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## ILC2s and fungal allergy

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### Abstract

Innate lymphoid cells (ILCs) have emerged recently as an important component of the immune system and the cell type that regulates mucosal immune responses and tissue homeostasis. Group 2 ILCs (ILC2s), a subset of ILCs, reside in various tissues and are characterized by their capacity to produce type 2 cytokines and tissue growth factors. These ILC2s play an important role in allergic immune responses by linking signals in the atmospheric environment to the immune system. Fungi are one of the major allergens associated with human asthma, and animal and *in vitro* models using the fungal allergens have provided significant information toward our understanding of the mechanisms of allergic disease. In mouse models of fungus-induced allergic airway inflammation, IL-33, IL-25, and TSLP are released by airway epithelial cells. Lung ILC2s that respond to these cytokines quickly produce a large quantity of type 2 cytokines, resulting in airway eosinophilia, mucus production, and airway hyperreactivity even in the absence of adaptive immune cells. Evidence also suggests that ILC2s interact with conventional immune cells, such as CD4<sup>+</sup> T cells, and facilitate development of adaptive immune response and persistent airway inflammation. ILC2s are also present in respiratory mucosa in humans. Further investigations into the biology of ILC2s and their roles in the pathophysiology of allergic diseases will provide major conceptual advances in the field and may provide useful information toward development of new therapeutic strategies for patients.

### Keywords

Allergy; Asthma; Cytokines; Fungi; Innate lymphoid cells

### Introduction

After the initial discovery and characterization of Th1- and Th2-type CD4<sup>+</sup> T cells approximately 25 years ago, T cells were thought to play a central role in regulation of immune responses and pathophysiology of diseases by producing a repertoire of cytokines.<sup>1</sup> However, the recent discovery of innate lymphoid cells (ILCs) added another layer of complexity and provided a major shift in this paradigm.<sup>2</sup> ILCs have emerged as important

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Conflict of interest

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effector cells of the immune system that are involved in pathogen clearance, lymphoid organogenesis, tissue remodeling and immune pathology through production of distinct sets of cytokines and growth factors. These cells are derived from a common lymphoid progenitor, exhibit lymphoid morphology, but express no phenotypic markers of conventional immune cells.<sup>3</sup>

ILCs have been categorized into three groups, including group 1 ILCs (ILC1s), group 2 ILCs (ILC2s), and group 3 ILCs (ILC3s), based on their cytokine profiles and the transcription factors that are utilized for their development and function.<sup>4</sup> ILC1s comprise IFN- $\gamma$ -secreting ILCs that use transcription factor T-bet for lineage commitment. ILC2s comprise type 2 cytokine-producing ILCs that require transcription factor GATA3 for their development and function. ILC3s comprise IL-17- and/or IL-22-producing ILCs that are dependent on transcription factor ROR $\gamma$ t for lineage specification. This article will focus on ILC2s and discuss their roles in allergic immune responses induced by fungal allergens. While major progress has also been made regarding the roles for ILC2s in viral infection and regulation of tissue homeostasis and metabolism, they are discussed elsewhere in detail<sup>5</sup> and will be described only briefly in this article.

## Fungi in allergic diseases and disease models

Allergic asthma is generally mediated by dysregulated production of Th2-type cytokines, such as IL-4, IL-5 and IL-13, although other cell types, such as Th17 cells, have also been implicated.<sup>6</sup> The interactions between mucosal epithelia and innate and adaptive immune cells were recently proposed as the underlying causes of dysregulated production of Th2-type cytokines in allergic diseases.<sup>7</sup> In particular, the epithelium-derived cytokines IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) have emerged as potential links between environmental exposure to allergens and type 2 immune responses.<sup>8–11</sup> For example, intraperitoneal injection of IL-33 in mice increases serum levels of IgE and Th2 cytokines and promotes airway eosinophilia and mucosal hyperplasia.<sup>12,13</sup> Inhalation of IL-33 induces type 2 lung inflammation even in T cell- and B cell-deficient animals.<sup>14</sup> TSLP activates DCs to polarize naïve T cells towards the pro-inflammatory Th2 cells that produce IL-4, IL-5, and IL-13 as well as TNF- $\alpha$ .<sup>15–17</sup> Mice expressing TSLP in the lungs develop spontaneous airway inflammation with characteristics similar to human asthma.<sup>18</sup>

While a number of environmental factors are associated with asthma and allergic diseases, allergen exposure likely plays a key role in triggering and exacerbating asthma and allergy symptoms.<sup>19</sup> In particular, exposure to airborne allergens derived from animals, arthropods, and molds is considered to be an important risk factor.<sup>20–22</sup> In humans, an association between fungal exposure, in particular to *Alternaria* and *Aspergillus*, and asthma is recognized clinically and epidemiologically.<sup>23,24</sup> Furthermore, severe asthma and life-threatening acute exacerbations of asthma have also been associated with increased airborne exposure to *Alternaria*.<sup>25–27</sup> However, the molecular mechanisms involved in fungal exposure and development of allergic immune responses are not well understood. Fungi secrete or contain numerous biologically active substances, including chitin,<sup>28</sup>  $\beta$ -glucan<sup>29</sup> and proteases.<sup>30</sup> Fungi likely contribute to the majority of protease activity in house dust samples.<sup>31</sup> Certain fungal allergens are also proteases themselves, including Asp f 5, f 6 and

f 11 from *Aspergillus*,<sup>23</sup> suggesting that proteases may be a key link between fungal exposure and Th2-type immune responses. Indeed, the protease activities produced by fungi were sufficient<sup>32,33</sup> and necessary<sup>31</sup> for induction of robust allergic airway inflammation induced by fungal organisms. Moreover, a prototypic cysteine protease, such as papain, enhanced Th2-type sensitization to bystander antigens.<sup>34</sup>

The conventional animal model of Th2-type immunity and “allergy” involved intraperitoneal or subcutaneous sensitization of mice with a model antigen ovalbumin (OVA) and an alum adjuvant, followed by airway challenge with the OVA antigen. More recent models used natural allergens, such as extracts of fungi and authentic proteases, which were directly administered into the airways of naïve and/or sensitized animals. These changes in the experimental model systems as well as new discoveries in basic science facilitated a major paradigm shift in our understanding of the mechanisms of type 2 immune responses in the airways.

## ILC2s

In the early 2000s, ILC2s were first described in mice as non-B/non-T cells that secrete IL-5 and IL-13 in response to IL-25.<sup>35,36</sup> When mice were infected with the parasite *Nippostrongylus brasiliensis* or exposed to the fungus *Aspergillus*, increased expression of IL-25 in the gut and lung was observed.<sup>35,36</sup> A subsequent study showed that these IL-25-responsive innate immune cells play important roles in *N. brasiliensis* worm expulsion.<sup>37</sup> In 2010, ILC2s were isolated and characterized by several investigators, and they were independently named as natural helper cells, nuocytes, and innate helper 2 cells.<sup>38–40</sup> Later a consensus report designated them as group 2 ILCs (ILC2s).<sup>4</sup>

ILC2s arise from the common lymphoid progenitors (CLPs) in the bone marrow and, like other ILCs, require the transcriptional inhibitor Id2 for their development.<sup>38</sup> Id2 inhibits the activity of the E proteins, which are implicated in differentiation of B cells and T cells.<sup>41</sup> The transcription factor promyelocytic leukemia zinc finger protein (PLZF) then mediates generation of an ILC precursor that gives rise to ILC1, ILC2 and ILC3 but not conventional natural killer (NK) cells.<sup>42</sup> The transcription factor ROR $\alpha$  is critical for further development of ILC2s from the Id2-dependent ILC precursor. Indeed, ROR $\alpha$ -deficient “Staggerer” mice, which carry a spontaneous mutation in the *Rora* gene, show severely impaired expansion of ILC2s as well as cerebellar developmental defects<sup>43</sup>; the other ILC subsets are not affected.<sup>44</sup> Mice that have received bone marrow from the “Staggerer” mice to circumvent their neurological defects have been used as a model for ILC2-deficient mice.<sup>45</sup> While GATA3 is required for the generation of the ILC precursor, it is also required for maintenance and effector functions of ILC2s.<sup>46,47</sup>

ILC2s do not express conventional cell surface markers for T cells, B cells, NK cells, myeloid cells, and DCs; thus, they are designated lineage-negative (Lin<sup>-</sup>). Mouse ILC2s express ST2 (IL-33 receptor), CD127 (IL-7R  $\alpha$ -chain), ICOS, CD117 (c-kit), Thy1, IL-17RB (IL-25 receptor), CD44 and CD25 (IL-2R  $\alpha$ -chain); the expression levels of these molecules varies depending on the anatomical location and activation states of the cells.<sup>45</sup> Mouse ILC2s are widely distributed in the tissues, including fat-associated lymphoid

clusters (FALC), mesenteric and mediastinal lymph nodes, liver, spleen, intestine, bone marrow, visceral adipose tissue and lung. Thus, ILC2s appear to be critically positioned to maintain homeostasis by responding rapidly to environmental cues, including metabolic stress and nutrient intake, and poised to rapidly respond to damage or stress in mucosal tissues. Functionally, ILC2s are considered to be the counterpart of Th2-type CD4<sup>+</sup> T cells. They characteristically produce type 2 cytokines, such as IL-5, IL-13 and IL-9, as well as certain growth factors, such as amphiregulin.<sup>48</sup> Amphiregulin is a member of the epidermal growth factor (EGF) family that promotes epithelial cell growth.<sup>49</sup>

ILC2s normally reside in the lungs of naïve non-sensitized animals; these ILC2s are Lin<sup>-</sup> and generally express various cell surface markers, including CD117, CD122 (IL-2R β-chain), CD25, CD127, Ly5.2, Thy1, Sca-1, ST2, CD69, CD9, CD38, MHC class II, CD44 and ICOS.<sup>40,49-51</sup> These cell markers have been used to identify and isolate ILC2s among the Lin<sup>-</sup> populations in the lung of naïve mice (Fig. 1A). Importantly, lung ILC2s are present in *Rag2*<sup>-/-</sup> mice (i.e. deficient in T cells) and *ST2*<sup>-/-</sup> mice (i.e. deficient in IL-33R), suggesting that they do not require T cell help or IL-33 for their development. In contrast, mice that are deficient in IL-2 receptor common γ-chain (cγ) or IL-7R α-chain lack mature ILC2s, consistent with their dependency on IL-7 for their development. Lung ILC2s are a rare cell population. In wild-type C57BL/6 mice, lung ILC2s represent only 0.25–1% of total live cells in the lung. ILC2s are located in collagen-rich regions close to the confluence of medium-sized blood vessels and airways, but not in alveolar areas of the lung.<sup>52</sup> Resting lung ILC2s show morphology similar to that of resting lymphocytes, with no apparent intracellular granule structures<sup>50</sup> (Fig. 1B). Once they are activated by cytokines such as IL-33, lung ILC2s increase in size and display pronounced endoplasmic reticulum and Golgi apparatus, suggesting that they are a highly-activated cell type.

Human ILC2s are typically Lin<sup>-</sup>, CD45<sup>+</sup>, CD127<sup>+</sup>, NKp44<sup>-</sup>, CD25<sup>+</sup> and CD161<sup>+</sup>.<sup>49,53-55</sup> Distinct expression of two prototypic Th2-type CD4<sup>+</sup> T cell markers, namely chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes (CRTH2) and IL-33 receptor ST2, is a unique feature of human ILC2s, and these molecules are useful to differentiate human ILC2s from other human ILCs.<sup>4</sup> Human ILC2s are reportedly found in peripheral blood, lung, BAL fluid, nasal tissue, tonsil, gut and skin of normal healthy individuals.

## Regulation and functions of ILC2s

Resting lung ILC2s show high mRNA expression levels of *Gata3*, *Rora*, *Cd69*, *Il2ra*, *Il2rg*, *Il4ra*, *Il7r*, *Il17rb*, *Il1rl1*, *Il5*, and *Il13*,<sup>40,51,52,56</sup> consistent with transcriptional regulation of this cell type and its effector function. ELISPOT assays revealed IL-5-producing lung ILC2s when cultured without stimuli,<sup>52</sup> suggesting constitutive (but minimal) production of IL-5 by resting ILC2s. Importantly, this constitutive expression of IL-5 by ILC2s may play an important role in regulating eosinophil homeostasis in various organs.<sup>52,57</sup> Growing evidence also links ILC2s with metabolic homeostasis, obesity, and dietary stress.<sup>57-59</sup> For example, deficiency of ILC2-derived IL-5 and IL-13 led to increased adiposity and insulin resistance.<sup>57</sup> ILC2s also responded to the daily cycle of caloric intake, resulting in cytokine expression associated with circadian rhythms.<sup>52</sup>

ILC2s are activated by cytokines, such as IL-25, IL-33 and TSLP,<sup>38–40,53,54</sup> which are derived from epithelial cells and certain immune cells. Activated lung ILC2s produce IL-4, IL-5, IL-9, IL-13 and GM-CSF.<sup>50,51,60</sup> In particular, IL-5 and IL-13 protein are produced in large quantities by ILC2s, perhaps beyond the levels that are produced by Th2-type CD4<sup>+</sup> T cells, making these cells a unique “factory” of cytokines.<sup>50</sup> In *in vitro* culture systems, IL-33 activates lung ILC2s probably more potently than IL-25 to produce IL-5 and IL-13.<sup>50,51,61</sup> In certain experiments, IL-25 and TSLP did not activate lung ILC2s by themselves, but they synergistically enhanced cytokine production by ILC2s in the presence of other cytokines, such as IL-33.<sup>51</sup> IL-25 and IL-33 also promote expansion and/or migration of lung ILC2s, as intraperitoneal or intranasal administration of IL-25 or IL-33 increased ILC2 cell numbers in lung tissues and draining lymph nodes *in vivo*.<sup>40,62</sup>

Lung ILC2 activities can also be regulated by IL-2-family cytokines. *In vitro*, neither IL-2 nor IL-7 alone induces significant IL-5 and IL-13 production by ILC2s. However, these two cytokines synergistically enhance IL-33- and IL-25-induced proliferation and type 2 cytokine production by lung ILC2s.<sup>49–51</sup> ILC2s were a dominant source of IL-9 in mice exposed to the protease papain.<sup>60</sup> IL-9 produced by ILC2s may have an autocrine positive feedback effect on ILC2s since lung ILC2s cultured with IL-9 increased production of type 2 cytokines.<sup>60,63</sup> Finally, TL1A, a TNF superfamily member, has also been reported to induce ILC2 cell expansion.<sup>64</sup>

Besides cytokines, lung ILC2s can be regulated by lipid mediators that are presumably generated during allergic inflammation by mast cells, eosinophils, and other inflammatory cells.<sup>65</sup> *In vitro*, leukotriene D<sub>4</sub> (LTD<sub>4</sub>) potently stimulates mouse lung ILC2s to produce not only IL-5 and IL-13 but also a large amount of IL-4; IL-4 is not generally produced by ILC2s stimulated with IL-33.<sup>66</sup> Intra-nasal administration of LTD<sub>4</sub> led to expansion of IL-5-producing ILC2s in the lung *in vivo*. Furthermore, prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) has also been shown to induce migration and functions of human ILC2s through the CRTH2 receptor.<sup>67</sup>

## Roles of ILC2s in innate type 2 responses to fungi

Initial studies on ILC2s demonstrated their roles in innate immunity against a variety of infectious organisms. For example, ILC2s play critical roles in protective immunity against helminth infection,<sup>38–40</sup> in influenza-induced lung inflammation and airway hyperreactivity (AHR),<sup>68</sup> and in respiratory epithelial repair after influenza infection.<sup>49</sup> Therefore, it is reasonable to speculate that ILC2s may be involved in immune responses to fungal organisms or their products.

To investigate this hypothesis, *Alternaria* extract was administered once into the airways of naïve non-sensitized BALB/c mice.<sup>69</sup> Th2 cytokine levels, including IL-5 and IL-13, were increased in as early as 6 h after *Alternaria* exposure. Interestingly, within 1 h after receiving *Alternaria* extract, IL-33 levels increased markedly in BAL fluids, preceding the increases in IL-5 and IL-13. The production of IL-5 and IL-13 in *Alternaria*-exposed mice was similar in *Rag1*<sup>-/-</sup> and wild-type mice, suggesting that these type 2 cytokine responses are independent of adaptive immunity. In contrast, *Alternaria*-induced increases in IL-5 and IL-13 were abolished in mice deficient in IL-33 receptor ST2. Altogether, these findings

suggested that IL-33 likely mediates rapid production of Th2-type cytokines in the airway mucosa through an innate mechanism(s). Robust *Alternaria*-induced IL-33-dependent type 2 cytokine responses were also observed in other studies.<sup>70,71</sup> Interestingly, among the various allergen extracts tested in these studies, only *Alternaria* induced significant IL-33 release into BAL fluids, suggesting that *Alternaria* has unique biological properties.

IL-33 was also involved in airway pathological changes in response to *Alternaria* when non-sensitized naïve mice were exposed repeatedly to *Alternaria* for a week.<sup>50</sup> Mice exposed to *Alternaria* for 7 days demonstrated marked peribronchial infiltration of inflammatory cells, epithelial hyperplasia, and pronounced airway eosinophilia. The magnitude of airway eosinophilia in *Rag1*<sup>-/-</sup> mice was roughly comparable to that in the wild type BALB/c mice for up to 5 days; airway eosinophilia further increased at a later time point in the wild type but not in *Rag1*<sup>-/-</sup> mice. Airway eosinophilia on day 5 or day 7 was significantly inhibited by >80% in the *ST2*<sup>-/-</sup> mice as compared to the wild type mice. These findings suggested that airway type 2 immune responses to *Alternaria* exposure consist of at least two arms: an initial innate immune response and a subsequent involvement of adaptive immunity; both arms are IL-33 dependent.

Subsequent studies established that the lung ILC2 population is the cell type that mediates this rapid type 2 cytokine production and pathological changes in response to *Alternaria* allergens.<sup>50</sup> *In vitro* culture of lung cells from naïve mice with IL-33 induced robust production of IL-5 and IL-13. Importantly, the lung cells that produced IL-5 did not express authentic markers for T cells (CD3, CD4, CD8), B cells (B220), macrophages (F4/80), mast cells/basophils (FcεRIα), or granulocytes (Gr-1), but they expressed Thy1.2, CD25 and CD44. When Lin<sup>-</sup>CD25<sup>+</sup>CD44<sup>hi</sup> lung cells were cultured with IL-33, they produced large quantities of IL-5 and IL-13. By FACS analysis, Lin<sup>-</sup>CD25<sup>+</sup>CD44<sup>hi</sup> cells highly expressed ST2, CD127 (IL7Rα), Sca-1, CD69, Thy1.2, CD9, CD38, and ICOS, consistent with published the characteristics of ILC2s.<sup>4</sup> The lung ILC2 cell population was nearly absent in the lungs of *Il7r*<sup>-/-</sup> C57BL mice, suggesting a critical role for IL-7 to induce and/or maintain ILC2s.

To directly examine the involvement of ILC2 cells in airway responses to *Alternaria*, a reconstitution approach was undertaken. When ILC2-deficient *Il7r*<sup>-/-</sup> mice received lung ILC2 cells from a donor, the ILC2 population was clearly detected in the lungs of the recipients, suggesting a successful homing (Fig. 2A). Importantly, *Il7r*<sup>-/-</sup> mice that were reconstituted with ILC2s and exposed to *Alternaria* extracts showed marked increases in eosinophil numbers and levels of IL-5 and IL-13 proteins (Fig. 2B). Pathologically, peribronchial infiltration of inflammatory cells, epithelial hyperplasia, and increased mucus production were observed in *Il7r*<sup>-/-</sup> mice reconstituted with ILC2s and exposed to *Alternaria* (Fig. 2C). Together, these results demonstrate the potent capacity of lung ILC2s in mediating IL-5 and IL-13 production, type-2 airway inflammation and pathological features of asthma upon exposure to *Alternaria* even in the absence of adaptive immune cells, such as T cells and B cells. Furthermore, in another study, a single intranasal exposure to *Alternaria* induced rapid production of CysLT that could activate ILC2s through CysLT1R, suggesting that an IL-33-independent pathway may also be involved.<sup>66</sup> In mice that were already sensitized to ryegrass allergen, exposure to *Alternaria* enhanced

proliferation and/or recruitment of both ILC2s and CD4<sup>+</sup> T cells and promoted airway inflammation and pathology.<sup>72</sup> The roles of ILC2s in innate type 2 responses were also demonstrated by mouse models using authentic proteases, such as papain and bromelain as surrogates of allergen proteases, as well as *Aspergillus* proteases.<sup>51,73</sup>

Chitin is a polysaccharide constituent of fungi. Exposure of naïve mice to chitin particles induced expression of IL-25, IL-33 and TSLP, which activated lung ILC2s and induced IL-5 and IL-13 production and subsequent accumulation of eosinophils and alternatively activated macrophages.<sup>74</sup> In the absence of all three epithelial-derived cytokines, ILC2s failed to produce IL-5 or IL-13. Interestingly, genetic ablation of ILC2s enhanced IL-1 $\beta$ , TNF $\alpha$ , and IL-23 expression, increased activation of IL-17A-producing  $\gamma\delta$ T cells, and induced prolonged neutrophilic inflammation of the airways. Thus, chitin-elicited activation of ILC2s may promote type 2 immune response and airway eosinophilia while it may suppress  $\gamma\delta$ T cell-mediated neutrophilic airway inflammation.

A major question remains how fungi and authentic proteases trigger production of epithelium-derived cytokines, such as IL-33. Allergen could be recognized by the immune system via three major mechanisms: 1) engagement of pattern recognition receptors, 2) molecular mimicry of TLR signaling complex molecules, and 3) proteolytic activity.<sup>75,76</sup> In particular, TLR4 likely plays a critical role in type 2 immune responses to inhaled HDM allergens,<sup>77–79</sup> low-dose LPS in the airways,<sup>80</sup> and papain injected into skin.<sup>81</sup> Furthermore, inhaled proteases promoted fibrinogen cleavage; the fibrinogen cleaved products in turn served as ligands for TLR4.<sup>82</sup> IL-33, IL-25 and TSLP were produced by airway epithelial cells when they were cultured with *Alternaria* and proteases *in vitro*.<sup>69,83,84</sup> IL-33 and TSLP were produced quickly in the lungs of naïve mice exposed to cysteine proteases, such as bromelain and papain.<sup>73</sup> However, mice deficient in TLR4 or a protease-activated receptor 2 (PAR2) developed comparable levels of airway eosinophilia compared to WT mice. Importantly, upon exposure to proteases, uric acid (UA) was rapidly released into the airway lumen, and removal of this endogenous UA by uricase prevented type 2 immune responses.<sup>73</sup> Conversely, exogenous UA promoted secretion of IL-33 by airway epithelial cells *in vitro*, and administration of UA into the airways of naïve animals induced extracellular release of IL-33, followed by activation of ILC2s. These findings provide mechanistic insights into the development of type 2 immunity to fungi and allergen proteases and suggest that an endogenous danger signaling molecules, such as UA, may play key roles in regulating type 2 immune responses in respiratory mucosa. Clearly, further studies are necessary to elucidate the molecular mechanisms involved in cytokine production by airway epithelial cells when they are exposed to allergens.

## Role of ILC2s in chronic airway inflammation induced by fungi

ILC2s may interact with other immune cells and play roles in chronic airway inflammation. For example, ILC2 cell numbers were not maintained in *Rag2*<sup>-/-</sup> mice infected with helminths or challenged with the protease papain,<sup>39,60</sup> suggesting that adaptive immune cells (presumably T cells) are required for ILC2 expansion, migration or survival. Naïve CD4<sup>+</sup> T cells supported proliferation and type 2 cytokine production by ILC2s in an IL-2-dependent manner.<sup>85</sup> Conversely, lung ILC2s enhanced CD4<sup>+</sup> T cell proliferation and promoted

production of type 2 cytokines *in vitro*.<sup>86</sup> The interaction between ILC2s and CD4<sup>+</sup> T cells likely involved the costimulatory molecule OX40L and IL-4, which was mainly derived from ILC2s. Nonetheless, ILC2-deficient mice effectively produced Th2 cell-mediated allergic airway inflammation in an OVA model, suggesting that ILC2s may promote the initiation of Th2 cell differentiation whereas they do not seem to be required for activation of established memory Th2 cells.<sup>87</sup> ILC2s may also interact with other immune cells. A more recent study showed that activation and proliferation of lung ILC2s in mice exposed to the protease papain were reduced in mice that are deficient in IL-4, specifically in the basophil compartment.<sup>88</sup> Furthermore, the initial description of ILC2s in FALC demonstrated that ILC2-derived IL-5 and IL-6 may promote IgA antibody production by B1 B cells.<sup>38</sup> Thus, ILC2s and their products have the potential to interact with a variety of immune cells, including CD4<sup>+</sup> T cells, basophils and B cells, and ILC2s may need to be considered as a key part of the immune cell network involved in allergic immune responses.

To investigate the immunological mechanisms in fungus-mediated chronic airway inflammation, mice were exposed to multiple airborne allergens, including the fungals allergen *Alternaria* and *Aspergillus* as well as HDM, for a prolonged period.<sup>89</sup> Chronic and multiple exposures to these allergens induced a robust increase in BAL eosinophils, which peaked in 4 weeks; eosinophils made up approximately 70% of total BAL cells. Plasma concentrations of antigen-specific IgE and IgG1 antibodies continued to rise for at least 8 weeks. Increased lung levels of type 2 cytokines, including IL-4, IL-5, and IL-13, were also observed. Notably, a marked increase in the lung levels of IL-33, approximately 10-times more than a baseline level, was observed in 4 weeks. In this model, unlike the acute exposure models as described above, increases in BAL eosinophils and cytokines as well as in plasma IgE were abolished in *Rag1*<sup>-/-</sup> mice, suggesting a critical role for adaptive immunity. Nonetheless, the number of ILC2s increased by >2-fold when animals were exposed to allergens for 4 weeks, suggesting their potential involvement.

The functions of these ILC2s during chronic inflammation were examined more in detail by using cytokine reporter mice, including the IL-5 reporter IL-5<sup>+/-venus</sup> mice and IL-13 reporter IL-13<sup>+/-eGFP</sup> mice. In PBS-exposed mice, a small fraction of ILC2s expressed IL-5 or IL-13; no other cell populations within the Lin<sup>-</sup> population expressed these cytokines. Importantly, when mice were exposed to allergens, both the proportion and absolute number of IL-5- or IL-13-producing ILC2s increased by approximately 4-fold. Unlike ILC2s, IL-5 or IL-13 signals were undetectable within the CD3<sup>+</sup> cell population in PBS-exposed animals. When mice were exposed to allergens, the prevalence of IL-5- or IL-13-positive cells increased dramatically; these cytokine-positive CD3<sup>+</sup> T cells expressed CD4. The absolute number of type 2 cytokine-positive CD4<sup>+</sup> T cells was approximately 5 times more than that of the cytokine-positive ILC2s.<sup>89</sup> Similarly, during adaptive immune responses in mice exposed to HDM or OVA, both ILC2s and CD4<sup>+</sup> T cells were sources of IL-5 and IL-13.<sup>62,90</sup> Altogether, both ILC2s and CD4<sup>+</sup> T cells likely contribute to the increased IL-5 and IL-13 production in mice after prolonged airway exposure to airborne allergens. However, the relative contributions of these cell types still need to be elucidated, considering that ILC2s might have a greater capacity to produce type 2 cytokines than CD4<sup>+</sup> T cells on a per cell basis.<sup>38</sup>



Recent studies provide mechanistic insights as to how ILC2s might regulate adaptive immunity and chronic airway inflammation. For example, adoptive transfer of both ILC2 and CD4<sup>+</sup> T cell populations, but not each population alone, into *Il7ra*<sup>-/-</sup> mice resulted in induction of a robust antigen-specific type 2-cytokine response and airway inflammation when mice were exposed to protease allergens, suggesting synergistic interactions between ILC2s and CD4<sup>+</sup> T cells during development of adaptive immune responses.<sup>86</sup> In another study, intranasal administration of papain stimulated both ILC2s and Th2 cells, causing allergic lung inflammation and elevated levels of IgE antibodies.<sup>91</sup> This process was severely impaired in ILC2-deficient mice. Indeed, ILC2-derived IL-13, but not IL-4, was critical as it promoted migration of activated lung DCs into the draining lymph node where they primed naive T cells to differentiate into Th2 cells. Importantly, both papain-induced ILC2 activation and Th2 cell differentiation were dependent on IL-33.

ILC2s and IL-33 also played key roles in the persistence of airway remodeling and AHR induced by fungal and other allergens.<sup>92</sup> Elimination of T cells in mice with allergen-induced chronic airway inflammation resulted in resolution of airway inflammation, but AHR or remodeling continued without T cells. Elimination of both T cells and ILC2 or blockade of IL-33 resulted in resolution of airway inflammation and AHR. Importantly, epithelial IL-33 activated ILC2s to produce IL-13, which in turn promoted production of IL-33 and ST2 expression by airway epithelial cells. Thus, during a chronic phase of airway inflammation, epithelial cells and ILC2s may form a positive feedback loop, resulting in continued airway remodeling and AHR even in the absence of antigens or adaptive immunity.

Finally, corticosteroid resistance is associated with persistent asthma and poses a major problem in the treatment of patients with severe asthma. In a mouse model of airway inflammation, in which mice were sensitized with OVA and then challenged with OVA, treatment with dexamethasone generally attenuated airway inflammation. In contrast, when mice were challenged with OVA plus IL-33, increased numbers of ILC2s and considerable production of type 2 cytokines persisted despite dexamethasone treatment.<sup>93</sup> These steroid-resistant ILC2s are likely induced by TSLP that is produced during allergic inflammation of the airway and then activates STAT5 and Bcl-xL in ILC2s. Altogether, these studies suggest that involvement of ILC2s in allergic airway inflammation is unlikely to be limited to the innate immune response but extends to regulation of chronicity and persistence of airway inflammation induced by allergens. The current working model to describe the roles of ILC2s in fungus- and protease-induced type 2 airway immune responses is provided in Fig. 3.

## Concluding remarks

After identification and characterization of ILC2s in 2010,<sup>38–40</sup> our knowledge of the biology of this novel cell type has expanded quickly. ILC2s are resident in various normal tissues. Although small in number, ILC2s likely play major roles in innate immunity and disease processes by producing large quantities of type 2 cytokines and tissue growth factors. In mice, ILC2s show both pathological and protective functions in virus- or allergen-induced allergic immune responses. Furthermore, ILC2s may also work with other

immune cells, such as CD4<sup>+</sup> T cells, DCs, and epithelial cells, and promote development and persistence of allergic airway inflammation.

However, the research in ILC2s is still in its infancy, and many questions remain to be addressed. For example, ILC2s contribute to the initiation and persistence of fungus-mediated allergic immune responses in mice. However, little is known about whether and how ILC2s are involved in the chronic and recurrent airway inflammation that is observed in human patients with asthma and other allergic diseases. The increased prevalence of ILC2s in nasal polyp tissues from patients with chronic rhinosinusitis<sup>55,94,95</sup> and in peripheral blood specimens from patients with asthma<sup>96</sup> suggest that ILC2s are likely involved. At the cellular level, the interactions between ILC2s and other immune and tissue cells such as mast cells and epithelial cells, as well the mechanisms involved in these interactions, need to be studied. Our knowledge of the processes involved in migration and tissue localization of ILC2s is also lacking. Finally, information is limited regarding the molecular mechanisms that explain how airway exposure to fungi and other allergens results in increased production and secretion of pro-type 2 cytokines, such as IL-33, leading to activation of ILC2s and other inflammatory cells in airway mucosa. Thus, a further understanding of the biology of ILC2s, their roles in disease status, and the molecular and cellular mechanisms involved in the activation and persistence of ILC2s will help us to better understand the mechanisms of asthma and other allergic airway diseases and to develop novel therapeutic options for these diseases.

## Abbreviations

<b>AHR</b>	airway hyperreactivity
<b>CLPs</b>	common lymphoid progenitors
<b>cy</b>	common $\gamma$ -chain
<b>CRTH2</b>	chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes
<b>FALC</b>	fat-associated lymphoid cluster
<b>ILCs</b>	innate lymphoid cells
<b>ILC1s</b>	group 1 ILCs
<b>ILC2s</b>	group 2 ILCs
<b>ILC3s</b>	group 3 ILCs
<b>Lin<sup>-</sup></b>	lineage-negative
<b>LTD<sub>4</sub></b>	leukotriene D <sub>4</sub>
<b>NK</b>	natural killer
<b>OVA</b>	ovalbumin
<b>PGD<sub>2</sub></b>	prostaglandin D <sub>2</sub>
<b>PLZF</b>	promyelocytic leukemia zinc finger

**TSLP** thymic stromal lymphopoietin**References**

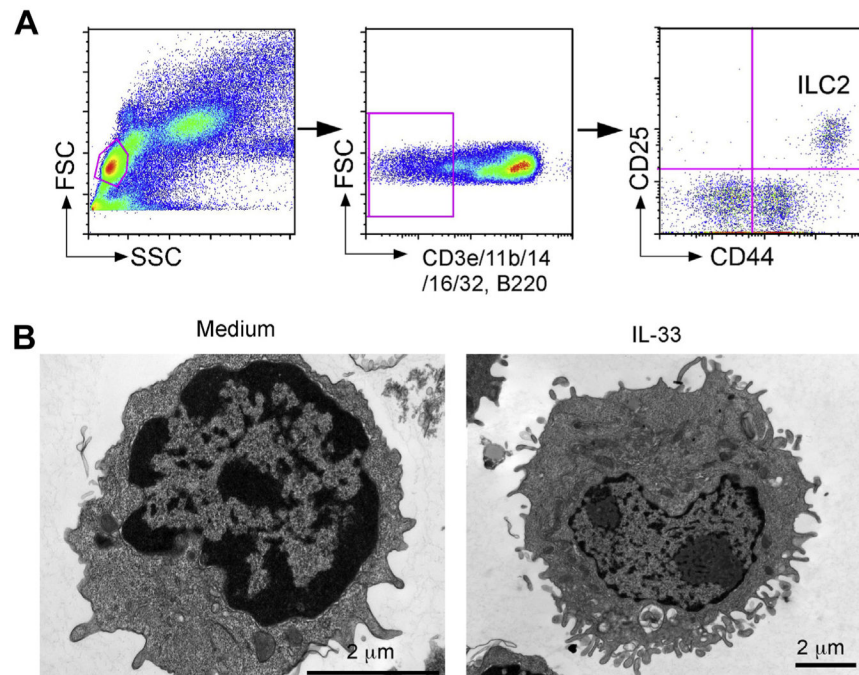
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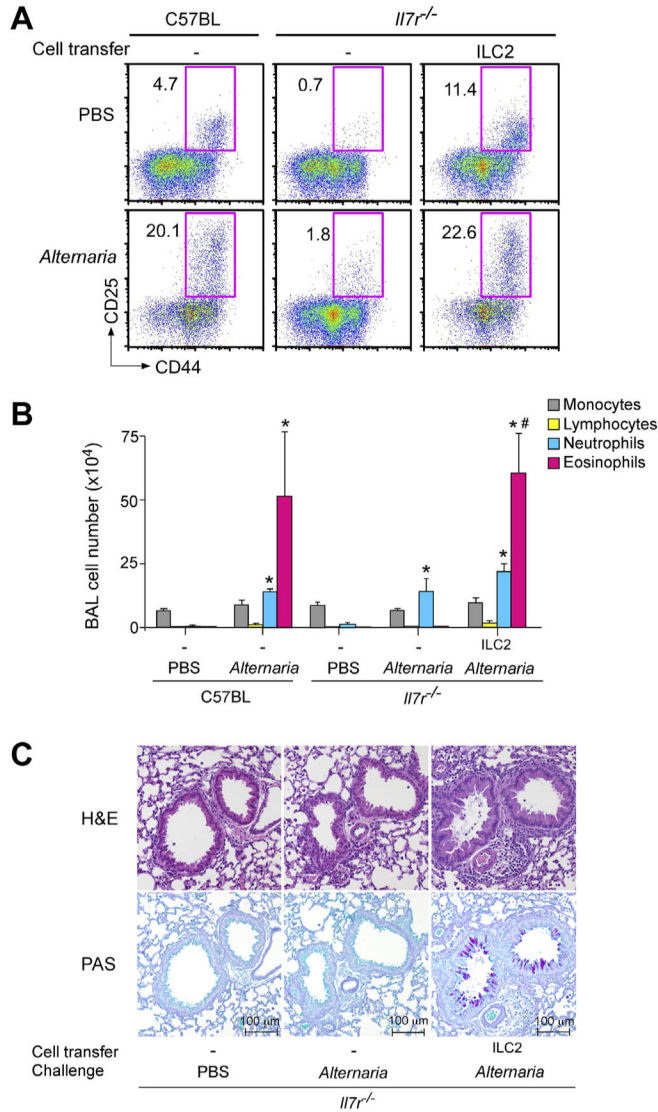
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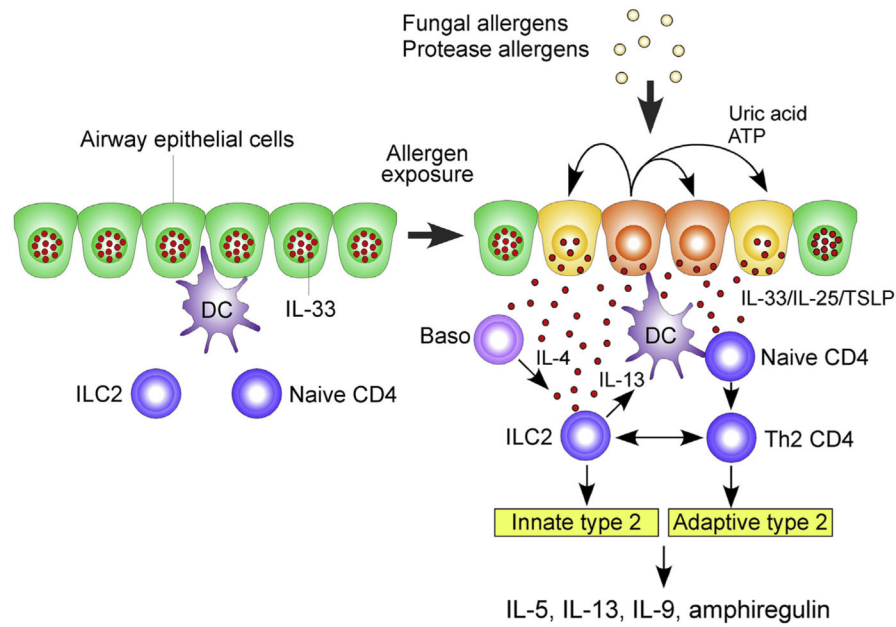


**Fig. 1.** Lung ILC2s respond vigorously to IL-33 and produce a large quantity of IL-5 and IL-13 *in vitro*. (A) Gating strategy and identification of ILC2s in lung single cell suspensions from naïve BALB/c mice. (B) Morphology of lung ILC2s. Lung ILC2s were cultured with medium alone or IL-33 and examined under electron microscopy. Original magnifications; 25,000× (medium alone, left) and 12,000× (IL-33, right).





**Fig. 2.** Lung ILC2s mediate eosinophilic airway inflammation and pathology in mice exposed to fungal allergen *Alternaria*. (A) ILC2s were isolated from the lungs of naïve C57BL/6 mice, and they were adoptively transferred to naïve *Il7r<sup>-/-</sup>* mice, which are deficient in mature CD4<sup>+</sup> T cells and ILCs. Mice were then exposed intranasally three times to PBS or *Alternaria* extract over 6 days. Transferred ILC2s are identified as Lin<sup>-</sup>CD25<sup>+</sup>CD44<sup>hi</sup> cells. The FACS plots were gated on Lin<sup>-</sup> cells. (B) Cell number and differentials in BAL fluids were determined. (C) Representative histology (upper panels: H&E staining, lower panels: PAS staining) of the mice as described in B.



**Fig. 3.** Working model to describe the roles of ILC2s in type 2 immune response in the airway exposed to fungal and protease allergens. In the resting condition, IL-33 is stored in the nucleus of airway epithelial cells. Exposure to fungal or protease allergens induce the extracellular release of IL-33 and production of IL-25 and TSLP by the airway epithelium. Autocrine secretion of ATP and uric acid likely play a key role in regulating epithelial release of IL-33. IL-33, IL-25 and TSLP activate ILC2s to produce a large quantity of type 2 cytokines, such as IL-5, IL-13, IL-9, and amphiregulin. Basophil-derived IL-4 may facilitate ILC2 production of cytokines. ILC2-derived IL-13 enhances antigen uptake and migration of dendritic cells and promotes proliferation and differentiation of Th2-type CD4<sup>+</sup> T cells. In addition, ILC2s and Th2-type CD4<sup>+</sup> T cells may interact directly to sustain production of type 2 cytokines. Abbreviations: Baso, basophils; DC, dendritic cells; TSLP, thymic stromal lymphopietin.