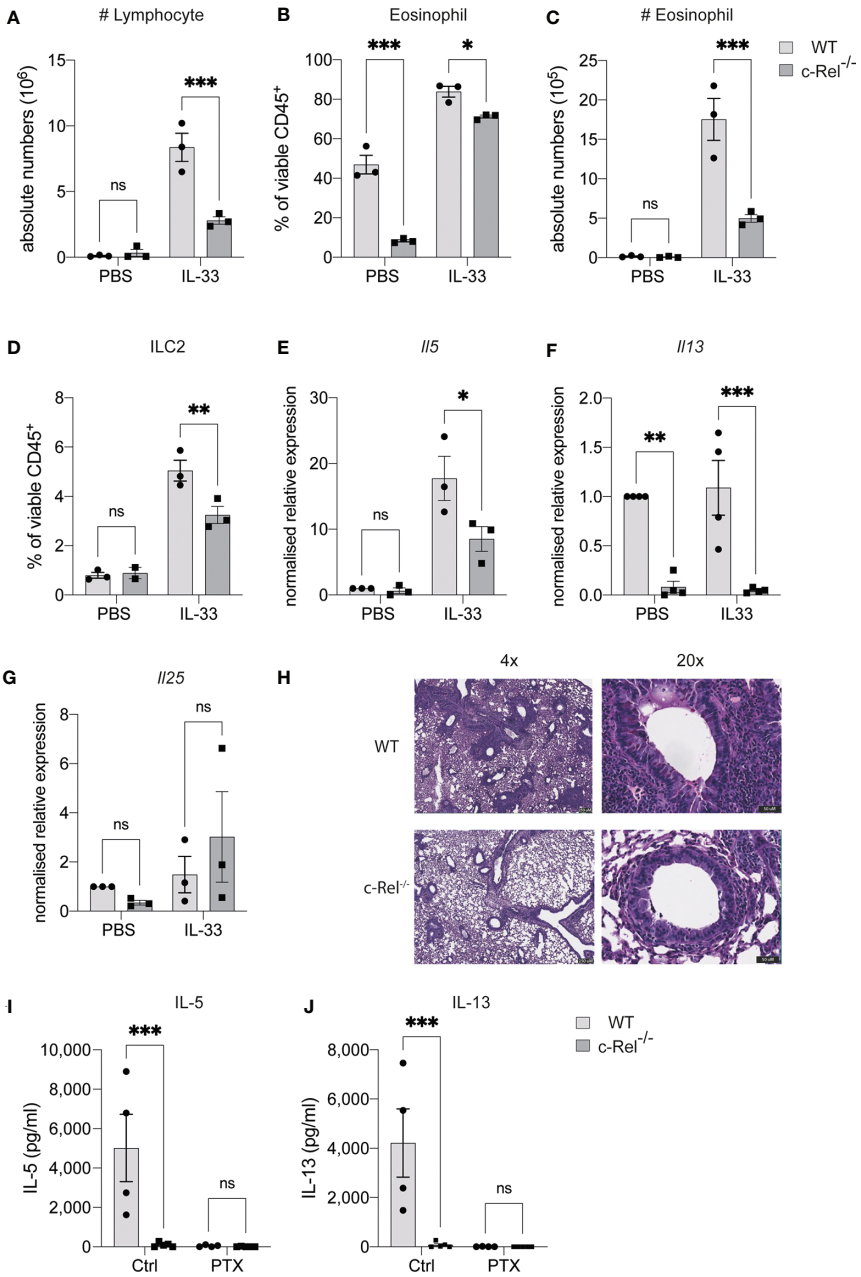

FIGURE 5 | c-Rel is critical for ILC2-mediated lung inflammation, induced by IL-33. **(A)** Absolute numbers of $CD45^+$ lymphocytes in the bronchoalveolar lavage (BAL) fluid following PBS (control) or rIL-33 administration for three days. **(B)** Quantification of Siglec-F^+ $CD11c^-$ eosinophils in BAL among viable $CD45^+$ cells. **(C)** Absolute numbers of BAL eosinophils. **(D)** Quantification of $CD127^+$ KLRG-1^+ lung ILC2s (gated on Lin^- $CD90^+$ $CD25^+$ $ST2^+$) among viable $CD45^+$ cells. **(E–G)** qPCR analysis of the indicated genes from lung tissue following PBS or rIL-33 administration. **(H)** PAS-stained lung histology visualized using 4x (left) and 20x (right) magnification. **(I, J)** ELISA analysis of the indicated cytokine from the supernatant of *ex vivo* lung ILC2s cultures in the presence of IL-2, IL-7, IL-25 and IL-33, with or without a c-Rel inhibitor, pentoxifylline. All graphs are representative of at least two independent experiments with 3-5 per group. Error bars represent ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. ns, non-significant.

we highlight that c-Rel is important for ILC2-dependent lung inflammation in response to papain and IL-33, with this finding confirming and extending previously published work on the role of c-Rel in promoting ovalbumin-alum induced airway inflammation (64).

c-Rel is responsible for many important immune cell functions controlled by regulating the expressions of a wide range of genes (65), including *Il13* (66) and is therefore also likely to be implicated in IL-13-producing Th2-mediated responses associated with asthma and allergy. Further, the loss

of p50 alone, or in combination with the absence of c-Rel is associated with impaired effector CD4 T cell survival (67) and Th2 cell functions following ovalbumin challenge (13), respectively. p50-deficient mice failed to mount lung allergic responses due to reduced GATA3 expression in Th2 cells and impaired type 2 cytokine secretion (13). Given p50 can dimerize with c-Rel (65), this points to a potential role of c-Rel in Th2 cell functions, including the possible involvement of impaired c-Rel dependent Th2 cells functions as part of the explanation for the defective inflammatory responses observed in our c-Rel-deficient mice. However, a comparative analysis of IL-5 and IL-13 production in murine experimental asthma showed that major producers of these type 2 cytokines are ILC2s, rather than Th2 cells (5). Furthermore, in line with our data showing that in cultures of *ex vivo* c-Rel-deficient mice ILC2s or pulmonary WT ILC2s treated with the c-Rel inhibitor, pentoxifylline, both sets of cultured cells failed to produce IL-5 and IL-13. This makes it very likely that the response observed in our *in vivo* asthma model experiments is largely mediated by c-Rel-dependent ILC2s.

NF-κB is not only expressed by immune cells (68), but also non-immune cells such as epithelial cells and stromal cells (69). It has been previously shown that transgenic mice expressing active IκB kinase (IKK) β in airway epithelial cells develop allergic airway disease due to activation of NF-κB signaling that in turn elevates Th2 and ILC2 responses during lung inflammation (70). Conversely, selectively preventing IKK-dependent NF-κB activation in mouse intestinal epithelial cells impairs Th2 responses following helminth infection, resulting in susceptibility (71). Thus, these findings point to an important role of epithelial cell-intrinsic NF-κB signals in mediating the type 2 response. Since we used global c-Rel knockout mice in the present study, we acknowledge that the immune response generated in our lung inflammation models may not be entirely ILC2-intrinsic if the c-Rel-mediated epithelial cell activation is contributing to the response observed. However, we observed equivalent expression of the epithelial cell-derived cytokines *Il25 and Il33* in papain-induced inflamed lungs of control and c-Rel$^{-/-}$ mice, indicating that c-Rel deletion in airway epithelial cells does not affect upstream activation component of the inflammatory response.

Asthma and allergic diseases are rapidly increasing worldwide, highlighting a crucial need for a novel anti-inflammatory drug that can efficiently modulate the type 2 response, known to be central to immune responses responsible for lung inflammation. In addition to Th2 cells, ILC2s are key producers of IL-5 and IL-13. Unlike Th2 cells, ILC2s mainly reside at the interface between the host and environment, such as in the submucosa of lungs, initiating inflammation in response to allergens. Accordingly, the localization of ILC2s provides a strategic approach to specifically target ILC2s to attenuate airway hyperreactivity associated with asthma. Our results suggest that a c-Rel-specific inhibitor such as the IT-603 and IT-901 compounds (72, 73) may offer a novel therapeutic strategy to inhibit IL-33-induced ILC2 activity in diseases such as asthma and allergies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Monash University Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

Designed the study and conceptualization: CZ, SS, and AZ. Performed the experiments: AZ, SS, JR, GR, and JN. Analyzed and interpreted experimental data: AZ, and SS. Performed the EMSA experiment: RG. Provided mouse strains: TSF, and SG. Wrote and drafted the manuscript: AZ. Provided comments, edited and reviewed the manuscript: CZ, SS, SG and AZ. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.667922/full#supplementary-material

Supplementary Figure 1 | (A) Gating strategies of BM ILC2s (cells, single cells, live cells, CD45$^+$ Lin$^-$ $\alpha_4\beta_7^+$). αLPs were defined as c-KIT^{low} CD25$^-$, ChILPs were defined as c-KIT^{high} CD25$^-$ and ILC2Ps were defined as c-KIT^{low} CD25$^+$. **(B)** Representative histogram plots of GATA expression in BM-derived Lin$^-$ $\alpha_4\beta_7^+$ ILC2s (gated on CD45$^+$ Lin$^-$). **(C)** Quantification of GATA3 expression in BM-derived ILC2s. Error bars represent ± SEM. BM, bone marrow; αLP, alpha lymphoid progenitor; ChILP, common helper innate lymphoid progenitors; ILC2Ps, innate lymphoid cell 2 progenitor; ns, non-significant.

Supplementary Figure 2 | (A) Representative flow cytometry plots of cultured BM-derived ILC2s of WT and c-Rel$^{-/-}$ mice. FACS-sorted BM ILC2Ps were cultured in ILC media in the presence of IL-2, IL-7 and IL-25 for 14 days. On d14, expanded cells were assessed for ILC2 markers (CD45$^+$ Lin$^-$ CD25$^+$ SCA-1$^+$). The top plot shows CD45$^+$ Lin$^-$ ILC2s (gated on live cell), whilst the bottom plot shows CD25$^+$ SCA-1$^+$ cells (gated on parent CD45$^+$ Lin$^-$ cells).

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Children With Asthma Have Impaired Innate Immunity and Increased Numbers of Type 2 Innate Lymphoid Cells Compared With Healthy Controls

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Background: Asthma is the most frequent cause of hospitalisation among children; however, little is known regarding the effects of asthma on immune responses in children.

Objective: The present study aimed to evaluate cytokine responses of peripheral blood mononuclear cells (PBMCs), PBMC composition and lung function in children with and without asthma.

Methods: Using a case-control design, we compared 48 children with asthma aged 3-11 years with 14 age-matched healthy controls. PBMC composition and cytokine production including interferon (IFN)-γ, interleukin (IL)-1β, IL-5 and IL-6 following stimulation with rhinovirus-1B (RV1B), house dust mite (HDM) and lipopolysaccharide (LPS) were measured. Lung function was assessed using impulse oscillometry and nitrogen multiple breath washout.

Results: The frequency of group 2 innate lymphoid cells were significantly higher in asthmatics and PBMCs from asthmatics had deficient IFN-γ production in response to both RV1B and LPS compared with controls ($P<0.01$). RV1B-induced IL-1β response and HDM-stimulated IL-5 production was higher in asthmatics than controls ($P<0.05$). In contrast, IL-1β and IL-6 were significantly reduced in response to HDM and LPS in asthmatics compared to controls ($P<0.05$). Children with asthma also had reduced pulmonary function, indicated by lower respiratory reactance as well as higher area of-reactance and lung clearance index values compared with controls ($P<0.05$).

Conclusion: Our study indicates that children with asthma have a reduced lung function in concert with impaired immune responses and altered immune cell subsets. Improving

our understanding of immune responses to viral and bacterial infection in childhood asthma can help to tailor management of the disease.

Keywords: asthma, children, innate immune response, innate lymphoid cells, lung function

INTRODUCTION

Asthma is the most prevalent chronic childhood condition (1, 2). Children with asthma frequently experience exacerbations, and the majority of exacerbations in children are associated with viral infections (3). While rhinoviruses (RVs) are the most frequent precipitants of virus-associated exacerbations in children (4), asthma exacerbations may also be triggered by invasive bacterial infection (5). Indeed, there is evidence showing strong associations between levels of household lipopolysaccharide (LPS) and asthma exacerbations (6–8) and reduced lung function (7). However, in children with asthma, a greater understanding of the mechanisms underlying the immune response to different stimuli, and factors contributing to increased risk of infection are required.

There are numerous immune cells, including neutrophils, eosinophils, natural killer (NK) cells, dendritic cells (DCs), lymphocytes as well as structural cells such as epithelial cells, that may contribute to an altered immune response in childhood asthma. Recent studies have demonstrated that innate lymphoid cells (ILCs) also have a key role in the development of virus-induced asthma exacerbations (9). Group 2 ILC (ILC2) secrete interleukin (IL)-5, IL-9 and IL-13 in response to IL-25 and IL-33 stimulation (9, 10), and circulating ILC2 levels are increased in adults with asthma. However, the role of ILC2 in childhood asthma is less clear.

The aims of this study were to compare cytokine responses of peripheral blood mononuclear cells (PBMCs) (including interferon (IFN)-γ, IFN-λ, IL-1β, IL-5 and IL-6) stimulated with RV1B, LPS and house dust mite (HDM) in asthmatic children with healthy controls and to characterise immune cell subsets (ILCs, eosinophils, neutrophils, lymphocytes, NK cells and DCs) in whole blood. We further investigated the relationship between immune cell subset populations and PBMC cytokine responses. Additionally, we aimed to compare the lung function parameters between children with and without asthma.

MATERIALS AND METHODS

Study Design and Participants

This was an observational, case-control study including children with asthma aged 3-11 years (n=48) and healthy, age-matched controls with no previous diagnosis of asthma or history of respiratory conditions (n=14). Children with asthma were recruited *via* attendance to the emergency department or admission to the John Hunter Children's Hospital and Maitland Hospital, following an exacerbation of asthma. This study includes baseline data obtained from asthmatic children participating in a 26-week clinical trial evaluating the effects of a high fruit and vegetable diet on asthma exacerbation (ACTRN12615000851561). Healthy controls were recruited *via* flyers placed in community centres and at the University of Newcastle (UoN). The study was conducted at the Hunter Medical Research Institute (HMRI), Newcastle, Australia, between September 2015 and March 2019. All participants were screened for eligibility prior to enrolment.

Inclusion criteria for children with asthma were physician diagnosis of asthma; recent exacerbation/s (≥1 exacerbation in past 6 months or ≥2 in the past 12 months) and stable asthma at the visit. Exclusion criteria included other respiratory conditions, diagnosed intestinal disorders, or consumption of nutritional supplements (in previous 4 weeks). Inclusion and exclusion criteria were the same for the control group, with the exception that controls had no history of asthma or wheeze. All subjects were consuming a low fruit and vegetable diet (≤3 serves of fruit and vegetables per day (assessed over past week). The study was approved by the HNEH Ethics Committee (15/06/17/4.03) and registered with the UON Human Research Ethics Committee. Written informed parental consent and child assent (where applicable), was obtained prior to participation in the study.

Clinical Assessment

All participants fulfilling the inclusion criteria attended HMRI for clinical assessment and blood collection following a 12 hour overnight fast. Clinical assessments included anthropometry, nitrogen multi-breath washout (MBW), impulse oscillometry (IOS) and blood collection. For details, refer to supplement.

Laboratory Methods

Immunoglobulin E (IgE) specific antibodies against 4 allergens (dust mites, mixed moulds, mixed grasses, and mixed animal epithelial) were measured in plasma using an ImmunoCAP Fluorenzyme assay (Pathology North, Newcastle, NSW Australia).

PBMCs were isolated from whole blood by density gradient method (11) using Ficoll-PaqueTM PLUS (GE Healthcare, Sydney, Australia) and cultured with and without RV1B, LPS or HDM for 48 hours. The concentrations of IFN-γ, IL-1β and IL-6 in the culture supernatants were analysed using bead-based multiplex assay (BD Bioscience, Sydney, Australia) and IFN-λ and IL-5 concentrations in the culture supernatants were measured using high-sensitivity commercial ELISA assays (R&D Systems, Sydney, Australia) as per the manufacturer's recommendations.

Quantification of major immune cell subsets in whole blood, including eosinophils and neutrophils, T lymphocytes, DCs, NK cells plus B cells and ILCs was performed by multi-parametric flow cytometry using the Fortessa LSR-X20 (BD Biosciences, Sydney, Australia).

For full laboratory analysis methods see supplement.

Statistical Analysis

Statistical analyses were performed using STATA 15 (StataCorp, College Station, Texas, USA). Data are reported as mean ± standard deviation or median (interquartile range). Significant differences between groups were determined using independent *t*-test (parametric data) or Wilcoxon Rank Sum tests (non-parametric data). Subgroup analyses on immune responses of PBMCs were performed on a subset of participants with negative RAST results (no history of allergy) as well as those with no history of OCS use. In adjusted comparisons, age, weight and height were adjusted using General Linear Model. Pearson's Chi-squared test or Fisher's exact test (expected cell sizes< 5) were used to test equality of proportions between groups. Exploratory analysis between immune cell subset populations and PBMC cytokine responses were assessed using Spearman's correlations. Significance was accepted when $P<0.05$.

RESULTS

Subject Characteristics

Demographic and clinical characteristics of the children with asthma and healthy controls enrolled in the study are listed in **Table 1A**. There were no significant differences in age, weight, height, BMI and BMI z score among the asthmatic children and healthy controls. The proportion of males was significantly higher in children with asthma compared to the healthy controls. Furthermore, the history of eczema (60.4% versus 0%, *P<0.0001)* or hayfever (60.4% versus 14.3%, *P<0.01)* was greater in the asthma group compared with the control group. Similarly, there were more children with history of food allergy in the asthmatics than in healthy controls, which appeared to be marginally significant (P=0.056). There were also more subjects with asthma that had a positive RAST result to dust mite (allergen specific IgE 15-50 kUA/L) ($P<0.001$).

Children with asthma were well-controlled at the time of sample collection [median asthma control score of 24 (21, 25)] (**Table 1B**). Of 48 subjects in the asthmatic group, 58.3% had ≥1 hospital admission for acute asthma exacerbation in the previous 12 months. Asthma medication use in the previous 12 months is presented in **Table 1B**.

TABLE 1A | Subject demographics and clinical characteristics.

Variable	Asthma (n=48)	Healthy controls (n=14)	*P*-value
Gender (Male: Female)	35:13	5:9	**0.023**
Age (years)	5.25 (3.82, 6.99)	6.58 (4.99, 8.38)	0.136
Age 3-6 years, n (%)	32 (66.7)	9 (64.3)	1.00
Age 7-11 years, n (%)	16 (33.4)	5 (35.7)	
Weight (kg)	21.50 (16.70, 25.75)	23.55 (18.70, 30.70)	0.215
Height (cm)	116.25 (103.45, 124.70)	122.16 (115.50, 133.30)	0.079
BMI z-score	0.05 ± 0.19	-0.04 ± 0.05	0.814
BMI percentile	51.70 ± 31.63	50.14 ± 33.36	0.872
Current food allergy, n (%)	11 (22.9)	0 (0)	0.056
History of Eczema*, n (%)	29 (60.4)	0 (0)	**<0.001**
History of Hayfever^, n (%)	29 (60.4)	2 (14.3)	**0.002**
Positive RAST Results‡, n (%)	n=47	n=13	
Dust Mite	29 (61.7)	0 (0)	**<0.001**
Animal mix	4 (8.5)	0 (0)	0.413
Grass mix	5 (10.6)	0 (0)	0.335
Mould mix	1 (2.1)	0 (0)	0.810

*Data are presented as mean ± SD or median (interquartile). BMI z-scores and percentiles were calculated using the Centre for Disease Control and Prevention (CDC) 2000 Growth Charts. Difference between groups analysed by the Wilcoxon Rank sum test (non-parametric data), two-sample t-test (parametric data) or Pearson's Chi-squared test/ Fisher's exact test (testing equality of proportions). P<0.05 considered statistically significant. *Based on parental response to "Has your child ever had eczema?" ^Based on parental response to "Has your child ever had a problem with sneezing, or a runny or blocked nose when he/she DID NOT have a cold or the flu?". ‡Plasma allergen-specific immunoglobulin E level between 15.0-50 kUA/L. Bold values indicate statistically significant difference (P<0.05) noted between the two groups.*

Ex Vivo Stimulation of Cytokine Production by PBMCs

Cytokine responses of PBMCs to Human Rhinovirus-1B stimulation

PBMCs from children with asthma stimulated with RV1B produced significantly less IFN-γ compared with PBMCs from healthy controls ($P<0.01$) (**Figure 1** and **Table 2**). A similar trend was observed for IFN-λ, although the difference did not reach statistical significance. In contrast, the concentrations of IL-1β in the supernatants of PBMCs infected with RV1B were significantly higher in children with asthma than in the healthy controls ($P<0.05$) (**Figure 1** and **Table 2**). The RV1B induced IL-6 response was also higher in asthmatics than healthy controls; however, this was not significant.

Cytokine responses of PBMCs to House Dust Mite stimulation

HDM-induced IL-1β and IL-6 production were significantly lower in children with asthma than healthy controls ($P<0.001$) (**Figure 2** and **Table 2**). In contrast, the production of type 2 cytokine, IL-5, following HDM exposure was significantly higher in asthmatic children than in age-matched healthy controls ($P<0.01$) (**Figure 2** and **Table 2**). However, there were no significant differences in HDM-induced IFN-γ, and IFN-λ production in PBMCs obtained from asthmatic children compared with those from healthy controls (*P>0.05)*.

TABLE 1B | Clinical characteristics of children with asthma.

Characteristics	Asthma (n=48)
Asthma control score, median (IQR)	24 (21, 25)
≥1 hospital admission due to exacerbation in previous 12 months, n (%)	28 (58.3)
Medication use, n (%)	
OCS intermittent	21 (43.7)
ICS or ICS/LABA combination	33 (68.7)
ICS intermittent*	7 (14.5)
ICS maintenance^	22 (45.8)
ICS/LABA maintenance ^	4 (8.3)
SABA only	12 (25.0)

*IQR, interquartile; OCS, oral corticosteroids; ICS, inhaled corticosteroids; LABA, long-acting β2-agonist; SABA; short-acting β2-agonist. *Reported to have been taken intermittently or on an as-needed basis. ^Reported to have been taken for most of the previous 12 months.*

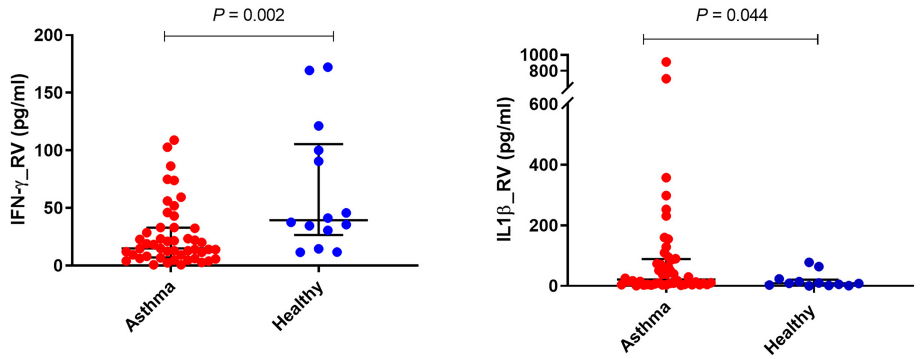

FIGURE 1 | Effects of RV1B stimulation on cytokine responses of PBMCs. PBMCs from children with asthma (n=48) and healthy controls (n=14) were exposed to media or RV1B (MOI=20) for 48h. Bars represent median (interquartile range). Data adjusted for the levels in uninfected PBMCs (control). Plot represents median with interquartile ranges and individual's values are represented by dots. PBMC, peripheral blood mononuclear cells; RV, rhinovirus; IFN, interferon.

TABLE 2 | Cell supernatant cytokine secretion of PBMCs in response to different stimuli in children with asthma and age-matched non-asthmatic group.

Cytokine *(pg/ml)*	Asthma (n=48)	Healthy controls (n=14)	*P*-value
PBMCs stimulated with Rhinovirus-1			
IFN-γ	14.88 (7.38, 32.72)	39.40 (30.59, 100.1)	**0.002**
IFN-λ	5.13 (1.87, 13.41)	6.63 (3.22, 89.46)	0.192
IL-1β	21.25 (5.08, 88.15)	8.29 (2.25, 18.54)	**0.044**
IL-5	0.51 ± 0.27	0.48 ± 0.26	0.649
IL-6 *(ng/mL)*	1.43 (0.31, 8.93)	0.48 (0.19, 2.46)	0.372
PBMCs stimulated with House Dust Mite			
IFN-γ	1.11 (0.77, 1.35)	0.99 (0.78, 1.14)	0.932
IFN-λ	2.18 (1.05, 4.55)	3.84 (2.1, 6.08)	0.159
IL-1β	14.25 (8.88, 49.04)	76.99 (52.88, 114.88)	**<0.001**
IL-5	6.62 (1.10, 22.86)	0.93 (0.54, 1.75)	**0.005**
IL-6 *(ng/mL)*	2.43 (1.00, 12.16)	30.90 (19.58, 56.81)	**<0.001**
PBMCs stimulated with Lipopolysaccharide			
IFN-γ	1.66 (1.06, 5.53)	41.14 (16.29, 55.50)	**<0.001**
IFN-λ	2.58 (0.88, 7.12)	2.21 (1.33, 3.06)	0.655
IL-1β	1888.58 (1173.98, 5863.56)	10681.23 (7967.57, 15169.19)	**<0.001**
IL-5	0.53 (0.25, 0.78)	0.60 (0.37, 0.79)	0.711
IL-6 *(ng/mL)*	73.26 (47.64, 119.08)	127.31 (82.20, 146.08)	**0.021**

Data are presented as median (interquartile range) or mean ± SD. All variables adjusted for the levels in uninfected PBMCs (control). Difference between groups analysed by Wilcoxon Rank Sum test (non-parametric data) or two-sample t-test (parametric data). PBMC, peripheral blood mononuclear cells; IFN, interferon; IL, interleukin. Bold values indicate statistically significant difference (P<0.05) noted between the two groups.

Cytokine responses of PBMCs to Lipopolysaccharide stimulation

There was significantly less IFN-γ produced by LPS stimulated PBMCs from children with asthma compared with those from the controls (*P*<0.001) (**Figure 3** and **Table 2**); however, no significant difference was found in LPS- induced IFN-λ production in PBMCs obtained from children with asthma compared with PBMCs from controls. Furthermore, PBMCs stimulated with LPS produced the same pattern of IL-1β and IL-6 response observed for HDM with PBMCs from asthmatic children producing significantly lower IL-1β and IL-6 compared with PBMCs from healthy controls (*P*<0.001 and *P*<0.05, respectively) (**Figure 3** and **Table 2**).

Subgroup analyses

A subgroup analysis on children with negative RAST results was performed on 19 children with asthma and 13 healthy controls. Production of IFN-γ in response to both RV1B and LPS was lower in asthmatic children than healthy controls (*P*= 0.018 and *P*= 0.009, respectively). Additionally, HDM stimulated IL-1β and IL-6 production (*P*=0.002 and *P*= 0.001, respectively) and as well as LPS induced IL-1β release were found to be lower in asthmatics compared to the healthy controls (*P*=0.005) (**Table S3**).

A subgroup analysis was also carried out on a subset of participants with no history of OCS use (asthmatics=27, healthy controls= 14). Children with asthma had deficient IFN-γ production in response to both RV1B and LPS compared with healthy controls (P=0.003 and P<0.0001, respectively). Moreover, IL-1β and IL-6 production in response to both HDM (*P* <0.001 and P<0.001, respectively) and LPS (*P* <0.001, *P*=0.029, respectively) were found to be lower in asthmatic children than in their age-matched counterparts (**Table S4**).

Peripheral Whole Blood Immune Cell Profiles

Immune cell subset frequency was assessed in PBMCs from 17 asthmatics and 11 healthy children. Asthmatic children showed a significantly higher number of ILC1 and ILC2 compared to healthy controls [(51.09 (39.04, 100.62) *versus* 21.91 (13.04, 67.60), *P*<0.05 and 151.80 (81.16, 335.17) versus 8.40 (6.52, 11.46), *P*<0.001, respectively]. Moreover, while there were no significant differences in numbers of ILC3 with natural cytotoxicity receptor (NCR+) between the two groups, the number of circulating ILC3 NCR- was significantly higher in children with asthma compared to healthy controls [25.00 (2.52, 70.99) *versus* 0.0 (0, 0.53), *P*<0.001] (**Figure 4** and **Table 3**). The frequency of granulocytes, DCs, NK cells and lymphocytes were similar in the two groups (**Table 3**).

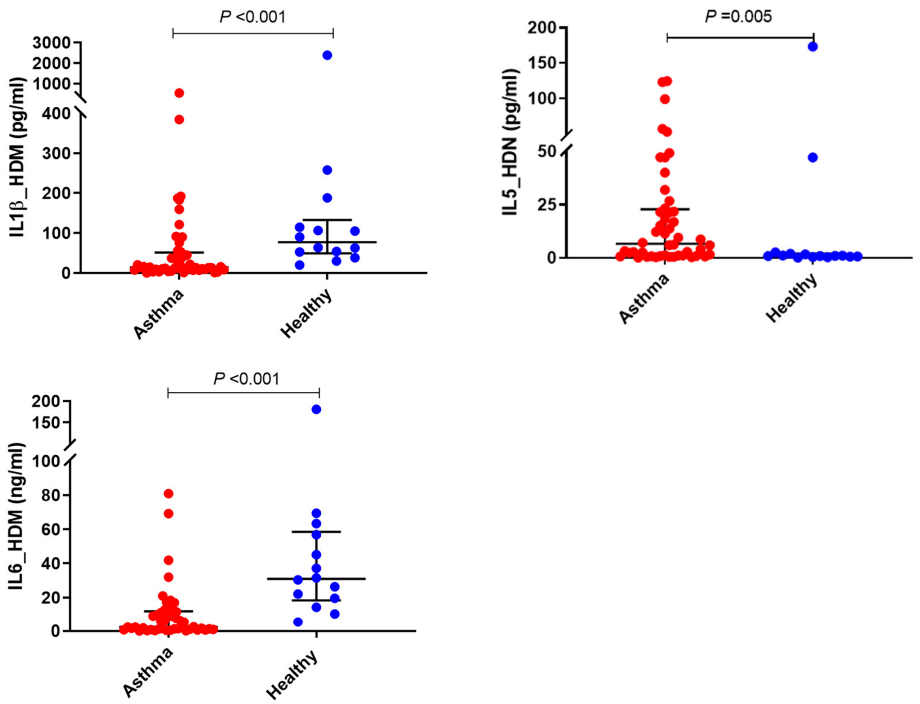

FIGURE 2 | Effects of HDM stimulation on cytokine responses of PBMCs. PBMCs from children with asthma (n=48) and healthy controls (n=14) were exposed to media or HDM for 48h. Bars represent median (interquartile range). Data adjusted for the levels in uninfected PBMCs (control). Plot represents median with interquartile ranges and individual's values are represented by dots. PBMC, peripheral blood mononuclear cells; HDM, house dust mite; IL, interleukin.

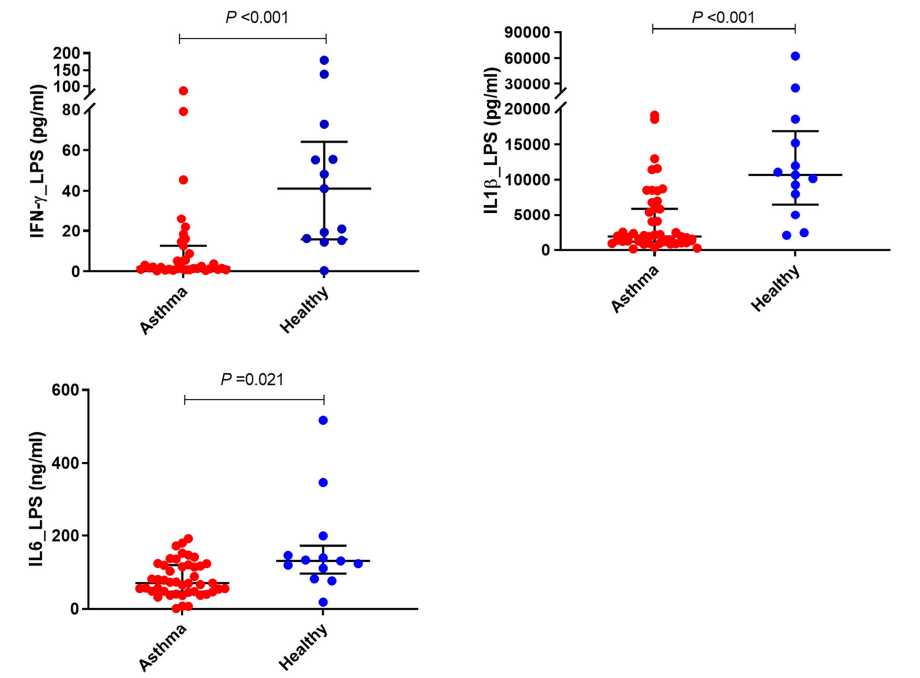

FIGURE 3 | Effects of LPS stimulation on cytokine responses of PBMCs. PBMCs from children with asthma (n=48) and healthy controls (n=14) were exposed to media or LPS for 48h. Bars represent median (interquartile range). Data adjusted for the levels in uninfected PBMCs (control). Plot represents median with interquartile ranges and individual's values are represented by dots. PBMC, peripheral blood mononuclear cells; LPS, lipopolysaccharide; IFN, interferon.

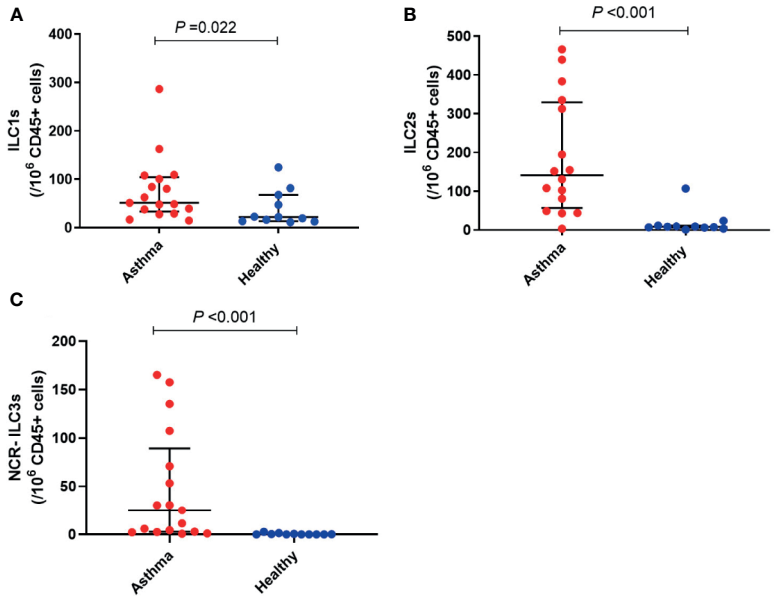

FIGURE 4 | Frequency of **(A)** ILC1, **(B)** ILC2 and **(C)** NCR- ILC3 in children with asthma and healthy controls. Bars represent median (interquartile range). Immune cell phenotyping was performed in whole blood using a lyse-wash procedure. Cells are per 10^6 $CD45^+$ cells. Plot represents median with interquartile ranges and individual's values are represented by dots. ILCs, innate lymphoid cells; NCR, natural cytotoxicity receptor.

TABLE 3 | Frequency of circulating immune cell subsets in children with asthma and age-matched non-asthmatic group.

Cell Subsets	Asthma (n=17)	Healthy controls (n=11)	*P*-value*
Innate lymphoid cells[1]			
ILC1	51.09 (39.04, 100.62)	21.91 (13.04, 67.60)	**0.022**
ILC2	151.8 (81.16, 335.17)	8.40 (6.52, 11.46)	**<0.001**
ILC3 NCR-	25 (2.52, 70.99)	0 (0, 0.53)	**<0.001**
ILC3 NCR+	0 (0, 0.69)	0 (0, 0)	0.052
Granulocytes			
Eosinophils	111914.8 ± 142033.3	63698.76 ± 34578.89	0.141
Neutrophils	280270.5 ± 137243	345993 ± 169330.6	0.865
Dendritic cells			
BDCA-1	30476.19 (16366.61, 37001.29)	6503.078 (1912.066, 43572.04)	0.111
BDCA-3	4444.444 (2646.28, 21046.3)	14217.13 (1572.327, 73949.58)	0.706
pDCs	26904.76 (19498.61, 57909.61)	16758.32 (3783.01, 40094.34)	0.352
NK cells	41905.82 (33153.01, 68353.91)	38138.79 (13537.55,56780.51)	0.543
B cells	432004.3 (412312.2, 634458.6)	287007.8 (234278.1, 486687)	0.068
T cells			
$CD4^+$ T cells	528823.5 (517112.3, 591268.7)	562661.8 (502188.2, 591235.2)	0.724
Activated $CD4^+$	34910.71 (27368.62, 46767.48)	34416.15 (25020.48, 79951.78)	0.795
Treg cells	36234.1 (34276.74, 39402.04)	32471.75 (27356.96, 40535.74)	0.249
$CD8^+$ T cells	189591.7 ± 140576.2	206728.8 ± 104836.4	0.732
Activated $CD8^+$	674.48 (247.83, 1730.77)	1056.32 (684.84, 4197.76)	0.094
TCR-beta T cells	742566.8 (683641.6, 832381)	768274.4 (729063.5, 838161.9)	0.759
γδ-T cells	81940.52 (58436.9, 115443.6)	60867.0 (48278.07, 89825.17)	0.249

*Data are presented as mean ± SD or median (interquartile range). [1]Innate lymphoid cells and granulocytes are per 10^6 $CD45^+$ cells. Dendritic cells are per 10^6 human leukocyte antigen$^+$ cells. B cells, T cells and NK cells adjusted are per 10^6 $CD3^+$ cells. *Difference between groups analysed by Wilcoxon Rank Sum test (non-parametric data) or two-sample t-test (parametric data). P < 0.05 considered statistically significant. ILCs, innate lymphoid cells; NCR, natural cytotoxicity receptor; BDC, blood dendritic cells; PDC plasmacytoid dendritic cells; NK cells, natural killer cells; TCR-β, T cell receptor-β; Treg cells, T regulatory cells. Bold values indicate statistically significant difference (P<0.05) noted between the two groups.*

Associations

Correlation analysis between peripheral blood immune cell numbers and cytokine responses of PBMCs revealed an inverse association between the frequency of ILC1 and LPS-induced IL-1β (r_s=-0.45, *P*=0.030). Furthermore, the frequency of ILC2 was inversely correlated with LPS-induced IL-1β and IL-6 (r_s=-0.43, *P*=0.035; and r_s=-0.45, *P*=0.030, respectively), and HDM-induced IL-6 (r_s=-0.54, *P*=0.006). Inverse associations were

also found between the prevalence of ILC3 NCR- and RV1B-induced IFN-γ (r_s=-0.48, *P*=0.019), HDM-induced IL-6 (r_s=-0.77, *P*<0.0001), and LPS-induced IL-1β (r_s=-0.48, *P*=0.018). Moreover, the frequency of B cells was inversely associated with RV1B-induced IFN-λ (r_s=-0.45, *P*=0.035). In addition, the frequency of TCR- β cells was positively associated with LPS-induced IL-1β (r_s=0.44, *P*=0.031).

Lung Function Assessments

Impulse Oscillometry

IOS variables included in the analysis were airway reactance (Xrs), respiratory resistance (Rrs) (all measured at 5, 10, 15 and 20 Hz), as well as respiratory impedance measured at 5 Hz (Z5), and reactance area (Ax). Children with asthma demonstrated lower reactance at 5, 10, 15 and 20 Hz compared with healthy controls (**Table 4**). Whereas, Ax values were greater in asthmatics than in healthy controls (**Table 4**). No difference was found in respiratory resistance and respiratory impedance between the two groups. After adjusting for age, weight and height, the difference between airway resistance at 5Hz and 20Hz (R_5-R_{20}) was significantly higher in asthmatics than in healthy controls (*P*<0.05). Additionally, the differences in airway reactance remained significant after adjusting for age, weight and height, while Ax value was no longer different between the groups.

Nitrogen Multiple-Breath Washout

Lung clearance index $(LCI)_{2.5}$ values were on average 1.09 units (95% confidence interval: 0.09- 2.09, *P*< 0.05) higher in children with asthma compared with healthy controls (**Table 4**). No significant difference was detected in functional residual capacity between the groups. Adjusting for age, weight and height did not alter the results.

DISCUSSION

For the first time, this study has demonstrated that children with asthma had deficient IFN-γ production in response to both RV1B and LPS infection, compared with the healthy controls. RV1B induced IL-1β response was higher in asthmatics than healthy controls. HDM-stimulated IL-5 production was also significantly greater in asthmatic children than in healthy controls. In contrast, both IL-1β and IL-6 production were significantly lower in response to HDM and LPS in children with asthma. Furthermore, the frequency of ILC1, ILC2, and ILC3 NCR- were significantly higher in children with asthma compared to healthy controls, while other immune cells such as granulocytes, DCs, B cells and T-cells were present in whole blood in similar numbers. These results indicate that innate immune dysfunction in asthma is not limited to adults and may explain the increased susceptibility of asthmatic children to viral and bacterial respiratory infections.

In the present study, we observed that *ex vivo* RV1B infection of PBMCs from asthmatic children resulted in >2.6 times less IFN-γ production compared with PBMCs from healthy controls. Whereas, RV1B-induced IL-1β production was 2.5-fold higher in asthmatic cells than in healthy controls, which suggests inflammasome induction (12). Our results suggest that diminished IFN-γ production, as well as overproduction of inflammatory cytokines (IL-1β) in children with asthma, may be involved in their high susceptibility to lower airway symptoms caused by RV infection. These findings are in concordance with another study in pre-school children (13) that showed reduced serum IFN-α levels in children with asthma compared to healthy controls. In line with our findings, numerous studies in adults reported that following RV exposure PBMCs from adult patients with asthma produced lower levels of IFN-γ compared with cells from healthy controls (14, 15).

IFNs can decrease susceptibility of host cells to viral infection, and an inverse association between IFN-γ production and viral load has been shown previously (3, 16). The antiviral activities of IFNs are mediated directly through the inhibition of viral replication in cells and indirectly through the induction of cytokines and chemokines, which results in recruitment of NK cells as well as CD4 and CD8 T cells (17). Diminished antiviral IFN responses in asthma could be the main mechanism for

TABLE 4 | Comparison of lung function parameters between children with asthma and age matched non-asthmatic group.

Variable	Asthma (n=27)	Healthy controls (n=13)	*P*-value	*Adjusted P**
X_5 Hz z-score	0.56 ± 0.45	0.93 ± 0.53	**0.028**	**0.043**
X_{10} Hz z-score	0.63 (-0.18, 0.83)	0.96 (0.64, 1.61)	**0.031**	**0.034**
X_{15} Hz z-score	1.47 (0.65, 2.23)	2.28 (1.87, 2.72)	**0.009**	**0.012**
X_{20} Hz z-score	-0.23 ± 1.24	0.64 ± 1.06	**0.036**	**0.023**
R_5 Hz z-score	-0.37 ± 0.91	-0.52 ± 0.83	0.624	0.622
R_{10} Hz z-score	-0.22 ± 0.99	-0.30 ± 0.73	0.810	0.713
R_{15} Hz z-score	-0.39 (-0.89, 0.30)	-0.35 (-0.63, 0.49)	0.711	0.986
R_{20} Hz z-score	-0.04 ± 1.06	0.12 ± 0.61	0.601	0.664
R_5-R_{20}	-0.33 ± 0.51	-0.65 ± 0.44	0.064	**0.036**
Z_5 Hz z-score	-0.42 ± 0.87	-0.60 ± 0.80	0.533	0.535
Ax (kPas/L)	1.01 (0.57, 1.67)	0.57 (0.20, 0.73)	**0.013**	0.085
$LCI_{2.5}$	7.65 ± 1.36	6.56 ± 0.56	**0.034**	**0.006**
FRC	1.27 (0.88, 1.54)	1.16 (1.07, 1.34)	0.953	0.385

*Lung function was measured using Impulse Oscillometry and Nitrogen Multiple- Breath washout. Data are presented as mean ± SD or median (IQR). Difference between groups analysed by Wilcoxon Rank Sum test (non-parametric data) or two-sample t-test (parametric data). Patients with missing and/or invalid data were excluded for each variable. *Age, weight and height were adjusted using General Linear Model. P<0.05 considered statistically significant. X, respiratory reactance; R, respiratory resistance; Z, respiratory impedance; AX, area of reactance; LCI, lung clearance index; FRC, Functional residual capacity. Bold values indicate statistically significant difference (P<0.05) noted between the two groups.*

enhanced susceptibility to respiratory viral infection (18). This mechanism could be an explanation for asthmatic patients having more severe lower respiratory tract symptoms and declines in lung function of greater duration and severity following RV infection (18).

IFN-γ also appears to be a key mediator of LPS-induced immune responses (19). We observed a deficient IFN-γ response to LPS in asthmatic children compared with age-matched healthy controls. Similarly, Contoli *et al.* showed primary bronchial epithelial cells and alveolar macrophages from adult asthmatic patients produced lower levels of IFN-λ following LPS stimulation than healthy controls (5). IL-6 and IL-1β production by PBMCs in response to HDM and LPS were impaired in children with asthma in our study. There is apparent controversy about the immune responses to LPS in patients with asthma. A previous study showed that following LPS exposure, PBMCs from adult asthmatic patients produced more IL-1β than cells from healthy controls (20), while, another study in adults reported that asthmatic patients have defective innate immune responses to LPS demonstrated by lower LPS-induced IL-1B response in asthmatics compared to healthy participants (21). Recent studies have highlighted the role of toll-like receptors (TLRs) in the innate and adaptive immune responses. TLRs are involved in the initial immune response to pathogens or environmental stimuli and are broadly expressed by a variety of tissues and cell types (22, 23). Previous studies have demonstrated an association between TLRs and the pathogenesis of asthma (22). One of the well-characterised TLRs that can recognise ligands such as LPS and HDM is TLR4. It has been shown that the expression of TLR4 on PBMCs is diminished in asthmatic patients (22). Our finding of a significantly lower level of IL-6 and IL-1B in response to HDM and LPS in the asthmatic group is in concordance with the previous study that reported lower expression of TLR4 in asthma (22).

This study also demonstrated that HDM-stimulated IL-5 release was significantly higher in children with asthma than in healthy controls. This is in consistent with previous studies that reported children and adolescents with asthma had enhanced TH2 cytokines responses to HDM in comparison with healthy controls (24–26). Previous research suggested that HDM can trigger a TH2-type response in the T cells that specifically recognize the allergen but that allergen-responsive T cells might be lower in healthy subjects than in patients with asthma. It can be suggested that the impaired inflammatory response observed in patients with asthma is due to an imbalance between type I and type II cytokines.

There are several additional points that warrant consideration. The number of children with sensitization to dust mite was significantly higher in children with asthma compared with healthy controls (61.7% vs. 0%, respectively). Increased IgE antibodies to allergens was found to be correlated with increased risk for lower respiratory tract symptoms with viral infections such as rhinovirus in patients with asthma (16). To examine whether allergy status is one of the causes of the observed significant differences in immune responses of PBMCs, we performed a subgroup analysis on children with negative RAST results (n= 32) and differences in the following variables remained statistically significant between the two groups: RV1B induced IFN-γ (P= 0.018), HDM stimulated IL-1β and IL-6 production (P=0.002, P= 0.001, respectively) and as well as LPS induced IFN-γ and IL-1β release (P= 0.009 and P=0.005, respectively) (**Table S3**). These findings confirm that the deficient IFN responses observed in children with asthma is not related to their allergy status. However, it should also be noted that no significant difference was observed in HDM-specific IL-5 responses between the two groups after adjusting for allergy status. Our results indicate that HDM-induced IL-5 production can be considered as a predictor for the presence of atopy in children with asthma, as has been suggested by others (27). Additional questions of concern relate to the effects that corticosteroids may have on the observed differences in immune responses of PBMCs. Prior studies have reported immune-suppressive characteristics of steroids (28, 29). Twenty-one children in asthma group (43.7%) used intermittent oral corticosteroids, while none of the healthy controls used corticosteroids for any health conditions. Our subgroup analysis of subjects with no history of OCS use (n=41) revealed the following: IFN-γ production in response to both RV1B and LPS remained to be significantly lower in children with asthma compared with healthy controls (P=0.003 and P<0.0001). Additionally, IL-1β and IL-6 production in response to both HDM and LPS were remained lower in asthmatic children than in their age-matched counterparts (P <0.001) (**Table S4**). These findings are in line with previous *in vitro* studies that reported corticosteroids inhibit viral-induced cytokines but do not inhibit interferons (30). However, the interaction between corticosteroids and virus infection is controversial and some studies demonstrated that treatment with corticosteroids induced viral replication in the epithelial cells by suppressing type I and type III IFN production (31). It is also worth noting that in our study there was an apparent increase in RV1B induced IFN-λ production in subjects with no history of OCS; however, this was not significantly different between the two groups. These data indicate the need to further *in vivo* and *in vitro* studies in the role of corticosteroids in innate immune responses and activation of immune cells in asthma.

We also measured the frequency of whole blood immune cell subsets and found a higher prevalence of ILC1, ILC2, and ILC3 NCR- in children with asthma compared to healthy controls. These findings are consistent with previous studies that also reported higher prevalence of ILC2 in blood from adult asthmatic patients than healthy controls (9, 10, 32), which was associated with worse asthma control (10). However, in another study, ILC2 were on average 50% lower in the blood of children with acute asthma compared with healthy controls (33). Compelling evidence indicates that ILCs have significant roles in asthma development, specifically virus-induced asthma (9), as they link the innate and the adaptive immune responses within the hypersensitive airway (10). The airway epithelial cells, as the first natural barrier, protect the body from external antigens. In response to pathogen recognition, allergen exposure or viral infection, epithelial cell-derived cytokines such as IL-25 and IL-33 are released. Following secretion, IL-25 and IL-33 can bind to their receptors on the surface of ILC2 and affect the growth and proliferation of these cells (9, 34). Activated ILC2 cells are potent secretors of Th2 cytokines (e.g., IL-5, IL-13)

(9, 34), which results in airway hyperactivity, mucus overproduction, airway smooth muscle constriction as well as airway remodelling (35).

In an exploratory analysis, we examined the relationship between peripheral blood immune cell numbers and cytokine responses of PBMCs to RV1B, HDM, and LPS stimulation. We found that the number of ILC2 and ILC3s NCR- was inversely correlated with HDM-induced IL-1β. LPS-induced IL-1β was found to be inversely correlated with ILC1s, ILC2 and ILC3s NCR-, while, positively associated with TCR+ cells. Inverse correlations were also found between ILC2 and IL-6 from LPS-stimulated PBMCs, as well as between ILC3s NCR- and IFN-γ production in response to RV1B stimulation. These findings suggest that increased circulating ILCs, in particular, ILC2 might be involved in dysregulated innate immune responses in asthmatic children. Additionally, we found that the frequency of B cells was inversely associated with RV1B-induced IFN-λ. This inverse correlation confirms the previous observation that virus-induced IFN-λ can reduce proliferation of B cells in a dose-dependent manner (36). To our knowledge, this is the first study to suggest a relationship between whole blood immunophenotype and innate immune responses of PBMCs. Previous research has explored the association between cord blood immune cell subsets and airway immune mediators and reported a positive association between activated CD4 and CD8 T cells and TNF-α, while regulatory T cells and CD4 T cells were reported to be inversely correlated with IL-1β (37).

Our study also revealed that compared with healthy controls, asthmatic children had lower lung reactance (X_{rs}) values, while area of reactance was found to be significantly higher in asthmatics than in healthy controls. Moreover, after adjusting for age, weight and height, asthmatic children had a greater R_5-R_{20} value compared with controls. This is consistent with previous studies that reported peripheral airway IOS indices (including A_x and R_5-R_{20}) were correlated with asthma control in both children and adults (38–41). Our findings are also in line with previous research showing that X_{rs} predicted values were significantly lower in asthmatics than in controls (42). In a cohort of 162 children aged 2-5 years, lower z-scores of reactance X_5 were observed in persistent asthmatics compared with children with intermittent asthma (43). Our results showed that functional impairment of the airways is present, even in young children with stable asthma. The ability to predict loss of asthma control in children can decrease asthma related mobility and mortality. Thus, IOS can be used to identify paediatric patients with stable asthma who are at risk of losing asthma control.

The measurements from nitrogen MBW showed that, as expected, $LCI_{2.5}$ was higher in asthmatic children than in healthy controls. These results are in agreement with previous research (44, 45). Baseline LCI was found to be significantly higher in school-aged children with asthma compared with healthy age-matched controls (40). Similarly, another study demonstrated that clinically stable paediatric patients with asthma had a significantly greater LCI value compared to healthy controls, which persisted after salbutamol use (41). These findings suggest that ventilation inhomogeneity is present in the airways of asthmatic children even in those with stable asthma. LCI has been found be useful in the recognition of early lung disease as well as in the prediction of lung function in children (46–49). Further studies are needed to assess the role of LCI in tracking the progression of early airway remodelling in children with asthma.

There are several limitations to our study, including the relatively small sample size of the control group. The rate of recruitment was lower than we had anticipated, with objections to the venous blood draw being the primary reason for parental refusal. Although, the sample size in this study was comparable to similar studies conducted in adults (50, 51) and children (52). Nonetheless, the study was adequately powered to detect important differences in circulating ILC subsets in children with asthma compared with healthy controls, and associations between immune cell subsets and responses to virus, HDM and LPS exposure, which are important observations.

A key strength of the present study is that the children with and without asthma were matched for age, which is known to affect immune responses (53). Additionally, asthma was defined by a doctors diagnosis (54). Another strength of this study is the exploration of the associations between whole blood immunophenotype and innate immune responses of PBMCs for the first time in children with asthma.

In summary, our study indicates that children with asthma have impaired innate immune responses, which may explain the high frequency of viral-induced acute exacerbations in this population. Furthermore, asthma was associated with increased frequency of ILC subsets, which could contribute to airway inflammation and tissue remodelling. Increased understanding of innate immune responses may facilitate the development of therapeutic strategies to prevent acute asthma exacerbations in young children with asthma.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://ogma.newcastle.edu.au/vital/access/%20/manager/Repository/uon:37141.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by HNEH Ethics Committee (15/06/17/4.03). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTION

LW, BB, MS, AC, MJ, and PW were involved in design of the study. BB, BH, and RM conducted the study. KN was involved in developing the laboratory methods for PBMC isolating and culture. MS, AC, and BH were involved in developing laboratory methods for cell quantification. BH and KN performed the experiments. BH performed the analysis and

drafted the manuscript with input from BB, MS, AC, and LW. BB and LW verified the analytical methods. All authors discussed the results and commented on the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.664668/full#supplementary-material

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The Role of Innate Lymphoid Cells in Chronic Respiratory Diseases

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The lung is a vital mucosal organ that is constantly exposed to the external environment, and as such, its defenses are continuously under threat. The pulmonary immune system has evolved to sense and respond to these danger signals while remaining silent to innocuous aeroantigens. The origin of the defense system is the respiratory epithelium, which responds rapidly to insults by the production of an array of mediators that initiate protection by directly killing microbes, activating tissue-resident immune cells and recruiting leukocytes from the blood. At the steady-state, the lung comprises a large collection of leukocytes, amongst which are specialized cells of lymphoid origin known as innate lymphoid cells (ILCs). ILCs are divided into three major helper-like subsets, ILC1, ILC2 and ILC3, which are considered the innate counterparts of type 1, 2 and 17 T helper cells, respectively, in addition to natural killer cells and lymphoid tissue inducer cells. Although ILCs represent a small fraction of the pulmonary immune system, they play an important role in early responses to pathogens and facilitate the acquisition of adaptive immunity. However, it is now also emerging that these cells are active participants in the development of chronic lung diseases. In this mini-review, we provide an update on our current understanding of the role of ILCs and their regulation in the lung. We summarise how these cells and their mediators initiate, sustain and potentially control pulmonary inflammation, and their contribution to the respiratory diseases chronic obstructive pulmonary disease (COPD) and asthma.

Keywords: pulmonary inflammation, airway inflammation, obstructive lung disease, COPD, asthma, NK cells, innate lymphoid cells (ILC)

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CHRONIC INFLAMMATORY LUNG DISEASES ARE AN ESCALATING GLOBAL HEALTH ISSUE

COPD is an irreversible chronic inflammatory lung disease that is the third leading cause of death worldwide (1). Patients with COPD exhibit airflow limitation, progressive deterioration in lung function and experience exacerbations; an acute worsening of their symptoms, often driven by lung infection (2). The major risk factor for COPD is cigarette smoking, although other risks such as environmental pollution or premature birth increasingly contribute to COPD susceptibility (3). COPD is underpinned by chronic inflammation, resulting in lung pathologies such as emphysema due to alveolar tissue destruction, and chronic bronchitis arising from goblet cell metaplasia and mucus overproduction (4). Inducible bronchus-associated lymphoid tissue (iBALT) often develops in COPD, particularly in advanced disease (5). COPD is heterogeneous and various disease processes, inflammatory cells (macrophages, neutrophils, cytotoxic T cells, T helper (Th)-1/17 cells) and cytokines are involved (6).

Asthma is a mostly reversible inflammatory airway disease affecting around 300 million people worldwide, where exaggerated swelling and narrowing of the conducting airways (airway hyperresponsiveness; AHR) is triggered in susceptible individuals by the inhalation of environmental particles (7–9). Asthma is differentiated into subtypes——allergic or non-allergic, and by severity——mild-intermittent, mild, moderate, or severe or by the dominant inflammatory response——eosinophilic or neutrophilic. The most common type of asthma is allergic or eosinophilic asthma, which is characterised by a type 2 immune response (driven by cytokines IL-4, IL-5 and IL-13) and IgE-mediated hypersensitivity (10). Conversely, during non-type 2 asthma, neutrophils, alongside a Th1/Th17 skewed response, predominate (11, 12). Severe asthma, which is predominantly neutrophilic, affects 5-10% of patients and is often unresponsive to standard corticosteroid-based therapies (13, 14). Asthma-COPD overlap (ACO) is a syndrome where patients exhibit characteristics of both asthma and COPD (15), complicating the study of inflammatory lung diseases.

This review will focus on ILCs and their involvement in COPD and asthma.

THE EMERGENCE OF ILCs IN IMMUNITY

ILCs are a somewhat newly identified family of innate immune cells that have garnered intense recent attention and our understanding of their biological roles is rapidly progressing. ILCs are mainly tissue-resident (16) and enriched at mucosal sites such as the respiratory, gastrointestinal and reproductive tracts, where they act as first responders to pathogens, aiding the innate immune system to launch a rapid defence, in addition to having roles in tissue repair and homeostasis (17). ILCs closely resemble Th cells in their development and function (18). They lack conventional antigen receptors, instead recognising non-specific danger signals, microbial compounds and cytokines (18), yet can also develop immunological memory (19). While ILCs and T cells have overlapping functions, ILCs perform additional non-redundant roles in priming adaptive immune responses (20). Like their T cell counterparts, ILCs are implicated in chronic inflammation, autoimmunity, and cancer (21–23).

The ILC family comprises five main subsets, which include natural killer (NK) cells, lymphoid tissue inducer (LTi) cells (which play a key role in the development of lymphoid tissues), ILC1, ILC2 and ILC3. ILCs have characteristics and functions that resemble adaptive $CD4^+$ Th cell subsets. ILCs are classified into three main groups: group 1 (ILC1 and NK cells), group 2 (ILC2) and group 3 (ILC3 and LTi cells), which correspond to Th1 (NK cells correspond to CD8+ cytotoxic T cells), Th2 and Th17 cells respectively (24), based on similar transcription factors and functional profiles (25–27). ILCs derive from the common lymphoid progenitor and primarily develop in the foetal liver or in the bone marrow after birth (28). ILC1, ILC2 and ILC3, but not conventional NK cells, develop from $Id2^+$ common helper-like innate lymphoid precursor cells (29), whereas conventional NK cells likely branch off earlier in development (30). Tissue-resident ILCs can be replenished from bone marrow or lymphoid organ precursors however, they are predominantly maintained through local self-renewal and expansion at tissue sites (16). While little is known about how ILC1s populate the lung, ILC2s and ILC3s arise in the lung shortly after birth, with ILC2 seeding dependent on production of IL-33 by type II alveolar epithelial cells (31) and ILC3s on insulin-like growth factor 1 from alveolar fibroblasts (32). ILCs are lineage-negative, lacking common lymphoid and myeloid lineage markers, and this feature is used to distinguish them by flow cytometry. ILCs are highly plastic and can change their phenotype and function depending on environmental signals, and their identification can also be complicated by their maturity (33).

PHENOTYPIC FEATURES OF ILC SUBSETS

ILC1s and NK cells require the transcription factor T-bet for their development; however, NK cells additionally utilise Eomes (34). ILC1s and NK cells secrete interferon-gamma (IFN-γ) and tumour necrosis factor alpha which are key in the defence against intracellular pathogens. NK cells employ both a cytotoxic ($CD56^{dim}$ subset) and cytokine ($CD56^{bright}$ subset) response (35, 36). Both ILC2s and ILC3s have the potential to differentiate into ILC1 or ILC1-like cells (37, 38). Indeed, STAT-1, a key transcription factor activated during bacterial and viral infections, has been found to skew the differentiation of ILCs toward ILC1 while suppressing ILC2 and ILC3 responses (39).

ILC2s are dependent on the transcription factor GATA-3 and support Th2 immune responses *via* production of type 2 cytokines such as IL-4, IL-5, and IL-13 (40), which are essential for defence against extracellular parasites but can also drive allergic responses. ILC2s are the predominant ILC subset in the steady-state lung, where they secrete amphiregulin to promote pulmonary wound healing after infection, suggesting a homeostatic function (41). In mice, two distinct ILC2 populations have been characterized: natural ILC2s that are identified as $Lineage^-ST2^+KLRG1^{int}$ and classified as homeostatic, tissue-resident and IL-33-responsive; and, inflammatory ILC2s, which are undetectable at the steady-state but expand in response to IL-25 and can be distinguished as $Lineage^-ST2^-KLRG1^{hi}$ cells (42). ILC2s are activated by IL-33, IL-25, thymic stromal lymphopoietin (TSLP) and other danger signals produced by the airway epithelium (43, 44), with further support from prostaglandin D_2 signalling through the CRTH2 receptor (40). Additionally, p38 MAPK has been found to positively regulate ILC2 function (45) while TGF-β is thought to program development *via* induction of ST2 expression in ILC2 progenitors (46). IL-1β is critical for ILC2 plasticity by inducing T-bet expression and promoting conversion into ILC1s in response to the Th1 cytokine IL-12 (47).

ILC3s and LTi cells require the transcription factor RORγt for their induction, and generate Th17-like responses, producing the cytokines IL-17, IL-22, and GM-CSF (24, 48). LTi cells also play

an important role in lymphoid organogenesis in foetal development (49–51). ILC3s can be further sub-grouped by the expression of Natural Cytotoxicity Receptors (NCRs) such as NKp46 (NCR^- or NCR^+) (52, 53); while the expression of CCR6 and T-bet distinguishes effector cytokine profile ($NCR^-CCR6^+T\text{-}bet^-$ produce IL-17, $NCR^+CCR6^-T\text{-}bet^+$ produce IFN-γ) (54). IL-18 can induce ILC3 proliferation and IL-22 production through NF-κB (55), while RANKL expression on ILC3s negatively regulates ILC3 cytokine production (56). Interestingly, the Th2 transcription factor GATA-3 is also critical for the induction and maintenance of ILC3s (57, 58) and therefore unsurprisingly, ILC2s have the potential to differentiate into IL-17-producing ILC3-like cells (59–61).

ILC SUBSETS IN COPD AND ASTHMA

ILC1 and NK Cells

ILC1 and NK Cells Are Indicators of COPD Severity

Recent studies suggest that an increased frequency of ILC1s in the peripheral blood of COPD patients correlates with disease severity and increased exacerbation risk (37, 62), and therefore may be utilised as a biomarker for disease progression. Furthermore, ILC1s as well as ILC3s are expanded in the lung of severe COPD patients (63). ILCs tend to localise to lymphoid aggregates in the lungs of COPD patients and smokers, whereas they are found in the parenchyma in healthy individuals (62). Cigarette smoke induces pulmonary ILC1s in a mouse model of COPD (62). ILC1s, alongside Th1 and CD8+ T cells can produce IFN-γ which is implicated in COPD pathogenesis by inducing elastolytic proteases and nitric oxide production by alveolar macrophages, leading to emphysema (64–66) (**Figure 1**). Furthermore, human ILC2s exhibit plasticity *in vitro* as well as *in vivo* when transferred to humanised mice, where they differentiate into ILC1s in the presence of IL-1β and IL-12 during pulmonary inflammation (63) and this is implicated in COPD exacerbations (37) (**Figure 1**).

On the other hand, NK cell-mediated destruction of lung tissue is implicated in COPD as NK cell cytotoxicity is enhanced in the lung of COPD patients, correlating with worsened lung function and emphysema (68) (**Figure 1**). Lung dendritic cells, *via* IL-15Rα signalling, prime NK cell cytotoxicity in the COPD lung, which may represent a therapeutic target (73). NK cell cytokine production is also implicated; in mice, cigarette smoke triggers NK cell pro-inflammatory cytokine release (74) by promoting their expression of the IL-33 receptor, ST2, while inhibiting type 2

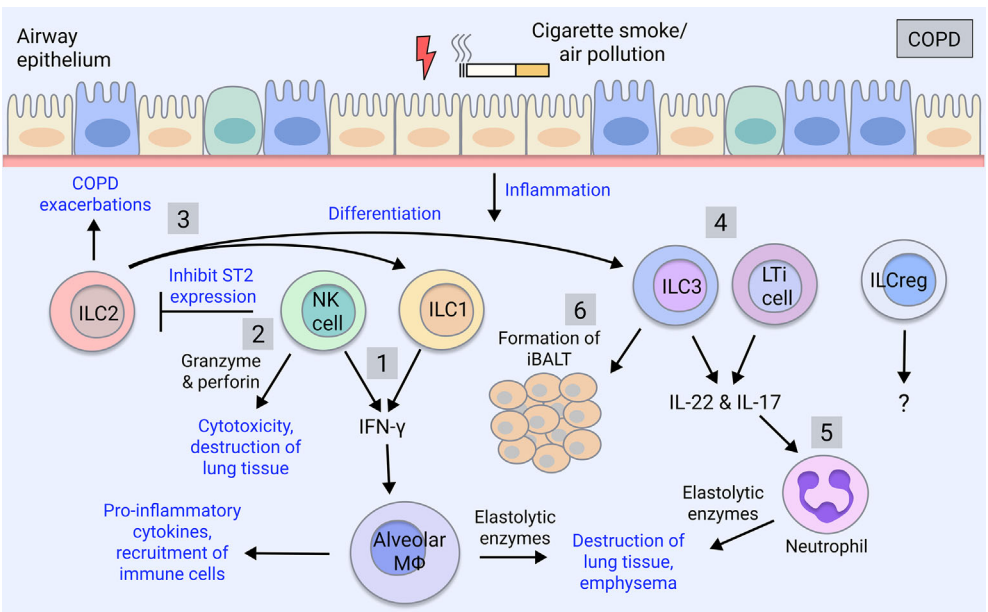

FIGURE 1 | ILC involvement in COPD. COPD is caused by cigarette smoking and insults such as air pollutants. COPD patients exhibit increases in group 1 and group 3 ILCs, which correlate with severity and exacerbations, whereas ILC2 numbers are reduced (63). 1) ILC1 and NK cells produce the pro-inflammatory cytokine IFN-γ, which activates alveolar macrophages causing the release of inflammatory mediators (67). Macrophages secrete proteases (MMPs, cathepsins) inducing the destruction of the lung parenchyma thereby contributing to emphysema (67). 2) NK cell cytotoxic activity through secretion of granzyme and perforin induces death of lung tissue, furthering emphysema (68). NK cells also inhibit the production of ILC2 through downregulation of their ST2 receptor (69). 3) ILC2s promote Th2 inflammation during COPD exacerbations or differentiate into ILC1-like cells in the presence of IL-1β and IL-12 during lung inflammation (37, 63, 70). They potentially also differentiate into ILC3s (59–61). 4) ILC3 and LTi cells produce IL-17 and IL-22, which are elevated in COPD patients, driving pathogenesis (71, 72). 5) IL-17 induces the maturation and recruitment of neutrophils, which are expanded in COPD patients, and *via* their release of proteases (neutrophil elastase, cathepsin G, proteinase-3), contribute to mucus secretion and alveolar destruction (6). 6) ILC3 and LTi cells contribute to the formation of iBALT, which is a feature of advanced COPD (5) and is the site of ILC localisation in COPD lungs (62). ILC_{regs} are yet to be understood in the regulation of COPD pathogenesis.

responses through downregulating ILC2 expression of ST2 (69) (**Figure 1**). Therefore, NK cells likely contribute to lung emphysematous destruction and inflammation in COPD.

NK Cells Have an Ambiguous Role in Asthma

While the contributions of ILC1s in asthma are currently unknown, ILC1s may be relevant to neutrophilic asthma or ACO, which warrants investigation. Meanwhile, NK cells can be both beneficial and detrimental in allergic and severe asthma. NK cells promote resolution of inflammation *via* inducing apoptosis of eosinophils and protecting against viral-induced inflammation (75, 76) (**Figure 2**). Furthermore, the immunomodulatory role of NK cells is impaired in severe asthma, with NK cells showing reduced lipotoxin A_4-mediated clearance of eosinophils (84). Additionally, NK cell-mediated eosinophil clearance is inhibited by corticosteroids, implicating the loss of NK cell cytotoxicity in severe and steroid-resistant asthma (85). Conversely, NK cells can drive asthma-like allergic airway inflammation by inducing type 2 cytokine production (86–90). However, NK cells played neither a positive nor negative regulatory role in a house dust mite (HDM) model (91). Given that most human studies implicate immunomodulatory rather than pro-inflammatory NK cell functions, it is speculated that this may not be directly recapitulated in mouse models, so further clarification is needed.

ILC2s

ILC2 Involvement in COPD and Exacerbations

ILC2s can convert to ILC1s in the setting of COPD, suggesting skewing towards type 1 inflammation in this disease (37) (**Figure 1**). However, ILC2s themselves have also been implicated in COPD by promoting type 2 inflammatory responses (92), although it is unclear if ACO patients, who exhibit an intermediate type 2 cytokine profile (93), were included in this cohort. Interestingly, ILC2s have been shown to mediate neutrophil recruitment in a model of cigarette smoke-induced COPD and their deficiency protected against emphysema yet promoted fibrosis through elevation of IL-13 and IL-33 (94). Furthermore, ILC2s have been implicated in promoting Th2 adaptive responses during acute COPD exacerbations (70) (**Figure 1**).

ILC2s Are Major Players in Allergic Asthma

In allergic asthma, which is commonly associated with type 2 inflammation, there are increases in ILC2s in the peripheral blood compared to healthy individuals or those with allergic rhinitis (95–97), and ILC2s are expanded in the lung of patients with severe asthma and associated eosinophilia (84, 98). In sputum analyses of eosinophilic asthma patients, ILC2s are strongly induced alongside alternatively-activated 'M2' macrophages, whereas numbers of alveolar macrophages are

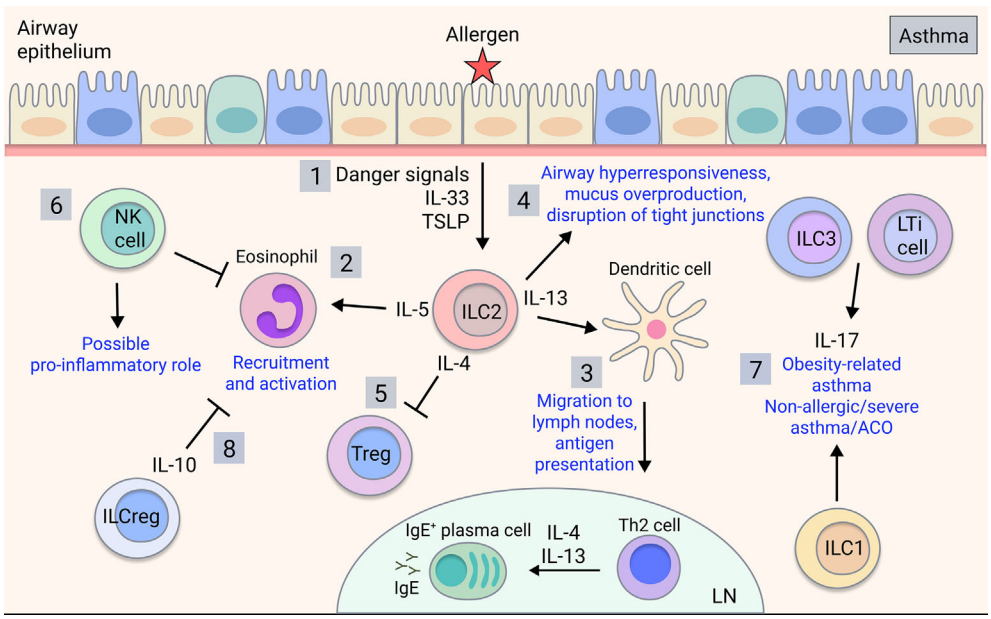

FIGURE 2 | ILC involvement in asthma. Upon allergen detection by airway epithelium, 1) ILC2s are activated by signals released by the airway epithelial cells and other activated immune cells, producing type 2 cytokines such as IL-4, IL-5, and IL-13 in allergic asthma. 2) IL-5 is key for eosinophil recruitment and activation in the lung (77) and 3) IL-13 mediates dendritic cell migration to the lymph nodes, promoting T cell differentiation into effector Th2 cells, which mediate B cell class-switching and IgE production (78). 4) ILC2-derived IL-13 also acts on the airway epithelium to induce airway hyperresponsiveness, mucus overproduction and disruption of barrier integrity (43, 79, 80). 5) ILC2-derived IL-4 may potentially inhibit T_{reg} production in asthma (81). 6) NK cells play an ambiguous role in asthma with both disease-driving and disease-modulatory activity shown. 7) ILC3s/LTi cells and possibly ILC1s contribute to obesity-related asthma and potentially non-allergic, severe asthma or ACO through production of IL-17 (82). 8) ILC_{regs} may regulate asthma by inhibiting eosinophil recruitment through IL-10 (83).

unchanged (99). Meanwhile, neutrophilic asthma patients exhibit increases in ILC1s and ILC3s along with inflammatory 'M1' macrophages (99) (**Figure 2**). ILC2s are critical in allergic airway inflammation, driving pathology alongside and independent of the adaptive immune system (100). Eosinophil recruitment is induced by ILC2 production of IL-5 (77) whereas their production of IL-13 triggers AHR, mucus overproduction and disruption of epithelial integrity (43, 79, 80) (**Figure 2**). ILC2-derived IL-13 also promotes dendritic cell migration and subsequent Th2 cell induction (78). ILC2 production of IL-4 blocks T_{reg} induction in food allergy responses (81), however this has yet to be shown in asthma. Interestingly, a subset of $CCR10^+$ ILC2s that exhibit ILC1-like characteristics and have the capacity to produce IFN-γ, were protective in both allergic and non-allergic severe asthmatic patients by limiting Th2 cytokine secretion and downregulating type 2 responses (101), demonstrating a protective function.

Numerous studies have explored the mechanisms of ILC2 regulation in allergic asthma. The neuropeptide neuromedin U, has been found to powerfully induce an asthma-like response and activate ILC2s (102), suggesting involvement of the neuroimmune axis. IL-1β, arginase 1, lipotoxin A_4, maresin 1, Nrf2, FABP5, IL-35, IFN-γ and PD-1 have all been shown to restrict ILC2 responses and promote resolution of allergic lung inflammation or prevent AHR through various mechanisms (79, 103–109). Furthermore, in allergic asthma, ILC2s are controlled by the transcription factor IRF7, a key regulator of anti-viral responses (110). On the other hand, neutrophils are reported to control allergic airway inflammation in a mouse HDM asthma model through the inhibition of ILC2 responses and G-CSF modulation (111). Collectively, this suggests that ILC2s may be a viable therapeutic target in asthma.

ILC3 and LTi Cells

Increased Il-17 Levels Implicate Group 3 ILCs in COPD Pathogenesis

In COPD, proportions of lung ILC subsets are skewed toward NCR^- ILC3s/LTi cells whereas non-COPD individuals have balanced proportions, with ILC2s and NCR^- ILC3s in greatest abundance (112) (**Figure 1**). NCR^- ILC3s are also enriched in severe COPD patients (63). In COPD patients and smokers, a highly migratory subset of ILC3s expressing neuropilin-1 receptor is found within the lung and associates with iBALT *via* induction of ICAM-1 and VCAM-1 on mesenchymal stromal cells (113) (**Figure 1**). In mice, ILC3s and ILC1s are increased in response to cigarette smoke exposure, whereas ILC2s are diminished (94). ILC3s are early producers of IL-17 and IL-22, which are implicated in COPD pathogenesis. IL-17 is elevated in the peripheral blood of COPD patients (114, 115) and steroid-resistant COPD (116), and COPD exacerbations have been associated with IL-17 and neutrophilic infiltration (71). IL-22 meanwhile, is elevated in COPD patient lungs and contributes to experimental COPD (72). Additionally, improvements in lung outcomes and comorbidities have been observed in cigarette smoke-exposed IL-17-deficient mice (117, 118). Given the association between ILC3 and IL-17 production, it is surprising that this link has not been addressed in COPD but could be a promising therapeutic avenue.

Group 3 ILCs May Be a Player in Neutrophilic Asthma and ACO

Currently, there are limited reports on ILC3s and LTi cells in asthma, however, ILC3-mediated production of IL-17 may contribute (**Figure 2**). IL-17 is implicated in severe asthma, neutrophilic asthma, asthma exacerbations and airway remodelling involving the recruitment of neutrophils (119, 120) and may contribute to ACO given that IL-17 levels are increased in ACO patients (121). Furthermore, the development of AHR in obesity-related asthma in mice relied on IL-17-producing ILC3s and the NLRP3 inflammasome, and ILC3s were expanded in the BAL of severe asthma patients (82). Therefore, *via* IL-17, ILC3s have a potential role in distinct asthma endotypes, although further studies are warranted in human disease.

ILCregs

Regulatory ILCs and Anti-Inflammatory Cytokine Production

Recently, a novel group of regulatory ILCs (ILC_{reg}) have been identified in gut, characterised by expression of the immunoregulatory cytokines IL-10 and TGF-β (122). While ILC_{regs} share mechanistic similarities with regulatory T cells (T_{reg}), they exhibit a unique transcriptome profile and lack the typical T_{reg} transcription factor FoxP3 (122). Furthermore, ILC_{regs} are transcriptionally distinct from typical ILCs, lacking common ILC transcription factors and can be distinguished as $Lineage^-CD45^+CD127^+$ cells and by production of IL-10 (122). Moreover, recent reports show that *ex vivo* human ILC1s and ILC2s can also potently produce IL-10 (123). In mice, an induced ILC_{reg} population derived from ILC2s, likely in response to retinoic acid, was observed in the lungs in HDM-induced allergic airway inflammation, with a similar population of cells also detected in human airway tissue (124). Likewise, a subset of ILC2s that produces IL-10 ($ILC2_{10}$) has been directly implicated in decreasing eosinophil recruitment to the injured lung (83) (**Figure 2**). While there is evidence that immunoregulatory ILC subsets are implicated in asthma, this has yet to be reported in COPD.

THE EFFECTS OF THERAPIES ON ILC RESPONSES

Recent guidelines recommend that adults with asthma receive a combination therapy comprising inhaled corticosteroids and long-acting β-agonists (125), and for severe asthma, inclusion of biologicals targeting type 2 responses (126). In COPD, while corticosteroids are ineffective, the recommendation for COPD exacerbations is a triple inhaled therapy containing corticosteroids, long-acting β_2-agonists, and long-acting muscarinic antagonists (127). Studies are beginning to reveal how these agents regulate ILC activity.

Dexamethasone is reported to inhibit type 2 cytokine production from ILC2s (128–131); however, IL-7 and TSLP induce resistance *via* IL-7Rα and STAT5 (129). Conversion of resting CD45RA+ ILC2s to inflammatory CD45RO+

ILC2s is suppressed by corticosteroids; however, once present, inflammatory ILC2s are resistant (132). Furthermore, inflammatory ILC2s are increased in the lung and blood of patients with chronic asthma correlating with disease severity and corticosteroid-resistance (132). In paediatric patients with severe therapy-resistant asthma, ILC2s, eosinophils and Th2 cells are increased, whereas Th17 cells and IL-17+ ILCs are unchanged. Systemic but not inhaled corticosteroids reduced ILC2s and Th2 cells as well as symptoms, despite persistence of IL-17+ cells and eosinophils (133). Collectively these studies suggest that ILC2 activity can be controlled by steroid therapy, but the inflammatory environment may alter steroid responsiveness. Anti-IL-5Rα therapy in patients with severe steroid-dependent asthma reduced blood and sputum eosinophils and IL-5Rα+ ILC2 but not total ILC2. While the functional relevance of IL-5Rα+ ILC2 is unclear, these changes were associated with improved asthma control and lung function (134).

ILC2s express the gene encoding β2-adrenergic receptor, with deficiency of this gene in mice inducing ILC2s and inflammatory responses (135). Furthermore, IL-33-induced ILC2 expansion and IL-5 and IL-13 production in lung were reduced by β2-agonist treatment, suggesting that β2-adrenergic receptor signalling limits the proliferation and function of ILC2s (135). With respect to cholinergic pathways, neuromedin U strongly activates ILC2s and amplifies IL-25-dependent lung inflammation (102, 136, 137). Moreover, the NMUR1 neuromedin U receptor is expressed on ILC2 and its deficiency attenuates ILC2 number and function in allergic airway inflammation (102). While little is known of the effects of muscarinic antagonists on ILCs or whether they express the receptors, in a papain-induced model of airway inflammation, the long-acting muscarinic antagonist tiotropium indirectly suppressed ILC2 activation by reducing IL-4 production from basophils (138). Clearly more studies are required to determine how therapies affect ILC phenotype and function, with these likely providing new insights into the regulation of these cells in chronic lung inflammation.

CONCLUSION

Innate lymphoid cells are an incompletely understood accomplice in the maladapted inflammatory environment that promotes chronic respiratory diseases. They potentially play a significant role in disease pathogenesis given their rapid response to pathogenic or environmental stimuli and may be the missing gap where conventional cell-based therapies have failed. However, the identification of ILCs is complex, making their study difficult, and their phenotype may be further complicated by their plasticity, microenvironment, and stages of differentiation. Although ILC manipulation in chronic respiratory diseases represents a significant challenge, understanding the intricacy of ILC regulation and the signals used by other tissue-resident cells to control their responses may provide a means of targeting them indirectly. The emergence of regulatory ILCs suggests another level of disease control, however these cells may be impaired or overwhelmed in chronic inflammatory settings. Nonetheless, ILCs represent a developing field in disease research. Understanding the role of ILCs during chronic inflammatory airway diseases like COPD and asthma will provide insight into the complexity of these diseases, how they are initiated and the manner in which they transition into a chronic state. Additionally, understanding how ILCs are regulated, how they respond to conventional treatments and furthermore how they regulate immune responses may allow us to devise strategies to switch their disease-driving capabilities into disease-modulatory actions.

AUTHOR CONTRIBUTIONS

MH conceptualised the article, contributed to the writing of the first draft and provided manuscript revisions. AH wrote the first draft, revised the manuscript and developed original figures. TG and ET amended the manuscript and provided extensive editorial input. All authors contributed to the article and approved the submitted version.

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The NF-κB Transcription Factor c-Rel Modulates Group 2 Innate Lymphoid Cell Effector Functions and Drives Allergic Airway Inflammation

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Group 2 innate lymphoid cells (ILC2s) play a key role in the initiation and orchestration of early type 2 immune responses. Upon tissue damage, ILC2s are activated by alarmins such as IL-33 and rapidly secrete large amounts of type 2 signature cytokines. ILC2 activation is governed by a network of transcriptional regulators including nuclear factor (NF)-κB family transcription factors. While it is known that activating IL-33 receptor signaling results in downstream NF-κB activation, the underlying molecular mechanisms remain elusive. Here, we found that the NF-κB subunit c-Rel is required to mount effective innate pulmonary type 2 immune responses. IL-33-mediated activation of ILC2s *in vitro* as well as *in vivo* was found to induce c-Rel mRNA and protein expression. In addition, we demonstrate that IL-33-mediated activation of ILC2s leads to nuclear translocation of c-Rel in pulmonary ILC2s. Although c-Rel was found to be a critical mediator of innate pulmonary type 2 immune responses, ILC2-intrinsic deficiency of c-Rel did not have an impact on the developmental capacity of ILC2s nor affected homeostatic numbers of lung-resident ILC2s at steady state. Moreover, we demonstrate that ILC2-intrinsic deficiency of c-Rel alters the capacity of ILC2s to upregulate the expression of ICOSL and OX40L, key stimulatory receptors, and the expression of type 2 signature cytokines IL-5, IL-9, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Collectively, our data using *Rel*$^{-/-}$ mice suggest that c-Rel promotes acute ILC2-driven allergic airway inflammation and suggest that c-Rel may contribute to the pathophysiology of ILC2-mediated allergic airway disease. It thereby represents a promising target for the treatment of allergic asthma, and evaluating the effect of established c-Rel inhibitors in this context would be of great clinical interest.

Keywords: group 2 innate lymphoid cell (ILC2), IL-33, c-Rel, type 2 immunity, airway inflammation

INTRODUCTION

Group 2 innate lymphoid cells (ILC2s) mediate early type 2 immune responses and thereby exert key roles in the initiation and orchestration of anti-helminth immunity as well as allergic inflammation (1–5). ILC2s exhibit striking functional similarity to adaptive type 2 T helper (Th2) cells. Like Th2 cells, they depend on the transcription factor GATA3 and produce type 2 signature cytokines such as interleukin (IL)-5 and IL-13 upon activation driving eosinophil recruitment as well as goblet cell hyperplasia and mucus production, respectively (6). ILC2s are located at barrier surfaces including the lung and, contrary to Th2 cells, lack the expression of specific antigen receptors (6). They instead become activated in an antigen-independent fashion in response to environmental cues such as alarmins IL-25, IL-33, and/or thymic stromal lymphopoietin (TSLP) that are released upon tissue perturbation (6).

Among these alarmins, IL-33 has been described as the most potent activator of lung ILC2s (7). IL-33 signals through the heterodimeric IL-33 receptor (IL-33R) composed of the ligand-binding chain ST2 and IL-1 receptor accessory protein (IL-1RacP) (8). While it is known that activating IL-33R signaling results in downstream nuclear factor (NF)-κB activation, the underlying molecular mechanisms in ILC2s remain largely elusive (8). NF-κB signaling is mediated by homo- or heterodimers of proteins of the Rel/NF-κB transcription factor superfamily including NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel (9). Despite sharing a common DNA recognition motif, knockout mice lacking individual Rel/NF-κB family members exhibit non-redundant phenotypes (10, 11). Moreover, differential expression patterns in tissues and responses to receptor signals as well as target gene specificity indicate that distinct NF-κB subunits exert unique physiological roles (10, 11). While stimulation of lung ILC2s with IL-33 results in phosphorylation of RelA and treatment with a pan-NF-κB inhibitor impairs ILC2 effector functions (12–15), RelB was shown to be an intrinsic repressor of ILC2s (16). On the contrary, the function of c-Rel in ILC2s remains undefined. c-Rel has been shown to promote type 2 immune responses upon ovalbumin (OVA)-induced allergic airway inflammation (17). Furthermore, inhibition of c-Rel in a mouse model of house dust mite-mediated allergic inflammation resulted in reduced levels of IL-13 and airway hyper-reactivity as well as lung inflammation (18, 19) and inhibited eosinophil recruitment in an OVA model of chronic asthma (20). Since ILC2s are main drivers of allergic asthma, we aimed to investigate whether c-Rel exhibits similar effects during ILC2-driven allergic airway inflammation.

In the present study, we found that deficiency in c-Rel severely diminished early pulmonary ILC2-driven type 2 responses to intranasal IL-33 administration. We further demonstrate that c-Rel expression and activation in ILC2s are positively regulated by IL-33.

MATERIALS AND METHODS

Mice

C57BL/6J wild-type (WT) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in house. $Rel^{-/-}$ mice have been previously described and were kindly provided by Dr. Steve Gerondakis (Monash University) (21). All animals were maintained on a C57BL/6J background and were bred and housed under specific pathogen-free conditions with *ad libitum* access to food and water. Experiments were conducted with adult female age-matched mice (8–12 weeks) in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University.

In Vivo Stimulation

Mice were anesthetized with isoflurane followed by intranasal administration of either phosphate-buffered saline (PBS) as a control or 500 ng carrier-free recombinant murine IL-33 (rmIL-33, R&D Systems) per mouse in a total volume of 40 µl. Mice were challenged with PBS or IL-33 for three consecutive days (d0, d1, and d2), and lungs were isolated and analyzed 24 h after the last treatment.

Lung Histopathology

Lungs were inflated with 10% buffered formalin, fixed overnight, and transferred to 70% ethanol. Fixed lungs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin following standard procedures. A histologic disease score from 0 to 4 was attributed based on peribronchial, perivascular, and parenchymal immune cell infiltration.

Preparation of Single-Cell Suspensions From Tissue

Lungs were isolated, finely minced, and digested in Roswell Park Memorial Institute (RPMI)-1640 containing 5% fetal bovine serum (FBS), 0.2 mg/ml of Liberase™ TM (Roche), and 0.1 mg/ml of DNase I (Roche) at 37°C. Digested lungs were homogenized with a 5-ml syringe attached to an 18G needle, filtered through a 70 µM cell strainer, and washed with PBS. Red blood cells were lysed using Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma-Aldrich), and cells were washed with fluorescence-activated cell sorting (FACS) buffer (PBS + 2% FBS). Small intestines were isolated, opened longitudinally, cut in small pieces, and vortexed in gut buffer (Hanks' Balanced Salt Solution (HBSS) + 2% FBS + 15 mM of HEPES) to remove fecal matter. Intestinal epithelial cells were removed by incubation in gut buffer containing 5 mM of EDTA for 20 min. Lamina propria was subsequently digested in RPMI-1640 supplemented with 5% FBS, 15 mM of HEPES, 0.1 mg/ml of Liberase™ TM (Roche), and 0.1 mg/ml of DNase I (Roche) at 37°C for 15 min. Digestion suspensions were filtered through a 70 µM cell strainer to obtain a single-cell suspension, and cells were washed with FACS buffer.

Flow Cytometry

Pelleted single-cell suspensions were resuspended in 2.4G2 hybridoma supernatant dilution and incubated for 15 min on ice to block Fc receptors. Cells were subsequently stained with antibody dilutions prepared in PBS supplemented with 2% FBS for 30 min on ice. Dead cells were excluded by staining with Fixable Viability Dye eFluor™ 780 (eBioscience) following the manufacturer's instructions. Intracellular staining was performed using the FoxP3/Transcription Factor Staining

Buffer Set (eBioscience) according to the manufacturer's protocol. Stained cell suspensions were acquired on a BD FACSCanto™ II System (BD Biosciences), a BD LSRFortessa™ Cell Analyzer (BD Biosciences) or sorted on a BD FACSAria™ III Cell Sorter (BD Biosciences). Flow cytometry data were analyzed using FlowJo X (BD Biosciences). All antibodies used for flow cytometry analyses are listed in **Table 1**.

Isolation and Expansion of Bone Marrow Group 2 Innate Lymphoid Cell Progenitors

Bone marrow ILC2 progenitors were isolated and expanded as described previously with minor modifications (22). Briefly, bone marrow from the tibias and femurs was pooled, subjected to red blood cell lysis using Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma-Aldrich), and sorted as lineage (CD3ε, CD5, CD11b, CD11c, Gr1, Ly6G, CD45R (B220), NK1.1, TCRβ, TCRγδ, and Ter-119)-negative, Sca-1$^+$c-kit$^-$CD25$^+$ cells. Isolated cells were expanded in complete ILC2 medium supplemented with recombinant murine IL-2, IL-7, IL-25, and IL-33 (all 50 ng/ml; R&D Systems) and TSLP (20 ng/ml; R&D Systems). After 2–3 weeks of expansion, cells were rested for 72 h in IL-2 and IL-7 (both 10 ng/ml), washed, and incubated in complete ILC2 medium without cytokines for 4 h before use in experiments.

TABLE 1 | Antibodies.

TARGET	CLONE	SOURCE
Flow cytometry antibodies		
CD3ε	145-2C11	eBioscience
CD5	53-7.3	eBioscience
CD11b	M1/70	eBioscience
CD11c	N418	eBioscience
CD19	eBio1D3	eBioscience
CD25	PC61.5	eBioscience
CD26	H194-112	eBioscience
CD45	30-F11	BioLegend
CD45R (B220)	RA3-6B2	eBioscience
CD90.2 (Thy-1.2)	53-2.1	BioLegend
CD117 (c-Kit)	2B8	eBioscience
CD127	A7R34 or SB/199	BioLegend
CD172a (SIRPa)	P84	eBioscience
CD252 (OX40L)	RM134L	BD Biosciences
CD275 (ICOSL)	HK5.3	eBioscience
CD278 (ICOS)	7E.17G9	BD Biosciences
c-Rel	1RELAH5	eBioscience
F4/80	BM8	eBioscience
FcεR1α	MAR-1	eBioscience
GATA3	TWAJ	eBioscience
GM-CSF	MP1-22E9	BioLegend
IL-5	TRFK5	BioLegend
IL-9	RM9A4	BioLegend
IL-13	eBio13A	eBioscience
Ki-67	SolA15	eBioscience
KLRG1	2F1	eBioscience
Integrin α4β7	DATK32	BioLegend
Ly-6A/E (Sca-1)	E13-161.7	BioLegend
Ly6C	AL-21	BD Biosciences
Ly6G/Ly6C (Gr-1)	RB6-8C5	eBioscience
Ly6G	1A8	BD Biosciences
MHC Class II (I-A/I-E)	M5/114	BD Biosciences
NK1.1	PK136	eBioscience
Siglec-F	E50-2440	BD Biosciences
IL-33R	RMST2-2	eBioscience
TER-119	TER-119	eBioscience
XCR-1	ZET	eBioscience
Western Blot antibodies		
c-Rel	D4Y6M	Cell Signaling Technology
GAPDH	14C10	Cell Signaling Technology
Histone H2A	D6O3A	Cell Signaling Technology
Anti-rabbit IgG, HRP-linked	polyclonal	Cell Signaling Technology

Isolation of Lung and Small Intestinal Group 2 Innate Lymphoid Cells

Single-cell suspensions from lung and small intestine were stained with respective surface antibodies and viability dye as described above and sorted on a BD FACSAria™ III Cell Sorter (BD Biosciences) as live CD45$^+$Lin$^-$Thy-1$^+$ST2$^+$CD25$^+$ or live CD45$^+$Lin$^-$Thy-1$^+$CD127$^+$KLRG1$^+$ cells, respectively. Isolated cells were cultured for 18 h in complete ILC2 medium containing recombinant murine IL-7 (10 ng/ml; R&D Systems), washed, and rested for 4 h in complete ILC2 medium without cytokines before being used in experiments.

Western Blotting Analysis

Bone marrow-derived ILC2s were stimulated for the indicated time points with rmIL-33, and sub-cellular fractionation was performed as previously described (23). Briefly, pelleted cells were resuspended in 900 µl of PBS/0.1% NP-40 containing protease inhibitor and triturated with a micropipette to lyse the cell membranes. Three hundred microliters of lysate was collected (WC=whole-cell lysate). The remaining 600 µl were centrifuged at 13,000 *g* for 10 s, and supernatant was collected (C=cytoplasmic fraction). Finally, the remaining pellet, containing intact nuclei, was resuspended in 300 µl of PBS/0.1% NP-40 with protease inhibitor. All fractionated samples were probe-sonicated for 15 s at 60% amplitude. Protein was quantified using the Bio-Rad Protein Assay (#500-0006), as per manufacturer's instructions. Prior to loading samples were denaturated in 3× Laemmli buffer containing sodium dodecyl sulfate (SDS) and 15% β-mercaptoethanol at 95°C for 5 min. 15 µg of protein for whole-cell lysate and cytoplasmic fraction samples, or 30 µl/one-tenth of the total nuclear fractionated samples, was separated on 8% polyacrylamide gels, with a 3% polyacrylamide stacking gel. Proteins were wet-transferred onto nitrocellulose membranes. For immunoblotting, membranes were probed with anti-c-Rel; anti-GAPDH or anti-H2A (all Cell Signaling Technology) in 0.1% T-TBS + 5% milk powder followed by incubation with anti-rabbit IgG-HRP (Cell Signaling Technology). The details of the antibodies are listed in **Table 1**.

ImageStream Analysis

Isolated lung ILC2s were left for 4 h in medium without cytokines and left untreated or stimulated with rmIL-33 (10 ng/ml; R&D Systems) for the indicated time points. Cells were fixed and permeabilized using the FoxP3/Transcription factor staining kit (eBioscience) according to the manufacturer's instructions and stained with anti-c-Rel, anti-CD25, and DAPI (NucBlue™ Fixed Cell ReadyProbes™ Reagent, Life Technologies). Samples were run on an ImageStreamX Mark II imaging flow cytometer (Amnis), and nuclear translocation of c-

Rel in $CD25^+$c-Rel^+DAPI^+ cells was analyzed using the similarity feature of the IDEAS software (Amnis). All antibodies are listed in **Table 1**.

RNA Extraction and Quantitative Real-Time PCR

Total RNA from cultured ILC2s was extracted using the Quick-RNA MicroPrep Kit (Zymo Research) according to the manufacturer's instructions. For preparation of total lung RNA, tissue was mechanically disrupted in a MagNA Lyser (Roche) followed by RNA extraction with TRIzol™ Reagent (Life Technologies) and clean-up using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. cDNA was prepared using $Oligo(dT)_{12-18}$ Primer (Life Technologies) and SuperScript™ III Reverse Transcriptase (Life Technologies). qRT-PCRs were performed with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Transcript expression was normalized to *Hprt* expression levels and quantified using the comparative $2^{-\Delta\Delta CT}$ method. Data are depicted as either relative expression or relative fold change compared to the mean of the control group. Primers used in this study were designed using the PrimerQuest Tool (Integrated DNA Technologies) and purchased from Integrated DNA Technologies. Sequence details of primers are provided in **Table 2**.

Protein Quantification

IL-5, IL-9, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in tissue culture supernatants were determined using the respective mouse DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. Absorbance was measured using an Enspire™ 2300 Multilabel Reader (PerkinElmer).

Intracellular Cytokine Staining

To determine intracellular cytokine production by isolated murine lung ILC2s, sorted cells in complete ILC2 medium (see above) were stimulated in 96-well round-bottom plates (15,000 cells/well) for 48 h with medium only; recombinant murine IL-2, IL-7, IL-9, and IL-33 (10 ng/ml; R&D Systems); or combinations thereof. GolgiPlug (BD Biosciences) was added for the last 6 h of culture. To analyze cytokine production following *in vivo* stimulation, lung cell suspensions (1×10^6 cells/well in a 96-well plate) in complete ILC2 medium were stimulated with Cell Stimulation Cocktail (eBioscience) according to the manufacturer's instructions in the presence of GolgiPlug (BD Biosciences). Cells were stained with respective surface antibodies and viability dye as described above, and intracellular cytokine staining was performed using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Stained cells were acquired on a BD LSRFortessa™ Cell Analyzer (BD Biosciences) and analyzed using FlowJo X (BD Biosciences). All antibodies used for flow cytometry analyses are listed in **Table 1**.

Statistical Analyses

All data were analyzed with GraphPad Prism software (GraphPad Software). p-Values below 0.05 were defined as statistically significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Unless otherwise indicated, figures display means ± standard deviation (SD). Experiment sample sizes (n), experiment replicate numbers, and statistical tests used are included in the respective figure legends.

RESULTS

c-Rel Is a Critical Mediator of Innate Type 2 Immune Responses

To investigate the role of c-Rel during ILC2-driven allergic airway inflammation, we challenged WT and c-Rel-deficient ($Rel^{-/-}$) mice intranasally with either PBS or IL-33 for three consecutive days and analyzed parameters of airway inflammation (**Figure 1A**). Pulmonary tissue histology showed that, when exposed to IL-33, lack of c-Rel resulted in a significant reduction in perivascular and peribronchial immune cell infiltration into lung tissue as compared with WT mice (**Figures 1B, C**). These findings could be recapitulated by flow cytometry, showing markedly decreased numbers of pulmonary $CD45^+$ leukocytes in c-Rel-deficient animals after IL-33 treatment (**Figure 1D**). Moreover, while challenge of WT mice with IL-33 resulted in a significant increase in total numbers of type 2 immunity-associated cell populations including eosinophils, type 2 dendritic cells (DC2s), and ILC2s, numbers were significantly diminished in $Rel^{-/-}$ mice (**Figure 1D** and **Supplementary Figures 1A–D**). Furthermore, c-Rel deficiency led to reduced expression of lung *Il5* transcripts with a trend towards lower *Il13* levels (**Figure 1E**). In addition, the type 2-associated chemokines *Ccl17* and *Ccl22* were greatly reduced in $Rel^{-/-}$ compared with WT mice (**Figure 1F**). In summary, compared with WT animals, c-Rel-deficient mice mount a reduced innate type 2 immune response with a significant reduction in pulmonary leukocyte infiltration, eosinophilia, DC2s, and ILC2s. Our results thus suggest that c-Rel is critical for the development of IL-33-driven allergic lung inflammation.

TABLE 2 | qRT-PCR primers.

Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')	Reference
Ccl17	GGAAGTTGGTGAGCTGGTATAA	GATGGCCTTCTTCACATGTTTG	Duerr et al. (22)
Ccl22	CTTCTTGCTGTGGCAATTCAG	TCACTAAACGTGATGGCAGAG	Duerr et al. (22)
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG	Hernandez et al. (24)
Il5	CTCTGTTGACAAGCAATGAGACG	TCTTCAGTATGTCTAGCCCCTG	Mohapatra et al. (25)
Il13	GCAGCATGGTATGGAGTGT	TATCCTCTGGGTCCTGTAGATG	this study
Rel	GGATCAACTGGAGAAGGAAGATT	ATGGACCCGCATGAAGAATAG	this study

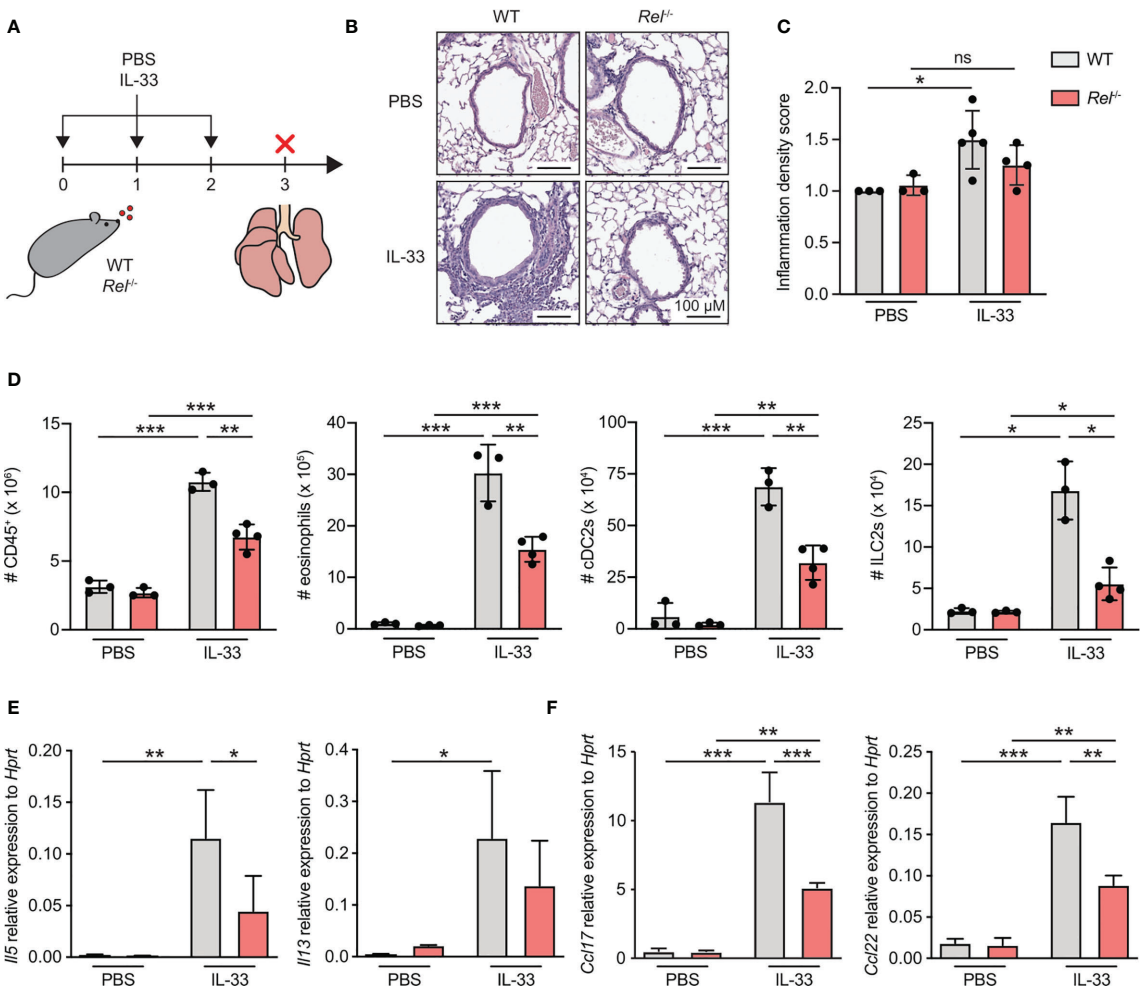

FIGURE 1 | c-Rel is a critical mediator of innate type 2 immune responses. **(A)** Wild-type (WT) and c-Rel-deficient (*Rel*$^{-/-}$) mice were intranasally challenged for three consecutive days (day 0, 1, and 2) with phosphate-buffered saline (PBS) as a control or IL-33 (500 ng/mouse). Lungs were isolated and analyzed 24 h after the last challenge (day 3). **(B)** Microscopy of lung sections stained with hematoxylin and eosin (H&E) from WT (left panel) and *Rel*$^{-/-}$ mice (right panel) treated with PBS or IL-33. Scale bars, 100 µM. **(C)** Pathology score of inflammatory infiltration density assessed microscopically from H&E-stained lung sections. ns, not significant. **(D)** Total numbers of CD45^{+} leukocytes, eosinophils, type 2 conventional dendritic cells (cDC2s), and group 2 innate lymphoid cells (ILC2s) in lungs of WT (gray) or *Rel*$^{-/-}$ (red) mice were determined by flow cytometric analysis. Expression levels of **(E)** *Il5* and *Il13* as well as **(F)** *Ccl17* and *Ccl22* in whole lung tissue of WT (gray) and *Rel*$^{-/-}$ (red) animals were assessed by qRT-PCR. Data are representative of two independent experiments with n = 3–4 mice per group. Data are shown as mean ± SD with *p < 0.05, **p < 0.01, ***p < 0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test.

c-Rel-Deficient Group 2 Innate Lymphoid Cells Exhibit No Intrinsic Defects in Their Developmental Capacity

To assess whether lack of c-Rel results in ILC2-intrinsic alterations that may explain why *Rel*$^{-/-}$animals mount a strongly reduced innate type 2 immune response, we analyzed ILC2 progenitor numbers in the bone marrow of c-Rel sufficient (*Rel*$^{+/+}$), heterozygous (*Rel*$^{+/-}$), and c-Rel-deficient (*Rel*$^{-/-}$) littermate control animals. We observed that mice of all three genotypes harbored comparable numbers of common lymphoid progenitors (CLPs), as well as the more downstream common helper ILC progenitors (CHILPs), and ILC2 progenitors (ILC2Ps) (**Figure 2A** and **Supplementary Figure 2A**). In addition, there was no difference in total numbers of mature lung ILC2s (**Figure 2B**). Together, these results indicate that c-Rel is dispensable for the development of ILC2s in the bone marrow and that c-Rel deficiency does not affect homeostatic numbers of lung-resident ILC2s.

IL-33-Driven *Ex Vivo* Activation of Group 2 Innate Lymphoid Cells Induces c-Rel Expression

Although it is well established that IL-33 signaling *via* ST2 triggers downstream activation of NF-κB pathways (8), the specific roles of the NF-κB subunit c-Rel in ILC2s remain unknown. We therefore analyzed the direct effect of IL-33 on c-Rel expression and activation in ILC2s. *Ex vivo* culture of bone marrow-derived

(**Figure 3A** and **Supplementary Figure 3A**) as well as lung ILC2s (**Figure 3B** and **Supplementary Figure 2B**) with IL-33 resulted in a rapid increase in c-Rel transcripts levels compared with unstimulated cells. Consistently, lung c-Rel protein levels increased in a dose-dependent manner and remained elevated for at least 72 h of culture with IL-33 (**Figures 3C, D**). Furthermore, c-Rel protein expression in lung, small intestine, and bone marrow-derived ILC2s was significantly elevated over levels found in unstimulated cells as early as 4 h following stimulation with IL-33 and was further induced 24 h post stimulation (**Figures 3E, F** and **Supplementary Figure 3B**). Taken together, these findings show that IL-33 potently induces c-Rel expression in ILC2s derived from different anatomical locations, indicating a tissue-spanning role of the IL-33–c-Rel axis.

c-Rel Translocates to the Nucleus Upon *Ex Vivo* Activation of Group 2 Innate Lymphoid Cells by IL-33

To determine whether c-Rel is activated upon IL-33 stimulation, we assessed nuclear translocation in ILC2s upon *ex vivo* IL-33 stimulation by Western blotting using cytoplasmic and nuclear ILC2 lysates (**Figure 4A**) as well as by ImageStream analysis (**Figures 4B, C**). With both methods, we observed that c-Rel remained confined to the cytoplasm in unstimulated ILC2s, while activation with IL-33 resulted in translocation of c-Rel from the cytoplasm to the nucleus, indicating a potential role of c-Rel in transcriptional regulation of ILC2 effector functions.

c-Rel Expression Is Induced in Lung Group 2 Innate Lymphoid Cells Following Intranasal Challenge With IL-33

To assess if c-Rel expression is also increased upon *in vivo* activation of ILC2s, we induced allergic airway inflammation by treating mice intranasally with IL-33 for 3 days. c-Rel-deficient mice that underwent the same treatment were used as negative controls. c-Rel transcript levels were significantly higher in lung tissues of IL-33-challenged mice compared with mice that received PBS, while no transcripts were detected in the lungs of control $Rel^{-/-}$ mice (**Figure 5A**). Consistent with ILC2s stimulated *ex vivo*, intranasal administration of IL-33 in WT mice resulted in a 10-fold induction of c-Rel expression in lung ILC2s over PBS-treated mice (**Figures 5B, C**). Taken together, these findings show that c-Rel expression is induced upon *ex vivo* stimulation of ILC2s as well as during IL-33- and allergen-induced allergic airway inflammation.

c-Rel Deficiency Limits Group 2 Innate Lymphoid Cell Effector Functions Following *Ex Vivo* Activation

Besides activating signals provided by alarmins, ILC2s require cues from STAT5-activating cytokines such as IL-2, IL-7, or TSLP, as well as costimulatory signals for optimal activation and exertion of effector functions (6, 26). We therefore assessed whether observed reduction in $Rel^{-/-}$ ILC2 numbers during allergic airway inflammation may stem from lowered sensitivity to activating signals due to diminished expression of the respective surface receptors. To this end, we assessed surface expression levels of CD25 (IL-2Rα), CD127 (IL-7R), and other key activating ILC2 receptors as well as the ILC2 master transcription factor GATA3 in isolated pulmonary WT and $Rel^{-/-}$ ILC2s at steady state and after *ex vivo* stimulation with IL-33 in the presence or absence of IL-2 or IL-7. We found that c-Rel deficiency did not result in diminished expression of surface CD25, CD127, or GATA3 by ILC2s at steady state or upon IL-33-mediated activation under the tested conditions (**Figures 6A** and **Supplementary Figure 4A**). Moreover, the IL-33R chain ST2 and the costimulatory receptor ICOS (**Figure 6B**) were slightly enhanced upon ILC2 activation in c-Rel-deficient ILC2s when compared with WT cells.

Besides ICOS, murine ILC2s also express ICOS ligand (ICOSL), whose interaction with ICOS promotes ILC2 survival and effector cytokine production upon IL-33-mediated activation (27, 28). Furthermore, constitutive as well as activation-induced expression of other costimulatory ligands and receptors including DR3, GITR, and OX40L on ILC2s have been reported (13, 29–32). We therefore assessed whether c-Rel regulates the expression of costimulatory ligands and/or receptors that promote ILC2 functions directly as well as are expressed by ILC2s to aid in instructing adaptive immune responses. While no changes in DR3

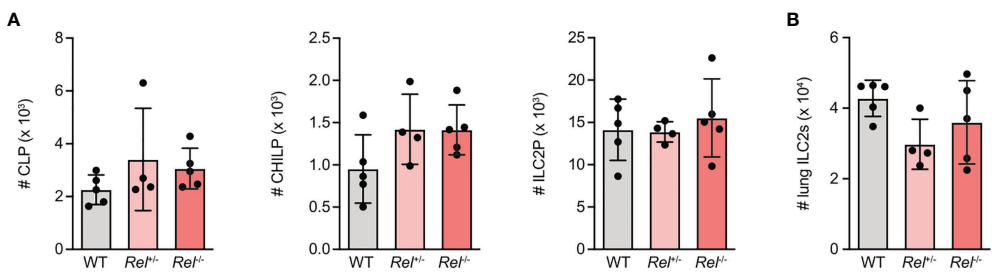

FIGURE 2 | c-Rel-deficient group 2 innate lymphoid cells (ILC2s) exhibit no intrinsic defects in their developmental capacity. Flow cytometric analysis of total numbers of **(A)** ILC2 precursor populations CLP (left), CHILP, (middle) and ILC2 progenitor (ILC2P) (right) as well as **(B)** pulmonary ILC2s in wild-type (WT), heterozygous $Rel^{+/-}$, and homozygous $Rel^{-/-}$ littermate control mice. Data points are representative of two independent experiments with n = 4–5 mice per treatment group **(A, B)**. Data are shown as mean ± SD as determined by one-way ANOVA followed by Tukey's multiple comparisons test. CLP, common lymphoid progenitor; CHILP, common helper ILC2 progenitor; ILC2P, ILC2 progenitor.

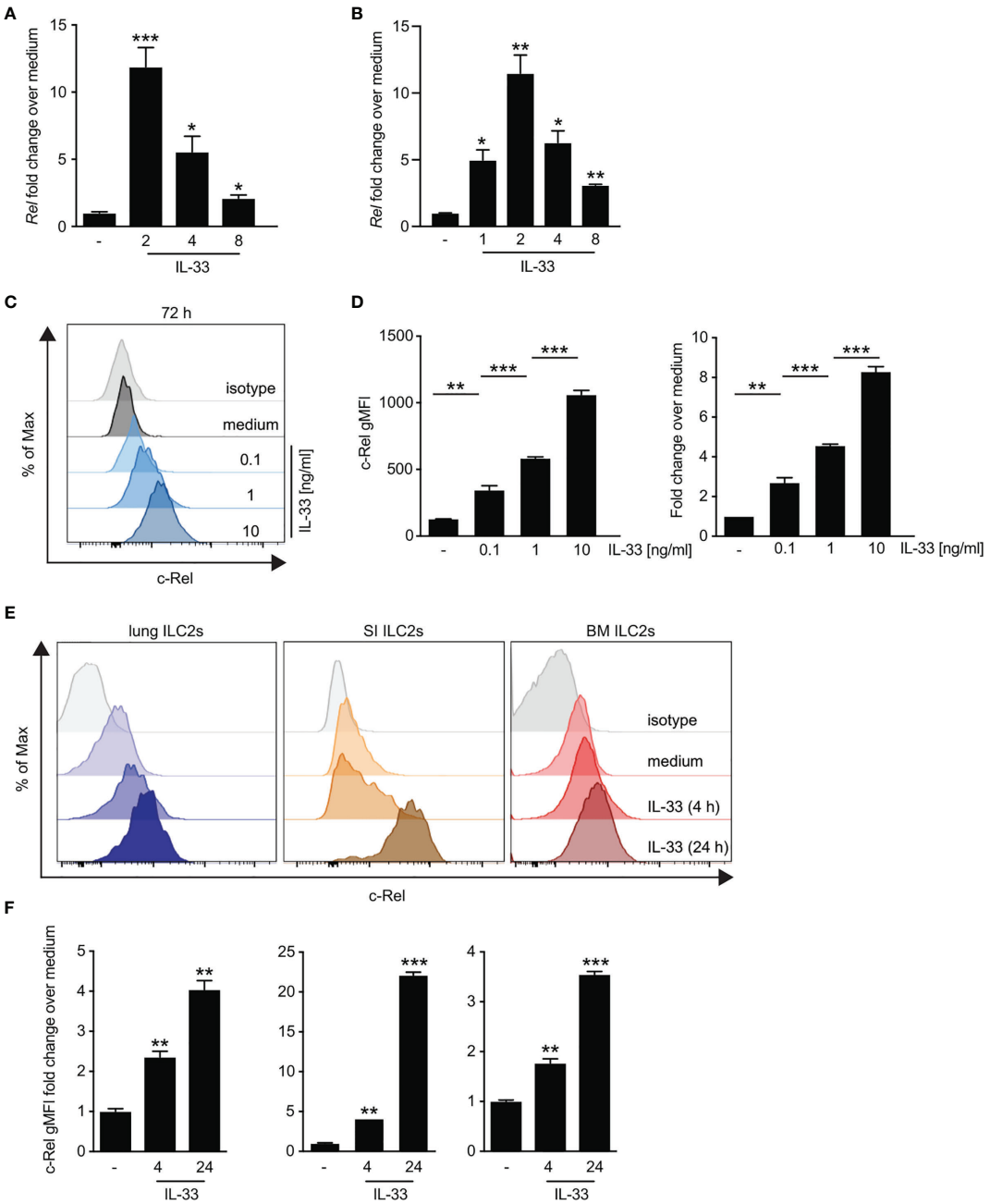

FIGURE 3 | IL-33-driven *ex vivo* activation of group 2 innate lymphoid cells (ILC2s) induces c-Rel expression. **(A–D)** Murine lung, small intestine, or bone marrow-derived ILC2s were isolated by flow cytometry and left unstimulated (–) or cultured in the presence of recombinant murine IL-33 for the indicated time points. Kinetics of c-Rel (*Rel*) transcript expression in **(A)** *ex vivo* expanded bone marrow-derived ILC2s stimulated with IL-33 (10 ng/ml) or **(B)** primary murine lung ILC2s. Asterisks indicate significance over untreated control. Transcript levels were assessed by qRT-PCR and are depicted as fold change over unstimulated (–) ILC2s. Asterisks indicate significance over untreated control. **(C, D)** Flow cytometric analysis of intracellular expression of c-Rel of isolated lung ILC2s after 72 h of *ex vivo* stimulation with medium (–) or IL-33 (0.1, 1, and 10 ng/ml). c-Rel staining is shown as **(C)** histogram plots in comparison with an isotype control antibody (gray), **(D)** gMFI (left panel), or fold change over unstimulated ILC2s (right panel). Asterisks indicate significance over unstimulated control. **(E)** Representative histogram plots of intracellular flow cytometric staining of c-Rel in lung (left), small intestine (middle), or bone marrow-derived (right) ILC2s left untreated (medium) or cultured with IL-33 (10 ng/ml) for 4 or 24 h. c-Rel staining is shown in comparison with staining with an isotype control antibody (gray). **(F)** c-Rel staining intensity in lung (left), small intestinal (middle), or bone-marrow-derived (right) ILC2s quantified as gMFI fold change over unstimulated cells. Asterisks indicate significance over respective untreated controls. Data are representative of at least two independent experiments including two or more biological replicates. Data are shown as mean ± SD with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as determined by two-tailed t-test (unpaired). gMFI, geometric mean fluorescence intensity.

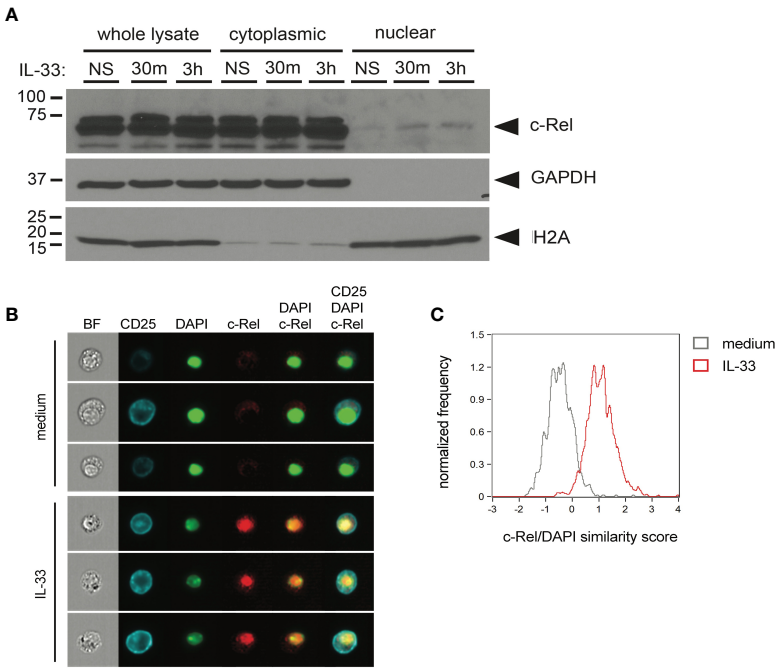

FIGURE 4 | c-Rel translocates to the nucleus upon *ex vivo* activation of group 2 innate lymphoid cells (ILC2s) by IL-33. **(A)** *Ex vivo* expanded bone-marrow derived ILC2s were left unstimulated (NS) or cultured with IL-33 (100 ng/ml) for 30 min or 3 h. Whole-cell, cytoplasmic, and nuclear lysates were probed for c-Rel by Western blotting. Expression of GAPDH and H2A served as fractionation and loading controls. **(B, C)** ImageStream analysis of isolated lung ILC2s left unstimulated (medium) or cultured with IL-33 (10 ng/ml) for 3 h. **(B)** Representative images (from left to right) of bright-field (BF), CD25, DAPI, and c-Rel as well as merged images of DAPI + c-Rel and CD25 + DAPI + c-Rel. **(C)** Quantification of nuclear translocation of c-Rel by overlay of c-Rel/DAPI similarity scores of untreated (gray histogram) and IL-33-activated ILC2s (red histogram). Data are representative of at least two independent experiments.

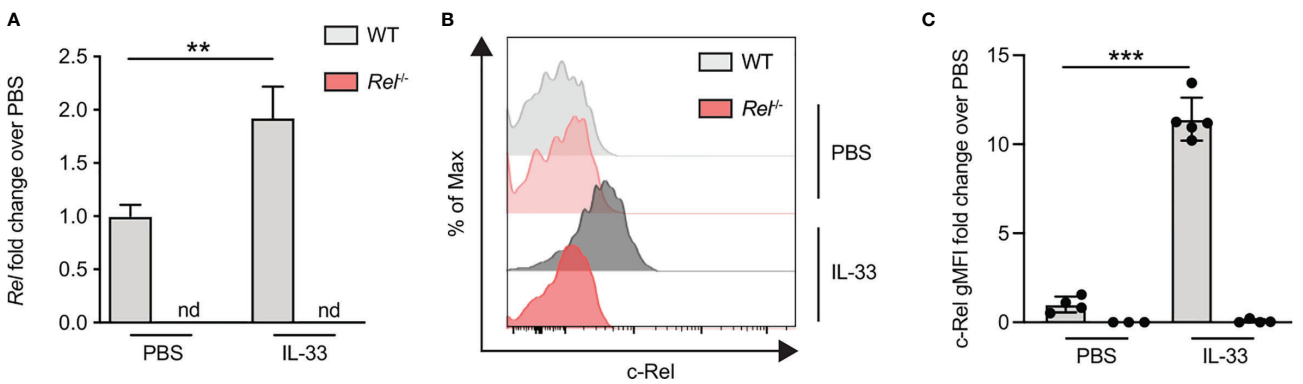

FIGURE 5 | c-Rel expression is induced in lung group 2 innate lymphoid cells (ILC2s) following intranasal challenge with IL-33. Wild-type (WT) (gray) and *Rel*$^{-/-}$ (red) mice were intranasally challenged for three consecutive days (days 0, 1, and 2) with either phosphate-buffered saline (PBS) or IL-33 (500 ng/mouse), and lungs were analyzed 24 h after the last administration. **(A)** Lung c-Rel transcript levels in WT and *Rel*$^{-/-}$ mice following intranasal challenge were determined by qRT-PCR, and **(B, C)** intracellular expression of c-Rel protein in lung ILC2s was analyzed by flow cytometry. **(B)** Representative histogram plots of intracellular c-Rel staining in pulmonary WT and *Rel*$^{-/-}$ ILC2s following intranasal challenge with PBS or IL-33 quantified as **(C)** gMFI fold change over the PBS-treated control group. Data points are representative of at least two independent experiments with n = 3–5 mice per treatment group. Data are shown as mean ± SD with $^{**}p < 0.01$, and $^{***}p < 0.001$ as determined by one-way ANOVA followed by Tukey's multiple comparisons test or by two-tailed t-test (unpaired). gMFI, geometric mean fluorescence intensity; nd, not detectable.

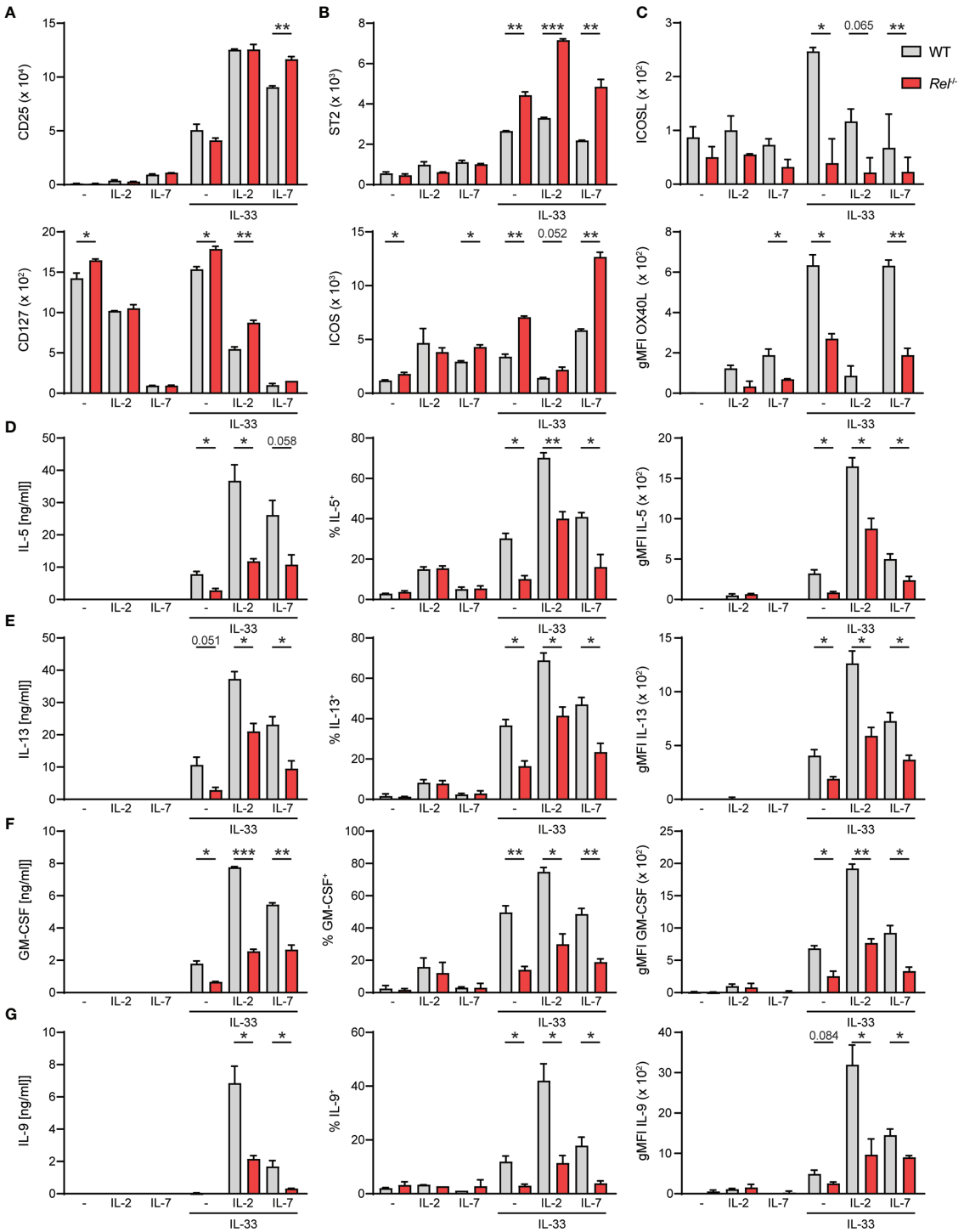

FIGURE 6 | c-Rel deficiency limits group 2 innate lymphoid cell (ILC2) effector functions following *ex vivo* activation. Lung ILC2s from wild-type (WT) and *Rel*$^{-/-}$ mice were isolated by flow cytometry and cultured *ex vivo* in the absence (–) or presence of IL-2, IL-7, or IL-33 and combinations thereof (all 10 ng/ml). Expression levels of surface **(A)** CD25 and CD127, **(B)** ST2 and ICOS, and **(C)** ICOSL and OX40L on isolated WT (gray) and *Rel*$^{-/-}$ (red) lung ILC2s after 24-h stimulation under indicated conditions. Analysis of **(D)** IL-5, **(E)** IL-13, **(F)** granulocyte-macrophage colony-stimulating factor (GM-CSF), and **(G)** IL-9 expression by WT (gray) and *Rel*$^{-/-}$ (red) lung ILC2s left untreated or cultured with IL-33 for 48 h. Cytokine concentration in culture supernatants is depicted in the left panel, frequencies of cytokine-producing cells are depicted in the middle panel, and cytokine expression levels are depicted as gMFI in the right panel. Data points are representative of two independent experiments with two biological replicates for each stimulation condition. Data are shown as mean ± SD with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as determined by two-tailed t-test (unpaired). gMFI, geometric mean fluorescence intensity.

or GITR expression were observed (data not shown), deficiency in c-Rel resulted in significantly reduced expression of surface ICOSL as well as OX40L (**Figure 6C**), indicating that c-Rel might further enhance ILC2 functions by driving ICOSL expression and also potentially drive adaptive type 2 responses by positively regulating surface OX40L levels.

Earlier studies demonstrated that c-Rel promotes cell cycle progression and survival in activated lymphocytes (21, 33–36). We therefore analyzed the proliferative capacity of lung ILC2s at steady state or upon IL-33-driven activation by Ki-67 staining after 48 h of activation. Ki-67 expression levels in lung ILC2s isolated from WT and *Rel*$^{-/-}$ mice were comparable upon IL-33 stimulation, indicating that c-Rel does not impact early expansion of IL-33-activated ILC2s (**Supplementary Figure 4B**).

ILC2s mediate their effector mechanisms by rapidly secreting large amounts of type 2 signature cytokines upon activation. To determine whether c-Rel directly regulates lung ILC2 type 2 signature cytokine production, we determined the concentrations of IL-5, IL-13, GM-CSF, and IL-9 in culture supernatants of ILC2s that were left unstimulated or were cultured with IL-2, IL-7, or IL-33 alone, or with indicated combinations (**Figures 6D–G**). We additionally assessed frequencies of cytokine-producing ILC2 and intracellular cytokine expression levels. All measured type 2 cytokines were induced upon activation of ILC2s with IL-33, and expression was further elevated when ILC2s were stimulated with IL-33 together with IL-2 or IL-7. Importantly, effector cytokine levels in the culture supernatant were significantly reduced in the absence of c-Rel (**Figures 6D–G**, left panel), which may be a direct result of significantly lower frequencies of cytokine-producing ILC2s (**Figures 6D–G**, middle panel) as well as significantly diminished production of cytokines within those cells (**Figures 6D–G**, right panel). Collectively, our data show that c-Rel positively regulates the expression of costimulatory ligands as well as ILC2 effector cytokines, thereby driving innate and potentially also adaptive type 2 immune responses.

c-Rel Drives Type 2 Effector Responses During IL-33-Induced Allergic Airway Inflammation

To further decipher the *in vivo* impact of c-Rel on ILC2 function, WT and *Rel*$^{-/-}$ mice were challenged intranasally for three consecutive days with PBS as a control or IL-33 (**Figure 7A**). Mice were sacrificed and lung ILC2 surface expression levels of CD25, CD127, and ST2 (**Figure 7B**), ICOS as well as ICOSL and OX40L were determined. Additionally, we assessed intracellular expression levels of GATA3 (**Figure 7F**) and frequencies of Ki-67^{+} proliferating lung ILC2s following *in vivo* challenge. In accordance with our *ex vivo* stimulation results, no significant differences were observed in the expression of the key stimulatory ILC2 receptors CD25, CD127, and ST2 and ICOS (**Figures 7B, C**). Importantly, both ICOSL and OX40L were significantly downregulated in the absence of c-Rel, suggesting an *in vivo* role of c-Rel in the regulation of ILC2 costimulation (**Figures 7D, E**). While GATA3 expression was comparable in ILC2s of WT and c-Rel-deficient mice (**Figure 7F**), frequencies of proliferating ILC2s were significantly reduced in the absence of c-Rel (**Figure 7G**).

To determine the effect of c-Rel on ILC2 effector cytokine production, lung cells from challenged animals were activated with phorbol myristate acetate (PMA)/ionomycin; and intracellular levels of IL-5 (**Figure 7H**), IL-13 (**Figure 7I**), GM-CSF (**Figure 7J**), and IL-9 (**Figure 7K**) in ILC2s were analyzed. While production of all cytokines was induced, frequencies of cytokine-expressing ILC2s were significantly reduced in *Rel*$^{-/-}$ mice following *in vivo* challenge (**Figures 7H–K**, top panel). Furthermore, intracellular cytokine expression levels, measured as gMFI, were markedly lower in the absence of c-Rel. Together, these data show that c-Rel is critical for ILC2 proliferation as well as type 2 effector cytokine production during allergic airway inflammation and might potentially drive subsequent adaptive immune responses by promoting the expression of OX40L.

DISCUSSION

ILC2s are critical mediators of early type 2 immune responses and allergic airway disease. They depend on an intricate network of transcriptional regulators to ensure efficient activation and execution of effector functions. Rel/NF-κB transcription factors modulate immune responses by regulating the expression of hundreds of genes involved in lymphoid cell development, proliferation, survival, and immune cell effector functions (37).

We here observed that the NF-κB transcription factor c-Rel promotes type 2 immunity in an ILC2-driven mouse model of allergic airway inflammation. Challenge of c-Rel-deficient mice resulted in reduced lung immune cell infiltration and diminished numbers of key type 2 immune cell populations including ILC2s, cDC2s, and eosinophils. Consistent with reduced ILC2 numbers, markedly lower transcript and protein levels of the type 2 signature cytokine IL-5 were observed, which is critical for the recruitment of eosinophils to the lung during allergic airway inflammation (38). Moreover, c-Rel deficiency led to lower pulmonary IL-13 transcript and protein levels, a hallmark cytokine of type 2 immunity. Attenuated induction of the type 2 chemokine ligands CCL17 and CCL22 in the lungs of *Rel*$^{-/-}$ mice following exposure to IL-33 was observed as well, which may be a direct result of reduced numbers of CCL17- and CCL22-producing DC2s (39). Moreover, CCL17 and CCL22 have been described previously to promote ILC2 chemotaxis, and reduced levels might affect positioning of ILC2s within the airways, which could contribute to the observed reduction of type 2 responses (40). In addition, we demonstrate that c-Rel deficiency in ILC2s leads to markedly reduced IL-9 and GM-CSF production *in vitro* and *in vivo*. IL-9 has been shown to increase ILC2 fitness (41) and reinforces innate and adaptive type 2 immune responses (42, 43). In addition, GM-CSF has been shown to drive type 2 immunity (44) and allergic sensitization (45). This suggests that the attenuated response of c-Rel-deficient mice to IL-33 may be the result of defective activation and cytokine production by ILC2s.

Consistent with earlier reports showing that c-Rel is dispensable for normal hemopoiesis and lymphocyte

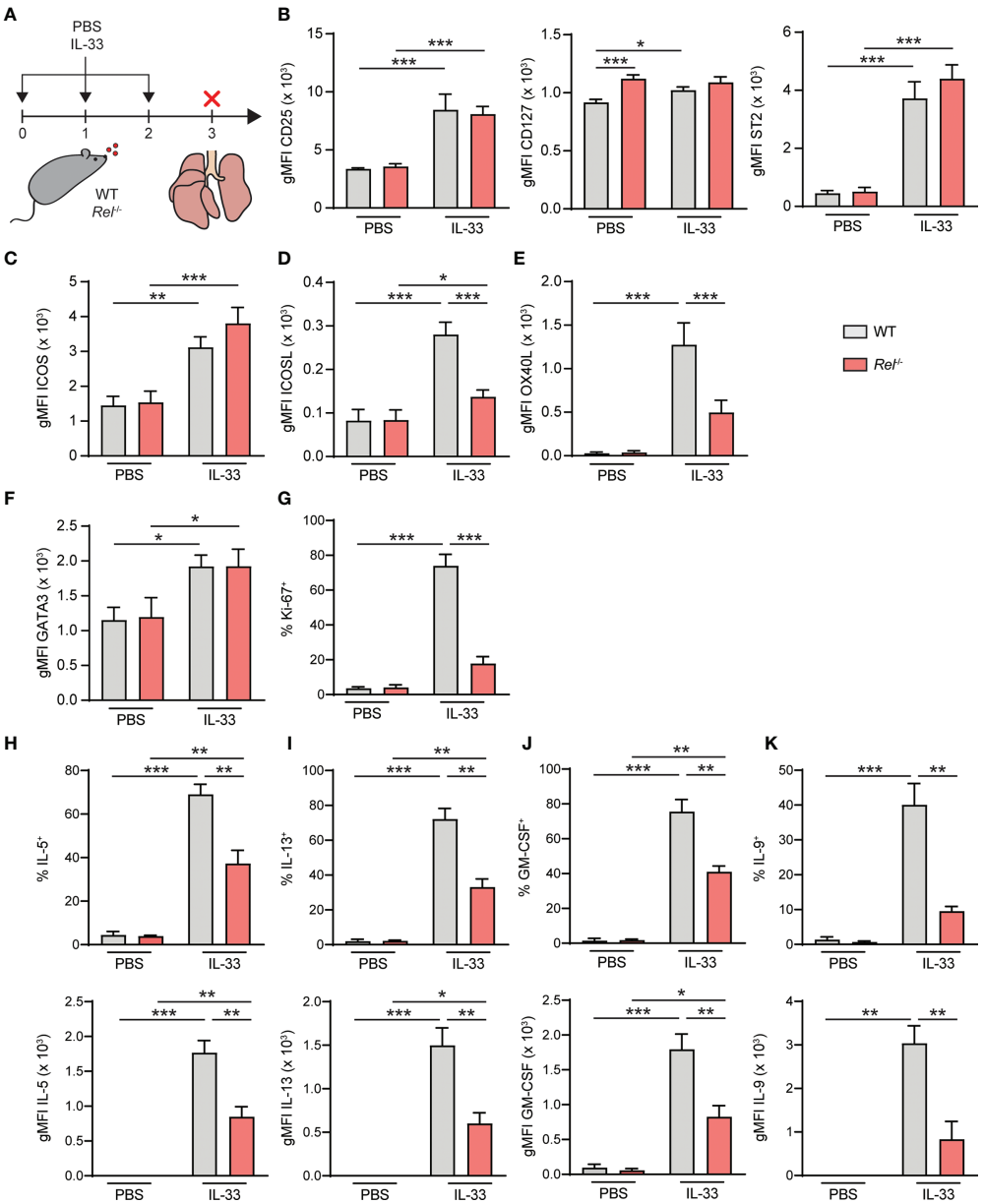

FIGURE 7 | c-Rel drives group 2 innate lymphoid cell (ILC2) effector responses during IL-33-induced allergic airway inflammation. **(A)** Wild-type (WT) and *Rel*$^{-/-}$ mice were challenged intranasally for 3 days (days 0, 1, and 2) with phosphate-buffered saline (PBS) or IL-33 (500 ng/mouse). Mice were sacrificed, and lungs were analyzed 24 h after the last administration. Flow cytometry analysis of surface expression levels of **(B)** CD25, CD127, and ST2, **(C)** ICOS, **(D)** ICOSL, and **(E)** OX40L, and intracellular expression levels of **(F)** GATA3 in lung ILC2s following intranasal challenge of WT (gray bars) and *Rel*$^{-/-}$ (red bars) mice. **(G)** Frequencies of Ki-67^{+} WT (gray bars) and *Rel*$^{-/-}$ (red bars) lung ILC2s following *in vivo* challenge. Analysis of intracellular expression of **(H)** IL-5, **(I)** IL-13, **(J)** granulocyte-macrophage colony-stimulating factor (GM-CSF), and **(K)** IL-9 in WT (gray bars) and *Rel*$^{-/-}$ (red bars) in lung ILC2s following intranasal PBS or IL-33 challenge and *ex vivo* restimulation with phorbol myristate acetate (PMA)/ionomycin. Frequencies of cytokine-expressing cells are depicted in the top panel; and expression levels, measured as gMFI, are shown in the bottom panel. Data points are representative of two independent experiments with two biological replicates for each stimulation condition. Data are shown as mean ± SD with *p < 0.05, **p < 0.01, and ***p < 0.001 as determined by two-tailed t-test (unpaired). gMFI, geometric mean fluorescence intensity.

development, the absence of c-Rel did not result in altered numbers of ILC2 precursors (21). In addition, numbers of lung ILC2s were comparable in naive WT and *Rel*$^{-/-}$ mice, indicating no homeostatic defects due to c-Rel deficiency that might contribute to the inability of c-Rel-deficient mice to mount an efficient type 2 response upon IL-33 challenge.

Our data show that c-Rel expression is potently induced in a dose-dependent manner in pulmonary ILC2s at both transcript and

protein levels following activation with IL-33. Upregulation of c-Rel protein levels was also observed in IL-33-stimulated ILC2s isolated from other tissues, indicating a general role of c-Rel in ILC2 function. Importantly, c-Rel expression was induced in lung ILC2s following intranasal challenge with IL-33, suggesting a physiological function during allergic airway inflammation. We furthermore demonstrated nuclear translocation of c-Rel in lung ILC2s upon IL-33 stimulation, and since c-Rel harbors a transcription transactivation domain, it can thereby act as a direct transcriptional activator once in the nucleus (37).

Our data suggest that the attenuated response of c-Rel-deficient mice to IL-33 may be the result of defective activation and expansion of ILC2s. IL-33 acts in concert with signals provided by costimulatory STAT5-activating cytokines IL-2 and IL-7, and costimulatory surface molecules to promote ILC2 survival, expansion, and exertion of effector functions (6, 26). Previous work demonstrated that c-Rel upregulates the expression of IL-2Rα (CD25) in T cells (21, 33, 46, 47), and it is known that IL-2 enhances ILC2 survival as well as activation-mediated expansion and type 2 cytokine production (48–50). Moreover, earlier studies demonstrated that c-Rel positively modulates cell cycle progression and the expression of anti-apoptotic proteins in activated T and B cells (21, 33–36). In addition, genes encoding the type 2 signature cytokines IL-4 and IL-13 were proposed as putative c-Rel target genes based on differential transcript expression observed in gain-of-function and loss-of-function experiments in T cells (51). While our work showed no effect of c-Rel on ILC2-intrinsic expression of the cytokine receptors CD25 and CD127, we observed a strong reduction of IL-5, IL-9, IL-13, and GM-CSF production upon IL-33 stimulation *in vitro* and *in vivo*. Similar to T cells, ILC2s express activating costimulatory molecules, including DR3, GITR, and ICOS, that promote ILC2 function in disease settings as well as during homeostasis (13, 29–31, 52, 53). While no reduction in ICOS expression was observed, ICOSL and OX40L expression was found to be significantly lower in c-Rel deficient ILC2 after activation. This could explain the markedly reduced ILC2 response in the absence of c-Rel during experimental acute allergic airway inflammation.

Interestingly, WT and c-Rel deficient ILC2s proliferated equally well *in vitro*. In contrast, the proliferative potential was found to be altered *in vivo*. Since c-Rel is expressed in a variety of cells within the hematopoietic compartment (10, 54), it cannot be excluded that c-Rel deficiency impacts effector functions of other innate as well as adaptive pulmonary immune cell populations such as IL-33-reactive macrophages, DCs, or $CD4^+$ T helper cells, which could potentially promote ILC2 activation by providing costimulation and/or activating cytokines. IL-33 was shown to induce IL-2 expression in DCs (55) and IL-2 is known to strongly enhance ILC2 survival as well as activation-mediated expansion and type 2 cytokine production (48–50). Moreover, interaction of ILC2 with $CD4^+$ T cells has been suggested to enhance the ability of $CD4^+$ T cells to produce IL-2, thereby reinforcing ILC2 activation (49). Interestingly, c-Rel deficiency was shown to alter the ability of $CD4^+$ T cells to produce IL-2 (46, 56). It is therefore possible that c-Rel deficiency also alters the capacity of $CD4^+$ T cells to produce IL-2 in response to IL-33, which could contribute to the altered proliferative capacity of ILC2 observed *in vivo*.

Our data using $Rel^{-/-}$ mice suggest that c-Rel promotes acute ILC2-driven allergic airway inflammation and suggests that c-Rel may contribute to the pathophysiology of ILC2-mediated allergic airway disease. It thereby represents a promising target for the treatment of allergic asthma, and evaluating the effect of established c-Rel inhibitors in this context would be of great clinical interest. In addition, while RelA deficiency results in embryonic lethality and $RelA^{-/-}$ cells exhibit severe defects regarding survival, proliferation, and effector functions (57), mice lacking c-Rel are viable and only show limited immunological defects (21). Thus, targeting c-Rel specifically may avoid the adverse side effects that have halted advancement of pan-NF-κB inhibitors for therapeutic applications (19, 58). IL-33-dependent ILC2 activation has also been reported during cancer (59, 60), viral infections (22, 61, 62), and murine models of autoimmune diseases (63–66); and given the observed induction of c-Rel expression in ILC2s from different tissues, modulation of c-Rel function in these conditions may be worth evaluating.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because no datasets were generated as listed in 2. Requests to access the datasets should be directed to jorg.fritz@mcgill.ca.

ETHICS STATEMENT

The animal study was reviewed and approved by the Canadian Council on Animal Care, McGill University, ethics committee.

AUTHOR CONTRIBUTIONS

JF and BM designed the intellectual framework of the study and the layout of the manuscript. BM, SK, and CD performed *in vivo* allergic airway inflammation models, and BM and SK conducted *ex vivo* cell culture experiments. BM, SK, CD, and DL analyzed data. MM carried out cellular fractionation and Western blotting analysis. LR performed histological scoring. SV and SG provided critical reagents and mouse strains. BM and JF designed experiments and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.664218/full#supplementary-material

Supplementary Figure 1 | Gating strategies for the identification of pulmonary type 2 cell populations. **(A)** Multicolor flow cytometry gating strategies to identify murine pulmonary **(B)** eosinophils (single, live $CD45^+SSC^{hi}Siglec\text{-}F^+CD11c^-$), **(C)** cDC2s (single, live $CD45^+CD64^-$ $Lin^-MHCII^+CD26^+CD11c^+XCR1\text{-}CD172a^+$) as well as **(D)** ILC2s (single, live $CD45^+$ $Lin^-Thy\text{-}1^+CD127^+KLRG1^+$). cDC2, conventional type 2 dendritic cell.

Supplementary Figure 2 | Gating strategies for the identification of bone marrow ILC2 progenitor populations and isolation of murine lung ILC2s. Flow cytometry gating strategies to **(A)** identify bone marrow CLP (single live $Lin^-CD127^+Flt3^+a4b7^-$), CHILP (single live $Lin^-CD127^+Flt3^+a_4b_7^+CD25^-$) and ILC2P (single live Lin-$CD127^+Flt3^+a_4b_7^+$ $CD25^+$) populations and **(B)** isolate murine lung ILC2s (single live Lin-$CD45^+Thy\text{-}1^+ST2^+CD25^+$). CLP, common lymphoid progenitor; CHILP, common helper ILC2 progenitor; ILC2P, ILC2 progenitor.

Supplementary Figure 3 | Gating strategies for the isolation of murine bone marrow-derived ILC2 progenitors and small intestinal ILC2s. Flow cytometry gating strategies to isolate **(A)** bone marrow-derived ILC2 progenitors (single Lin^- $Sca\text{-}1^+c\text{-}Kit^-$ $CD25^+$) and **(B)** murine small intestinal ILC2s (single live $CD45^+Lin^-$ $KLRG1^+CD127^+$).

Supplementary Figure 4 | Absence of c-Rel does not affect GATA3 expression or early ILC2 proliferation upon *ex vivo* IL-33 challenge. Isolated WT (grey bars) and $Rel^{-/-}$ (red bars) lung ILC2s were left untreated (-) or cultured with IL-2, IL-7, IL-33 (all 10 ng/ml) or indicated combinations for 48 hours and analyzed by flow cytometry. **(A)** GATA3 expression, shown as gMFI. **(B)** Frequencies of Ki-67-expressing lung ILC2s. Data points are representative of two independent experiments with two biological replicates for each stimulation condition. Data are shown as mean ± SD with $^{**}p < 0.01$ as determined by two-tailed t test (unpaired). gMFI, geometric mean fluorescence intensity.

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